



Intramural Activities

Volume 2

October 1, 1988-September 30, 1989

1989 annual report

Division Of

Cancer Etiology



89 annual report

Division Of

Cancer Etiology

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ANNUAL REPORT
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

TABLE OF CONTENTS

OFFICE OF THE DIRECTOR (VOLUME 1)		Page No.
Director's Overview		
Administrative Highlights		1
Scientific Highlights		7
Activities in the Office of the Director		30
<u>Project Reports:</u>		
CP 03509	Carcinogenesis Chemotherapy and Biological Markers in Nonhuman Primates	37
CP 04548	Registry of Experimental Cancers/WHO Collaborating Center for Tumors of Laboratory Animals	40
CP 05551	Liposomes as Carriers for Anti-HIV Agents	43
CP 05576	Expression of <u>ras</u> and Collagenase in Primary Tumors vs Metastases	46
CP 05578	Tumor-Endothelial Cell Interaction; Basement Membrane Degradation	51
CP 05608	Purification and Further Characteristics of 92kDa Gelatinase	55
CP 05609	Gelatinase/Type IV Collagenase Response in Normal and Neoplastic Cells to TPA	58
CP 05621	The Involvement of <u>ras</u> Oncogenes in Chemical Carcinogenesis	61

BIOLOGICAL CARCINOGENESIS PROGRAM (BCP)

Report of the Associate Director	63
----------------------------------	----

Laboratory of Cellular and Molecular Biology

Summary Report	73
----------------	----

Project Reports:

CP 04930	Biology of Natural and Induced Neoplasia	87
CP 04940	Viruses and Transforming Genes in Experimental Oncogenesis and Human Cancer	90
CP 04941	Biochemical Characterization of Retroviruses	100
CP 04976	Chromatin Radiosensitivity in Cancer Predisposition and Neoplasia	104
CP 05060	Mechanisms of Carcinogenesis In Vitro: Oncogenic Transformation of Human Cells	108
CP 05062	Transforming Genes of Naturally-Occurring and Chemically-Induced Tumors	113
CP 05063	Studies on HHV-6, EBV and HIV	116
CP 05164	Oncogenes, Growth Factor Pathways and Hematopoietic Cell Signal Transduction	121
CP 05366	The Role of Proto-Oncogenes Encoding Growth Factor Receptor in Neoplasia	126
CP 05457	Growth Factor Receptors in Transformation	129
CP 05461	Mechanism of Activation of <u>dbl</u> Oncogene: Structural/Functional Analysis of KGF	133
CP 05463	Participation of Growth Factors and Oncogene Products in Growth Regulation	136
CP 05469	Identification of New Tyrosine Kinase Oncogenes	139
CP 05472	Structural Characterization of Putative Growth Factor Receptor Gene <u>c-erbB-2</u>	141
CP 05514	Analysis of PDGF Receptor Role in Neoplastic Transformation	143

		Page No.
CP 05546	Structural and Functional Characterization of v- <u>sis</u> Gene Product	146
CP 05547	Role of PDGF Expression in the Neoplastic Process	148
CP 05548	Development of Expression Cloning System for Oncogene cDNAs	151
CP 05549	Protein Kinases in Growth Factor-Mediated Cell Activation and Transformation	154
CP 05596	Purification and Characterization of Epithelial Cell Mitogens	157
CP 05597	Role of Inositol Lipid Turnover in EGF-Induced Cell Growth	160
CP 05598	Autocrine Mechanism for v- <u>sis</u> Transformation	164
	Contract Narrative	167

Laboratory of Molecular Oncology

	Summary Report	169
--	----------------	-----

Project Reports:

CP 04963	Toward a Molecular Description of Malignant Transformation by p21 <u>ras</u> Oncogenes	181
CP 05120	Expression of Retroviral and Oncogene Proteins in Bacteria	185
CP 05238	Transforming Genes of Acute Leukemia Viruses and Their Cellular Homologues	189
CP 05295	Studies on the Activation of Oncogenes in Viruses and Human Tumors	195
CP 05440	Site-Directed Mutagenesis of <u>ras</u> Oncogenes	200
CP 05441	Characterization of the Gene Product of the c- <u>myc</u> Locus and the c- <u>ets</u> Locus	203
CP 05442	Human <u>ETS</u> Genes in Human and Cancer Genetics	208

		Page No.
CP 05443	Proto-Oncogene Expression During Cell Differentiation and Development	211
CP 05484	Proto-Oncogene <u>ets</u> in Sea Urchin and <u>Xenopus Laevis</u>	215
CP 05485	Application of Monoclonal Antibodies to the Study of Oncogene Products	218
CP 05563	Introduction of the HIV <u>tat</u> Gene into Lymphoid Cells	222
CP 05564	Analysis of HIV Gene Expression	225
CP 05565	Study of the Biochemical and Functional Properties of the <u>ets</u> Genes	228
CP 05566	Study of the Biological and Biochemical Function of <u>ets</u> Proto-Oncogenes	231
CP 05569	Effect of <u>c-myc</u> on Cellular Gene Expression	235
CP 05570	Expression of HIV-1 Structural Gene Proteins in Prokaryotic Vectors	238
CP 05571	Studies of E26 Avian <u>v-ets</u> and its Cellular Homologue in Mouse Cells	244
CP 05572	Isolation of Potential Oncogenes from Teleost Tumors	247
CP 05574	Characterization of <u>Drosophila</u> <u>Melano-gaster</u> <u>ets</u> and <u>ets</u> -like Genes	252
CP 05585	Gene Expression in Colon Carcinoma and Polyposis	256
CP 05586	Molecular Cloning of the Feline Immunodeficiency Virus	259
CP 05587	Search for <u>ets</u> -related Sequences in Yeast DNA	261
CP 05588	Development of <u>ets</u> -2 Transgenic Mice	264
CP 05589	Transformation of Primary Cells: Cooperation of <u>ets</u> with Other Oncogenes	267
CP 05590	Characterization of the Products of <u>ets</u> Gene Family	270

		Page No.
CP 05591	Biological Characterization of the <u>ets</u> Proteins	273
CP 05592	Expression of HIV-2 <u>env</u> Gene Products <u>E. coli</u>	276
CP 05593	Transcriptional Regulation of the Human <u>ETS-2</u> Oncogene	278
CP 05594	Identification of Potential Suppressor Genes for <u>ras</u> Transformation	282
CP 05595	DNA Topoisomerase I Activity in Retroviruses	285

Laboratory of Molecular Virology

Summary Report		289
----------------	--	-----

Project Reports:

CP 05216	<u>Ras</u> Oncogene Regulation in Yeast	293
CP 05254	Regulation of Gene Expression	296
CP 05391	Transcriptional Analysis of the JC Virus Enhancer	298
CP 05392	Regulation of Transcription by Large T-Antigen	300
CP 05394	Enhancer Elements in B-Lymphocytes and T-Lymphocytes	302
CP 05605	Transformation by Human CMV	305
CP 05606	Sjogren's Syndrome in HTLV-I Transgenic Mice	307
CP 05607	Activation of Cellular Genes in HTLV-I Transgenic Mice	309

Laboratory of Tumor Cell Biology

Summary Report		311
----------------	--	-----

Project Reports:

CP 05534	Monocyte/Macrophages and Accessory Cells in Pathogenesis of HIV-1 Infection	331
----------	---	-----

	Page No.	
CP 05535	Retrovirus Infection, Treatment, Prevention and Etiology of TSP	336
CP 05536	Immune Response to HIV: Neutralizing Antibody and Vaccine Development	343
CP 05537	Immunopathogenesis of Human RNA and DNA Viruses	348
CP 05538	Structure and Function of HIV Genome	353
CP 05539	Determinants of the Latency and Pathogenicity of Human Retroviruses	357
CP 05560	Induction of Lymphotoxin Expression by HTLV-I Infection	360
CP 05614	Immunobiology of HIV-1: Neutralizing Antibody and Vaccine Development	363
CP 05615	Biology of HIV-1 in Chimpanzees and an Accidentally Infected Lab Worker	367
CP 05616	Anti-HIV Factors in Animal Sera and CD4 Anti-receptor Therapy for HIV-1	371
CP 07148	Studies on T-Cell Malignancies, Lymphomas and AIDS	375
CP 07149	Molecular Biological Studies on Human Pathogenic Viruses	385
Contract Narratives		395

Laboratory of Tumor Virus Biology

Summary Report		407
----------------	--	-----

Project Reports:

CP 00543	Characterization of the Papillomaviruses	412
CP 00565	Transforming Activities and Proteins of the Papillomaviruses	418
CP 00898	Role of Human Papillomaviruses in Human Carcinogenesis	422
CP 05420	Transformation by Polyomaviruses	426

		Page No.
CP 05481	Biochemical Regulation of Tyrosine Protein Kinases	428
CP 05482	Control of Papillomavirus Late Transcription	432
CP 05518	Transformation and Gene Regulation of the Hamster Papovavirus	437

Laboratory of Viral Carcinogenesis

Summary Report		441
----------------	--	-----

Project Reports:

CP 05326	HLA Antigens: Structure, Function, and Disease Association	446
CP 05328	Immunologic Studies of the Human T-Cell Lymphoma Virus	449
CP 05367	The Genetic Structure of Natural Populations of Past and Present	452
CP 05382	Genes Involved in Preneoplastic Progression	457
CP 05383	Membrane Signal Transduction in Tumor Promotion	462
CP 05384	Genetic Analysis of Human Cellular Genes in Neoplastic Transformation	466
CP 05385	Molecular Genetic Analysis of Feline Cellular Genes: A Comparative Approach	473
CP 05389	Reproductive Strategies in Animal Species Emphasizing Developmental Biology	479
CP 05414	Characterization of Retroviruses (Type-D and SIVs) Isolated from Primates	484
CP 05417	Characterization and Expression of <u>raf</u> Oncogenes in Normal and Tumor Cells	488
CP 05434	Immunology of AIDS and AIDS-Related Diseases	492
CP 05528	Mechanisms of the HTLV-I and BLV rex Proteins	495

	Page No.	
CP 05529	Genetic and Molecular Organization of the MHC in the Domestic Cat	499
CP 05531	Functional Characterization of the Relationship Between <u>raf</u> and Protein Kinase C	504
CP 05532	Effect of <u>raf</u> Family Protein Kinases on Cell Physiology	507
CP 05533	Domains Involved in Regulation of <u>raf</u> Activity	511
CP 05580	Human Genetic Loci Which Influence Susceptibility to HIV Infection and Pathology	515
CP 05581	Role of Kinase Oncogenes in Growth Factor Abrogation and c- <u>myc</u> Regulation	523
CP 05582	Growth Modulation and Analysis of Chemically-Induced Tumors	526
CP 05583	Regulation of Equine Infectious Anemia Virus Gene Expression	529
CP 05584	Genomic Organization in Nonhuman Primates and Other Comparative Genetic Studies	532
CP 05618	Construction of a Novel Class of Retro- viral Vector Using BLV and HTLV-I	535
CP 05619	Role of Secondary Oncogenes in Plasmacytoma Acceleration by Avian v- <u>myc</u>	538
CP 05620	Development of Vaccines and Antivirals Against Retrovirus Infection in Primates	541

Biological Carcinogenesis Branch

Summary Report	544
----------------	-----

DNA Virus Studies I

Summary Report	556
Grants Active During FY 89	563
Contracts Active During FY 89	570

DNA Virus Studies II

Summary Report	571
Grants Active During FY 89	578
Contracts Active During FY 89	587

RNA Virus Studies I

Summary Report	588
Grants Active During FY 89	596

RNA Virus Studies II

Summary Report	606
Grants Active During FY 89	614

AIDS Virus Studies

Summary Report	622
Grants Active During FY 89	627
Contracts Active During FY 89	629

Research Resources

Summary Report	630
Contracts Active During FY 89	632

CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM (CPCP) (VOLUME II)Laboratory of Biology

Summary Report	633
----------------	-----

Project Reports:

CP 04629	Regulation of Stages of Carcinogenesis Induced by Chemical or Physical Agents	642
CP 04673	The Immunobiology of Carcinogenesis	648
CP 05499	Chromosome Alterations and Proto-Oncogene Transposition in Carcinogenesis	657
CP 05552	Lymphokine Modulation of Human Cervical Epithelial Cell Carcinogenesis	662

Laboratory of Cellular Carcinogenesis and Tumor Promotion

Summary Report	667	
<u>Project Reports:</u>		
CP 04504	Model Systems for the Study of Chemical Carcinogenesis at the Cellular Level	672
CP 04798	Metabolism and Mode of Action of Vitamin A	678
CP 05177	Use of Immunological Techniques to Study Interaction of Carcinogens with DNA	683
CP 05178	Cellular and Tissue Determinants of Susceptibility to Chemical Carcinogenesis	688
CP 05270	Molecular Mechanism of Action of Phorbol Ester Tumor Promoters	692
CP 05445	Molecular Regulation of Epidermal-Specific Differentiation Products	700

Laboratory of Chemoprevention

Summary Report	705	
<u>Project Reports:</u>		
CP 05051	Biology and Molecular Biology of Transforming Growth Factor-beta	709
CP 05396	Development of Methods to Study the Functions of TGF-beta	714
CP 05398	Characterization of Latent Forms of TGF-beta	717
CP 05550	Immunohistochemical Localization of Transforming Growth Factor-beta	720
CP 05617	Transcriptional Control of TGF-beta Genes	724

Laboratory of Comparative Carcinogenesis

Summary Report		727
<u>Project Reports:</u>		
CP 04542	Chemistry of Nitroso Compounds & Other Substances of Interest in Cancer Research	734
CP 04582	Mechanisms of Inorganic Carcinogenesis: Nickel	738
CP 05092	Transplacental Carcinogenesis and Tumor Promotion in Nonhuman Primates	741
CP 05093	In Vitro Studies on Organ Specificity in Transplacental Carcinogenesis	745
CP 05299	Interspecies Differences in Transplacental Carcinogenesis and Tumor Promotion	749
CP 05301	Biology and Pathology of Natural and Experimentally Induced Tumors	755
CP 05303	Pathogenesis and Promotion of Natural and Induced Tumors	759
CP 05352	Metabolic and Pharmacological Determinants in Perinatal Carcinogenesis	763
CP 05353	Sensitivity Factors in Special Carcinogenesis Models	766
CP 05399	Oncogene Expression in Chemically Induced Tumors	769
CP 05487	Carcinogenesis and Mutagenesis by Fecapentaenes	774
CP 05488	Mechanisms of Inorganic Carcinogenesis: Cadmium	777
CP 05524	Effects of Chemical Carcinogens on Gene Expression	781

Laboratory of Experimental Carcinogenesis

Summary Report	875
<u>Project Reports:</u>	
CP 04986	Molecular Basis of Steroid Hormone Action 795
CP 05262	Cellular Evolution of Chemically Induced Rat Hepatomas 799
CP 05263	Computer Analysis of Carcinogenesis by Two-Dimensional Gel Electrophoresis 803
CP 05283	Conditional Expression of Mammalian Genes 807
CP 05317	Opal Suppressor Phosphoserine and the 21st Naturally Occurring Amino Acid 810
CP 05373	Purification and Characterization of a Rat Hepatic Proliferation Inhibitor 814
CP 05450	Chromatin Structure and Gene Expression 818
CP 05453	Cellular and Molecular Aspects of Hepatocarcinogenesis 821
CP 05496	Metabolism and Disposition of IQ in Monkeys 825
CP 05553	Expression of Cytochrome P-450s and Their Role in Mutagenesis and Carcinogenesis 827
CP 05555	Aminoacyl-tRNAs in HIV and Other Retroviral Infected Cells 830
CP 05556	Computer-Assisted Design of Recognition Peptides 833
CP 05558	Cellular Proteins in Oncogene Transformed Rat Liver Epithelial Cells 836
CP 05559	Plasma Membrane Proteins in Normal and Neoplastic Rat Hepatocytes 839
CP 05599	Mechanism of Fibrogenesis and Cirrhosis in Rat Liver 842
CP 05600	Role of Multidrug Resistance Genes in Hepatocarcinogenesis 845

	Page No.
CP 05601 Analysis of POMC Tissue-Specific Expression and Glucocorticoid Repression	848
CP 05602 Analysis of Zn Finger DNA Binding Domains	851
 <u>Laboratory of Experimental Pathology</u>	
Summary Report	857
<u>Project Reports:</u>	
CP 04491 Quantitative Studies on Concurrent Factors in Neoplastic Transformation	860
CP 05274 Respiratory Carcinogenesis by Chemical and Physical Factors	862
CP 05276 Growth Control in Epithelial Cells and Its Alteration in Carcinogenesis	866
 <u>Laboratory of Human Carcinogenesis</u>	
Summary Report	869
<u>Project Reports:</u>	
CP 05192 Repair of Carcinogen-Induced DNA Damage in Human Cells	885
CP 05409 Control of Growth and Differentiation of Human Bronchial Epithelial Cells	889
CP 05426 Characterization and Mode of Action of the <u>raf</u> Subfamily of Oncogenes	894
CP 05435 Development of Techniques for the Measurement of Carcinogen-Adducts in Humans	898
CP 05480 Genetic Polymorphisms and Allelic Sequence Deletions in Human Lung Cancer	902
CP 05505 In Vitro Transformation of Human Bronchial Epithelial Cells	906
CP 05508 Isolation of Tumor Suppressor Genes by Subtraction Libraries	911
CP 05541 Growth of Human Hepatocytes	913

		Page No.
CP 05542	Assessment of Tobacco Smoke Genotoxicity	916
CP 05543	Tumor Suppression and Somatic Cell Genetics	919
CP 05610	Oxy-radicals and Aldehydes in Carcinogenesis	921
CP 05611	In Vitro Studies of Human Mesothelioma	925
CP 05612	Tumor Suppression in Monochromosome Transfer Hybrids	929
CP 05613	Tissue Equivalent Models for Studying Cellular Subtractions and Carcinogenesis	932
	Contract Narratives	935

Laboratory of Molecular Carcinogenesis

	Summary Report	947
	<u>Project Reports:</u>	
CP 04496	Chromosomal Proteins and Chromatin Function	953
CP 04517	DNA Repair in Human Cancer-Prone Genetic Diseases	957
CP 05086	Phenotyping of Cytochrome P-450s in Animal and Human Tissues	963
CP 05125	Preparation and Characterization of Monoclonal Antibodies to P-450	966
CP 05318	Structural Characterization of Cytochrome P-450	969
CP 05436	Mutagen Activation Analysis with Expressed P-450s and Monoclonal Antibodies	972
CP 05521	Polymorphic Drug Oxidation: The Human and Rat Debrisoquine 4-Hydroxylase Gene	975

	Page No.
CP 05522 Structure and Characterization of Human Thyroid Peroxidase	978
CP 05561 Transcriptional Regulation of Cytochrome P-450 Genes	981
CP 05562 Assessment of Human P-450 Catalytic Activities by cDNA-Directed Expression	985
CP 05603 Regulation of Cytochrome P-450	990
CP 05604 Immunopharmacological Identification and Regulation of Cytochrome P-450	993

Chemical and Physical Carcinogenesis Branch

Summary Report	995
----------------	-----

Biological and Chemical Prevention

Summary Report	1001
Grants Active During FY 89	1008
Contracts Active During FY 89	1013

Carcinogenesis Mechanisms

Summary Report	1014
Grants Active During FY 89	1035

Diet and Nutrition

Summary Report	1048
Grants Active During FY 89	1055

Molecular Carcinogenesis

Summary Report	1058
Grants Active During FY 89	1086

Smoking and Health

Summary Report	1102
Grants Active During FY 89	1112
Contracts Active During FY 89	1113

Chemical Research Resources

Summary Report	1114
Contracts Active During FY 89	1118

Radiation Effects Branch

Summary Report	1119
Grants Active During FY 89	1137
Contracts Active During FY 89	1145

EPIDEMIOLOGY AND BIOSTATISTICS PROGRAM

Report of Associate Director	1147
------------------------------	------

Biostatistics Branch

Summary Report	1167
Summary Report of Progress on Research Contracts	1176
Research Contracts Active During FY 89	1177

Project Report:

CP 04265	Consulting in Statistics and Applied Mathematics	1178
CP 04267	Research in Mathematical Statistics and Applied Mathematics	1184
CP 04269	Biomedical Computing - Consultation, Research and Development, Service	1188
CP 04475	Skin Cancer and Solar Radiation Program	1191
CP 04500	Methodologic Studies of Epidemiology	1196
CP 04779	Field Studies in High Risk Areas	1202
CP 05498	Consulting on Epidemiologic Methods	1210

Clinical Epidemiology Branch

Summary Report	1215
----------------	------

Project Reports:

CP 04377	Familial, Congenital, and Genetic Factors in Malignancy	1234
CP 04400	Clinical Epidemiology of Cancer	1245
CP 05139	NIH Interinstitute Medical Genetics Program: The Genetics Clinic	1254

		Page No.
CP 05146	Morbidity in Childhood Cancer Survivors and Their Offspring	1258
CP 05194	National Cancer Mortality Studies by Computer	1262
CP 05279	Development of Epidemiologic Data Resources	1266
CP 05280	Carcinogenic Effects of Ionizing Radiation	1269
CP 05329	Hepatitis B Virus (HBV) and Liver Cancer in Army Veterans of WWII	1272

Environmental Epidemiology Branch

Summary Report		1277
Summary Report of Progress on Research Contracts: Environmental Studies Section		1286
Contracts Active During FY 89		1291
<u>Project Reports:</u>		
CP 04378	U.S. Cancer Mortality Survey and Related Analytic Studies	1293
CP 04410	Studies of Persons at High Risk of Cancer	1298
CP 04411	Cancer and Related Conditions in Domestic Animals: Epidemiologic Comparisons	1307
CP 04480	Studies of Occupational Cancer	1310
CP 05128	Diet and Nutrition in Cancer Etiology	1318
CP 05400	Epidemiology of Human Lymphotropic Viruses: ATL, AIDS and Cancer	1326
CP 05526	Analytical Investigations of Selected Issues in Human Cancer	1346

Radiation Epidemiology Branch

Summary Report 1359

Summary Report of Progress on Research Contracts 1371
Contracts Active During FY 89 1379

Project Reports:

CP 04481 Studies of Radiation-Induced Cancer 1381

CP 05368 Studies of Drug-Induced Cancer and 1412
Multiple Primary Cancers

Extramural Programs Branch

Summary Report 1419

Grants Active During FY 89 1439

Contracts Active During FY 89 1455

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

October 1, 1988 through September 30, 1989

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

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

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

A recognizable difference exists between the role of HPV-16 DNA in animal cells and in humans. Whereas in NIH 3T3 cells recombinant HPV is capable of inducing malignant transformation, the same HPV in the identical plasmid will only immortalize human genital cells and has led us to conclude that other etiologic agents or cofactors must be involved for progression to malignancy. This conclusion reaffirms the epidemiological conclusion that HPV-16 or HPV-18 in women is not sufficient to assure the development of a high grade lesion into a carcinoma because only a small percentage of all such cases progress to

cancer. Thus, we conclude that HPV can be considered necessary but not sufficient to cause cervical cancer. With this result and using the HPV models developed within the laboratory, we are studying four interrelated facets of the role of HPV in the transformation of human genital epithelial cells: 1) determination of carcinogens--chemical, physical, or viral--that alter HPV or cellular genes in such a way as to result in cancer; 2) study of differentiation in human foreskin and exocervical epithelial cells and the interruption of terminal differentiation by immortalization and carcinogenesis; 3) molecular analysis of HPV-immortalized cells to determine the nature of the immortalizing gene and to study cellular regulation of HPV gene expression; and 4) sensitivity of HPV immortalized and tumorigenic cells to the lymphokine leukoregulin developed in this laboratory.

Because neither immortalized foreskin keratinocytes or cervical cells are tumorigenic in nude mice, we have concluded that HPV may be necessary but not sufficient to cause cervical cancer. The use of cervical cells from the squamo-columnar zone (demonstrated by the keratin pattern) is particularly relevant in our studies because HPV-associated squamous cell carcinoma are found in that area. Therefore, a number of chemical and biological carcinogens have been added in an attempt to convert these cells to the malignant state. Thus far, only the addition of activated v-Ha-ras, an oncogene found in some cervical cancers expressing HPV-16 or -18, to HPV-16-immortalized human cervical cells has resulted in malignancy as proven by the formation of cystic squamous cell carcinomas by HPV-16-Ha-ras cells in nude mice. This two-stage model utilizing relevant human cells demonstrates that HPVs play a critical role in cervical malignancy and provides a system for elucidating critical cellular changes associated with progression to malignancy.

To further investigate HPV-induced immortality as well as cellular events brought about by integrated HPV DNA, a number of recombinant plasmids have been constructed with HPVs. Included are other HPVs with a high or moderate association with cervical cancer and some with a low or no oncogenic association. These various HPVs have been transfected into foreskin-derived keratinocytes. Only those HPVs associated with malignancy, i.e., 16, 18, 31 and 33, are able to convert cells to immortality; HPVs-1, -5, -6 and -11 induce small colonies that senesced similarly to non-transfected cells. The immortal cells contain integrated and transcriptionally active viral genomes. In contrast, cells transfected with the non-immortalizing HPVs contain only episomal viral DNA. The difference in activity between the two types of HPVs is consistent with the pathogenesis of cervical carcinomas. Attempts to convert non-immortalizing HPVs to ones capable of integrating into the host genome have failed.

A common characteristic of the immortalizing recombinant HPV DNAs is the presence of an interruption in either the E1 or E2 open reading frame (ORF). The plasmid HPV DNA pMHPV-16d contains a frameshift mutation in ORF E1 (splitting it into two ORFs) not found in other isolates of HPV-16; pHPV-18, pHPV-31, and pHPV-33 have vector sequences cloned into ORFs E1, E2/E4, and E1/E2, respectively. To determine whether interruption of ORF E1 in HPV-1a, -5, or -11 would confer the ability to integrate and immortalize keratinocytes, plasmids were digested with restriction enzymes that cleaved within this ORF. Transfection with these restricted plasmids did not induce immortality, even though HPV DNA was present in the selected but mortal cells. Thus,

interruption within the E1 ORF was also not sufficient to account for immortalization. Plasmids pHPV-5 and -11, contain interruptions in E7 and L1 due to molecular cloning. Because E7 and L1 are intact in all recombinant HPVs that immortalized keratinocytes, one or both of these ORFs might be required. To test this possibility, pHPV-5 and -11 DNAs were digested with restriction enzymes that separated vector from HPV sequences; HPV sequences were isolated and subsequently self-ligated to restore the integrity of these ORFs. The ligated DNAs (consisting mainly of concatameric molecules) were also ineffective in immortalizing human keratinocytes in two separate attempts when cotransfected with the neomycin resistance gene. As a positive control, pHPV-31 and -33 DNA were treated in the same manner; immortalization still occurred. Therefore, restoration of the interrupted sequences of pHPV-5 or -11 is not sufficient to restore their ability to immortalize.

It has been suggested that E6 and E7 are the gene(s) responsible for immortalization by HPV in human epithelial cells. All immortalized recombinant HPV DNAs contain an intact upstream regulatory region and E6 and E7 ORFs. Immortalizing HPV DNAs also all contain a splice site within the E6 ORF allowing production of an E6 mRNA, whereas non-immortalizing types do not. However, the effect of the E6 protein on cell growth has not been defined. Moreover, the HPV sequence in pSHPV-18 was interrupted in ORF E1 by insertion of the vector pSV2neo, which contains a strong polyadenylation signal. Thus, it is possible that E6 and E7 are the only intact genes expressed because the other HPV-18 ORFs are separated from the upstream regulatory region. To test this possibility, RNA from HPV-18-immortalized human keratinocytes was hybridized to two subgenomic fragments of HPV-18 DNA. The first included the intact E6 and E7 ORFs and a partial E1 ORF which ends at the EcoRI cloning site; the second contained the rest of E1, E2, E4, E5, and L2 and most of L1. The E6/E7 probe hybridized to the same transcripts in three HPV-18-immortalized cell lines as did the intact 7.9-kbp HPV genome. In contrast, the second probe failed to hybridize to any messages in one line and hybridized very weakly in the two other lines. Therefore, at least one HPV-18-immortalized line transcribes RNAs only from the intact E6 and E7 ORFs. This suggests that the immortalization function is limited to one or both of these genes.

Control of HPV mRNA expression in immortalized lines is also being investigated. One cervical line, HCX16-5, with a single form of HPV-16 synthesizes HPV-16 mRNA species that are 0.4 kb smaller than those consistently observed for the other lines; the predominate species was 1.2 kb instead of 1.6. Analysis of the mRNA with sub-genomic HPV-16 probes suggested that the 1.2 kb species in HCX-16-5 was a mixture of two truncated forms of the 1.6 kb mRNA; either the E6 or E5 region was missing. This was confirmed by examination of a cDNA library from line HCX-16-5. Of 19 clones 13 were truncated in E5; three were missing E6; two others were missing both. Sequence analysis of the clones missing E6 suggest that a minor promoter exists in E7. Moreover, four clones truncated in E5 contained poly(A)-tails at different sites 400 to 450 bp upstream of the standard HPV-16 early mRNA poly(A)-site. Because line HCX-16-5 is the only one that lacks the complete L2 and L1 ORFs, these regions may contain sequences that regulate termination of the early mRNA.

Cervical carcinomas differ from other carcinomas in that they become less differentiated with proliferation in vitro or in vivo. A mechanism by which

HPV might contribute to the carcinogenic process is by altering normal cell differentiation. Normally the epithelium of the cervix consists of a stratified squamous epithelium. Only the basal cell layer divides. As cells differentiate they become flattened in appearance and gene expression is modulated. For example, genes for structural proteins such as keratins, involucrin, and filaggrin are up-regulated, whereas expression of genes encoding extracellular matrix proteins, such as collagen type IV and laminin, are down-regulated. The progressive development of squamous carcinoma from normal squamous epithelium is characterized by alterations in normal differentiation. These are termed cervical intraepithelial neoplasia grades 1-3; they are graded according to the percentage of epithelial thickness containing undifferentiated basal cells. A technique that permits in vitro to in vivo correlations by transplantation of intact cell monolayers onto the body wall of nude athymic immunodeficient mice demonstrates that HPV immortalized cells differentiate aberrantly, mimicking cervical intraepithelial neoplasia. However, no invasion occurs. In vitro growth characteristics are consistent; cells stratify poorly and a subpopulation is resistant to terminal differentiation.

In summary, morphologic studies suggest that a majority of HPV-immortalized lines at low passage retain the ability to undergo squamous differentiation. Possibly these cells are already partially differentiated. With increasing passage some lines become unable to differentiate normally, are dysplastic and resemble cervical intraepithelial neoplasia grades 1-3. The morphologic changes correlate with a reduced rate of terminal differentiation in terms of anchorage independence, with long-term cultures having a higher percentage of cells able to survive in suspension than cells at earlier passages. The morphologic changes also correlate with altered expression of several RNAs important in normal squamous differentiation. Steady state levels of RNA for involucrin and keratin 1 are reduced. These two genes are normally expressed in supra basal cells during squamous differentiation. In contrast the laminin gene which is down-regulated during keratinocyte differentiation is expressed at high levels in immortalized lines.

Specific chromosome changes have been demonstrated in several forms of cancer but only 1% of the cytogenetic data are concerned with primary epithelial tumors which represent 80% of all human cancer. In general, chromosome changes in carcinoma are more complex compared to those in hematologic malignancies or sarcomas. This complexity and karyotypic heterogeneity may obscure pathologically relevant primary changes. Consequently, newly developed in vitro models with keratinocyte or cervical epithelial cells are useful for discerning chromosome changes critical to cell immortality and progression to malignancy.

Human exocervical epithelial cells immortalized by HPV16 DNA become tumorigenic only after transfection with an activated v-Ha-ras oncogene. A tumorigenic cell line (HCX-16-2HR) is aneuploid, with structural alterations frequently seen in spontaneous solid tumors. These include an isochromosome originating from a deleted long arm of chromosome 1 not found in HPV-16-immortalized non-tumorigenic cells, a chromosome 17 with an interstitial deletion of the long arm, and a complex rearrangement involving chromosomes 11 and 20 with loss of the short arm of chromosome 11. Balance of genes on chromosomes 1 and 11 is important for the expression of tumorigenicity as both chromosomes contain suppressor genes for malignancy. The HCX-16-2HR line also

exhibits abnormalities of chromosome 21. The short arm of chromosome 21 has an enlarged satellite region exhibiting heavy silver nitrate staining indicative of an increased dosage and activity of ribosomal genes and the long arm has a rather large terminal homogeneously staining region (HSR).

Gene amplification whose presence is manifested by two chromosomal abnormalities and double minutes (DM) can involve specific proto-oncogenes in a particular form of cancer. HSR can persist at the initial site of formation or can be found translocated to a new chromosomal location. Gene amplification is considered a phenomenon related to tumor progression. However, the evidence provided with the HCX-16-2HR cervical cell line, as well as with several human keratinocytes lines analyzed, also implicates the induction of HSR and DM in the initial stages of transformation. HPV-16 integration was examined in the HCX-16-2HR line and viral sequences were detected at aberrant chromosome locations on both the short and the long arms of chromosome 21. The integration site on the long arm of chromosome 21 coincides with the location of HSR and is near the ets-2 proto-oncogene, thus providing a new example of HPV integration near a proto-oncogene. It appears that HPV integration causes the formation of HSR and the resulting gene amplification. Thus, further isolation and cloning of the flanking sequences of the HPV-16 integrated amplified region may provide new insight to the mechanism of DNA amplification and its role in the acquisition of cell immortality.

Integration of the HPV genome into the cellular DNA can also have repercussions on the initial stages of cell transformation leading to genomic instability and uncontrolled cell multiplication. The persistence of the integrated HPV and continuous transcription of viral genes is required for the perpetuation of the transformed phenotype as well as for tumor progression. The results obtained with HeLa cells derived from a cervical carcinoma are consistent with this concept. At the integration site of HPV-18 on chromosome 8 near the myc proto-oncogene, amplification of viral sequence was observed in the absence of myc gene rearrangement or amplification. However, this region contains sequences which have not been previously identified and whose role in the development of this malignancy remains to be studied.

Keratins are known markers of epithelia and, thus, are important to HPV containing cells. Two type II keratin genes were localized at the same region on chromosome 12. The gene for keratin 1, a specific marker for terminal differentiation in mammalian epithelia, is also located at the same site on chromosome 12. Keratins 13, 14 and 16, all type I genes, are located at the same site on chromosome 17. Therefore, chromosomal localization demonstrates that large clusters of keratin genes occur at defined loci on these two chromosomes. There are still at least 14 other epithelial keratin genes as well as several hair-specific keratin genes to map, so it cannot yet be ruled out that keratin genes are localized on chromosomes other than 12 and 17.

The progress made in understanding the role of chromosomal alterations in cancer has been possible because of the rapid advancement in molecular biology which resulted in the isolation and localization of genes involved in neoplastic development. By in situ hybridization two proto-oncogenes, pkc, a raf-related sequence, and erbB-2 a cellular analog of v-erbB, were localized on chromosomes X and 7, respectively. The location of pkc on the short arm of chromosome X (p11.2-11.4) corresponds to the breakpoint of the specific

translocation, t(X;18) in soft tissue sarcomas. The locus of the erbB-2 proto-oncogene on chromosome 17(q12-21.32) is within the same band of the breakpoint in reciprocal translocation t(15;17) specific for acute promyelocytic leukemia. In addition to these proto-oncogenes, a receptor-like gene for a platelet-derived growth factor designated T11 and two type II keratin genes were mapped on chromosomes 4(q11-12) and 12(q11-12), respectively.

The sensitivity of several HPV-16 DNA-immortalized HCX lines of epithelial cervical cells to natural lymphocytotoxicity and modulation of the sensitivity by leukoregulin have been compared in relation to the time of establishment of the cells in culture and their transfection with HPV-16 DNA. Early passage cells were evaluated after culturing for 11 to 18 weeks, while the later passage cells were examined after 30 weeks in culture. Both early and late passage HPV-16-immortalized cervical epithelial cells are resistant to NK lymphocytotoxicity. Leukoregulin caused a slight increase ($P < 0.05$) in susceptibility to NK lymphocytotoxicity in the early passage HCX-16-2S cervical cells. Leukoregulin up-regulation of sensitivity to NK lymphocytotoxicity is absent in the late passage NK-resistant cells. On the other hand, both early and late passage immortalized cervical epithelial cells are sensitive to LAK lymphocyte cytotoxicity. Leukoregulin, moreover, markedly up-regulates the sensitivity to LAK lymphocytotoxicity, especially with the late passage cervical epithelial cells at lymphocyte/target cell ratios of 10:1 and 25:1. The differential sensitivity of early and late passage HPV-16-immortalized cervical epithelial cells to leukoregulin up-regulation of NK lymphocytotoxicity is seen with each cell line, independent of previous chemical carcinogen co-treatment or serum selection of the immortalized cells. Although leukoregulin's slight augmentation of sensitivity to NK lymphocytotoxicity is seen only with the early passage cells, leukoregulin increases the sensitivity of both the early and late passage HPV-immortalized cervical epithelial cells to LAK lymphocytotoxicity. The increased susceptibility to LAK lymphocytotoxicity over that of NK is 1.5- to 4-fold higher for the late passage compared to the early passage HPV-immortalized cervical epithelial cells. This is observed with HPV-16 DNA immortalized cervical epithelial cells developed in the presence (HCX-16-2S and HCX-16-5S) or the absence (HCX-16-1/MNNG and HCX-16-5) of serum.

The response of the early passage HPV-16-immortalized cervical cells is very similar to that of cervical epithelial cells not transfected with HPV-16 DNA, while the response of the late passage HPV-16-transfected cervical cells is similar to that of HPV-16-positive QGU cervical carcinoma cells. QGU cervical carcinoma cells are very resistant to NK and leukoregulin failed to induce susceptibility, but like the HPV-16 DNA-immortalized cervical epithelial cells, the carcinoma cells are LAK-sensitive and leukoregulin markedly up-regulates their sensitivity. Thus, late passage HPV-16-immortalized cervical epithelial cells exhibit a greater leukoregulin up-regulation in sensitivity to LAK than to NK lymphocyte cytotoxicity, mimicking the pattern of leukoregulin-induced up-regulation observed with the HPV-16-positive cervical carcinoma cells.

Tay et al. have shown that few NK cells are present in cervical epithelia with HPV infections and in cervical intraepithelial neoplasia. The in vitro resistance of HPV-16-immortalized cervical epithelial cells, like HPV-16-positive cervical carcinoma cells, to NK lymphocyte killing and its inability

to be up-regulated by leukoregulin suggests that NK cells alone may be ineffective in the destruction of dysplastic and neoplastic cervical cells. NK lymphocytes, however, can function in an immunomodulatory capacity, not only modulating other effector cells, but by regulating their own activity. They do so by producing and releasing an assortment of cytokines, such as gamma IFN, IL-2, and leukoregulin which act to amplify and regulate the immune response. IFN and IL-2 can act directly on the NK cells to enhance their killer activity, while leukoregulin instead modulates natural lymphocytotoxicity activity by up-regulating the sensitivity of tumor target cells to NK and LAK lymphocyte killing.

Interferon, particularly alpha IFN, has been used in the clinical treatment of human genital papillomavirus infections. The response rate of these patients to IFN treatment is dependent on the HPV type with HPV-16/-18 infections showing a lower response rate. IFN can be applied topically but is usually administered systemically. In the latter situation, it is difficult to know whether IFN, which can both up-regulate effector lymphocytes and down-regulate target cell sensitivity, is acting directly on the abnormal target cell, on the immune effector cell, or both. Our investigations show that leukoregulin treatment of late passage HPV-immortalized cells has no effect on the sensitivity of the cells to NK cytotoxicity. On the other hand, both the HPV-immortalized cells and the cervical carcinoma cells are very sensitive to IL2-activated killer LAK cells and this sensitivity is increased by leukoregulin. Since it has been shown that LAK precursor cells do exist in vivo and that their activity can be enhanced by exogenous IL-2, it is possible that LAK effector cells may play a significant role in vivo in the immune response against cervical neoplasia. NK cells may function as immunomodulators, releasing substances that activate and amplify LAK effector cells, as evidenced by the in vitro reactivity of LAK cells to the cervical target cells and their corresponding up-regulation by leukoregulin.

In addition to leukoregulin-induced up-regulation of target cell sensitivity to NK and LAK lymphocytotoxicity, leukoregulin increases target cell plasma membrane permeability and tumor cell uptake of anti-cancer drugs. Together with the present observations this indicates that leukoregulin alone or in combination with anti-viral or anti-cancer drugs, has the potential to play a significant role in mediating the successful treatment of HPV-16 infections and prevention of cervical dysplasia and neoplasia. The development of HPV-immortalized human cervical epithelial cells to study cervical carcinogenesis provides a valuable model system to define the physiological and biotherapeutic role of leukoregulin and other cytokines in the prevention and control of cervical dysplasia and neoplasia.

Characterization of the molecular and biological pathways of leukoregulin action this past year focused upon further elucidation of the mechanism of action and potential usefulness of this direct-acting anti-cancer lymphokine. Examination of the physiologic role of leukoregulin demonstrates that its ability to upregulate target cell sensitivity to natural killer cell immunocytotoxicity extends to lymphokine-activated killer (LAK) lymphocyte cytotoxicity and to T-lymphocyte killing of allogeneic human tumor cells. Current results demonstrate that in addition to increased plasma membrane permeability leukoregulin modulates cell surface antigen expression concomitant with up-regulation of target cell sensitivity to natural lymphocyte cytotoxicity. Continued evaluation of leukoregulin's ability to

facilitate the target cell uptake of pharmacologically active molecules indicates that the leukoregulin membrane channel freely admits molecules as large as 20,000 daltons and that the increase is accompanied by augmented pharmacologic action. In a third area, the response of virus replication to leukoregulin virus expression has been studied in cells acutely infected with herpes simplex virus and in lymphoid cells chronically infected with human immunodeficiency virus.

The up-regulation of target cell sensitivity to natural cytotoxicity also occurs in T-lymphocyte-directed cytotoxicity as studied in collaboration with C. Slingsluff and H. L. Seigler in the Department of Surgery at Duke University. Up-regulation of tumor cell sensitivity by T cytotoxic lymphocytes, however, is directed against allogeneic not autologous cells in investigations with melanoma target cells and effector lymphocytes derived from the individual with the melanoma. Human cytotoxic T-lymphocyte (CTL) populations specifically cytotoxic for autologous human melanoma cells were generated in vitro and assayed in a 4 hour chromium release assay. In the absence of leukoregulin, allogeneic melanoma cells were not lysed (10% lysis at an effector:target cell ratio of 5:1). Pretreating allogeneic melanoma cells or NK-sensitive K562 leukemia cells with leukoregulin increased lysis of the allogeneic target cells 17 and 67% by CTL. Autologous normal peripheral blood lymphocytes were also subject to CTL lysis when pretreated with leukoregulin (50% at an E:T of 40:1 with leukoregulin; 2% without leukoregulin). Modulation of cytotoxicity was dose-related and was most effectively inhibited by autologous cold target cells, but persisted in the presence of monoclonal antibody (w6/32) to HLA class I antigens. Lysis of autologous melanoma was not increased by pretreatment with leukoregulin. Leukoregulin appears to mediate lysis of melanoma, NK targets, and normal peripheral blood lymphocytes by tumor-specific CTL. Modulation of CTL cytotoxicity is leukoregulin dose-related and non-MHC restricted. These observations suggest that CTLs have the capacity to react with more than one target cell receptor configuration or that the configuration of the target cell recognition site is rapidly regulated by leukoregulin.

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

The efficacy of leukoregulin up-regulation of drug uptake was further evaluated by measuring tumor cell replication in the presence of enhanced chemotherapeutic drug uptake and virus replication in acute and chronically infected cells. In K562 human leukemia cells treated for 30 minutes with leukoregulin, there is a synergistic increase in the proliferation inhibitory

action of doxorubicin. Collaborative studies with Charles Taylor, M.D., at the University of Arizona Cancer Center also demonstrate a synergistic increase in the cytotoxicity of vincristine in human 8226 multiple myeloma cells. More importantly, a two-log increase occurs in cell killing by vincristine of multiple drug-resistant variants of the 8226 myeloma cells treated with leukoregulin; e.g., leukoregulin increases the sensitivity of the multiple drug-resistant cells to a 100-fold smaller concentration of vincristine. This is an important finding as few agents have been found that enhance the sensitivity of cells exhibiting multiple drug resistance to pharmacologic control. In another area of potential biotherapeutic usefulness, we have demonstrated in a collaborative study with John Hooks, Ph.D. at the National Eye Institute that leukoregulin in the presence of acycloguanosine (Acyclovir) decreases infectious herpes simplex virus production by two logs from human WISH amnion cells acutely infected with herpes simplex virus. The reduction in virus titer occurs without any effect on the production of infectious virus by leukoregulin alone, demonstrating for the first time, in an acute virus infection, a cytokine-specific increase in drug targeting and pharmacologic anti-viral action.

Leukoregulin also is able to modulate the expression of human immunodeficiency virus in chronically infected human lymphoid cells. Collaborative studies with Guido Poli, M.D. in the laboratory of A. Fauci, M.D., at the National Institute of Allergy and Infectious Diseases indicate that leukoregulin exerts a differential effect on human immunodeficiency virus expression in ACH2 T-lymphocyte cells and U1 histiocytic lymphoma cells. Leukoregulin inhibits the induction of virus expression by other cytokines in ACH2 cells and by itself is able to induce virus expression in U1 cells as measured by viral reverse transcriptase. These observations are of interest in these models of latent virus infection as they permit investigation of the ability of cytokines to modulate the expression of latent human immunodeficiency virus for the purpose of therapeutic control and for the study of its role in carcinogenesis.

The intramural research program summarized in this report has been accomplished because of the multidisciplinary focus on target cell changes. The two Sections within the Laboratory, with independent programs, study cell surface and DNA alteration. These approaches are relevant to projects of both Sections and are integrated when appropriate. One example is the concerted effort by both Sections on cellular changes and their modulation induced by HPV and host factors at both chromosome and molecular levels. During the past year members of the Laboratory have served as advisory experts on editorial boards of major cancer journals. They have also functioned as organizers of international meetings, invited plenary speakers to international or national meetings, peer reviewers of research projects for national organizations, and collaborators in new research initiatives within and outside the United States. The overall goal of our studies and related activities is to gain an insight to the carcinogenesis process in order to prevent or intervene in the development of specific cancer in humans.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP04629-24 LB

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Regulation of Stages of Carcinogenesis Induced by Chemical or Physical Agents

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

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

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biology

SECTION

Somatic Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

5.8

PROFESSIONAL

3.8

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.)

A series of human epithelial cell models has been developed relevant to the study of the role of human papillomaviruses (HPV). HPVs associated with invasive cervical cancer can immortalize cervical cells. Although the HPV proteins that are also observed in cervical cancer are produced, no tumor has yet resulted after transplanting immortal cells into nude mice. Currently, the cellular response of target cells is being investigated with respect to alterations in differentiation and karyotype, cellular regulation of HPV gene expression, and changes caused by exogenous agents that convert HPV immortal cells to tumor-forming cells. A technique that permits in vitro to in vivo correlations by transplantation of intact monolayers onto the body wall of nude mice demonstrates that HPV-immortalized cells differentiate aberrantly, mimicking cervical intraepithelial neoplasia. However, no invasion occurred. In vitro growth characteristics were consistent; cells stratified poorly and a subpopulation was resistant to terminal differentiation. Control of HPV mRNA expression in immortalized lines is also being investigated. One cervical line with a single form of HPV-16 produces truncated early mRNA species that contain no E5 sequences as the result of using alternative termination sites. The lack of L1 and L2 sequences in these cells suggests that these genes have regulatory elements controlling proper mRNA termination. The addition of activated v-Ha-ras to immortalized HPV-16 cervical cells resulted in the formation of squamous cell carcinomas with invasive elements in nude mice. Some areas were similar to cervical intraepithelial neoplasia often observed in vitro. Chromosome alterations in these cells included an increase of 1q, often seen in carcinomas. The ability to obtain tumors indicates that HPVs play a critical role in cervical cancer.

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

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

Objectives:

This study investigates factors and mechanisms required for neoplastic transformation of human and other cells relevant to the etiology and prevention of cancer. The specific objectives are: (1) to define the role of chemical, physical, and biological agents pertinent to the process of carcinogenesis; (2) to study aberrant differentiation of immortalized and tumorigenic cells both in vitro and in vivo; (3) to characterize the chromosomal alterations associated with carcinogenesis; (4) to evaluate the relationships between DNA metabolism and carcinogenesis.

Methods Employed:

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

Major Findings:

A number of pure chemical carcinogens as well as tobacco smoke condensate have been added to HPV-16- or HPV-18-immortalized epithelial cells. Although toxicity, morphologic changes and chromosome alteration have been noted, no tumors have been observed when the cells were injected into animal.

The only conversion of HPV-immortalized cells to the malignant state, thus far, has been obtained by transfecting Ha-ras. Recently, two laboratories

have demonstrated that some invasive cervical carcinomas have an activated Harvey-ras gene. Because the activated ras gene was found in conjunction with both HPV-16 and HPV-18 DNA, the possibility exists that activation of ras is sufficient to convert the papilloma-containing exocervical cells into a tumor producing line. Moreover, in many cervical tumors ras activation is also accompanied with amplified and/or overexpressed c-myc gene. Therefore, a cervical cell line (HCX) immortalized by transfection with recombinant HPV-16 DNA was cotransfected with either the v-Ha-ras or v-myc gene to determine if either gene was cocarcinogenic with HPV-16. Only cells subjected to the v-Ha-ras gene rapidly progressed to a tumorigenic state. Thus, this is the first in vitro model commencing with normal cervical cells for studying human cervical squamous cell carcinoma. These tumors were consistent with the derivation of cells (squamous-columnar junction) that represent the target for malignant transformation. The tumor, upon culturing, had a morphology similar to that of cells prior to injection into animals. The tumor karyotype was consistent with their human origin and similar to the original HPV-ras line. The HPV-ras line's most significant alteration compared to the immortal non-tumorigenic line was an increase in the number of long arms of chromosome one. Such an alteration is often observed in cervical cancer preceding or coincidental with stromal invasion. The experimental induced tumors invaded both stromal and muscular tissue.

Because these HCX-16-ras cells consistently produce tumors, the cells were analyzed for alteration in HPV-16 structure or expression. Southern blot analyses of high molecular weight DNA from HCX-16-2 and HCX-16-2HR confirmed that the specific HPV-16 BamHI fragments including at least one HPV-16 intact copy were identical. Thus, the ras gene was not responsible for any rearrangements of HPV-16 sequences. Because E6 and E7 genes are usually expressed in cervical cancer it was important to determine whether their expression had been altered by introduction of the ras gene was determined. E7 may have a possible function in progression to or in the maintenance of the malignant state. The expression of E6 or E7 protein was unaffected by the expression of v-Ha-ras. Thus, the progression to neoplasia did not require an increase in the expression of these two genes.

The state of the cotransfected viral Ha-ras gene was also determined. Intact and non-intact viral Ha-ras BamHI fragments are found in the transformed line as well as the tumor-derived lines. The intact BamHI (6.2kbp) fragment demonstrates that the viral long terminal repeat is still linked to the ras sequence providing optimal conditions for v-Ha-ras expression. Just as the HPV-16 genome was unaltered, c-Ha-ras locus also appears unaltered. The presence of two bands in all the HCX-16-2HR lines examined at approximately 6.6kbp is consistent with a polymorphism of alleles of c-Ha-ras often linked to susceptibility to cancer. As expected, the ras protein (p21) compared to the parental line HCX-16-2 indicating that the transfected v-Ha-ras was expressed.

These in vitro results are consistent with the hypothesis that the development of cervical cancer requires, in addition to specific HPV types commonly found in severe dysplasia or carcinoma, diverse cofactors or cocarcinogens. This two-stage model utilizing relevant human cells demonstrates that HPVs play a

necessary role in cervical malignancy and provides a system for elucidating critical cellular changes associated with progression to malignancy.

In terms of state of differentiation cervical carcinomas differ from other carcinoma, such as skin carcinomas, in that morphologically they are less differentiated. A technique was adapted that permitted in vitro - in vivo correlations by transplanting cell monolayers, removed intact from culture dishes by digestion with dispase, beneath a dorsal skin-muscle flap of nude mice. Normal human foreskin keratinocytes (HKC) and HCX formed a well-differentiated stratified squamous epithelium. The majority of HPV-immortalized HKC cell lines at early passage (<100 population doublings) formed a thin keratinizing squamous epithelium in which dysplastic changes were not apparent. However, several HPV-16 and HPV-18-immortalized cell lines differentiated aberrantly demonstrating changes that mimicked cervical intraepithelial neoplasia grades 1 to 3. No invasion of the adjacent connective tissue was observed. Changes in squamous differentiation became apparent or more severe with increasing passage of these cell lines. Similar results occurred in vitro as HPV-immortalized cell lines stratified poorly and contained a subpopulation resistant to differentiation promoted by serum or loss of anchorage dependence. HPV-immortalized cells exhibited altered expression of several RNAs (involucrin, keratin 1, laminin) which is important in normal squamous differentiation. Thus, HCX and HKC that contain integrated and transcriptionally active HPV DNAs exhibit progressive alterations in differentiation both in vitro and in vivo. Using this model it will be possible to identify the underlying causes associated with pathologic alterations characteristic of cervical intraepithelial neoplasia and to determine the differentiation markers signaling loss of differentiation characteristic of cervical squamous cell carcinoma.

Control of HPV-16 expression might be expected to influence the phenotype of transformed epithelial. Therefore, factors that influence transcription of HPV genes are being examined. Particularly important are the E6/E7 genes because they are important for immortalization and cell proliferation. The predominant HPV-16 mRNA species in 6 of 7 human cervical cell lines immortalized by recombinant HPV-16 DNA was 1.6 kb, spanned bases 97-226, 409-880, and 3357-4234, and contained E6*, E7, E1/E4, and E5. But in HCX-16-5 it was 1.2 kb; a relatively low level of 1.6 kb was also detected. Moreover, other prominent species in HCX-16-5 were similarly shorter. HCX-16-5 was also the only line that contained a single HPV-16 DNA integration site and no intact 7.9 kbp BamHI fragment. The integrated HPV-16 fragment spanned the 3'-end of L1, the long control region, the entire early region, and the 5'-end of L2. Further analysis of the mRNA with sub-genomic HPV-16 probes suggested that 1.2 kb species in HCX-16-5 is a mixture of two truncated forms of the 1.6 kb species; either the E6 or E5 region was missing. A cDNA library of line HCX-16-5 was constructed to examine more precisely the structure of its viral mRNA. Twenty-one HPV-16-specific clones were isolated from approximately 10⁵ cDNAs. Analysis with subgenomic probes indicated that 2 were missing E6 and truncated in the E5 region; of the remaining 19, 13 were truncated in E5, 3 were missing E6, and 3 contained both. Sequence analysis of the 5'-end of several clones with E6 show that they begin between bases 110-119, just downstream of p97, the E6 promoter. Two clones that are missing E6 begin in E7, 122 and 162 bases upstream of the E1 splice donor site at 880. This

suggests that HPV-16 contains a promoter in E7. Furthermore, the poly(A)-tail in one clone with E5 begins at 4234 just downstream from the poly(A)-signal at 4314, the standard termination region of the early messages. Poly(A)-tails of four clones truncated for E5 begin at 3759, 3761, 3776, and 3820, 30-91 upstream from the end of E2. These data suggest that the lack of complete L1 or L2 sequences changes the predominant termination site of the early messages.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP04673-18 LB

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

The Immunobiology of Carcinogenesis

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

PI:	C. H. Evans	Chief, Tumor Biology Section	LB	NCI
Others:	P. Furbert-Harris	IRTA Fellow	LB	NCI
	E.C. Farley	Visiting Fellow	LB	NCI
	F. D'Alessandro	Visiting Fellow	LB	NCI
	P. D. Baker	Microbiologist	LB	NCI
	A. C. Wilson	Chemist	LB	NCI
	J. L. Hooks	Chief, Immunology Section	LI	NEI
	G. Poli	Visiting Fellow	OD	NIAID

COOPERATING UNITS (if any)

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 Department of Medicine, University of Arizona (C. Taylor)
 Department of Chemistry, Weizmann Inst. Sci. (Z. Fishelson)

LAB/BRANCH

Laboratory of Biology

SECTION

Tumor Biology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5.5

PROFESSIONAL:

4.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Lymphokines, interleukins, and other immunological hormones, i.e., the secretory bioregulatory macromolecules of lymphocytes, macrophages, and other leukocytes, are being studied to define their effective anticarcinogenic and tumor cell growth inhibitory activities. Leukoregulin, a lymphokine isolated during the course of this project, can prevent carcinogenesis and inhibit tumor cell growth. Anticarcinogenic action is direct, irreversible and occurs without cytotoxicity. Inhibition of tumor cell growth is primarily reversible but can become irreversible due to increased susceptibility of preneoplastic and neoplastic cells to cytolytic destruction by natural killer cells resulting from leukoregulin target cell interaction. Leukoregulin at very high concentrations is also directly cytolytic for tumor cells. The direct-acting anticarcinogenic activity of leukoregulin is more potent than the tumor cell inhibitory activity; but by also being able to increase target cell sensitivity to the cytoreductive action of naturally cytotoxic lymphocytes, leukoregulin can be an effective homeostatic mechanism for control of carcinogenesis at its later stages of development. Leukoregulin-induced changes in plasma membrane permeability are partially dependent upon extracellular ionic calcium and are accompanied by increased calcium flux, the rapid opening and closing of plasma membrane single ion channels and translocation of protein kinase C from the cytosol to the plasma membrane which may be important events in the molecular pathway, resulting in inhibition of tumor and other abnormal cell proliferation by this immunologic hormone. The recent identification of the ability of leukoregulin to increase the uptake of pharmacologically active molecules in leukoregulin-sensitive cells provides a new area of potential therapeutic usefulness for this lymphokine and a new approach to drug delivery - biotherapeutic targeted drug delivery and pharmacologic up-regulation by target cell-specific cytokines.

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

C. H. Evans	Chief, Tumor Biology Section	LB	NCI
F. D'Alessandro	Visiting Fellow	LB	NCI
E. Farley	Visiting Fellow	LB	NCI
P. Furbert-Harris	IRTA Fellow	LB	NCI
P. D. Baker	Microbiologist	LB	NCI
A. C. Wilson	Chemist	LB	NCI
J. L. Hooks	Chief, Immunology Section	LI	NEI
G. Poli	Visiting Fellow	OD	NIAID

Objectives:

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

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

Methods Employed:

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

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

Major Findings:

Characterization of the molecular and biological pathways of leukoregulin action this past year focused upon further elucidation of the mechanism of action and potential usefulness of this direct-acting anti-cancer lymphokine. Examination of the physiologic role of leukoregulin demonstrates that its ability to up-regulate target cell sensitivity to natural killer cell immunocytotoxicity extends to lymphokine-activated killer (LAK) lymphocyte cytotoxicity and to T lymphocyte killing of allogeneic human tumor cells. Current results demonstrate that in addition to increased plasma membrane permeability leukoregulin modulates cell surface antigen expression concomitant with up-regulation of target cell sensitivity to natural lymphocyte cytotoxicity. Continued evaluation of leukoregulin's ability to facilitate the target cell uptake of pharmacologically active molecules indicates that the leukoregulin membrane channel freely admits molecules as large as 20,000 daltons and that the increase is accompanied by augmented pharmacologic action. In a third area, the response of virus replication to leukoregulin, virus expression has been studied in cells acutely infected with herpes simplex virus and in lymphoid cells chronically infected with human immunodeficiency virus.

Leukoregulin interaction with tumor cells up-regulates their sensitivity to LAK cytotoxicity similar to the previously established ability of the lymphokine to increase the sensitivity of carcinoma, leukemia, and sarcoma cells to natural killer lymphocyte cytotoxicity. One to two units of leukoregulin/ml increases target cell sensitivity two- to tenfold at effector to target cell ratios as small as 1:1. Leukoregulin up-regulation of LAK cytotoxicity, as observed in up-regulation of NK cytotoxicity, is opposite the action of gamma interferon which down-regulates target cell sensitivity to lymphocytotoxicity. The up-regulation of target cell sensitivity by leukoregulin is unique; it does not occur in target cells exposed to tumor necrosis factor, interferons, or colony stimulating factors either alone or in combination.

The up-regulation of target cell sensitivity to natural cytotoxicity also occurs in T lymphocyte-directed cytotoxicity as studied in collaboration with C. Slingluff and H. L. Seigler in the Department of Surgery at Duke University. Up-regulation of tumor cell sensitivity by T cytotoxic lymphocytes, however, is directed against allogeneic not autologous cells in investigations with melanoma target cells and effector lymphocytes derived from the individual with the melanoma. Human cytotoxic T-lymphocyte (CTL) populations specifically cytotoxic for autologous human melanoma cells were generated in vitro and assayed in a 4 hour chromium release assay. In the absence of leukoregulin, allogeneic melanoma cells were lysed to a minimal extent (10% lysis at an effector:target cell ratio of 5:1). Pretreating allogeneic melanoma cells or NK-sensitive K562 leukemia cells with leukoregulin increased lysis of the allogeneic target cells 17 and 67% by CTL. Autologous, normal peripheral blood lymphocytes were also subject to CTL lysis

when pretreated with leukoregulin (50% at an E:T of 40:1 with leukoregulin; 2% without leukoregulin). Modulation of cytotoxicity was dose-related and was most effectively inhibited by autologous cold target cells, but persisted in the presence of monoclonal antibody (w6/32) to HLA class I antigens. Lysis of autologous melanoma was not increased by pretreatment with leukoregulin. Leukoregulin appears to mediate lysis of melanoma, NK targets, and normal peripheral blood lymphocytes by tumor-specific CTL. Modulation of CTL cytotoxicity is leukoregulin dose-related and non-MHC restricted. These observations suggest that CTLs have the capacity to react with more than one target cell receptor configuration or that the configuration of the target cell recognition site is rapidly regulated by leukoregulin.

The up-regulation of target cell sensitivity to natural lymphocyte cytotoxicity occurs concurrently with an increase in target cell plasma membrane permeability. Leukoregulin permeabilization of K562 cells, as measured flow cytometrically by the loss of intracellular fluorescein, is partially dependent on extracellular Ca^{++} . The increase in membrane permeability resulting from leukoregulin concentrations producing a 20-50% increase in permeability is reduced in the absence of extracellular Ca^{++} . At higher leukoregulin concentrations the membrane permeabilizing action of leukoregulin is independent of the presence of extracellular Ca^{++} . The partial dependence of lower concentrations of leukoregulin upon extracellular Ca^{++} is further demonstrated when K562 cells are incubated for 2 hours in Ca^{++} -free medium with a concentration of leukoregulin inducing a change in the permeability of one-third of the K562 cells. At that time if 1 mM extracellular Ca^{++} is added, 45 minutes later there is a 22% net increase in the loss of fluorescein from the cells compared to cells incubated for the same period without added extracellular calcium. If, however, the cells are washed before the addition of fresh medium containing ionic Ca^{++} but no leukoregulin there is no additional increase in membrane permeability.

In contrast to leukoregulin, calcium ionophore A23187 induces an increase in the plasma membrane permeability of K562 cells which is totally dependent on the presence of extracellular Ca^{++} . Calcium ionophore A23187 plasma membrane permeabilization, however, develops fully following the addition of extracellular 1 mM Ca^{++} regardless of whether the cells have been washed after exposure to calcium ionophore A23187 and prior to the addition of extracellular Ca^{++} . Phytohemagglutinin also induces a dose-dependent change in the plasma membrane permeability of K562 cells but in contrast to both leukoregulin and calcium ionophore A23187 the increase in plasma membrane permeability by phytohemagglutinin is independent of the presence of extracellular Ca^{++} . Comparison of leukoregulin, calcium ionophore A23187, and phytohemagglutinin at concentrations that after a 2 hour cell exposure produce approximately a half maximal change in the permeability of the K562 cell plasma membrane in the presence of extracellular Ca^{++} shows a similar divergence in their patterns of Ca^{++} dependence. In the absence of extracellular Ca^{++} , membrane permeability changes induced by leukoregulin are decreased but not eliminated, are eliminated for calcium ionophore A23187 and remain unchanged for phytohemagglutinin. Each agent at a concentration producing half maximal change in the permeability of K562 cell plasma membranes also inhibits the proliferation of K562 cells by 50-90% at 3 days. In the absence of extracellular Ca^{++} the proliferation inhibitory action of

leukoregulin is eliminated, the inhibition of cell proliferation by calcium ionophore A23187 is decreased and the proliferation inhibitory action of phytohemagglutinin on K562 cells is unchanged.

Cell populations exhibiting alterations in membrane permeability and cell proliferation contain a mixture of affected and non-affected cells at leukoregulin, calcium ionophore A23187 or phytohemagglutinin concentrations producing less than a maximal change. The responder and nonresponder cells are not generally easy to dissect out from a heterogenous population for further study. Through the use of flow cytometric cell sorting, however, individual populations can be isolated and the cell proliferation capacity of the affected and non-affected cells examined after exposure to a particular membrane permeablizing agent. The proliferation ability of K562 cells with leukoregulin-induced increased membrane permeability isolated by cell sorting, is inhibited for 1-3 days after which it returns to the same rate as exhibited by non-treated or non-affected cells. This ability of leukoregulin-treated cells to recover and resume proliferation is in contrast to the irreversible loss of proliferative capacity by calcium ionophore A23187 or phytohemagglutinin-treated K562 cells exhibiting increased plasma membrane permeability. Even calcium ionophore A23187 and phytohemagglutinin-treated K562 cells having no demonstrable increase in plasma membrane permeability exhibit some depression in their proliferation rate.

These results reveal that the cell permeablizing action of leukoregulin is partially dependent on the presence of extracellular Ca^{++} and that the increase in membrane permeability is followed by a reversible inhibition of cell proliferation. This contrasts with the calcium ionophore A23187 which produces a dose-dependent change in the plasma membrane permeability of K562 cells that is totally dependent on the presence of extracellular Ca^{++} and which results in an irreversible inhibition of cell proliferation. Leukoregulin modulation of membrane permeability and cell proliferation also differs from that induced by phytohemagglutinin transmembrane signaling as the membrane permeablizing action of phytohemagglutinin is independent of extracellular calcium and, like calcium ionophore A23187, leads to irreversible inhibition of K562 cell proliferation.

Calcium ionophores equilibrate Ca^{++} across biological membranes and also across subcellular organelles, e.g., mitochondria and microsomes. The membrane permeability changes induced by calcium ionophore A23187 that are measured by the efflux of fluorescein from labeled cells are due to the actual transport of Ca^{++} across the membrane and not just to the presence of the ionophore. This is demonstrated by experiments incorporating a wash step after exposure of K562 cells to calcium ionophore A23187 in the absence of extracellular Ca^{++} because membrane permeability changes are only observed following the subsequent addition of extracellular Ca^{++} . Therefore, calcium ionophore A23187 initially equilibrates across the plasma cell membrane and, when extracellular Ca^{++} is present, is followed by an influx of Ca^{++} into the cell. The resulting increase in intracellular Ca^{++} leads, in turn, to destabilization of the plasma membrane and an increase in membrane permeability. In contrast, when leukoregulin is washed from the cell prior to the addition of extracellular Ca^{++} , membrane permeability is not increased,

demonstrating that it is the extracellular nonbound leukoregulin and/or its interaction with the target cell that is calcium-dependent.

The membrane destabilizing action of leukoregulin at concentrations of the lymphokine producing a partial increase in membrane permeability of the K562 cell population is more dependent on extracellular Ca^{++} than the increase in membrane permeability at higher concentrations of leukoregulin. The explanation for the shift from calcium dependence to independence may be that at lower concentrations of leukoregulin, membrane destabilization requires extracellular Ca^{++} because the previously described leukoregulin-induced increase in intracellular Ca^{++} levels is not high enough to directly affect membrane destabilization without supplementation from an extracellular Ca^{++} source. At high leukoregulin concentrations the increase in intracellular Ca^{++} through mobilization from internal stores may be sufficient so that a contribution from extracellular Ca^{++} is not required. The kinetics of permeability change, the molecular size, hydrophilicity and partial Ca^{++} dependence of leukoregulin are, moreover, inconsistent with leukoregulin itself being a calcium ionophore. The membrane destabilizing action of the mitogen phytohemagglutinin, unlike both leukoregulin and calcium ionophore A23187, is totally independent of the presence of extracellular Ca^{++} showing again the unique nature of leukoregulin's mode of action.

These observations also demonstrate that exposure of K562 leukemia cells to any of the three modulators of membrane stability for two hours is sufficient to affect cell proliferation. The inhibition of cell proliferation, moreover, follows a pattern of sensitivity to extracellular Ca^{++} that is similar to the pattern of the changes in membrane permeability induced by these modulators of Ca^{++} metabolism. Two-hour exposure to leukoregulin in Ca^{++} -free medium abrogates leukoregulin's inhibition of cell proliferation, whereas inhibition by calcium ionophore A23187 is greatly reduced and the inhibition by phytohemagglutinin is unchanged. This suggests that the membrane permeability changes are directly related to the inhibition of cell proliferation induced by these modulators of intracellular calcium flux and membrane stability. The cell proliferation inhibition action of calcium ionophore A23187 is not eliminated in Ca^{++} -free medium in contrast to the abrogation of increased membrane permeability by calcium ionophore A23187 in the absence of extracellular Ca^{++} . This is probably because calcium ionophore A23187 is not completely removed from the cells by washing the cells in calcium-free medium because the ionophore has been inserted into the plasma membrane. When the cells are resuspended in normal Ca^{++} -containing medium for the cell proliferation assay, the membrane-bound ionophore then is able to transport the added extracellular Ca^{++} into the cell, increasing intracellular Ca^{++} and inhibiting the growth of the K562 cells.

Leukoregulin is a unique lymphokine that rapidly induces nonlethal membrane permeabilization in K562 cells. This form of membrane destabilization is partially dependent on extracellular Ca^{++} unlike the totally calcium-dependent target cell inhibitory action of many other products of cytotoxic T cells, e.g., cytolysin and perforin and lymphokines, e.g., lymphotoxin and interleukin-2. The membrane permeability changes induced by leukoregulin correlate closely with subsequent changes in cell proliferation. It is likely, however, that many steps are involved in the mechanism of

Leukoregulin-induced inhibition of cellular proliferation since calcium ionophore A23187 and phytohemagglutinin have similar dose-dependent membrane permeability changes but exert an irreversible action on cell proliferation.

The leukoregulin-induced increase in target cell membrane permeability is associated with but follows activation of plasma membrane ion channels. Cell conductance measured by the patch clamp technique demonstrates that after several minutes leukoregulin induces a transient expression of plasma membrane cation-selective ion channels in K562 cells. The opening of the cation-selective ion channels commences concurrently with the increased intracellular calcium ion flux induced by the leukoregulin but precedes the increase in plasma membrane permeability induced by the lymphokine. The molecular interrelationship between cation-selective ion channel activity and these changes in cellular biochemistry, particularly with regard to the inhibition of DNA synthesis which does not occur until 6 hours after leukoregulin interaction with the cell, remains unclear. The time course of leukoregulin-induced cation-selective ion channel activity, in particular its transient expression, makes it seem unlikely that the activated cation-selective ion channels per se are the ultimate cause of the increase in cell permeabilization to large molecular weight compounds such as fluorescein or propidium iodide. Given the complex nature of control of cell division and the observation that leukoregulin stops proliferation of the target cell in each position in the cell cycle, it is likely that membrane cation-selective ion channel induction is but a first step in a sequence of extracellular-intracellular events leading to plasma membrane destabilization and cytostasis.

The induction of membrane cation selective ion channel activity in target cells by leukoregulin differs in several important electrophysiological respects from that of other cytotoxic factors secreted by leukocytes that have been shown to increase membrane conductances: a) the channels produced are nearly an order of magnitude smaller in unit conductance, 50 pS compared to 400 pS; b) the perforin/cytolysin channel opens irreversibly, whereas the leukoregulin-induced channel opens and closes; c) gating of the leukoregulin-induced channel is voltage-dependent; d) the leukoregulin-induced channel does conduct K^+ . Virtually all leukocytes studied to date express some type of voltage-dependent conductance mechanism. Despite intensive investigation of these conductance mechanisms their roles in the effector and/or the target cells are, as yet, obscure. The phytohemagglutinin-activated calcium channel in human T-lymphocytes and in polymorphonuclear leukocytes are the only effector ligand-gated ion channels recognized to date. Leukoregulin is the first cytokine demonstrated to induce a transient membrane conductance in the target cell and in this respect it acts in a manner analogous to a neurohormone.

The increase in plasma membrane permeability in leukoregulin-treated target cells is accompanied by increased uptake of pharmacologically active macromolecules. The uptake of metabolic inhibitory antibiotics as large as 4000 daltons is augmented as much as 30-fold within 60 minutes after leukoregulin target cell interaction. Various sized preparations of fluorescein-labelled dextran demonstrate that molecules as large as 20,000

daltons are taken up more rapidly by leukoregulin-treated cells. Leukoregulin also facilitates tumor cell uptake of insulin and rapidly modulates growth factor receptors as indicated flow cytometrically by down-regulation of transferrin uptake in K562 leukemia cells. Modulation of target cell drug uptake and growth factor binding in combination with lymphocyte cytotoxicity provides new and potentially potent methods for controlling target cell function and in particular for more specific and potent tumor cell destruction.

The efficacy of leukoregulin up-regulation of drug uptake was further evaluated by measuring tumor cell replication in the presence of enhanced chemotherapeutic drug uptake and virus replication in acute and chronically infected cells. In K562 human leukemia cells treated for 30 minutes with leukoregulin there is a synergistic increase in the proliferation inhibitory action of doxorubicin. Collaborative studies with Charles Taylor, M.D., at the University of Arizona Cancer Center also demonstrate a synergistic increase in the cytotoxicity of vincristine in human 8226 multiple myeloma cells. More importantly, a two log increase also occurs in cell killing by vincristine of multiple drug resistant variants of the 8226 myeloma cells treated with leukoregulin; e.g., leukoregulin increases the sensitivity of the multiple drug resistant cells to a 100-fold smaller concentration of vincristine. This is an important finding as few agents have been found that enhance the sensitivity of cells exhibiting multiple drug resistance to pharmacologic control. In another area of potential biotherapeutic usefulness, we have demonstrated, in collaboration with John Hooks, Ph.D. at the National Eye Institute, that leukoregulin in the presence of acycloguanosine (Acyclovir) decreases infectious herpes simplex virus production by two logs from human WISH amnion cells acutely infected with herpes simplex virus. The reduction in virus titer occurs without any effect on the production of infectious virus by leukoregulin alone, demonstrating for the first time in an acute virus infection a cytokine-specific increase in drug targeting and anti-viral pharmacologic action.

Leukoregulin also is able to modulate the expression of human immunodeficiency virus in chronically infected human lymphoid cells. In collaboration with Guido Poli, M.D. in the Laboratory of A. Fauci, M.D., at the National Institute of Allergy and Infectious Diseases, leukoregulin has been found to exert a differential effect on human immunodeficiency virus expression in ACH2 T-lymphocyte cells and U1 histiocytic lymphoma cells. Leukoregulin inhibits the induction of virus expression by other cytokines in ACH2 cells and by itself is able to induce virus expression as measured in U1 cells by viral reverse transcriptase. These observations are of interest in these models of latent virus infection as they permit investigation of the ability of cytokines to modulate the expression of latent human immunodeficiency virus for the purpose of therapeutic control and for the study of its role in carcinogenesis.

Publications:

Barnett SC, Evans CH. Leukoregulin induced translocation of protein kinase C activity in K562 cells. Clin Exp Immunol 1988;73:505-9.

Evans CH. Leukoregulin. In Mitchell M, ed. Immunity to Cancer II. New York: A.R. Liss, 1988;259-70.

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Evans CH, Barnett SC, Gelleri BA, Furbert-Harris P, Sheehy PA, Barker JA, Baker PA, Wilson AC, Farley EK, D'Alessandro F. Biological and molecular characteristics of leukoregulin action. In: Groopman J, Evans CH, Golde D, eds. Mechanisms of action and therapeutic applications of biologicals in cancer and immune deficiency disorders. New York: A.R. Liss (In Press).

Evans CH, DiPaolo JA. Chemico-biological interactions in the immunologic modulation of initiated and promoted transformation. In: Elmore EL, Langenbach RJ, Barrett JC, eds. Tumor promoters: biological approaches for mechanistic studies and assays. New York: Raven Press, 1988;179-86.

Evans CH, Wilson AC, Gelleri BA. Preparative isoelectric focusing in ampholine electrofocusing columns versus immobiline polyacrylamide gel for the purification of biologically active leukoregulin. Anal Biochem 1989;177:358-63.

Furbert-Harris P, Evans CH. Leukoregulin up-regulation of tumor cell sensitivity to natural killer and lymphokine-activated killer cell cytotoxicity. Cancer Immunol Immunother (In Press).

Furbert-Harris PM, Evans CH, Woodworth CD, DiPaolo JA. Loss of leukoregulin up-regulation of NK but not LAK lymphocytotoxicity in human papilloma virus 16 DNA-immortalized cervical epithelial cells. JNCI (In Press).

Sheehy PA, Barnett SC, Evans CH, Barker JL. Activation of ion channels in tumor cells by leukoregulin, a cytostatic lymphokine. JNCI 1988;80:868-71.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05499-03 LB

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Chromosome Alterations and Proto-Oncogene Transposition in Carcinogenesis

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

PI:	N. C. Popescu	Research Microbiologist	LB	NCI
Others:	J. A. DiPaolo	Chief	LB	NCI
	M. Krause	Visiting Scientist	LCMB	NCI
	T. Matsui	Visiting Fellow	LCMB	NCI
	P. Bowden	Visiting Associate	LB	NCI
	D. Zimonjic	Guest Researcher	LB	NCI

COOPERATING UNITS (if any)

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

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TOTAL MAN-YEARS

1.0

PROFESSIONAL

0.9

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.)

Aneuploidy and structural cytogenetic alterations were identified in human papillomavirus (HPV) type 16 immortalized human exocervical cells. Chromosomes 11, 17, 19 and 21 were involved; these chromosomes are commonly affected in solid tumors, including cervical carcinomas. The formation of certain structural changes can be attributed to the viral integration into the cellular genome. HPV-16-immortalized exocervical cells became tumorigenic after transfection with v-Ha-ras oncogene. This transition was accompanied by the formation of an isochromosome derived from the long arm of chromosome 1. This alteration may lead to proto-oncogene imbalance and contributes to the expression of the malignant phenotype. On HeLa cells HPV-18 integration resulted in amplification of viral sequences and an increased expression of cellular genes at integration site. In addition, at the integration site on chromosome 8 near the *myc* gene, a new cellular sequence was detected. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a carcinogenic nicotine derivative, causes DNA damage in human and rodent cells as demonstrated by a sensitive cytological assay for sister chromatid exchange formation. Two proto-oncogenes *pKs* and *erbB-2*, T-11, a receptor-like gene for platelet-derived growth factor, and two type II keratin genes were localized on human chromosomes. The *pKs* gene was localized on the short arm of chromosome X (p11.2-11.4) and related sequence on chromosome 7 close to the centromere (p12-q11.2). *ErbB-2* was localized within bands q12-21.32 on chromosome 17 close to the breakpoint of a specific chromosome translocation (15;17) in acute promyelocytic leukemia. The T-11 gene was mapped on chromosome 4 (q11-12), and two keratin genes were mapped on chromosome 12q-11-13. The localization of these genes will permit study of neoplasms with alterations at these sites.

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

N. C. Popescu	Research Microbiologist	LB	NCI
J. A. DiPaolo	Chief	LB	NCI
M. Kraus	Visiting Scientist	LCMB	NCI
T. Matsui	Visiting Fellow	LCMB	NCI
P. Bowden	Visiting Associate	LB	NCI
D. Zimonjic	Guest Researcher	LB	NCI

Objectives:

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

Methods Employed:

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

Major Findings:

The detection of primary alterations which may constitute pathologically relevant changes is possible in cultured epithelial cells. Normal human exocervical cells transfected with human papillomavirus (HPV)-16 DNA evolved into non-malignant immortal lines. A line examined in detail was aneuploid and had several types of structural alterations, translocations, deletions, as well as homogeneously staining regions (HSR). Some alterations appear to be caused by viral integration as demonstrated by in situ localization of HPV-16 integration at aberrant chromosomes in an immortalized line HCX-16-2. Transfection with v-Ha-ras oncogene resulted in the acquisition of tumorigenicity. The tumorigenic cells HCX-16-2HR had a new abnormality, an isochromosome originating from the long arm of chromosome 1. Alteration of chromosome 1 is the most common change in cervical cancer. Possibly oncogene dosage of this chromosome may be a contributing factor to the maintenance of continuous growth and tumorigenicity.

HPV-18 integration site on chromosome 8 in HeLa cells was examined in detail and both viral and cellular gene alterations were identified. E6, E7 and E1

open reading frames are amplified fivefold, and the late viral DNA region, the viral long control region, and cellular flanking sequences are amplified 15-fold.

Further analysis of the HPV-18 integration site on chromosome 8 revealed that this region also contains sequences which have not been previously identified. This new sequence is tentatively called pal-1, for papillomavirus-associated locus-1. The biological significance and the transforming potential of the new sequences remain to be elucidated.

Women who smoke have a higher incidence of cervical cancer than non-smokers. Two nicotine derivatives 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), as well as N'-nitrosornicotine, have been implicated in tobacco-induced human cancers because they are found in smoke condensate from cigarettes and because they produce tumors in mice, rats and hamsters. These carcinogens will be used in cocarcinogenesis studies with epithelial cells. To assess their ability to interact with the cellular DNA the induction of sister chromatid exchange (SCE) was examined in cultured normal human lymphocytes (HL) and Chinese hamster V79 cells. In V79 cells NNK produced a dose-dependent increase in SCE only with metabolic activation. In HL NNK induced a small but statistically significant increase in SCE with or without metabolic activation. These data provide the first evidence that NNK and/or its metabolic derivatives are able to induce DNA damage leading to SCE formation both in hamster and human cells. The differences in response between the two cell types suggests the existence of a difference in susceptibility associated with NNK metabolism and its interaction with cellular DNA.

Chromosome rearrangement is a mechanism that can affect either the expression or the function of proto-oncogenes. Chromosomal localization of proto-oncogenes is essential for studying alterations caused by chromosomal rearrangements. A cellular analog of v-erbB, erbB-2 (neu, HER-2), was identified in the Laboratory of Cellular and Molecular Biology. The close structural similarity to the epidermal growth factor receptor defined the predicted gene product of erbB-2 as a putative growth factor receptor molecule for an as yet unidentified ligand. Alterations of the erbB-2 gene in tumors, particularly mammary tumors, frequently appear to involve overexpression in the presence or absence of gene amplification. The erbB-2 gene was localized on normal chromosomes at region 17q12-21. For an independent confirmation of this localization and for a more precise mapping, a cell line designated Sp and derived from a normal donor carrying a constitutional t(15;17) was used. The breakpoint of this translocation is at the same band with the breakpoint of the translocation 15;17 specific for acute promyelocytic leukemia. On normal and translocated chromosomes 17 the erbB-2 gene locus was assigned within band q12 and subband 21.32.

The pKs gene, a raf-related sequence, was isolated from human liver cDNA. The pKs gene is distinct from c-raf-1 and its pseudo-oncogene c-raf-2, and has a related homolog detectable by Southern blot hybridization. After *in situ* hybridization with a pKs cDNA probe, two sites of labeling were observed on the X chromosome and number 7. Grain distributions at these sites showed that the pKs gene is located at Xp11.2-11.4 and its related sequences at 17p12-7q11.21. The number of proto-oncogenes on X chromosome is increasing and new

specific cancer cell alterations involving this chromosome are being identified. Dbl, another proto-oncogene, was also localized on the X chromosome. A specific Xp;18q translocation corresponds to the pKs gene location, suggesting the involvement of this gene in soft tissue malignancy. Also, due to its location, the pKs-1 gene might be useful in detecting DNA polymorphism as well as in mapping, diagnosis or isolation of genes associated with X-linked syndromes, particularly immunodeficiency syndromes.

A genomic cDNA of a novel gene, whose predicted product has a structure similar to platelet-derived growth factor (PDGF) and colony-stimulating factor, was isolated in the Laboratory of Cellular and Molecular Biology. This gene, designated T-11, was localized by in situ hybridization on chromosome 4q 11-12. This localization places the type α PDGF receptor gene on the same region of the c-kit proto-oncogene, a related receptor-like gene. Other genes of this subfamily have been localized on the distal half of chromosome 5q. There is evidence for a common ancestral origin of human chromosomes 4 and 5. These related receptor genes cluster near the centromere on the long arm of 4 or at the distal half of the long arm of 5. Thus, if the progenitor(s) of these genes is confined to a single ancestral chromosome, the breakup of linkage might be explained by an inversion within the long arm.

Human genomic DNA containing two type II keratin genes, one coding for keratin 1 (K1, a 68-kD basic protein) and another closely linked type II gene 10-15 kb upstream (K?, -gene product unknown), was isolated on a single cosmid clone in our laboratory. EcoRI restriction fragments of the cosmid were subcloned into pGEM-3Z, and specific probes comprising the C-terminal coding and 3' noncoding regions of the two genes were constructed. The type II keratin genes were localized by in situ hybridization of the subcloned probes to normal human lymphocyte chromosomes. Both the gene for human keratin 1, a specific marker for terminal differentiation in mammalian epidermis, and another closely linked unknown type II keratin gene (K?, 10-15 kb upstream of K1) are on the long arm (q11-13) of human chromosome 12. Other genes, such as human homeobox gene C8 which is involved in cellular differentiation, are present in this region of chromosome 12 and a coordinate regulation of keratin genes and homeobox genes may be important during embryonic development.

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

PROJECT NUMBER

Z01CP05552-02 LB

PERIOD COVERED

October 1, 1988 to Sept 30, 1989

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

Lymphokine Modulation of Human Cervical Epithelial Cell Carcinogenesis

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

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

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biology

SECTION

Tumor Biology

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.0

PROFESSIONAL

1.0

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

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

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

Human papillomavirus (HPV)-immortalized cervical epithelial cells and HPV-positive cervical carcinomas are being evaluated as a model of lymphokine modulation of epithelial cell sensitivity to natural immunologic cytotoxicity. The sensitivity of human cervical epithelial cells, immortalized by transfection with HPV-16 DNA, to lysis by NK and LAK lymphocytes, was evaluated at progressive stages of transformation. Both early (10-20 weeks) and late (>30 weeks) passage HPV-16-immortalized cells were resistant to NK but sensitive to LAK lymphocyte cytotoxicity at lymphocyte to cervical cell ratios ranging from 1:1 to 50:1 in a 4 hr ⁵¹Cr release assay. Treatment of early passage HPV-16-DNA-immortalized cells with 2.5 units/ml of the NK lymphocytotoxicity sensitizing lymphokine, leukoregulin, for 1 hr, induced modest sensitivity to NK (P <.05) but markedly up-regulated LAK sensitivity two- to threefold. At the later passages, leukoregulin up-regulation of sensitivity to NK was lost but remained to LAK lymphocytotoxicity. Similarly, an HPV-16-positive human cervical carcinoma cell line, QGU, was also resistant to NK and sensitive to LAK lymphocytotoxicity; leukoregulin failed to confer sensitivity to the NK-resistant QGU tumor cells and increased their sensitivity to LAK lymphocytotoxicity 1.5 to 2-fold. Although the HPV-immortalized cervical cells containing integrated HPV-16 DNA are not tumorigenic, they mimic the response of established HPV-16-positive cervical carcinoma cells. HPV-16-immortalized cervical epithelial cells provide a useful model for the study of cytokine modulation of dysplastic and neoplastic cervical epithelial cell sensitivity to natural lymphocytotoxicity.

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

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

Objectives:

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

Human papillomaviruses (HPV) infect squamous epithelium and are associated with the development of benign and malignant epithelial tumors. HPV type 16 is a suspected etiologic agent in the pathogenesis of cervical intraepithelial neoplasia and malignancy. In most premalignant and benign cervical lesions, HPV DNA exists in an episomal form, while in cervical cancers it is found integrated into the cellular genome. The cell-mediated effector arm of the immune response might be expected to have a key role in host defense against HPV infection and the possible development of a neoplastic state. Support for this is the presence of T-cells and the absence of a significant number of B-cells in cervical metaplasia and in normal cervical epithelium. However, in HPV infections and cervical intraepithelial neoplasia there is a general depletion of intraepithelial T lymphocytes with T4⁺ helper cells being more depleted than T8⁺ suppressor cells.

Because specific T-cell reactivity is partially or completely absent in patients with HPV infections and cervical intraepithelial neoplasia, and because substantial evidence exists for the role of natural killer (NK) lymphocytes in both viral infections and in neoplasia, investigation of the presence and function of this form of natural lymphocytotoxicity in these virus-associated diseases is relevant. Toward this end, Tay et al. have evaluated the presence of NK lymphocytes in tissue specimens from patients with HPV infection and cervical intraepithelial neoplasia. NK lymphocytes, positive for both the leu 7 (HNK-1) and leu 11 (NK-15) surface antigens, were present in most HPV infections and in half of the cervical intraepithelial neoplasia specimens tested.

HPV-16-immortalized human cervical epithelial (HCX) cell lines have been developed in this laboratory as a model for studying the role of HPV in the development of cervical cancer. Important characteristics of this model are that HPV-16 DNA is integrated and transcriptionally active, and although the cells are not tumorigenic in nude mice, they acquire additional transformed properties with cultivation. In this respect, these cells resemble dysplastic

cervical epithelial cells. Leukoregulin is a cytokine which increases tumor cell membrane permeability and drug uptake, inhibits tumor cell replication and up-regulates the sensitivity of tumor cells to killing by NK and lymphokine-activated killer (LAK) lymphocytes. The present investigation examines the sensitivity of HPV-16-immortalized human cervical epithelial cell lines at progressive stages of transformation to NK and LAK lymphocytotoxicity and the modulation of their sensitivity to lymphocytotoxicity by leukoregulin.

Methods Employed:

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

Major Findings:

The sensitivity of several HPV-16 DNA-immortalized HCX lines of cervical cells to natural lymphocytotoxicity and modulation of the sensitivity by leukoregulin was compared in relation to the time of establishment of the cells in culture and their transfection with HPV-16 DNA. Early passage cells were evaluated after culturing for 11 to 18 weeks, while the later passage cells were examined after 30 weeks in culture. Both early and late passage HPV-16-immortalized cervical epithelial cells were resistant to NK lymphocytotoxicity. Leukoregulin caused a slight increase ($P < 0.05$) in susceptibility to NK lymphocytotoxicity in the early passage HCX-16-2S cervical cells. Leukoregulin up-regulation of sensitivity to NK lymphocytotoxicity was absent in the late passage NK-resistant cells. On the other hand, both early and late passage immortalized cervical epithelial cells were sensitive to LAK lymphocyte cytotoxicity. Leukoregulin, moreover, markedly up-regulated the sensitivity to LAK lymphocytotoxicity, more so with the late passage cervical epithelial cells at lymphocyte/target cell ratios of 10:1 and 25:1. The differential sensitivity of early and late passage HPV-16-immortalized cervical epithelial cells to leukoregulin up-regulation of NK lymphocytotoxicity was seen with each cell line, independent of previous chemical carcinogen co-treatment or serum selection of the immortalized cells. Although leukoregulin's slight augmentation of sensitivity to NK lymphocytotoxicity was seen only with the early passage cells, leukoregulin

increased the sensitivity of both the early and late passage HPV-immortalized cervical epithelial cells to LAK lymphocytotoxicity. The increased susceptibility to LAK lymphocytotoxicity over that of NK was 1.5 to 4-fold higher for the late passage compared to the early passage HPV-immortalized cervical epithelial cells. This was observed with HPV-16 DNA immortalized cervical epithelial cells developed in the presence (HCX-16-2S and HCX-16-5S) or the absence (HCX-16-1/MNNG and HCX-16-5) of serum.

The response of the early passage HPV-16-immortalized cervical cells was very similar to that of cervical epithelial cells not transfected with HPV-16 DNA, while the response of the late passage HPV-16-transfected cervical cells was similar to that of HPV-16-positive QGU cervical carcinoma cells. QGU cervical carcinoma cells were very resistant to NK, and leukoregulin failed to induce susceptibility; but like the HPV-16 DNA-immortalized cervical epithelial cells the carcinoma cells were LAK-sensitive and leukoregulin markedly up-regulated their sensitivity. Thus, late passage HPV-16-immortalized cervical epithelial cells exhibited a greater leukoregulin up-regulation in sensitivity to LAK than to NK lymphocyte cytotoxicity, mimicking the pattern of leukoregulin-induced up-regulation observed with the HPV-16-positive cervical carcinoma cells.

Tay et al. have shown that few NK cells are present in cervical epithelia with HPV infections and in cervical intraepithelial neoplasia. The in vitro resistance of HPV-16-immortalized cervical epithelial cells, like HPV-16-positive cervical carcinoma cells, to NK lymphocyte killing and its inability to be up-regulated by leukoregulin suggests that NK cells alone may be ineffective in the destruction of dysplastic and neoplastic cervical cells. NK lymphocytes, however, can function in an immunomodulatory capacity, not only modulating other effector cells, but by regulating their own activity. They do so by producing and releasing an assortment of cytokines, such as gamma IFN, IL-2, and leukoregulin which act to amplify and regulate the immune response. IFN and IL-2 can act directly on the NK cells to enhance their killer activity, while leukoregulin instead modulates natural lymphocytotoxicity activity by up-regulating the sensitivity of tumor target cells to NK and LAK lymphocyte killing.

Interferon, particularly alpha IFN, has been used in the clinical treatment of human genital papillomavirus infections. The response rate of these patients to IFN treatment is dependent on the HPV type with HPV-16/-18 infections showing a lower response rate. IFN can be applied topically but is usually administered systemically. In the latter situation, it is difficult to know whether IFN, which can both up-regulate effector lymphocytes and down-regulate target cell sensitivity, is acting directly on the abnormal target cell, on the immune effector cell, or both. This study shows that leukoregulin treatment of late passage HPV-immortalized cells has no effect on the sensitivity of the cells to NK cytotoxicity. On the other hand, both the HPV-immortalized cells and the cervical carcinoma cells are very sensitive to IL-2-activated killer LAK cells and this sensitivity is increased by leukoregulin. Since it has been shown that LAK precursor cells do exist in vivo and that their activity can be enhanced by exogenous IL-2, it is possible that LAK effector cells may play a significant role in vivo in the immune response against cervical neoplasia. NK cells may function as

immunomodulators, releasing substances that activate and amplify LAK effector cells, as evidenced by the *in vitro* reactivity of LAK cells to the cervical target cells and their corresponding up-regulation by leukoregulin.

In addition to leukoregulin-induced up-regulation of target cell sensitivity to NK and LAK lymphocytotoxicity, leukoregulin increases target cell plasma membrane permeability and tumor cell uptake of anti-cancer drugs. Together with the present observations this indicates that leukoregulin alone or in combination with anti-viral or anti-cancer drugs has the potential to play a significant role in mediating the successful treatment of HPV-16 infections and prevention of cervical dysplasia and neoplasia. The development of HPV-immortalized human cervical epithelial cells to study cervical carcinogenesis provides a valuable model system to define the physiological and biotherapeutic role of leukoregulin and other cytokines in the prevention and control of cervical dysplasia and neoplasia.

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ANNUAL REPORT OF

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

October 1, 1988 through September 30, 1989

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

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

IN VITRO PATHOGENESIS SECTION: The In Vitro Pathogenesis (IVP) Section (1) develops relevant model systems for the study of all phases of the process of carcinogenesis; (2) defines regulatory mechanisms for the normal control of growth and differentiation and alterations in these controls induced by initiators and promoters; (3) produces, isolates, and studies initiated cells; (4) studies functional alterations in gene expression produced by initiators and promoters and the mechanism by which these functional changes occur; and (5) elucidates factors which determine susceptibility to carcinogenesis.

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

Regulation of Epidermal Growth and Differentiation and Relationship to Early Events in Epidermal Carcinogenesis: Cellular and molecular aspects of chemical carcinogenesis in lining epithelia are studied in mouse epidermis by in vivo and in vitro techniques. The initiation event in skin carcinogenesis correlates with an altered program of terminal differentiation of epidermal cells. In cell culture, epidermal differentiation is regulated by the concentration of extracellular calcium. Primary cultures with the characteristics of basal cells result from growth in medium with low levels of

calcium (0.02-0.09mM). Induction of terminal differentiation of normal cells by increasing the calcium concentration in the culture medium to >0.1mM causes a two- to threefold increase in the level of intracellular free calcium at 5 - 30 minutes. This plateau level is maintained for at least 24 hours. Cells of initiated lines which survive in medium with high calcium showed an altered response to increased external calcium, with a sharp four- to ninefold peak of intracellular free calcium in all cells within 2 minutes and a return to normal by 9 hours. These and other differences in intracellular calcium between normal and initiated keratinocytes may be related to alterations in phosphoinositide metabolism. The ras oncogene is highly correlated to the initiated or papilloma phenotype in epidermis. Introduction of an activated ras gene into normal keratinocytes alters their phenotype to that of papilloma cells which are resistant to the differentiation-inducing effects of phorbol ester tumor promoters. In culture, introduction of the v-fos oncogene into cells with an activated ras gene results in their conversion to malignancy. Expression of TGF- α by either papilloma or normal cells stimulates tumor growth without affecting tumor progression. TGF- β is elaborated by normal keratinocytes induced to differentiate by TPA or by increasing external calcium. The type of TGF- β secreted is altered by introduction of v-ras into normal keratinocytes.

Molecular Regulation of Epidermal-Specific Differentiation Products: cDNA clones corresponding to the major proteins expressed in mouse epidermis have been isolated and characterized. To identify sequences regulating the expression of these genes, vector constructs using different regions of the genomic clones to drive expression of the chloramphenicol acetyltransferase gene has revealed sequences which are induced to active transcription by specific calcium concentrations. A gene encoding a cysteine-rich protein, which is a major component of the cornified envelope, has been isolated and shown by in situ hybridization experiments to be expressed in the granular layer of the epidermis. Cloned keratin genes, constructed with inducible promoter sequences, have been introduced into epidermal tumor cells and the proteins are expressed. The chronic applications of retinoids on human skin in vivo causes a number of changes in epidermal differentiation markers including the induction of keratin 6 and keratin 13 and an increased number of cell layers expressing transglutaminase.

Determinants for Susceptibility to Carcinogenesis: The SENCAR mouse is unusually sensitive to skin carcinogenesis by initiation and promotion, while the BALB/c mouse is resistant. We have developed a technique of grafting epidermal and dermal cells to athymic nude mice to form a reconstituted skin and have developed a series of initiated SENCAR and BALB/c mouse epidermal cell lines with a variety of molecular lesions. Cell lines initiated with 7,12-dimethylbenz[a]anthracene (DMBA) contain an activated ras-Ha gene and form squamous papillomas when grafted along with primary dermal fibroblasts. Introduction of an activated fos gene into these cells and grafting results in squamous cell carcinomas. A cell line from SENCAR mouse epidermal cells initiated in culture with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) has no activated ras-Ha or other transforming oncogene detectible by transfection into NIH 3T3 cells, forms apparently normal skin when grafted, and has lost the ability to suppress papilloma formation by cells containing an activated ras-Ha gene. Grafting of MNNG-initiated cells into which an activated ras-Ha gene has been introduced results in squamous cell carcinomas, suggesting the molecular lesion leading to initiation of these cells is complemented by an

activated ras-Ha to produce malignant conversion. Introduction of an activated fos gene has no effect. SENCAR mice develop papillomas when treated with TPA alone, without exogenous chemical initiation; and SENCAR cells in culture produce foci resistant to Ca²⁺-induced terminal differentiation, suggesting the existence of a constitutively initiated epidermal cell population. Papillomas formed on SENCAR mice by treatment with promoter alone did not have an activated ras-Ha oncogene at codon 61, nor did four cell lines developed from such papillomas. Two of these lines formed papillomas when grafted and thus are the first cell lines producing papillomas which have no activated ras-Ha gene. These cell lines should be useful to elucidate the molecular lesions required for the papilloma phenotype and for malignant conversion.

Immunological Techniques to Study the Interaction of Carcinogens with DNA:

Antibodies specific for carcinogen-DNA adducts have been used to quantify DNA modification in biological samples substituted with 2-acetylaminofluorene (AAF), cisplatin and benzo(a)pyrene (BP) by quantitative immunoassays and by immunohistochemistry. Comparison of adduct and tumor dose responses in bladders and livers of mice fed AAF chronically showed linear adduct and liver tumor formation, but non-linear bladder tumor formation. Weibull "Hit Theory" analysis suggests that threefold more adduct-related events are required for tumorigenesis in the bladder as compared to the liver. Preneoplastic liver enzyme-altered foci have been shown to have reduced capacity for AAF-DNA adduct formation and an increased proliferative advantage. In CHO cells pulsed with BrdU at the time of AAF exposure, replicating regions of metaphase chromosomes have been shown to exhibit the highest AAF-DNA adduct concentrations. Cisplatin DNA adducts were measured in DNA from nucleated peripheral blood cells and in tissues obtained from cancer patients. A comparison of adduct levels with disease response for 72 ovarian and testicular cancer patients indicated that individuals with high adduct levels in blood cell DNA have a high rate of complete response to therapy. Cisplatin-DNA adducts were shown to be persistent in many tissues at least a year after the last therapy. BP-DNA antigenicity was measured in blood cell DNA of firefighters and controls. Factors which contributed to sample positivity were firefighting, the ingestion of charcoal broiled foods, daily alcohol consumption and smoking. Immunoaffinity columns, prepared with hydrocarbon or aromatic amine class-specific adduct antisera, are being used to concentrate DNA adducts in animal and human samples prior to chemical characterization.

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.

Two aspects have been studied in detail in the past year:

1. Retinoids in cell surface structure and biological activity. Vitamin A and its derivatives, the retinoids, are of interest in cancer research because they play an essential role in the maintenance of normal differentiation in

most epithelial tissues under normal physiological conditions and modulate growth of certain neoplastic cells by inducing differentiation. Vitamin A deficiency causes loosening of adhesive strength between the tracheal epithelium and the underlying connective tissue. Conversely, we have found that retinoic acid (RA) and other retinoids with biological activity in maintaining normal epithelial differentiation enhance the adhesiveness of cultured fibroblastic cells (NIH 3T3) as well as that of mouse epidermal cells to plastic dishes coated with specific extracellular matrix proteins, such as laminin and type IV collagen. Thus, the study of fibroblast cell adhesion induced by RA has become relevant to our understanding of the mechanism by which retinoids maintain epithelial cell differentiation. RA also decreases inositol transport into NIH 3T3 cells.

An investigation utilizing lectins which bind to specific cell surface carbohydrate groupings in glycoproteins of NIH 3T3 cells demonstrated a consistent increase by RA of phytohemagglutinin (PHA-L) binding to a glycoprotein of MW 130,000 (gp 130) as judged by SDS-PAGE analysis and Western blotting. Since protein staining was not affected and PHA-L specifically binds to β 1,6-linked N-acetylglucosamine residues, we conclude that in NIH 3T3 cells, RA probably increases transcription of the gene coding for UDP-N-acetylglucosaminyl transferase V, i.e., the enzyme responsible for the biogenesis of β -1,6-linked N-acetylglucosamine residues. The cytostatic activity of PHA-L was enhanced in RA-treated compared to control cells, consistent with higher lectin binding.

Since laminin and retinoic acid both induce differentiation of cultured human neuroblastoma cells, we studied the concentration of the laminin receptor and its mRNA in these cells by Western and Northern blot analysis. These investigations failed to show any significant effect of the retinoid on laminin receptor concentration and on its mRNA. However, the analysis of the cell surface glycoproteins by Western blotting and carbohydrate detection with lectins revealed a four- to sixfold increase in the binding of PHA-E to a band of approximate MW 68,000 (gp 68). Since the binding of other lectins was not affected, these data permit the conclusion that RA causes a specific increase in the concentration of β 1,4-linked "bisecting" N-acetyl-glucosamine in gp 68 of neuroblastoma cells. This effect is probably the result of an action of RA through its receptor on the expression of uridine diphosphate N-acetylglucosamine: gp 68 N-acetylglucosaminyl transferase III which specifically generates the β 1,4-linked "bisecting" glucosamine. These data suggest that RA may modulate the structure and possibly the activity of cell surface glycoproteins by specific effects on glycosyl transferases. Future work will focus on the identification of the gp 130 and gp 68 to study whether they are receptors for extracellular matrix components, as well as on determination of the glucosamine transferase activities III and V in neuroblastoma and in NIH 3T3 cells.

2. Effect of vitamin A depletion on mouse skin tumorigenesis. Studies in female SENCAR mice showed that vitamin A depletion inhibited (98%) tumor formation caused by painting DMBA and TPA. Dietary administration of retinoic acid caused tumor induction in otherwise tumorless mice. The schedule of administration of the DMBA (week 3) and TPA (weeks 4-13), adapted to permit tumor formation during vitamin A depletion, also permitted the prevalence of keratoacanthomas instead of papillomas.

MOLECULAR MECHANISMS OF TUMOR PROMOTION SECTION: The efforts of the Molecular Mechanisms of Tumor Promotion Section are directed toward understanding the early events in the interaction of phorbol ester tumor promoters with cells and tissues. Particular attention is being devoted to the analysis of the major phorbol ester receptor, protein kinase C. The bryostatins, macrocyclic lactones, activate protein kinase C and compete for phorbol ester binding. They only induce a subset of the typical phorbol ester responses, however. The biochemical mechanisms for these differences are being explored through immunoblotting of protein kinase C, comparison of phosphorylation patterns, tritiated bryostatin binding analysis, and comparison of effects on cloned and chromatographically separated protein kinase C isozymes. Other structural classes of protein kinase C modulators under investigation include 7-beta-meprounic acid 3-p-hydroxybenzoate, cyclic diglycerides, and phorbol derivatives modified in the tigliane ring structure or its functional groups. A second research direction is to identify the targets of irritant but non-promoting phorbol related derivatives. We have shown that resiniferatoxin acts as a selective ultrapotent capsaicin analog. Typical phorbol esters exert their inflammatory activity through both resiniferatoxin-sensitive and -insensitive pathways. The latter correlate better with promoting activity. Current efforts are directed at identification and characterization of resiniferatoxin receptors.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP04504-17 CCTP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

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

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

PI:	S. H. Yuspa	Chief	LCCTP	NCI
Others:	H. Hennings	Research Chemist	LCCTP	NCI
	J. Strickland	Research Chemist	LCCTP	NCI
	D. Greenhalgh	Guest Researcher	LCCTP	NCI
	U. Lichti	Guest Researcher	LCCTP	NCI
	A. Dlugosz	Guest Researcher	LCCTP	NCI
	F. Kruzevski	Special Volunteer	LCCTP	NCI
	E. Lee	Guest Researcher	LCCTP	NCI
	A. Player	IRTA Fellow	LCCTP	NCI
	M. Ueda	Visiting Fellow	LCCTP	NCI
	W. Weinberg	Guest Researcher	LCCTP	NCI

COOPERATING UNITS (if any)

BIOCON Inc, Rockville, MD (L.M. Endler); Johns Hopkins, Baltimore, MD (R. Tucker); Univ. of Arizona, Tucson, AZ (G. T. Bowden), Baylor College of Medicine (D. Roop).

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

9.0

PROFESSIONAL

6.0

OTHER:

3.0

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.)

Cellular and molecular aspects of chemical carcinogenesis in lining epithelia are studied in mouse epidermis by in vivo and in vitro techniques. The initiation event in skin carcinogenesis correlates with an altered program of terminal differentiation of epidermal cells. In cell culture, epidermal differentiation is regulated by the concentration of extracellular calcium. Primary cultures with the characteristics of basal cells result from growth in medium with low levels of calcium (0.02-0.09mM). Induction of terminal differentiation of normal cells by increasing the calcium concentration in the culture medium to >0.1mM causes a two- to threefold increase in the level of intracellular free calcium at 5 - 30 minutes. This plateau level is maintained for at least 24 hours. Cells of initiated lines which survive in medium with high calcium showed an altered response to increased external calcium, with a sharp four- to ninefold peak of intracellular free calcium in all cells within 2 minutes and a return to normal by 9 hours. These and other differences in intracellular calcium between normal and initiated keratinocytes may be related to alterations in phosphoinositide metabolism. The ras oncogene is highly correlated to the initiated or papilloma phenotype in epidermis. Introduction of an activated ras gene into normal keratinocytes alters their phenotype to that of papilloma cells which are resistant to the differentiation-inducing effects of phorbol ester tumor promoters. In culture, introduction of the v-fos oncogene into cells with an activated ras gene results in their conversion to malignancy. Expression of TGF- α by either papilloma or normal cells stimulates tumor growth without affecting tumor progression. TGF-B is elaborated by normal keratinocytes induced to differentiate by TPA or by increasing external calcium. The type of TGF-B secreted is altered by introduction of v-ras into normal keratinocytes.

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

S. Yuspa	Chief	LCCTP NCI
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D. Greenhalgh	Guest Researcher	LCCTP NCI
U. Lichti	Guest Researcher	LCCTP NCI
A. Dlugosz	Guest Researcher	LCCTP NCI
F. Kruszewski	Special Volunteer	LCCTP NCI
E. Lee	Guest Researcher	LCCTP NCI
A. Player	IRTA Fellow	LCCTP NCI
M. Ueda	Visiting Fellow	LCCTP NCI
W. Weinberg	Guest Researcher	LCCTP NCI
M. Sporn	Chief	LC NCI
D. Lowy	Chief	LCO NCI
S. Aaronson	Chief	LCMB NCI

Objectives:

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

Methods Employed:

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

nude or newborn mice. Genetic aberrations are studied by DNA transfection, gene cloning and sequencing, and nucleic acid hybridization and restriction analysis.

Major Findings:

A. Ca^{2+} Metabolism in Normal and Neoplastic Keratinocytes: The Underlying Biochemistry of Epidermal Neoplasia. Previous studies in this laboratory have shown that medium with 0.02-0.1 mM Ca^{2+} (low Ca^{2+}) selects for the growth of proliferating basal cells and that shifting to medium with 1.2 mM Ca^{2+} (high Ca^{2+}) concomitantly inhibits proliferation and induces terminal differentiation. Cell lines established from papillomas, from initiated skin, or after carcinogen exposure in culture (putative initiated cell lines) survive in high Ca^{2+} medium. In collaboration with Dr. Robert Tucker of Johns Hopkins University, intracellular-free calcium (Ca_i) levels were measured by use of a calcium-sensitive probe, Fura 2, in normal and initiated cells. Most normal keratinocytes respond to increased extracellular calcium by a gradual two- to threefold increase in Ca_i lasting for at least 28 minutes. A subpopulation displays a sharp peak of Ca_i at 2 minutes. In the initiated cells, the Ca_i level in low calcium medium was two- to threefold higher than that in normal cells, and all cells showed a four- to ninefold increase in Ca_i 2 minutes after external calcium was increased. As in the normal cells, the plateau level of Ca_i at 28 minutes was 2-3 times the initial level. The sharp peak in Ca_i at 2 minutes is dependent on factors in serum. The plateau at 28 minutes is dependent on the extracellular calcium concentration. The induction of terminal differentiation appears to be related to a plateau in Ca_i lasting at least 24 hours. Basal levels of Ca_i in normal keratinocytes remain elevated after 24 hours in high Ca^{2+} medium; the Ca_i in initiated cells returns to normal by 9 hours. The prolonged plateau in normal keratinocytes was prevented by either ouabain or low potassium medium, blockers of terminal differentiation. Increasing the calcium in the culture medium from 0.05 to 0.10, 0.12 or 1.2 mM resulted in increases in plateau values of Ca_i of 1.5-, 2.0-, and 2.6-fold respectively. Thus normal keratinocytes are highly responsive to incremental increases in their calcium environment, and this is an important signal for differentiation.

B. Signal Transduction in Normal and Neoplastic Keratinocytes. Ca^{2+} may act both by increasing intracellular Ca^{2+} and by stimulating phosphatidylinositol turnover, thus activating protein kinase C and elevating intracellular Ca^{2+} . Inositol turnover is stimulated with both 1 mM and 0.15 mM Ca^{2+} but is not sustained at the lower concentration of Ca^{2+} in normal cells. The profile of inositol metabolism is altered in initiated cells; in particular, the metabolite IP_3 is not increased substantially. Aluminum fluoride, which activates G proteins in the plasma membrane, induces a rapid increase in IP_3 . Thus, G proteins have a role in the regulation of inositol phosphate metabolism in epidermal cells. Aluminum fluoride alters the expression of differentiation markers in response to Ca^{2+} .

Bryostatins cause a prolonged down regulation of protein kinase C. Treatment with bryostatins inhibits keratinocyte differentiation at calcium concentrations from 0.11 to 0.13 mM. Based on immunofluorescence microscopy and immunoblotting, the induction of the differentiation-specific keratins K1 and K10, filaggrin, and a cornified envelope precursor protein are inhibited. The characteristic calcium-mediated morphological changes are also inhibited. These findings indicate that protein kinase C plays a key role in calcium-induced differentiation of primary keratinocytes.

Staurosporine is a putative inhibitor of protein kinase C and other protein kinases. Paradoxically, staurosporine induces differentiation in normal keratinocytes and in neoplastic keratinocyte cell lines. The effects of staurosporine are partially blocked by bryostatin. These results suggest that staurosporine activates protein kinase C in keratinocytes but causes additional effects on pathways involved in differentiation. Staurosporine may be a useful compound to inhibit tumor formation *in vivo*.

C. Malignant conversion in vitro. Activation of the ras oncogene is sufficient to initiate epidermal cells and produce papillomas. In order to study genes which may complement ras in producing malignant conversion, an assay has been developed which uses primary keratinocytes in which an activated ras gene has been introduced by a defective retroviral vector. When these cells are switched to high Ca^{2+} medium, they are blocked in their differentiation program but can no longer proliferate, leaving a lawn of non-proliferating, partially-differentiated cells in culture. When introduction of the ras oncogene is followed by exposure to carcinogens, foci evolve which can proliferate in high calcium medium. Foci which proliferate in high Ca^{2+} medium have been isolated and most are nontumorigenic, suggesting that the carcinogen-induced event is not malignant conversion but rather an intermediate stage in neoplastic progression. When normal keratinocytes were cultured in low Ca^{2+} medium, many cells died within 2-3 weeks while others formed rapidly growing foci which could be subcultured. These rapidly growing cells produced benign tumors when grafted to nude mice and possessed a heterozygous mutation in the c-ras^{Ha} gene. Fibroblast-conditioned low Ca^{2+} medium prevented focus formation, ras^{Ha} gene mutation and tumorigenicity. These results demonstrate that suboptimal culture conditions favor a spontaneous mutation in codon 61 of c-ras^{Ha} gene of keratinocytes.

To determine if a transforming growth factor contributes to malignant conversion, human transforming growth factor- α (hTGF- α) was introduced into cultured primary mouse epidermal cells or papilloma cells. Results indicated that expression of hTGF- α by either tumor cells (autocrine) or adjoining normal cells (paracrine) can stimulate tumor growth, particularly when tumor growth is suppressed by normal tissue. However, expression of this growth factor did not appear to influence tumor progression directly.

Co-infection of primary murine keratinocytes with helper-free (Psi 2) FBR or FBJ v-fos and v-ras^{Ha} retroviruses resulted in carcinomas when recipient cells were tested in nude mice. Introduction of only v-fos produced normal or hyperplastic skin, while v-ras^{Ha} produced papillomas. Thus, two oncogene events are sufficient to produce the malignant phenotype in epidermal cells. Malignant conversion by combined mutations in ras and fos genes are associated with elevated expression of the secreted proteases transin and urokinase. Benign tumors produced by a mutated ras gene do not express these proteases. The elaboration of secreted proteases may be associated with malignant conversion.

D. TGF- β effects. The influence of transforming growth factor- β (TGF- β) on normal and initiated keratinocytes is being explored in collaboration with Drs. Michael Sporn, Adam Glick, David Danielpour, and Anita Roberts of the Laboratory of Chemoprevention. TGF- β inhibits growth of both cell types, and this can be reversed by adding anti-TGF- β antibody to the medium. TGF- β is elaborated by

keratinocytes induced to differentiate by Ca^{2+} or TPA. In addition, retinoic acid increases TGF- β secretion in normal and initiated cells. Elevation of Ca^{2+} increases TGF- β_2 mRNA and peptide levels more than 10- to 20-fold, but decreases the expression of TGF- β_1 . Introduction of a v-ras^{Ha} gene blocks the induction of terminal differentiation by Ca^{2+} . In the v-ras^{Ha} cells, elevation of Ca^{2+} increases TGF- β_1 and suppresses TGF- β_2 peptide levels, even though the mRNA expression patterns are identical between the two cell types. These results indicate that an activated ras^{Ha} gene can regulate TGF- β expression at the post-transcriptional level. TGF- β_1 and β_2 inhibit growth of papilloma lines. TGF- β secretion is increased in papilloma lines in high Ca^{2+} . TGF- β_1 is the principal type secreted by initiated lines SP-1 and 308, suggesting the ras gene is regulating these lines as well. Retinoic acid (10⁻⁷M) increases secretion of TGF- β in normal cells and 308 and SP-1 cells, and also inhibits growth of all cell lines (perhaps via TGF- β).

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

PROJECT NUMBER

201CP04798-19 CCTP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

PI:	L. M. De Luca -	Research Chemist	LCCTP NCI
OTHERS:	M. Webber	IPA Appointee	LCCTP NCI
	R. Sinha	IRTA Fellow	LCCTP NCI
	D. Cai	Visiting Fellow	LCCTP NCI
	S. Ross	Biologist	LCCTP NCI

COOPERATING UNITS (if any)

NIDR, NIH (Y. Yamada, H. Kleinman); Microbiological Associates, Bethesda, MD. (M. Wenk)

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

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INSTITUTE AND LOCATION

NIH, NCI, Bethesda, MD 20892

TOTAL MAN-YEARS:

7.0

PROFESSIONAL:

5.0

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.)

(1) Retinoids enhanced the adhesiveness of several cell lines specifically to laminin-coated plates in a reversible manner. They also decreased inositol transport. Cell membrane protein profiles by SDS-PAGE did not show any retinoid-induced differences, but specific staining with lectins revealed an increase in PHA-L binding to a cell surface glycoprotein of MW 130,000 (gp 130). Since PHA-L binds specifically to α 1,6-linked N-acetyl-glucosamine, retinoic acid (RA) probably increases gene expression for transferase V, the enzyme responsible for this linkage. The cytostatic activity of PHA-L was enhanced in RA-treated cells, consistent with higher lectin binding. The human neuroblastoma cell line (LAN-1) responds to RA as well as laminin by extending neurites and by a slowdown in cell division. SDS-PAGE and Western blot analysis failed to show any effect of RA on the laminin receptor protein, using an antibody which detects the 68,000 receptor. Moreover, Northern blot analyses of the mRNA fraction also failed to show any increase in laminin receptor mRNA. Staining of SDS-PAGE gels after Western blotting and binding with various lectins clearly indicated a four-to sixfold increase in PHA-E binding to a specific cell surface glycoprotein of MW 68,000 (gp 68). Since PHA-E specifically binds to α 1,4-linked "bisecting" N-acetyl-glucosamine, RA probably affects transferase III activity in neuroblastoma cells. Thus, RA appears to control the degree of glycosylation of specific glycoprotein sites in different cells. (2) In vivo experiments, vitamin A-depleted female SENCAR mice show a requirement for dietary RA for skin tumor formation, with DMBA as the initiator and TPA as the promoter.

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

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

Objectives:

1. To investigate the mechanisms by which retinoids and tumor promoters modify cell surface properties, such as cell adhesiveness, and transport properties.
2. To study the effect of dietary vitamin A depletion on skin tumorigenesis.

Methods Employed:

1. To investigate the mechanisms by which retinoids and tumor promoters modify cell surface properties such as cell adhesiveness and transport properties.

A. Cell attachment assay.

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

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

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

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

B. Western blotting and lectin staining techniques for cell surface glycoproteins.

Cell surface glycoproteins are solubilized by 0.5% Nonidet, separated on SDS-PAGE, transferred to nitrocellulose filters and stained with specific biotinylated lectins which are revealed by avidin-complexed horseradish peroxidase, which eventually reacts with appropriate substrates to generate visible bands.

A polyclonal antibody against the laminin receptor (developed by Y. Yamada) was used to study the effect of RA on the receptor concentration in preparations of membrane glycoproteins. Specific hybridization probes to detect laminin receptor mRNA by Northern blot analysis were developed by Y. Yamada of the National Institute for Dental Research.

2. To study the effect of dietary vitamin A depletion on skin tumorigenesis.

Two regimens were tested to induce vitamin A deficiency. SENCAR mice were either 1) fed a vitamin A-deficient diet at 4 or 9 weeks of age or 2) their mothers were fed the diet from the time of birth of the experimental animals which were weaned onto the same diet. The latter regimen produced typical symptoms of vitamin A deficiency in the offspring by weeks 12-14 and all the mice died by week 19; the former regimen permitted sufficient accumulation of vitamin A to sustain life for up to 45 and 75 weeks, respectively, in the majority of mice. For our experiments, vitamin A depletion was produced by placing the mothers on the deficient diet at birth of the experimental animals. A single topical dose of 20 μ g of 7,12-dimethylbenzanthracene (DMBA) was used as the initiator at 3 weeks of age and 1 to 2 μ g of 12-O-tetradecanoylphorbol-13-acetate (TPA) once weekly as the tumor promoter for 10 weeks (from week 4-13 of the experiment). This regimen produced keratoacanthomas instead of the usual papillomas produced with initiation at 8 weeks of age.

Major Findings:

1. Findings of cell surface membrane studies.

Retinoic acid increased the attachment of NIH 3T3 cells specifically to laminin; it also decreased the transport of inositol. SDS-PAGE analyses of the 0.5% Nonidet solubilized cell surface proteins failed to show any changes in Ponceau S staining intensity. However, specific lectin staining of a glycoprotein (MW 130,000, i.e. gp 130) was shown with PHA-L which binds to β 1,6-linked N-acetyl glucosamine. Other lectins did not show a significant effect. Therefore, RA specifically increases the concentration of β 1,6-linked N-acetylglucosamine in gp 130. Since this linkage is generated by transferase V, it seems reasonable to suggest that RA increases transferase V gene expression in NIH 3T3 cells. The increased PHA-L binding to RA-treated NIH 3T3 cells was accompanied by an enhanced cell killing activity by this lectin for RA-treated compared to DMSO control cells.

Cultured human neuroblastoma cells (NAL-1) respond to RA or laminin treatment by forming neurites and by a slowdown in cell division rate. An analysis of the cell surface proteins of NAL-1 cells showed no major effect on Ponceau S staining of SDS-PAGE profiles. Moreover, Western and Northern blot analyses failed to show an increase of laminin receptor protein and mRNA concentrations. However, binding of PHA-E to a specific glycoprotein of MW 68,000 (gp 68) was increased four-to sixfold by RA treatment. Since PHA-E binds specifically to β -1,4-linked N-acetylglucosamine ("bisecting glucosamine"), RA probably increases gene expression for Transferase III, the enzyme that generates this linkage.

These data support the view that RA enhances carbohydrate branching, resulting in more complex carbohydrate structures on specific cell surface glycoproteins. Whether this mechanism is responsible for the observed enhanced cell attachment to laminin and/or decreased inositol transport remains to be investigated.

2. Study of the effect of dietary vitamin A depletion on skin tumorigenesis.

Our work on skin tumorigenesis in female SENCAR mice was preceded by a careful and extensive assessment of the effect of feeding a Purina diet from birth for periods of 0, 4 and 9 weeks. When these feeding regimens were followed by a vitamin A-deficient diet, it took 12, 45 and 75 weeks respectively for the onset of vitamin A deficiency to occur. Thus, the most rapid and reliable method was to feed the mother and the offspring the vitamin A-deficient diet from birth of the experimental animals. Application of DMBA (at week 3) and TPA (from weeks 4 through 13) was timed to permit the beginning of tumor formation during the critical time (at about week 12) when vitamin A deficiency symptoms begin to appear. Our work permits the following conclusions:

- A. Depletion of vitamin A strongly inhibits DMBA-TPA-induced skin tumorigenesis in female SENCAR mice, before any significant effect on epidermal thickness, number of nucleated cell layers and labeling indices.
- B. Most mice died without visible tumors by week 19 on the vitamin A-deficient diet, after loss of body weight and independently of whether or not they had received TPA.
- C. Switching of the diet at week 12 from vitamin A-deficient to RA, β -carotene or retinylpalmitate-containing diets caused tumor formation but only in DMBA TPA-treated mice. However, tumor incidence rates were always less than in Purina-fed mice.
- D. Most of the benign tumors were keratoacanthomas in both Purina and purified diet groups.

- E. Mice on the purified, RA-supplemented diet developed fewer benign tumors per mouse than Purina-fed mice.
- F. Carcinoma formation was similar in the Purina and purified diet + RA groups.

Publications:

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05177-08 CCTP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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:	O. Olivero	Fogarty Fellow	LCCTP	NCI
	S. Gupta-Burt	Biotechnology Fellow	LCCTP	NCI
	S. Yuspa	Chief	LCCTP	NCI
	E. Reed	Senior Investigator	MB	NCI
	A. Weston	Visiting Associate	LHC	NCI

COOPERATING UNITS (if any)

NCTR, Jefferson, AR (F. A. Beland); Natl. Hosp., Oslo, Norway (H. Huitfeldt); Johns Hopkins U., Baltimore, MD (P. Strickland); Columbia Univ., New York, NY (R. Santella).

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, MD 20892

TOTAL MAN-YEARS

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 unredacted type. Do not exceed the space provided.)

Antibodies specific for carcinogen-DNA adducts have been used to quantify DNA modification in biological samples substituted with 2-acetylaminofluorene (AAF), cisplatin and benzo(a)pyrene (BP) by quantitative immunoassays and by immunohistochemistry. Comparison of adduct and tumor dose responses in bladders and livers of mice fed AAF chronically showed linear adduct and liver tumor formation, but non-linear bladder tumor formation. Weibull "Hit Theory" analysis suggests that threefold more adduct-related events are required for tumorigenesis in the bladder as compared to the liver. Preneoplastic liver enzyme-altered foci have been shown to have reduced capacity for AAF-DNA adduct formation and an increased proliferative advantage. In CHO cells pulsed with BrdU at the time of AAF exposure, replicating regions of metaphase chromosomes have been shown to exhibit the highest AAF-DNA adduct concentrations. Cisplatin DNA adducts were measured in DNA from nucleated peripheral blood cells and in tissues obtained from cancer patients. A comparison of adduct levels with disease response for 72 ovarian and testicular cancer patients indicated that individuals with high adduct levels in blood cell DNA have a high rate of complete response to therapy. Cisplatin-DNA adducts were shown to be persistent in many tissues at least a year after the last therapy. BP-DNA antigenicity was measured in blood cell DNA of firefighters and controls. Factors which contributed to sample positivity were firefighting, the ingestion of charcoal broiled foods, daily alcohol consumption and smoking. Immunoaffinity columns, prepared with hydrocarbon or aromatic amine class-specific adduct antisera are being used to concentrate DNA adducts in animal and human samples prior to chemical characterization.

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

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

Objectives:

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

Methods Employed:

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

Major Findings:

In an attempt to elucidate the relationship between AAF-DNA adduct formation and tumorigenesis, adducts have been measured in livers and bladders of mice continuously fed 0.02% AAF in the diet. These studies, performed in

collaboration with Dr. F. Beland, compared adducts at 1 month of continuous AAF feeding with tumors at 18, 24 and 33 months. In both liver and bladder, DNA adduct formation was linear over a dose range between 5 and 150 mg AAF/kg diet, although adducts in bladder were 2-3 times higher than those in liver. Tumorigenesis in the liver was also linear, but in the bladder there were no tumors at doses lower than 60 mg/kg. Weibull ("hit" theory) analysis of the relationship between tumor incidence and adduct formation suggests that three adduct-related events are required for tumorigenesis in the bladder while only one is required for tumorigenesis in the liver. Further studies will investigate preneoplastic tissue specific adduct-related and non-adduct-related events.

In collaboration with Dr. H. Huitfeldt (University of Oslo), immuno-histochemical localization of DNA adducts has been studied in frozen sections of livers from male rats fed AAF. When replicating liver cells were examined in animals pulsed with BrdU, small clusters and individual replicating cells were shown not to contain adducts detectable by immunofluorescence. The presence of enzyme markers in these cells is currently under investigation, since they may be progenitor cells for the adduct-negative enzyme-altered foci.

When synchronized CHO cells were exposed to N-acetoxy-AAF in S phase and allowed to continue the cell cycle until arrested by colchicine at mitosis, the metaphase chromosome spreads obtained exhibited a non-random pattern of adduct distribution. This pattern was consistent for different experiments when exposure occurred at the same time. To investigate the replicative state of these chromosomes at the time of exposure, cells were co-incubated with bromodeoxyuridine (BrdU) and subsequent localization of replicated regions was achieved with anti-BrdU antisera. When N-acetoxy-AAF and BrdU exposure took place simultaneously early in S phase, the areas of high adduct concentration corresponded to areas of BrdU incorporation. When both compounds were administered late in S phase, the areas of replicative synthesis were different from those observed in the early S phase pulsing but the areas of high adduct concentration corresponded to the areas of BrdU incorporation in the same experiment. This suggests that more adducts are formed in chromosomal regions replicating at the time of exposure.

Cisplatin is a potent chemotherapeutic agent. An ELISA developed with cisplatin-DNA antisera is being used to quantitate adducts in DNA extracted from nucleated peripheral blood cells (wbc) of testicular and ovarian cancer patients receiving platinum drug therapy. Disease response data for 55 ovarian cancer patients and 17 poor prognosis testicular cancer patients showed that individuals forming high levels of cisplatin-DNA adducts in blood cell DNA were more likely to undergo complete remission than those forming fewer adducts or no adducts at all. Several tissues (brain, bone marrow, peripheral nerve, kidney, liver, spleen, lymph node and ovarian tumor) were obtained at autopsy from 8 individuals weeks to months after their most recent therapy. Adducts were assayed by ELISA and atomic absorbance spectrometry (AAS) (a collaboration with Dr. E. Reed of the Medicine Branch, NCI). In general, adducts were present in most tissues of the same individual, including tumor, and appeared to be persistent for many months after the last treatment.

An interlaboratory standardization of the benzo(a)pyrene-DNA ELISA by this Laboratory, Dr. A. Weston (LHC, NCI) and Dr. R. Santella (Columbia University) has produced a "standard assay" in which the results are being expressed as fmoles BP-DNA antigenicity. In collaboration with Dr. P. Strickland of Johns Hopkins Medical School, this ELISA was used to monitor the DNA of nucleated blood cells obtained from firefighters and age- and sex-matched controls in the Baltimore-Washington area. Factors which contributed to adduct formation as independent variables were consumption of charcoal broiled foods, daily alcohol consumption and smoking. This study has been extended to firefighters involved in the Montana forest fires in the summer of 1988 and laboratory volunteers who have consumed large quantities of charcoal broiled foods. In collaboration with Dr. A. Weston, immunoaffinity columns containing antisera which recognize DNA adducts of PAHs and aromatic amines are now being used to concentrate and purify adducts in animal and human samples before positive adduct identification is achieved by other methods.

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

PROJECT NUMBER

Z01CP05178-08 CCTP

PERIOD COVERED

October 1, 1988, to September 30, 1989

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

Cellular and Tissue Determinants of Susceptibility to Chemical Carcinogenesis

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

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

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Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.7

PROFESSIONAL:

1.7

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 SENCAR mouse is unusually sensitive to skin carcinogenesis by initiation and promotion while the BALB/c mouse is resistant. We have developed a technique of grafting epidermal and dermal cells to athymic nude mice to form a reconstituted skin and have developed a series of initiated SENCAR and BALB/c mouse epidermal cell lines with a variety of molecular lesions. Cell lines initiated with 7,12-dimethylbenz[a]anthracene (DMBA) contain an activated ras-Ha gene and form squamous papillomas when grafted along with primary dermal fibroblasts. Introduction of an activated fos gene into these cells and grafting results in squamous cell carcinomas. A cell line from SENCAR mouse epidermal cells initiated in culture with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) has no activated ras-Ha or other transforming oncogene detectable by transfection into NIH 3T3 cells, forms apparently normal skin when grafted, and has lost the ability to suppress papilloma formation by cells containing an activated ras-Ha gene. Grafting of MNNG-initiated cells into which an activated ras-Ha gene has been introduced results in squamous cell carcinomas, suggesting the molecular lesion leading to initiation of these cells is complemented by an activated ras-Ha to produce malignant conversion. Introduction of an activated fos gene has no effect. SENCAR mice develop papillomas when treated with TPA alone, without exogenous chemical initiation; and SENCAR cells in culture produce foci resistant to Ca2+-induced terminal differentiation, suggesting the existence of a constitutively initiated epidermal cell population. Papillomas formed on SENCAR mice by treatment with promoter alone did not have an activated ras-Ha oncogene at codon 61, nor did four cell lines developed from such papillomas. Two of these lines formed papillomas when grafted and thus are the first cell lines producing papillomas which have no activated ras-Ha gene. These cell lines should be useful to elucidate the molecular lesions required for the papilloma phenotype and for malignant conversion.

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

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

Objectives:

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

Methods Employed:

The SENCAR mouse was developed by a selective breeding protocol for enhanced susceptibility to skin carcinogenesis by initiation and promotion. Comparisons are made to BALB/c mice as a representative resistant strain. The epidermis can be separated from the dermis by flotation of the skin, dermis side down, on a solution containing trypsin. The separated epidermal cells are cultured in medium containing Ca^{2+} levels <0.1 mM to select for basal cells or >0.1 mM to induce terminal differentiation. Initiated cells can be selected from an excess of normal cells on the basis of the resistance of the former to Ca^{2+} -induced terminal differentiation. Papilloma cell lines were developed from papillomas produced on adult SENCAR and BALB/c mouse skin by initiation with DMBA or MNNG and promotion with TPA or, in the case of SENCAR, by promotion with TPA without chemical initiation. Papillomas were removed, minced, and cells were dissociated by treatment with collagenase, followed by trypsin, and cells were cultured in medium with low $[Ca^{2+}]$. A grafting system has been developed in athymic nude mice which can produce normal skin from cultured primary epidermal and dermal cells of newborn mice. This system makes possible grafting of mixtures of normal with initiated cells as well as epidermal and dermal cells from different mouse strains in the same graft. Cultured cells are released from flasks or dishes by treatment with trypsin, and a mixture of epidermal and dermal cells is centrifuged and the cell pellet is applied to the graft bed within a silicone chamber which separates the graft from the host skin. After one week, the chamber is removed, and the wound allowed to heal. Grafts are examined both grossly and histologically at sacrifice.

Major Findings:

Using the reconstituted skin grafting system which we developed, we have studied interactions among normal epidermal cells, dermal fibroblasts, and initiated cell lines from both SENCAR and BALB/c mice. Most studies have utilized line SP-1, which was derived from papillomas produced on SENCAR mouse skin by initiation with 7,12-dimethylbenz[*a*]anthracene (DMBA) and promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA) and has an activated ras^{Ha} oncogene with a mutation in codon 61 as shown by XbaI restriction endonuclease analysis.

Papilloma formation by SP-1 cells in grafts is strongly suppressed by normal SENCAR primary epidermal cells, but not by SENCAR primary dermal fibroblasts or BALB/c primary epidermal cells. Initiated cell line SCR722, which does not have an activated ras gene and makes an apparently normal epidermis when grafted, has lost the ability to suppress papilloma formation by SP-1 cells. SCR722 was derived from SENCAR mice initiated with N-methyl-N'-nitro-N-nitrosoguanidine. SCR722 has no activated oncogene capable of forming foci in NIH 3T3 cells but is converted to malignancy by introduction of an activated ras^{Ha} gene, implying that the molecular lesion leading to initiation in SCR722 is complementary to the ras^{Ha} gene for malignant conversion. Introduction of an activated fos gene into SCR722 does not change the "normal" skin phenotype of grafted cells.

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

Both in vivo tumorigenesis studies and in vitro focus formation studies have suggested that a population of endogenously initiated cells exists in SENCAR skin. To study the nature of the molecular lesion in these cells, we have examined DNA from papillomas derived from SENCAR mouse skin treated with TPA alone, i. e., without exogenous chemical initiation. All eight papillomas and one carcinoma produced by treatment with TPA alone were found to lack activation of the ras^{Ha} gene by an A to T transversion at codon 61. Cell lines, derived from four of these papillomas, also lacked such an activated ras^{Ha} gene. Each cell line has a basal cell morphology when cultured in medium with $[Ca^{2+}] = 0.05$ mM. When $[Ca^{2+}]$ in the medium is raised to 1.4 mM, each cell line assumes a differentiated morphology but continues to proliferate. Normal epidermal cells terminally differentiate and die under these conditions. If $[Ca^{2+}]$ in the medium is reduced to 0.05 mM following several weeks in medium with 1.4 mM $[Ca^{2+}]$, the cells rapidly resume a basal cell morphology. At the earliest passage tested, two of the cell lines formed squamous papillomas and two formed squamous cell carcinomas when grafted to athymic nude mouse hosts. Subcutaneous injections yielded results consistent with those of grafts. The papilloma phenotype appears to be unstable, since evidence of conversion to malignancy was seen. The conversion may have occurred through spontaneous activation of a transforming oncogene detected by NIH 3T3 transfection assays. The molecular lesion leading to initiation in these papillomas remains unknown but is clearly not activation of the ras^{Ha} gene by an A to T transversion at codon 61. The two cell lines forming papillomas when grafted are the first such cell lines we have developed which

do not have a ras^{Ha} gene activated at codon 61. These cell lines may be useful in identifying additional molecular lesions responsible for the papilloma phenotype, as well as in defining complementary molecular lesions resulting in conversion to malignancy.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05270-08 CCTP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Molecular Mechanism of Action of Phorbol Ester Tumor Promoters

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

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

COOPERATING UNITS (if any)

Boston U. School of Med., Boston, MA (A. J. Tauber); Arizona St. U. (G.R. Pettit, C. L. Herald, Y. Kamano); Upjohn Co., Kalamazoo, MI (K.L. Leach); U. of California, Riverside, CA (J.A. Traugh, D. Murray) Program Resources, Inc., Frederick, MD (J. Beutler).

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

Molecular Mechanisms of Tumor Promotion Section

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TOTAL MAN-YEARS

5.5

PROFESSIONAL

4.25

OTHER

1.25

CHECK APPROPRIATE BOX(ES)

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

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

The efforts of the Molecular Mechanisms of Tumor Promotion Section are directed toward understanding the early events in the interaction of phorbol ester tumor promoters with cells and tissues. Particular attention is being devoted to the analysis of the major phorbol ester receptor, protein kinase C. The bryostatins, macrocyclic lactones, activate protein kinase C and compete for phorbol ester binding. They only induce a subset of the typical phorbol ester responses, however. The biochemical mechanisms for these differences are being explored through immunoblotting of protein kinase C, comparison of phosphorylation patterns, tritiated bryostatin binding analysis, and comparison of effects on cloned and chromatographically separated protein kinase C isozymes. Other structural classes of protein kinase C modulators under investigation include 7-beta-meprounic acid 3-p-hydroxybenzoate, cyclic diglycerides, and phorbol derivatives modified in the tiglane ring structure or its functional groups. A second research direction is to identify the targets of irritant but non-promoting phorbol related derivatives. We have shown that resiniferatoxin acts as a selective ultrapotent capsaicin analog. Typical phorbol esters exert their inflammatory activity through both resiniferatoxin-sensitive and insensitive pathways. The latter correlate better with promoting activity. Current efforts are directed at identification and characterization of resiniferatoxin receptors.

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

Peter M. Blumberg	Research Chemist	LCCTP	NCI
M. Dell'Aquila	Staff Fellow	LCCTP	NCI
B. Warren	IRTA Fellow	LCCTP	NCI
L. Schuman	IRTA Fellow	LCCTP	NCI
A. Szallasi	Visiting Fellow	LCCTP	NCI
J. Barchi	Staff Fellow	LMC	NCI
S.H. Yuspa	Chief	LCCTP	NCI
H. Hennings	Research Chemist	LCCTP	NCI
M.C. Willingham	Research Biologist	LMB	NCI
V. Marquez	Research Chemist	LMC	NCI
Z. Szallasi	Visiting Fellow	LCCTP	NCI
F. Joo	Visiting Scientist	LNNS	NINDS

Objectives:

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

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

Methods Employed:

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

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

Major Findings:

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

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

Protein kinase C exists as a family of isozymes. We have expressed the α , β , γ , and δ isozymes in COS cells and isolated the α and β isozymes from mouse brain by hydroxyapatite chromatography. Binding analysis confirms that bryostatin 1 shows only small differences in affinity for these different isozymes.

Human A549 cells, like mouse C3H/10T1/2 cells, show disparate responses to the bryostatins for different endpoints. For example, whereas both bryostatin 1 and phorbol 12,13-dibutyrate inhibit EGF binding, only phorbol 12,13-dibutyrate induces arachidonic acid release. The extent of translocation and the rate of down regulation of protein kinase C in response to the bryostatins was examined in this cell system. Little difference in down regulation was observed. Bryostatin, moreover, induced a similar pattern of protein kinase C translocation to the phorbol esters, although the extent of translocation was less. Current efforts are focused in examining the role of the less well-characterized protein kinase C isozymes, δ , ϵ , and zeta, in bryostatin action.

A second major direction is to characterize novel classes of protein kinase activators to probe the requirements for enzymatic modulation. The triterpene 7-beta-hydroxymaprounic acid 3-p-hydroxybenzoate (MPA), from the Euphorbiaceae Maprounea africana, was found to inhibit phorbol ester binding in a natural products screen. We have now characterized its activity in detail. MPA functions as a potent protein kinase C agonist. In vitro, MPA both inhibited [³H]phorbol 12,13-dibutyrate binding to the enzyme reconstituted in phosphatidylserine:phosphatidylcholine (1:4) and stimulated enzymatic activity. The 50% effective dose was similar for both binding and activation - approximately 3 μg/ml. Furthermore, in C3H10T1/2 cells, MPA (10 μg/ml) stimulated [³H]arachidonic acid release to 80-90% of the level stimulated by phorbol 12,13-dibutyrate. Three characteristics of the action of MPA were in marked contrast to those of the phorbol esters, however. First, the potency of MPA showed little dependence on the lipid environment. Second, the inhibition of phorbol ester binding was time dependent, as was the stimulation of arachidonic acid release in the intact cells. Third, the kinetics of binding inhibition were suggestive of a mixed mechanism. These differences suggest that MPA may interact with protein kinase C in a different fashion than the phorbol esters. Current efforts are focused on determining whether these biochemical differences translate into unique behavior in biological systems.

In collaboration with the Laboratory of Medicinal Chemistry, Division of Cancer Treatment, NCI, cyclic diglycerides are being assessed for their activity on protein kinase C. The strategy is that these derivatives, being rigid molecules, will help define the 3-dimensional requirements for binding. Normal diglycerides, in contrast, are relatively unconstrained.

Structure-activity analysis of the phorbol esters has been largely limited to the variation in naturally produced compounds as a result of the complexity of synthetic chemistry of this class of molecules. Ongoing collaboration with a synthetic chemistry group under Paul Wender, Stanford University's Department of Chemistry, seeks to prepare novel analogs modified in residues, e.g., the 9-OH or 3-carbonyl, whose influence has previously been inaccessible to analysis. Compounds examined include 3 α -OH and 3 β -OH derivatives of phorbol and phorbol 12,13-dibutyrate derivatives with the C-20 hydroxymethyl group modified to make it more analogous to bryostatin.

The third major area of investigation has been to understand the mechanism of resiniferatoxin action. Resiniferatoxin is an extremely irritant diterpene present in the latex of several members of the genus Euphorbia. Its mechanism of action has been shown to be clearly distinct from that of the structurally related phorbol esters. Since resiniferatoxin possesses a 4-hydroxy-3-methoxyphenyl substituent, a key feature of capsaicin, the major pungent ingredient of plants of the genus Capsicum, we examined the ability of resiniferatoxin to induce typical capsaicin responses. We find that treatment of rats with resiniferatoxin, like treatment with capsaicin, caused hypothermia, neurogenic inflammation, and pain. These responses were followed by loss of thermoregulation, by desensitization to neurogenic inflammation, and by chemical and thermal analgesia, with cross-tolerance between

resiniferatoxin and capsaicin. Resiniferatoxin was 3-4 orders of magnitude more potent than capsaicin for the effects on thermoregulation and neurogenic inflammation. Resiniferatoxin was only comparable in potency to capsaicin, however, in the assay for induction of acute pain; and the desensitization to acute pain appeared to require less resiniferatoxin than did desensitization for the other responses. We conclude that resiniferatoxin acts as an ultrapotent capsaicin analog and hypothesize that it may distinguish between subclasses of capsaicin response.

We have examined the duration of desensitization following acute treatment with the maximal tolerated dose of RTX or capsaicin. Desensitization by RTX to neurogenic inflammation began to diminish by 7 days, whereas desensitization to pain and to induction of hypothermia persisted for several weeks. Interestingly, a partial hypothermic response returned within 24 hr if challenge was with RTX at 500-fold its ED_{50} for control animals; the animals, moreover, maintained their ability to thermoregulate in a hot environment. The time course of the morphological changes, ultrastructure and calcium staining, of dorsal root ganglion neurons was examined in parallel. The ultrastructural changes were evident by 4 hr and persisted for the duration of the experiments. Limited calcium staining was visible at 12 and 24 hr after treatment but then diminished. In comparison with capsaicin treatment, RTX caused more long lasting desensitization as well as a distinct spectrum of response.

A homovanillyl group is an essential structural feature of capsaicin and the most prominent feature distinguishing resiniferatoxin from typical phorbol-related compounds. We therefore examined the RTX-like activity of two 20-homovanillyl esters of diterpene derivatives with particular similarities to RTX. The potency of 12-deoxyphorbol 13-phenylacetate 20-homovanillate (dPP-HV) was comparable to RTX for local induction and desensitization of chemical pain but was 2-4 orders of magnitude less potent for the other RTX responses tested (stimulation and desensitization of neurogenic inflammation and hypothermia). Mezerein 20-homovanillate (Mez-HV) displayed very weak activity in pain induction and was inactive in the other assays. The parent derivatives (resiniferonol orthophenylacetate, 12-deoxyphorbol 13-phenylacetate and mezerein) were inactive in inducing RTX-like effects. Reciprocally, the presence of the 20-homovanillate ester reduced binding affinities to protein kinase C by 11-, 130-, and 690-fold for RTX, dPP-HV, and Mez-HV, respectively. Our findings provide further evidence for heterogeneity among capsaicin-sensitive pathways. Most significantly, our results demonstrate that esterification of phorbol-related diterpenes with homovanillic acid can yield capsaicin analogs with unique activities.

Tumor promoting phorbol esters are potent inflammatory agents for mouse skin, and the potential mechanistic role of inflammation in tumor promotion is under active investigation. Resiniferatoxin, a uniquely irritant phorbol-related diterpene, acts as a capsaicin analog to induce and then to block neurogenic inflammation. We have taken advantage of this property to probe the role of neurogenic inflammation in phorbol ester action in mouse skin. We find that pre-treatment of CD-1 mice with resiniferatoxin blocked the early (3 hr)

erythema and edema (6 hr) in response to phorbol 12-myristate 13-acetate (PMA), whereas the edema at later times (12-24 hrs) was only partially blocked. Since the efficiency of resiniferatoxin pretreatment decreased as a function of time if PMA was applied 24, 48 or 96 hr after resiniferatoxin administration, the late edema response to PMA may be a combination of increasing edema of non-neurogenic origin and the recovering neurogenic response due to the decreasing desensitization. For other phorbol esters, 12-deoxyphorbol mono- and diesters, and mezerein, variable proportions of neurogenic and non-neurogenic inflammation were observed as expected from the discrepancies between their inflammatory and tumor promoting activities. PMA-induced skin hyperplasia, unlike edema, was not inhibited by resiniferatoxin pretreatment, arguing that the hyperplasia does not require the early neurogenic inflammatory response. Distinction between inflammatory mechanisms may help to clarify the role of inflammation in tumor promotion.

We find that mouse strains with different sensitivities to phorbol ester induced promotion displayed marked differences in neurogenic edema responses to PMA application. In SENCAR strain, bred for its profound sensitivity to the promotion of skin tumors by phorbol esters, RTX pretreatment had little inhibitory effect: edema response to PMA was fully restored within 6 hr after application, although these mice were desensitized against xylene, a potent inducer of neurogenic inflammation for several days. On the other hand, in C57BL/6J mice, shown to be resistant to promotion by phorbol esters, edema response to PMA was totally eliminated by RTX pretreatment during the first 8 hrs after PMA administration. CD-1 and DBA/2J mice, reported to be susceptible to PMA promotion, responded similarly: edema response was blocked by RTX pretreatment partially during the early phase (up to 8 hrs) of inflammation. We hypothesize that the non-neurogenic part of PMA induced edema might correlate with the sensitivity to promoting action.

Current efforts are focused on demonstration and characterization of specific RTX receptors.

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Patents:

Blumberg PM. U.S. Patent (pending): Use of Resiniferatoxin and Analogs Thereof to Cause Sensory Afferent C-Fiber and Thermoregulatory Desensitization.

Blumberg PM. U.S. Patent (pending): New Class of Compounds Having a Variable Spectrum of Activities for Capsaicin-like Responses and Uses Thereof.

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

PROJECT NUMBER

Z01CP5445-05 CCTP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Molecular Regulation of Epidermal-Specific Differentiation Products

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

PI:	S. H. Yuspa	Chief	LCCTP NCI
Others:	D. Rosenthal -	Biotechnology Fellow	LCCTP NCI
	P. Steinert	Senior Investor	DB NCI
	D. Hohl	Visiting Fellow	DB NCI
	C. A. Huff	Howard Hughes Fellow	LCCTP NCI
	T. Kartasova	Visiting Fellow	LCCTP NCI

COOPERATING UNITS (if any)

Baylor College of Med., Houston, TX (D. Roop); University of Oslo, Oslo, Norway (H. Huitfeldt); German Cancer Center, Heidelberg, FRG (J. Schweizer); University of Michigan, Ann Arbor, MI (J. Voorhees).

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	3.2	PROFESSIONAL:	3.2	OTHER:	none
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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.)

cdNA clones corresponding to the major proteins expressed in mouse epidermis have been isolated and characterized. To identify sequences regulating the expression of these genes, vector constructs using different regions of the genomic clones to drive expression of the chloramphenicol acetyltransferase gene have revealed sequences which are induced to active transcription by specific calcium concentrations. A gene encoding a cysteine-rich protein, which is a major component of the cornified envelope, has been isolated and shown by in situ hybridization experiments to be expressed in the granular layer of the epidermis. Cloned keratin genes, constructed with inducible promoter sequences, have been introduced into epidermal tumor cells and the proteins are expressed. The chronic applications of retinoids on human skin in vivo causes a number of changes in epidermal differentiation markers, including the induction of keratin 6 and keratin 13 and an increased number of cell layers expressing transglutaminase.

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

S. H. Yuspa	Chief	LCCTP	NCI
D. Rosenthal	Biotechnology Fellow	LCCTP	NCI
P. Steinert	Senior Investigator	DB	NCI
D. Hohl	Visiting Fellow	DB	NCI
C. A. Huff	Howard Hughes Fellow	LCCTP	NCI
T. Kartasova	Visiting Fellow	LCCTP	NCI

Objective:

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

Methods Employed:

The isolation of cDNA clones is accomplished by the purification of epidermal mRNA, reverse transcription and cloning of double-stranded cDNA in the plasmid pBR322 or gtl1 expression vectors. The cDNA clones are characterized by hybridization-selection assays and by direct DNA sequence analysis. Genes are isolated by screening genomic libraries with nick-translated cDNAs and characterized by restriction endonuclease digestion and direct DNA sequence analysis. The expression of specific genes is monitored by RNA blot analysis and quantitated by slot-blot analysis. Transcripts for individual genes are detected in histological sections of skin by in situ hybridization with 35S-labeled RNA probes. Monospecific antisera are produced with synthetic peptides corresponding to unique sequences within each protein. The antisera are used to monitor normal and abnormal expression of these polypeptides by immunofluorescent staining, immunoblotting, and immunoprecipitation.

Major Findings:

The production of very specific antibodies with synthetic peptides that permit the detection of individual gene products and the development of an in situ hybridization technique that allows the localization of specific gene transcripts within cells in different layers of the epidermis have revealed the high degree of regulation that exists for various genes expressed at specific differentiation states in the epidermis. A full-length cDNA clone has been identified and shown to encode a precursor of a major component of the cornified envelope. This clone was found to correspond to a 1.3 kb mRNA detectable in the granular layer of normal human epidermis. The amino acid composition for this clone, deduced from the nucleotide sequence, is very similar to the amino acid composition of mature cornified envelopes and appears, therefore, to be the major component of the cornified envelope. The component, termed "loricrin," can be detected in

immunoblots of human epidermal extracts and is highly insoluble. Indirect immunofluorescence demonstrates the protein at the periphery of cells, mostly in the granular layer; additional studies indicate that it is located on the cytoplasmic side of the cell envelope. The identity of the clone has been confirmed by hybrid selection, translation *in vitro* and subsequent immunoprecipitation (studied in collaboration with Drs. Daniel Hohl and Peter Steinert (Dermatology Branch [DB], NCI).

The complete MK1 gene was constructed using three overlapping genomic clones. Three promoters, SV-40 early, mouse metallothionein I (MT-I) and mouse mammary tumor virus (MMTV), were introduced upstream of the MK1 protein coding sequence. These MK1 constructs were introduced into mouse cell lines, NIH 3T3 fibroblasts and SLC-1 carcinoma. MK1 expression in MK1-transfected cells was analyzed by immunofluorescent staining of fixed cells and Western blot analysis. Results showed that expression of the MK1 protein can be detected in NIH 3T3 fibroblasts by immunofluorescence when both constitutive (SV40-early) or inducible (MT-I) promoters drive the transcription of the MK1 gene. The MK1 protein, when expressed in NIH 3T3 fibroblasts, does not form filaments and stains with fluorescent antibodies as bright particles in the cytoplasm. On Western blot, only protein of approximately 50 kD can be detected with MK1 specific antibodies and only after induction with Cd²⁺ ions.

In order to elucidate the mechanism regulating the expression of these genes, members of each subset have been isolated and sequenced. In initial attempts to define sequences which regulate the expression of these genes, synthetic oligonucleotides corresponding to a 5' flanking region of the human differentiation-associated keratin gene (K1) have been inserted into a vector containing the chloramphenicol acetyltransferase gene. These studies have revealed a keratinocyte-specific enhancer; however, additional enhancement of this activity is not achieved by inducing differentiation. This may be due to the absence of a differentiation-specific enhancer in these sequences or to difficulties in inducing terminal differentiation in the cell cultures employed. In an attempt to overcome these problems, transgenic mice have been produced in collaboration with Dr. Su Chung (USUHS). These mice contain the differentiation-associated human keratin gene, HK1, which is flanked by 2 kilobases upstream and 3 kilobases downstream. This DNA fragment contains sufficient sequence information for tissue- and developmental-specific expression. Expression of the HK1 gene was compared with that of the endogenous mouse K1 (MK1) gene in transgenic mice using antibodies specific for each protein. Approximately 20-30% of the basal cells expressed the HK1 gene but not the endogenous MK1 gene. The failure of the HK1 gene to respond to negative factors regulating expression of the MK1 gene was investigated using primary epidermal cell cultures prepared from transgenic mice. As observed *in vivo*, only rare cells expressed the MK1 gene, while approximately 20% expressed the HK1 gene. In addition, in these cells retinoic acid inhibited calcium-induced expression of the MK1 gene but not the HK1 gene. This finding indicates that the 12 kb fragment containing the HK1 gene is lacking the sequences required for retinoic acid to exert inhibitory effects observed for the endogenous MK1 gene (studied in collaboration with Drs. D. Roop, Baylor College of Medicine, and P. Steinert (DB, NCI).

The expression of keratins K1 and K10 is specific to the differentiating suprabasal layers of the epidermis. K1 and K10 are also synthesized by keratinocytes when they are stimulated to differentiate *in vitro* in response to elevated calcium levels in the medium. Using transient transfection assays in primary mouse keratinocytes, we have found that a segment of the human K1 gene located 3' to the protein-coding region confers calcium inducibility and epidermal specificity to the reporter gene coding for chloramphenicol acetyl transferase (CAT). This *cis*-acting regulatory sequence is the first genetic element reported to be either calcium-inducible or epidermal-specific and should therefore prove to be an invaluable tool for the expression of any protein in the differentiating epidermis of intact animals.

The response of cultured human keratinocytes to retinoic acid (RA) was found to be partially reproduced *in vivo*. The effect of RA *in vitro* is to suppress differentiation-specific keratins K1 and K10 and to induce K13, which is normally confined to stratified internal epithelia. Skin biopsies from a clinical trial of RA were used to examine the effects of RA on human keratinocyte differentiation *in vivo*. Indirect immunofluorescence and histological examination showed that the RA can reprogram transglutaminase, K6 and K13 but not K1 and K10, markers of differentiation in intact skin. All of the RA-treated samples showed an increase in the total number of cell layers; 60% revealed a thickening of the stratum granulosum and 50% demonstrated parakeratosis (studied in collaboration with Drs. J. Weiss, Emory Clinic and C. Ellis, J. Voorhees, University of Michigan).

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ANNUAL REPORT OF

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

October 1, 1988 through September 30, 1989

The problem of the isolation, characterization, and biological role of transforming growth factors (TGFs) continues to be the major focus of our laboratory. Previously, we had shown the TGFs can be isolated from a variety of epithelial and mesenchymal tumors of murine, chicken and human origin, caused either by chemicals or viruses, or of spontaneous origin. All of these TGFs are acid stable, low molecular weight peptides. New methods to achieve purification have been developed in our laboratory. The experimental use of TGF-beta in wound healing has been a finding of major importance and has provided a great deal of encouragement to proceed further with the entire problem of the molecular biology and molecular genetics of these growth factors. Finally, we are now involved in a major attempt to integrate studies of retinoids into our current program of studies on peptide growth factors.

The present activities of the Laboratory of Chemoprevention are devoted almost exclusively to studying the chemistry and biology of the various type beta transforming growth factors. By now, 5 different molecular species of TGF-beta have been identified. These studies include the interactions of these peptide growth factors with the genetic apparatus of the cell, particularly oncogenes, as well as their interactions with low molecular weight regulatory agents, such as retinoids and steroids. The Laboratory is involved with the total spectrum of studies that can be done with growth factors, ranging all the way from mutating their chemical structure with the most advanced techniques of recombinant DNA research to evaluating their potential therapeutic usefulness as clinical agents in patients with defective wound healing.

As part of our interest in the roles of the various TGF-Bs in embryology, we have attempted to clone genes related to mammalian TGF-Bs from cDNA libraries of embryonic chicken and frog cells and tissues. This has resulted in the identification of 3 novel forms of TGF-B: TGF-Bs 3 and 4 in the chicken and TGF-B5 in the frog. TGF-Bs 1 and 2 have also been cloned from the chicken library. Comparison of the degree of identity between mammalian TGF-Bs 1, 2 and 3 and their avian counterparts has revealed greater than 99% sequence conservation in the mature, processed 112 amino acid C-terminal portion of the peptides; the TGF-B1's are 100% conserved, whereas there is a single amino acid substitution between chicken and human TGF-Bs 2 and 3.

Examination of the developmental regulation of expression of TGF-Bs 1-4 in chicken embryos has shown that TGF-Bs 2 and 3 are most prominently expressed.

TGF- β 1 and 4 mRNAs are expressed only in cultured chicken embryo chondrocytes and fibroblasts; these mRNAs are not detectable in RNA prepared either from whole embryos or from specific embryonic organs and tissues. In certain tissues, expression of TGF- β 2 and 3 mRNAs are controlled coordinately, while in other tissues, such as heart, expression of TGF- β 2 mRNA is highest at early stages of development, whereas that of TGF- β 3 increases throughout development until hatching.

Thus far, TGF- β 5 has been detected only in Xenopus laevis. Like all of the other TGF- β s, it is approximately 70% homologous to TGF- β s 1-4. It was cloned from an oocyte library, but is expressed most prominently from the neurula stage into adulthood. Interestingly, in the adult, the tissue expressing the highest level of TGF- β 5 was the lung. This will be pursued with in situ hybridization as well as immunohistochemical studies to try to identify the specific cell types expressing high levels of the peptide. Present plans include attempts to clone TGF- β 5 homologues in other species as well as to detect expression of other TGF- β s in Xenopus.

TGF- β s 2 and 5 have also been isolated from medium conditioned by Xenopus XTC cells. Using several steps of HPLC followed by electrophoresis on polyacrylamide gels, we have obtained the N-terminal amino acid sequence of each of these proteins. This represents the first characterization of TGF- β 5 as a protein and has shown that it is secreted in a latent form, like all other TGF- β s, and shares many types of biological activities with other TGF- β s such as inhibition of growth of epithelial cell lines, stimulation of anchorage-independent growth of NRK cells, and inhibition of antibody secretion by human B lymphocytes.

Given that there are 5 different types of TGF- β and that secretion of the different TGF- β types by cells is independently regulated, it has become necessary to develop specific assays for the different TGF- β s. Toward this end, sandwich ELISA assays specific for TGF- β 1 and TGF- β 2 have been developed. These assays quickly and accurately measure each of these two TGF- β s with no interference from non-TGF- β peptides or from other types of TGF- β . They are being used to measure the relative amounts of each of these two TGF- β types in various cells and tissues and to correlate synthesis and secretion of each of these TGF- β s with relative mRNA expression.

Most cells express TGF- β receptors and many cell types secrete TGF- β . As TGF- β has been shown to play a central role in tissue repair and bone remodeling, we have focussed on its effects on specific cells, which participate in these processes such as macrophages, fibroblasts, endothelial cells, immune cells, and osteoblasts. Moreover, since many pathological processes result from aberrant expression of peptides important in related physiological conditions, we have examined the expression of TGF- β in disease processes such as carcinogenesis, and fibroproliferative diseases such as arthritis and proliferative vitreo-retinopathy. Increased expression of TGF- β appears to accompany all of these processes and we are currently attempting to define its specific effects in each of these disease processes.

In vitro experiments have demonstrated that TGF- β 1 expression is regulated by TGF- β 1 itself (autoregulation) and by phorbol ester treatment of cells. Moreover the expression of TGF- β 1 and TGF- β 2 is reciprocally regulated in calcium-induced differentiation of primary keratinocytes. In addition treatment of cells with anti-estrogens and with retinoids has specific effects on TGF- β expression. To understand these effects at a molecular level, we have cloned and sequenced the 5' flanking regions of the TGF- β 1 gene. We have demonstrated that there are two distinct promoter regions of the TGF- β 1 gene, one upstream of the 5'-most transcriptional start site, and another between the two major transcriptional start sites. In contrast to some other genes which have two promoters, the two TGF- β 1 promoters are regulated in a parallel fashion by both TGF- β 1 and by phorbol esters. Each promoter contains TRE-like elements, the binding sites for the transcription factor complex of AP-1/Jun:Fos. By a variety of means we have demonstrated that TRE-elements are critical to both TGF- β 1 autoinduction and induction by phorbol esters. In addition, we have shown that TGF- β 1 induces expression of the c-jun promoter through a TRE-element in that promoter. These experiments begin to explain, at a molecular level, some of the interactions between oncogenes such as fos and jun and growth factors such as TGF- β 1. Moreover, their reciprocal effects on induction of each other's expression suggest that aberrant expression of either, as in carcinogenesis, might lead to increased expression of both.

By raising antibodies to peptides which represent regions of the TGF- β s which are not highly conserved, reagents specific for each TGF- β have been developed. These have been useful for detecting various TGF- β species on Western blots as well as in tissue sections by immunohistochemistry. The use of antibodies to both mature and precursor regions of TGF- β s provide additional evidence that a specific TGF- β is being localized, while affinity purification of antibodies increases sensitivity of the assays and demonstrates specificity.

Immunohistochemical localization of TGF- β s with these antibodies has shown that TGF- β s 1, 2, and 3 are expressed in the developing mouse embryo (with TGF- β 2 being the most abundant), while only TGF- β s 2 and 3 are expressed in the chick embryo. In some instances, such as the mouse heart, all 3 TGF- β s are expressed, while in other organs, such as the developing nervous system, only TGF- β s 2 and 3 are detected. TGF- β s 2 and 3 are also expressed in the nervous system of the chick embryo. The widespread localization of TGF- β s in both mouse and chick embryos suggest that it plays a role in morphogenesis and pattern development.

The localization of TGF- β s in normal adult mouse tissues such as heart, brain and kidney suggest that it plays a role in normal homeostasis of these tissues and we are looking for changes of expression of the TGF- β s in disease states. For example in a rat model of cardiac infarction there is a loss of immunoreactive TGF- β 1 from the infarcted myocytes, but an increase in staining in viable border zone myocytes with corresponding changes in mRNA levels. In a rat model of cardiac hypertrophy there is also an increase in TGF- β 1 mRNA. Alterations of levels of other TGF- β s are being investigated. If similar effects are seen in cultured cardiac myocytes, studies on the mechanism of this increase can be undertaken. Studies in which TGF- β is infused into infarcted hearts to determine its effects on cardiac repair are being initiated.

We have shown that the latent form of TGF-beta secreted by platelets is a 3-component complex in which active TGF-beta is non-covalently complexed with the remainder of its biosynthetic precursor and a third 140 KDa protein. Recombinant constructs also secrete TGF-beta in a latent form but this differs from the native form in that it lacks the 140 KDa component and appears to be undersialylated. In vitro studies indicate that the recombinant and native complexes show similar sensitivity to activation by chaotropic agents, but some of the gross physicochemical properties of the complexes are different, and the implications in terms of in vivo action are being analysed.

Clinically useful antiestrogens have been shown to induce TGF-beta secretion from breast cancer cell lines, and it has been proposed that the clinical efficacy of these agents against tumors may be due, at least in part, to their ability to induce local synthesis of this growth inhibitor. We have shown that antiestrogens will induce TGF-beta synthesis in fibroblasts by a novel mechanism not involving the estrogen receptor. This raises the possibility that antisteroids may induce TGF-beta synthesis in stromal elements of a tumor, and that the TGF-beta might inhibit tumor development in a paracrine manner. We have further shown that a novel synthetic progestin, gestodene, can also induce TGF-beta synthesis specifically in malignant breast cells. Thus, gestodene may have potential as a chemopreventive agent. In both systems, the induction of TGF-beta appears to be predominantly at a post-transcriptional level.

In summary, we are now exploring a very wide range of studies which involve many actions of TGF-B in both health and disease. Particular emphasis is being placed on the connection between TGF- β and the actions of agents that will be used for chemoprevention of cancer, with the goal of developing better agents to prevent the development of cancer by arresting or reversing the further progression of preneoplastic cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05051-11 LC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Biology and Molecular Biology of Transforming Growth Factor-beta

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

PI:	Anita Roberts	Staff Scientist	LC	NCI
OTHERS:	Sonia B. Jakowlew	Sr. Staff Fellow	LC	NCI
	David Danielpour	Guest Researcher	LC	NCI
	Paturu Kondaiah	Visiting Associate	LC	NCI
	Nannette Roche	Biologist	LC	NCI
	Pamela J. Dillard	Chemist	LC	NCI
	Kyung-Young Kim	Special Volunteer	LC	NCI

COOPERATING UNITS (if any)

Wilmer Ophthalmological Institute, Johns Hopkins University, Baltimore (Bert Glaser) Laboratory of Molecular Genetics, NICHD (Igor Dawid)

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

7

PROFESSIONAL:

4.0

OTHER:

3.0

CHECK APPROPRIATE BOX(ES)

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

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

The purpose of this project is to attempt to define the entire set of TGF-B's in terms of the chemistry, biology, and molecular biology of each of the component members and to try to understand species-specific mechanisms of selective expression of the various TGF-B's. With regard to the chemistry and molecular biology, the emphasis has been on comparison of amino acid and nucleotide homologies both between the different TGF-B's and between any particular TGF-B in different species including various mammals, as well as avian and amphibian species. Such comparisons identify certain invariant regions common to all TGF-B's which may be necessary for structural features as might be required in the fit of precursor and processed peptides in the latent form of TGF-B, or as might be necessary for receptor binding. The TGF-B family has now been expanded to 5 distinct peptides; the mature forms of these TGF-B's are each approximately 68 to 80 % homologous to each other. TGF-B's 3 and 4 have been cloned from a chicken embryo chondrocyte cDNA library and TGF-B5 has been cloned from a Xenopus oocyte cDNA library. In addition, TGF-B's 2 and 5 have been purified, sequenced, and characterized biologically from medium conditioned by Xenopus tadpole XTC cells. Analysis of the expression of the mRNAs corresponding to the various TGF-B's in embryos suggests not only that expression of each of the different TGF-B's is regulated independently, but also that expression of any particular TGF-B is regulated differently in different species. These studies are being correlated with investigations based on immunohistochemical staining and in situ hybridization to identify tissue and cell-specific localization.

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

Anita B. Roberts	Staff Scientist	LC	NCI
Sonia B. Jakowlew	Sr. Staff Fellow	LC	NCI
David Danielpour	Guest Researcher	LC	NCI
Paturu Kondaiah	Visiting Scientist	LC	NCI
Nanette B. Roche	Biologist	LC	NCI
Pamela J. Dillard	Chemist	LC	NCI
Kyung-Young Kim	Special Volunteer	LC	NCI

Objectives:

This project is directed toward three goals: 1) identifying new members of the TGF- β family and characterizing their specific patterns of expression; 2) determining the range of biological activities of the 5 known TGF- β 's, with particular emphasis on identifying unique activities of the various TGF- β 's, and 3) identifying specific pathological processes in which the TGF- β 's might play a role. As these peptides all play critical roles in embryogenesis, the search for new forms of TGF- β has focussed on embryonic cDNA libraries. Specific biological functions of the various TGF- β 's are also being investigated in embryos with special emphasis on embryonic induction.

Methods Employed:

Standard methods are utilized based on use of specific reagents such as iodinated TGF- β , antibodies to TGF- β , and cDNA probes for the various TGF- β 's. In addition, polymerase chain reaction (PCR) methodology is being employed both for the cloning of homologs of the various TGF- β 's in other species and for attempts to identify new members of the TGF- β family.

Major Findings:

As part of our interest in the roles of the various TGF- β 's in embryology, we have attempted to clone from cDNA libraries of embryonic chicken and frog cells and tissues genes related to mammalian TGF- β 's. This has resulted in the identification of 3 novel forms of TGF- β : TGF- β 's 3 and 4 in the chicken and TGF- β 5 in the frog. TGF- β 's 1 and 2 have also been cloned from the chicken library. Comparison of the degree of identity between the mammalian TGF- β 's 1, 2, and 3 and their avian counterparts has revealed greater than 99% sequence conservation in the mature, processed 112 amino acid C-terminal portion of the peptides; the TGF- β 1's are 100% conserved, whereas there is a single amino acid substitution between chicken and human TGF- β 's 2 and 3.

Examination of the developmental regulation of expression of TGF- β 's 1-4 in chicken embryos has shown that TGF- β 's 2 and 3 are most prominently expressed.

TGF- β 1 and 4 mRNAs are expressed only in cultured chicken embryo chondrocytes and fibroblasts; the levels of these mRNAs are not detectable in RNA prepared either from whole embryos or from specific embryonic organs and tissues. In certain tissues, expression of TGF- β 2 and 3 mRNAs are controlled coordinately, while in other tissues, such as heart, expression of TGF- β 2 mRNA is highest at early stages of development, whereas that of TGF- β 3 increases throughout development until hatching.

Thus far, TGF- β 5 has been detected only in Xenopus laevis. Like all of the other TGF- β 's, it is approximately 70% homologous to TGF- β 's 1-4. It was cloned from an oocyte library, but is expressed most prominently from the neurula stage into adulthood. Interestingly, in the adult, the tissue expressing the highest level of TGF- β 5 was the lung. This will be pursued with in situ hybridization as well as immunohistochemical studies to try to identify the specific cell types expressing high levels of the peptide. Present plans include attempts to clone TGF- β 5 homologues in other species as well as to detect expression of other TGF- β 's in Xenopus.

TGF- β 's 2 and 5 have also been isolated from medium conditioned by Xenopus XTC cells. Using several steps of HPLC followed by electrophoresis on polyacrylamide gels and transfer to membranes, we have obtained the N-terminal amino acid sequence of each of these proteins. This represents the first characterization of TGF- β 5 as a protein and has shown that it is secreted in a latent form, like all other TGF- β 's, and shares many types of biological activities with other TGF- β 's such as inhibition of growth of epithelial cell lines, stimulation of anchorage-independent growth of NRK cells, and inhibition of antibody secretion by human B lymphocytes.

Given that there are 5 different types of TGF- β and that secretion of the different TGF- β types by cells is independently regulated, it has become necessary to develop specific assays for the different TGF- β 's. Toward this end, sandwich ELISA assays specific for TGF- β 1 and TGF- β 2 have been developed. These assays quickly and accurately measure each of these two TGF- β 's with no interference from non-TGF- β peptides or from other types of TGF- β . They are being used to measure the relative amounts of each of these two TGF- β types in various cells and tissues and to correlate synthesis and secretion of each of these TGF- β 's with relative mRNA expression.

TGF- β and Disease

Most all cells express TGF- β receptors and many cell types secrete TGF- β . As TGF- β has been shown to play a central role in tissue repair and bone remodeling, we have focussed on its effects on specific cells which participate in these processes such as macrophages, fibroblasts, endothelial cells, immune cells, and osteoblasts. Moreover, since many pathological processes result from aberrant expression of peptides important in related physiological conditions, we have examined the expression of TGF- β in disease processes such as carcinogenesis, and fibroproliferative diseases such as arthritis and proliferative vitreo

retinopathy. Increased expression of TGF- β appears to accompany all of these processes and we are currently attempting to define its specific effects in each of these disease processes.

Publications:

- Carrington JL, Roberts AB, Flanders KC, Roche NS, Reddi HA. Localization and expression of transforming growth factor-beta during endochondral bone and marrow development. *J Cell Biol* 1988;107:1969-75.
- Connor TB, Roberts AB, Sporn MB, Danielpour D, Dart LL, Michels, RG, De Bustros S, Enger C, Glaser BM. Correlation of fibrosis and transforming growth factor-beta type 2 levels in the eye. *J Clin Invest* 1989;83:1661-6.
- Danielpour D, Dart LL, Flanders KC, Roberts AB, Sporn MB. Immunodetection and quantitation of the two forms of transforming growth factor-beta (TGF-beta 1 and TGF-beta 2) secreted by cells in culture. *J Cell Physiol* 1989;138:79-86.
- Flanders KC, Thompson NL, Cissel DS, Ellingsworth LR, Roberts AB, Sporn MB. Epitope-dependent immunohistochemical localization of transforming growth factor-beta. *J Cell Biol* 1989;108:653-60.
- Gabrielson EW, Gerwin BI, Harris CC, Roberts AB, Sporn MB, and Lechner JF. Stimulation of DNA synthesis in cultured human mesothelial cells by specific growth factors. *FASEB J* 1988;2:2717-21.
- Hannan RL, Kourembanas S, Flanders KC, Rogelj SJ, Roberts AB, Faller DV, Klagsburn M. Endothelial cells synthesize basic fibroblast growth factor and transforming growth factor-beta. *Growth Factors* 1989;1:7-17.
- Jakowlew S, Dillard PJ, Kondaiah P, Sporn MB, Roberts AB. Complementary deoxyribonucleic acid cloning of a novel transforming growth factor-beta mRNA from cultured primary chick embryo chondrocytes. *Molec Endocrinol* 1988;2:747-55.
- Jakowlew SB, Dillard PJ, Sporn MB, Roberts AB. Complementary deoxyribonucleic acid cloning of a mRNA encoding transforming growth factor-beta 4 from chick embryo chondrocytes. *Molec Endocrinol* 1988;2:1186-95.
- Jakowlew S, Dillard PJ, Sporn MB, Roberts AB. Nucleotide sequence of chicken transforming growth factor-beta 1(TGF- β 1). *Nucleic Acids Res* 1988; 16:8730.
- Kondaiah P, Van Obberghen-Schilling E, Ludwig R, Dhar R, Sporn MB, Roberts AB. cDNA cloning of porcine TGF-beta: evidence for alternate splicing. *J Biol Chem* 1988;263:18313-7.
- Lafyatis R, Remmers EF, Roberts AB, Yocum DE, Sporn MB, Wilder RL. Anchorage-independent growth of synoviocytes from arthritic and normal joints: stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. *J Clin Invest* 1989;83:1267-76.
- Morales TI, Roberts AB. TGF-beta regulates the metabolism of proteoglycans in bovine cartilage organ cultures. *J Biol Chem* 1988;263:12828-31.

Roberts AB, Flanders KC, Kondaiah P, Thompson NL, Van Obberghen-Schilling E, Wakefield L, Rossi P, de Crombrughe B, Heine U, Sporn MB. Transforming growth factor beta: biochemistry and roles in embryogenesis, tissue repair and remodeling, and carcinogenesis. *Recent Prog in Horm Res* 1988;44:157-97.

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Roberts AB, Sporn MB. Transforming growth factor-betas: a large family of multifunctional regulatory proteins. *J Anim Sci* 1988;66:67-75.

Smiddy WE, Glaser BM, Green W, Connor TB, Roberts AB, Lucas R, Sporn MB. Transforming growth factor beta: a biological chorioretinal glue. *Arch Ophthalmol* 1989;107:577-80.

Thompson NL, Flanders KC, Smith JM, Ellingsworth LR, Roberts AB, Sporn MB. Cell type specific expression of transforming growth factor beta-1 in adult and neonatal mouse tissue. *J Cell Biol* 1989;108:661-9.

Wahl SM, Hunt DA, Wong HJ, Dougherty S, McCartney-Francis N, Wahl LM, Ellingsworth L, Schmidt JA, Hall G, Roberts AB, Sporn MB. Transforming growth factor beta is a potent immunosuppressive agent which inhibits interleukin 1-dependent lymphocyte proliferation. *J Immunol* 1988;140:3026-32.

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

PROJECT NUMBER
 Z01CP05396-06 LC

PERIOD COVERED
 October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
 Development of Methods to Study the Functions of TGF-beta

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

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

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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.)

The goal of this project is to dissect the genes involved in the signal transduction pathways of TGF- β leading to inhibition of growth. Since insertional mutagenesis with a retrovirus is not an efficient way to isolate mutant cells, the chemical mutagen, nitrosoguanidine, was used to mutagenize mink lung cells. Mutant cells which have been isolated are resistant to inhibition of growth by TGF- β . Work has begun on construction of cDNA libraries from modified CDM-8 cells which are sensitive to inhibition by TGF- β . This will facilitate the isolation of revertants which are sensitive to inhibition by TGF- β by using a BrdU-Hoechst-visible light treatment. These cell lines will be used to identify genes involved in signal transduction by TGF- β . Another way to identify genes involved in signal transduction is also being developed. Since mutagenized cells are mixtures of TGF- β sensitive and resistant cells, the BrdU-Hoechst-visible light selection method was used to eliminate cells which are resistant to inhibition by TGF- β . The cells which are sensitive to growth inhibition by TGF- β are being treated with the retrovirus containing the suppressor gene from *E. coli* by cloning in the lambda vector. These studies will increase our understanding of the mechanisms of signal transduction of TGF- β leading to growth inhibition.

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

Shinichi Watanabe	Staff Fellow	LC	NCI
J. Michael Thomas	Biologist	LC	NCI

Objectives:

Since TGF- β inhibits the growth of certain cells, it is possible to isolate mutant cells which are resistant to the effect. This represents a unique opportunity to study signal transduction of the growth factor at a molecular level. Thus far, nothing is known regarding the identity of genes involved in the process of signal transduction by TGF- β . The objectives are to isolate mutant cells which are resistant to TGF- β inhibition and to use these cells for gene transfer experiments to identify genes involved in signal transduction. Another project involves the expression of TGF- β 3 in animal cells to produce a biologically active protein. This will demonstrate that TGF- β 3 which was identified by DNA hybridization only is indeed closely related functionally to the other TGF- β peptides.

Methods Employed:

Although standard molecular biological techniques have been used throughout, some unique techniques should be mentioned. Cells are mutagenized at high nitrosoguanidine concentrations 3 to 4 times (every 10 days) to make sure cells are haploid with respect to active genes. Back selection for TGF- β sensitive cells is done by growing the cells in bromodeoxyuridine for two cell cycles and allowing additional growth in the presence of Hoechst dye before visible light treatment. This additional Hoechst dye treatment increases the sensitivity of the cells to visible light about 10,000-fold. Also, a retrovirus carrying bacterial suppressor gene is being used for insertional mutagenesis. This will allow cloning the gene near the insertional site by the presence of the sup gene. For expression of TGF- β 3, the polymerase chain reaction was used to modify both the 5' and 3' ends to make sure that only the coding region of TGF- β 3 is inserted in the SV40-based vector. This treatment allowed use of the SV40 promoter and SV40 polyadenylation sites for expression of TGF- β 3 and permitted elimination of the inhibitory effect of the high GC content region of the TGF- β 3 gene.

Major Findings:

Nitrosoguanidine was used to mutagenize mink lung cells for isolating mutant cells which are resistant to TGF- β induced growth inhibition. This process was repeated several times to ensure the high recovery of mutant cells. As expected, the efficiency of mutation was one resistant cell in about 300,000. This high efficiency isolation of mutant cells which are resistant to the inhibitory effects of TGF- β allowed insertional mutagenesis to be performed after the TGF- β resistant cells were killed by BrdU-Hoechst-visible light treatment. This produced TGF- β resistant cells. Other technical findings are that mink

lung cells are very sensitive to the electroporation used for gene transfer experiments. About 5% of surviving cells could be transformed by a given gene. Also, the method for isolating revertant cells is very efficient. It requires that cells use bromodeoxyuridine for DNA synthesis and Hoechst dye sensitization of cells to visible light to kill TGF- β resistant cells. TGF- β 3 protein was made from mouse cells at about 100 ng/ml after cell cloning. The peptide was biologically active in the assays for inhibition of growth of mink lung cells and for formation of colonies of NRK cells in soft agar in the presence of EGF. The peptide was also recognized by an antibody raised to a peptide of TGF- β 3.

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

PROJECT NUMBER

Z01CP05398-06 LC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Characterization of Latent Forms of TGF-beta

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

PI:	Lalage M. Wakefield	Visiting Associate	LC	NCI
OTHERS:	Peter Brown	Staff Fellow	LC	NCI
	Christine Naugle	Special Volunteer	LC	NCI

COOPERATING UNITS (if any)

Genentech, Inc., San Francisco, Dr. Arthur Levinson;
 Rayne Institute, London, England, Dr. Anthony Colletta

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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)

Transforming growth factor-beta (TGF-beta) is a multi-functional peptide that regulates growth and differentiation of a wide variety of cell types. The purpose of this project is to determine the role that the endogenously-produced TGF-beta may play in the control of growth of normal and transformed cells, and to study the regulation of TGF-beta action in this context. Normal and transformed cells have been shown to secrete TGF-beta in a biologically inactive form, and we expect that activation of this latent form will be a critical regulatory step in TGF-beta action. We have characterized the latent form of TGF-beta released by platelets as a three-component noncovalent complex, and have demonstrated that recombinant TGF-beta is also synthesized in a latent form that differs from the native latent form in having only two protein components. The purified recombinant latent form is being used to study the biological activities of latent TGF-beta, the *in vivo* activation mechanism and the *in vivo* pharmacokinetics of the latent form. It is anticipated that this will be the clinically useful form of the molecule. We have also shown that certain pharmacological agents in the steroid hormone superfamily will induce secretion of TGF-beta from target cells in its biologically active form; this finding has important implications for the chemoprevention and treatment of human cancers.

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

Lalage M. Wakefield	Visiting Associate	LC	NCI
Peter Brown	Staff Fellow	LC	NCI
Christine Naugle	Special Volunteer	LC	NCI

Objectives:

The purpose of this project is to examine the role of TGF-beta in the control of normal cell growth and in the process of malignant transformation. The mechanism of action of this growth factor is being studied at a biochemical level. Particular emphasis is given to the activation of the latent forms of TGF-beta, and the induction of TGF-beta in normal and malignant tissues by steroid hormones and their antagonists, with a view to the development of novel agents for the prevention or treatment of cancer.

Methods Employed:

Latent forms of TGF-beta are being purified by fast liquid chromatography, using HIC, IMAC, ion exchange and gel filtration. TGF-beta activity is quantitated in a specific radioreceptor assay using iodinated ligand or by sensitive bioassay. Immunoprecipitation, Western blots, ELISA assays, native and denaturing electrophoresis are used to characterize the latent complexes.

Major Findings:Characterization of latent forms of TGF-beta

We have shown that the latent form of TGF-beta secreted by platelets is a 3-component complex in which active TGF-beta is non-covalently complexed with the remainder of its biosynthetic precursor and a third 140 KDa protein. Recombinant constructs also secrete TGF-beta in a latent form but this differs from the native form in that it lacks the 140 KDa component and appears to be undersialylated. *In vitro* studies indicate that the recombinant and native complexes show similar sensitivity to activation by chaotropic agents, but some of the gross physicochemical properties of the complexes are different, and the implications in terms of *in vivo* action are being analyzed. *In vivo*, the recombinant latent complex is cleared more rapidly than the active form of TGF-beta, suggesting that the recombinant complex may not be a suitable form for systemic delivery of TGF-beta in a clinical setting.

Induction of TGF-beta by Steroids and their Antagonists

Clinically useful antiestrogens have been shown to induce TGF-beta secretion from breast cancer cell lines, and it has been proposed that the clinical efficacy of these agents against tumors may be due, at least in part, to their ability to

induce local synthesis of this growth inhibitor. We have shown that antiestrogens will induce TGF-beta synthesis in fibroblasts by a novel mechanism not involving the estrogen receptor. This raises the possibility that antisteroids may induce TGF-beta synthesis in stromal elements of a tumor, and that the TGF-beta might inhibit tumor development in a paracrine manner. We have further shown that a novel synthetic progestin, gestodene, can also induce TGF-beta synthesis specifically in malignant breast cells. Thus, gestodene may have potential as a chemopreventive agent. In both systems, the induction of TGF-beta appears to be predominantly at a post-transcriptional level.

Publications:

Wakefield LM, Smith DM, Broz S, Jackson M, Levinson A, Sporn MB. Recombinant transforming growth factor-beta1 is synthesized as a two-component latent complex that shares some structural features with the native platelet latent TGF-beta complex. *Growth Factors* 1989;1:203-19.

Wakefield LM, Smith DM, Flanders KC, Sporn MB. Latent transforming growth factor-beta from human platelets: a high molecular weight complex containing precursor sequences. *J Biol Chem* 1988;263:7646-54.

Wakefield LM, Thompson NT, Flanders KC, O'Conner-McCount MD, Sporn MB. Transforming growth factor-beta: multifunctional regulator of cell growth and phenotype. *Ann NY Acad Sci* 1988;551:290-98.

Wakefield LM, Thompson NT, O'Conner-McCourt MD, Sporn MB. Transforming growth factor-beta: an endogenous inhibitor of cell growth. In: Maggi M, Johnston CA, eds. *Horizons in endocrinology*, Serono Symposia Publications. New York: Raven Press, 1988;52:21-9.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05550-02 LC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Immunohistochemical Localization of Transforming Growth Factor-beta

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

PI:	Kathleen C. Flanders	Sr. Staff Fellow	LC	NCI
OTHERS:	Ursula I. Heine	Staff Scientist	LCC	NCI
	Thomas Winokur	Sr. Staff Fellow	LC	NCI
	Bryan McCune	Biotechnology Fellow	LC	NCI
	David S. Cissel	Biologist	LC	NCI
	Larry Mullen	Animal Technician	LC	NCI

COOPERATING UNITS (if any)

NHLBI, Bethesda, Maryland (Ward Casscells)

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

6.0

PROFESSIONAL:

4.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

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

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

Most cells in culture both produce and respond to transforming growth factor- β (TGF- β), but little is known about the in vivo expression and action of this multifunctional peptide. The situation is further complicated by the recent identification of 5 different TGF- β s, all of which are approximately 70% homologous. Subtle differences in biological actions of these TGF- β s are beginning to be found. To investigate differences in expression we have raised polyclonal antibodies in rabbits to peptides corresponding to regions of the different TGF- β s. Peptides from areas of the mature protein as well as the precursor regions were used. All antibodies were tested in radioimmunoassays, enzyme-linked immunosorbent assays and Western blots to determine specificity and only antibodies which reacted with the specific TGF- β were used in immunohistochemical localization studies. In the mouse embryo TGF- β s 1, 2, and 3 have all been localized to developing organ systems, with specific expression of TGF- β s 2 and 3 in the nervous system. In the chick embryo TGF- β s 2 and 3 are predominantly expressed. In the mouse TGF- β 1 expression continues into adulthood and the expression of TGF- β s 2 and 3 in the adult are being examined. The role of TGF- β in pathological conditions is also being investigated. Changes in expression of TGF- β s in cardiac ischemia and hypertrophy have been found and the mechanisms of these changes are being examined in vitro. The efficacy of TGF- β as an agent to aid in healing of cardiac infarcts is also being investigated. We hope to perform similar studies to examine the role of TGF- β s 2 and 3 in brain ischemia. The expression of TGF- β s in breast and prostate carcinomas in human and animal models is being studied at the mRNA and protein levels, including tumors which were treated with retinoids or steroid hormones.

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

Kathleen C. Flanders	Sr. Staff Fellow	LC	NCI
Ursula I. Heine	Staff Scientist	LCC	NCI
Thomas Winokur	Sr. Staff Fellow	LC	NCI
Bryan McCune	Biotechnology Fellow	LC	NCI
David S. Cissel	Biologist	LC	NCI
Larry Mullen	Animal Technician	LC	NCI

Objectives:

The purpose of this project is to examine the roles of the various transforming growth factor-betas (TGF-Bs) in embryonic development, the maintenance of normal growth homeostasis, the pathogenesis of disease states, and the repair of several types of wounds. We are interested in differential expression of the TGF-Bs which might suggest unique biological functions for different TGF-Bs or suggest other factors that are involved in controlling this differential expression. To accomplish this we have raised antibodies to peptides corresponding to sequences of the mature and precursor regions of TGF-Bs 1, 2, 3, and 4 which each appear to recognize one specific TGF-B. Immunohistochemical studies with these antibodies have shown which TGF-Bs are most important in the development of the mouse and chick embryo. Localization of specific TGF-Bs in adult tissues provides a baseline for comparative studies of pathological disease states and suggests which form of TGF-B might be most effective in models of wound healing. Cell and organ culture systems are being developed to investigate the mechanisms of TGF-B action in these systems.

Methods Employed:

Polyclonal antisera were raised in rabbits to peptides corresponding to various regions of the TGF-Bs. Each antiserum was characterized for specificity by enzyme-linked immunosorbent assay, radioimmunoassay, and Western blots. Immunohistochemical staining was done with antibodies affinity purified against the immunizing peptide on paraffin-embedded tissues using the avidin-biotin peroxidase technique. Probes specific for each TGF-B are being tested in in situ hybridization experiments to enable correlation of tissue mRNA and protein levels. Immunoelectron-microscopy is being developed to allow subcellular localization of TGF-B in cultured cells and tissues.

Major Findings:Characterization of TGF-B Antibodies

By raising antibodies to peptides which represent regions of the TGF-Bs which are not highly conserved, reagents specific for each TGF-B have been developed. These have been useful for detecting various TGF-B species on Western blots as well as in tissue sections by immunohistochemistry. The use of antibodies to

both mature and precursor regions of TGF-Bs provide additional evidence that a specific TGF-B is being localized, while affinity purification of antibodies increases sensitivity of the assays and demonstrates specificity.

Role of TGF-Bs in Development

Immunohistochemical localization of TGF-Bs with these antibodies has shown that TGF-Bs 1, 2, and 3 are expressed in the developing mouse embryo (with TGF-B 2 being the most abundant), while only TGF-Bs 2 and 3 are expressed in the chick embryo. In some instances, such as the mouse heart, all 3 TGF-Bs are expressed, while in other organs, such as the developing nervous system, only TGF-Bs 2 and 3 are detected. TGF-Bs 2 and 3 are also expressed in the nervous system of the chick embryo. The widespread localization of TGF-Bs in both mouse and chick embryos suggest that it plays a role in morphogenesis and pattern development.

Role of TGF-Bs in Disease States

The localization of TGF-Bs in normal adult mouse tissues such as heart, brain and kidney suggest that it plays a role in normal homeostasis of these tissues and we are looking for changes of expression of the TGF-Bs in disease states. For example, in a rat model of cardiac infarction there is a loss of immunoreactive TGF-B1 from the infarcted myocytes, but an increase in staining in viable border zone myocytes with corresponding changes in mRNA levels. In a rat model of cardiac hypertrophy there is also an increase in TGF-B1 mRNA. Alterations of levels of other TGF-Bs are being investigated. If similar effects are seen in cultured cardiac myocytes, studies on the mechanism of this increase can be undertaken. Studies in which TGF-B is infused into infarcts to determine its effects on wound healing are being initiated. The immunohistochemical localization of TGF-Bs in cyclosporin-induced kidney fibrosis and brain ischemia are also beginning.

The expression of TGF-Bs in breast and prostate carcinomas in humans and animal models is also being studied at the protein and mRNA levels. Infiltrating lymphocytes show expression of TGF-B1 and there may be some increased expression in the breast carcinomas. The effects of retinoid and steroid hormone treatments of these tumors on TGF-B expression will also be investigated.

Publications:

Danielpour D, Dart L, Flanders KC, Roberts AB, Sporn MB. Immunodetection and quantitation of the two forms of transforming growth factor- β (TGF-B1 and TGF-B2) secreted by cells in culture. *J Cell Physiol* 1989;138:79-86.

Carrington JL, Roberts AB, Flanders KC, Roche NS, Reddi AH. Changes in accumulation, localization and expression of message for transforming growth factor-beta during endochondral bone and marrow development. *J Cell Biol* 1988;107:1969-75.

Flanders KC, Thompson NL, Cissel DS, Ellingsworth LR, Sporn MB, Roberts AB. Epitope-dependent immunohistochemical localization of transforming growth factor-beta. *J Cell Biol* 1989;108:653-60.

Hannan RL, Kourembanas S, Flanders KC, Rogelj SJ, Roberts AB, Faller DV, Klagsbrun M. Endothelial cells synthesize basic fibroblast growth factor and transforming growth factor beta. *Growth Factors* 1989;1:7-17.

Roberts AB, Flanders KC, Kondaiah P, Thompson NL, Van Obberghen-Schilling E, Wakefield L, Rossi P, de Crombrugge B, Heine U, Sporn MB. Transforming growth factor beta: biochemistry and roles in embryogenesis, tissue repair and remodeling, and carcinogenesis. *Recent Prog Horm Res* 1988;44:157-97.

Roberts AB, Thompson NL, Flanders KC, Wilder RL, Sporn MB. Transforming growth factor-beta: possible roles in carcinogenesis. *Brit J Cancer* 1988;57: 594-600.

Thompson NL, Bazoberry F, Speir EH, Casscells W, Ferrans VJ, Flanders KC, Kondaiah P, Geiser AG, Sporn MB. Transforming growth factor- β 1 in acute myocardial infarction in rats. *Growth Factors* 1989;1:91-9.

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

PROJECT NUMBER

Z01CP05617-01 LC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Transcriptional Control of TGF-beta Genes

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:	Adam B. Glick	NRSA Fellow	LC	NCI
	Seong-Jin Kim	Visiting Fellow	LC	NCI
	Takafumi Noma	Visiting Fellow	LC	NCI
	Fabienne Denhez	Visiting Fellow	LC	NCI
	Su Wen Qian	Visiting Fellow	LC	NCI
	Andrew Geiser	IRTA	LC	NCI
	Jeanne Miller	Special Volunteer	LC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

7.2

PROFESSIONAL

7.0

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.)

The purpose of this project is to gain insight into the molecular mechanisms of transcriptional control of TGF- β expression by cloning and subsequent analysis of the promoter regions of the different TGF- β genes. This is a new project. Thus far, only the TGF- β 1 promoter has been cloned and characterized; however, the 5' flanking regions of the human TGF- β 2 and TGF- β 3 genes have recently been cloned and their characterization should soon follow. This will facilitate molecular analysis of differentially regulated expression of TGF- β 's 1 and 2 as in induction of differentiation of keratinocytes. Analysis of the promoter activity of the human TGF- β 1 gene by assessment of the activity of transfected chimeric constructs of regions of the TGF- β 1 promoter linked to the bacterial gene encoding chloramphenicol acetyltransferase has shown that there are two distinct regions with promoter activity: one 5' of the first transcriptional start site at -453 to -323 and another between the two major transcriptional start sites. The 5' flanking region of the gene has neither a CAAT nor a TATA box but rather a region of many CCGCC repeats, some of which bind the transcription factor Sp1. Deletion analysis of both the first and second promoters demonstrates that the major sites required for autoinduction of TGF- β 1 gene activity or for induction by phorbol esters are TRE-like elements which bind the transcription factor complex AP-1/Jun:Fos. Further analysis of the promoter has shown that there are negative regulatory regions as well as enhancer-like elements, one of which is located in a highly conserved region of the first intron of the TGF- β 1 gene.

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

Anita B. Roberts	Staff Scientist	LC	NCI
Adam Glick	NRSA Fellow	LC	NCI
Seong-Jin Kim	Visiting Fellow	LC	NCI
Takafumi Noma	Visiting Fellow	LC	NCI
Fabienne Denhez	Visiting Fellow	LC	NCI
Su Wen Qian	Visiting Fellow	LC	NCI
Jeanne Miller	Special Volunteer	LC	NCI
Andrew Geiser	IRTA	LC	NCI

Objectives:

The object of this project is to clone and characterize the respective promoter elements from each of the 5 different TGF- β 's so that we can investigate the molecular mechanisms of differential control of expression of each of these TGF- β genes in response to a variety of treatments such as differentiating agents, glucocorticoids, retinoids, tumor promoters, etc. We also hope to examine the differences between the promoter of any one TGF- β , such as TGF- β 1, in different species, as this should provide insight into observations such as the high level of expression of TGF- β 1 in mouse embryos compared to its very low level of expression in chicken embryos.

Methods Employed:

Methods are those basic to molecular biology including cloning, dideoxy sequencing, S1 protection, and primer extension as well as analysis of expression of chimeric CAT constructions and methods basic to characterizing specific protein binding sites on DNA such as gel retardation assays and DNase1 footprinting analysis.

Major Findings:

In vitro experiments have demonstrated that TGF- β 1 expression is regulated by TGF- β 1 itself (autoregulation) and by phorbol ester treatment of cells. Moreover, the expression of TGF- β 1 and TGF- β 2 is reciprocally regulated in calcium-induced differentiation of primary mouse keratinocytes. In addition treatment of cells with anti-estrogens and with retinoids has specific effects on TGF- β expression. To understand these effects at a molecular level, we have cloned and sequenced the 5' flanking regions of the TGF- β 1 gene, made chimeric constructs of portions of this region linked to expression of the bacterial gene, chloramphenicol acetyltransferase, and analyzed for expression of these constructs after transfection into cells treated in a variety of ways.

These experiments have demonstrated that there are two distinct promoter regions of the TGF- β 1 gene, one upstream of the 5'-most transcriptional start site at -453 to -323, and another between the two major transcriptional start sites. In

contrast to some other genes which have two promoters, the two TGF- β 1 promoters are regulated in a parallel fashion by both TGF- β 1 and by phorbol esters. Each promoter contains TRE-like elements, the binding sites for the transcription factor complex of AP-1/Jun:Fos. By a variety of means including analysis of deletion constructs of the promoters, transactivation experiments, blocking of induction upon cotransfection with anti-sense jun or anti-sense fos, gel retardation, as well as DNase 1 footprinting with partially purified AP-1, we have demonstrated that the TRE-elements are critical to both TGF- β 1 autoinduction and induction by phorbol esters. In addition, we have shown that TGF- β 1 induces expression of the c-jun promoter through a TRE-element in that promoter.

These experiments begin to explain, at a molecular level, some of the interactions between oncogenes such as fos and jun and growth factors such as TGF- β 1. Moreover, their reciprocal effects on induction of each other's expression suggest that aberrant expression of either, as in carcinogenesis, might lead to increased expression of both.

Our future plans include investigation of possible effects of TGF- β on post-translational modifications of transcription factors. In addition, we hope to carry out analyses similar to those just described for TGF- β 1 to characterize the promoter regions of TGF- β 's 2 and 3 to begin to unravel the mechanisms of differential control of expression of these genes. This analysis will also focus on characterization of negative control elements as well as enhancer sequences in these promoters.

Publications:

Kim SJ, Glick A, Sporn MB, Roberts AB. Characterization of the promoter region of the human transforming growth factor- β 1 gene. *J Biol Chem* 1989;264:402-8.

Kim SJ, Jeang K-T, Glick A, Sporn MB, Roberts AB. Promoter sequences of the human TGF- β 1 gene responsive to TGF- β 1 autoinduction. *J Biol Chem* 1989;264:7041-5.

ANNUAL REPORT OF

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

October 1, 1988, through September 30, 1989

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.

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

Identification of specific transforming genes (oncogenes) in both human and experimental tumors has stimulated great interest and intensive efforts in many laboratories to clarify the roles of those genes in the pathogenesis of cancer. As many of the known oncogenes derive from normal elements of the mammalian genome, there is a real possibility that the biochemical mechanisms of neoplastic transformation may be definable by thorough analysis of the properties of the oncogenes and their cellular homologs. Of special interest in chemical carcinogenesis are oncogenes that behave as dominant genetic elements and that are activated to this behavior either by single-base mutations, such as the *ras* family of genes. This mechanism of activation can be caused, in principle, by genotoxic chemical carcinogens, which can be provisionally identified as such by their mutagenic or

blastogenic effects. The mechanisms of action of such agents, long considered to involve damage to DNA, may eventually be reconciled with molecular virology, and the crucial events in cellular transformation, by both chemical and biological agents, defined through analysis of the activation and behavior of oncogenes. Detection and critical evaluation of the roles of dominant oncogenes, especially mutant genes of the *ras* and *erbB* families, have become a major unifying theme in the research activities of several Sections within this Laboratory.

Research on the carcinogenicity of synthetic fecapentaene-12 (FP-12) has produced no evidence that this highly mutagenic natural product is capable of inducing neoplasms in either rats or mice, in the colon or in other tissues.

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

The Office of the Chief (1) organizes comparative research on mechanisms of chemical carcinogenesis in susceptible and resistant species of experimental animals; (2) arranges and fosters collaborative approaches to specific research projects involving several Sections within, and independent investigators outside, the Laboratory; and (3) provides general support and direction to the intramural research program of the Laboratory.

Research of the Primate Research Working Group involves non-human primates of the species *Erythrocebus patas*, *Macaca fascicularis*, and, in collaboration with Oak Ridge Associated Universities, *Saguinus oedipus*. This project is evolving from its previous observation/description phase (induced tumors as the principal experimental endpoint) to an analytic phase in which biochemical measurements predominate. There are at present four active projects that involve such measurements: (1) pharmacokinetics of nitrosamine distribution and elimination in *patas* monkeys, and the effect on pharmacokinetic parameters of concomitant ingestion of ethanol; (2) quantification by ³²P-postlabeling of carcinogen-DNA adducts in placental, fetal, and maternal tissues, following single or multiple exposures to carcinogens known to be present in the human environment. Assays are performed at different stages of gestation and at different intervals between exposure and sampling; (3) quantification of the potency of various drugs that are known promoters of carcinogenesis in non-squamous epithelia in rodents, both as promoters of carcinogenesis and as inducers of transcription of mRNA leading to increased levels of specific enzymes. This finding may provide means of prediction of tumor promoting activity and a way to extrapolate among species for at least one category of non-genotoxic carcinogens; (4) search for dominant oncogenes and recessive suppression genes in naturally-occurring and chemically-induced tumors in non-human primates.

The Perinatal Carcinogenesis Section (1) investigates the induction of cancer in experimental animals before birth and during infancy; (2) evaluates perinatal exposures to chemical carcinogens, inducers of xenobiotic metabolism, and tumor promoters as causative factors in pediatric and adult forms of human cancer; (3) studies the effects of exposure to carcinogens during pregnancy; and (4) investigates the relation of cellular differentiation to perinatal susceptibility to chemical carcinogens and to the consequent development of neoplasia.

A determining role for metabolic activation/detoxication in susceptibility to transplacental carcinogenesis has been definitively confirmed for a polycyclic aromatic hydrocarbon in a pharmacogenetic mouse model. Detailed mechanistic analysis of the phenomena underlying this relationship is now well underway, including measurement of the enzymatic activity of and gene expression for the relevant Phase I and Phase II enzymes, Western blot of protein levels, and assessment of DNA adducts by the ³²P-postlabeling method. A comparison of the induction kinetics in B6D2F₁ and D2B6F₁ fetuses from responsive and nonresponsive mothers, respectively, has shown that similar levels of AHH activity are attained following transplacental injection of MC regardless of the phenotype of the mother. However, fetuses from nonresponsive D2 mothers maintained their induced P-450IA1 levels, as measured enzymatically, for at least 48 hr, whereas values for fetuses from responsive mothers had declined to control levels by this time. A gene for an important Phase II enzyme, uridine diphosphoglucuronic acid transferase, has been found to be well expressed in fetal mouse liver. When this multifactoral analysis is eventually completed, it will provide a depiction of genetics-related modulation of tumorigenesis of unprecedented completeness.

The interaction of two important categories of human-exposure chemicals, ethanol and *N*-nitrosamines, is being studied in mice and patas monkeys. In mice, oral coadministration of ethanol with three different nitrosamines has resulted in significant increases in tumors initiated by the nitrosamine in three target organs. It has been postulated that this effect of ethanol is due to competitive blockage of nitrosamine metabolism in liver, leading to greater delivery of dose to sensitive targets. In confirmation of this hypothesis, coadministration of ethanol with *N*-nitrosodimethylamine resulted in a striking prolongation of the circulating half-life of the carcinogen. An even more dramatic retardation of clearance was seen in the patas monkey, suggesting that the findings may be extrapolatable to the human. The further hypothesis that the increase in circulating levels might result in a greater number of tumor initiating events was supported by the finding that ethanol cotreatment led to a three- to ninefold increase in amounts of promutagenic O⁶-methylguanine in lung DNA. Further analysis of these models will continue to elucidate not only the specific interactions between nitrosamine and ethanol, but also the more general task of evaluating, in terms of risk assessment, the net outcome of multifactor exposure of humans.

The expression of activated cellular oncogenes in chemically-induced tumors in rodents (rats, mice, and Syrian hamsters and in selected homologous human neoplasms, and the relationship of oncogene expression to progression from the normal to a neoplastic phenotype are being studied by transfection of NIH 3T3 and SHOK cells and by hybridization techniques including oligonucleotide probing for specific point mutations. Polymerase chain reaction (PCR) techniques are being employed to amplify selected genomic segments in order to search for subpopulations of cells in a given tissue in which activating oncogene mutations may be present. Such subpopulations of cells seem to occur naturally, with a point mutation in the *c-H-ras* gene, in the skin of SENCAR mice that have been bred for high susceptibility to squamous papilloma and carcinoma induction. The *c-neu* (HER-2, *c-erbB-2*) gene, is the only growth-factor receptor-protein kinase type oncogene known to be mutationally activated in an experimental tumor, the rat schwannoma. We are now able to confirm that activation of this gene also occurs in homologous tumors in the mouse and Syrian hamster as well, and gene cloning and sequencing studies are in progress to ascertain whether this also occurs in comparable human neoplasms.

Investigation of the relationship between morphogenetic differentiation and susceptibility to perinatal carcinogenesis consists of two parts: (1) application of *in vivo* somatic cell mutagenesis techniques to explore organ- and tissue-specificity in transplacental carcinogenesis; (2) definition of the role of morphogenetic differentiation of the permanent kidney during prenatal and early postnatal life as a determinant of the origin and behavior of tumors induced by perinatal exposure to chemical carcinogens. Mutagenesis studies have been expanded to include (as a control) a factor that is coded by an autosomal recessive gene, diaminopurine resistance (DAPr). This parameter has reinforced the overall conclusion that rodent fetal cells in primary culture have spontaneous and induced mutation frequencies that are much lower than the frequencies observed in many established cell lines. Efforts to precisely define the nutritional requirements of renal blastemal and epithelial cells *in vitro* have shown that blastemal cells are supported by a pituitary growth factor that is acid-stable, heat-sensitive, and more than 3,000 molecular weight, presumably a polypeptide, the nature of which is under study.

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

Malignant testicular interstitial cell tumors were induced in rats by repeated exposure to cadmium. Such exposure was also associated with a high incidence of hepatocytic metaplasia of the pancreas, a finding possibly related to the suppression of pancreatic carcinogenesis by cadmium in the rat. Likewise, cadmium was found to suppress liver tumor initiation and promotion in the B6C3F1 mouse. Cadmium feeding increased the incidence of prostatic tumors and leukemia in rats. Dietary zinc was found to have a variable effect on cadmium carcinogenesis. Zinc deficiency decreased the incidence of prostatic lesions and leukemias in rats fed cadmium, while increasing the number of neoplastic foci in the testes after cadmium injection. A suppression of cadmium-induced prostatic tumors by zinc deficiency was linked to increased incidence of prostatic atrophy in zinc-deficient animals, probably due to reduced testicular function, further indicating the key role of androgen input for cadmium-induced prostatic tumorigenesis. In this regard it was shown that prostatic accumulation and retention of cadmium are dependent on circulating testosterone levels. Metallothionein, the low molecular weight protein thought to confer tolerance to cadmium, was found to be absent not only in the testes, but also in the rodent prostate and ovaries. The testicular cadmium-binding protein (TCBP) detected in place of metallothionein was found, unlike metallothionein, to be uninducible by pretreatments that result in tolerance to cadmium testicular carcinogenicity, such as high dose zinc or low dose cadmium. Such treatments, however, prevented cadmium-induced cytotoxicity in isolated testicular interstitial cells. TCBP also appeared to enhance nuclear disposition of cadmium in isolated interstitial cells. Hence, the absence of metallothionein in target tissues of cadmium carcinogenesis may contribute to the susceptibility of these tissues in several ways.

Investigations of the effects of essential divalent metals on nickel carcinogenesis completed previously have led to two main conclusions: (1) The essential metals may inhibit Ni action at the tumor initiation stage; (2) The essential metals prevent Ni-caused inhibition of phagocytes and/or enhance cellular immunity; hence, inhibition of Ni carcinogenesis may be mediated by phagocytes.

Subsequently, three bioassays have been undertaken to check the importance of the tumor-initiating, tumor-promoting, and immunotoxic properties of Ni to its carcinogenic action. A single i.p. injection of Ni resulted in the initiation in high incidence of renal tumors that were promoted with sodium barbital. In contrast, a bioassay to test promotional activity of oral Ni toward renal tumors initiated with methyl(methoxymethyl)nitrosamine had negative results. In a third bioassay, addition of *M. bovis* antigen to i.m. Ni resulted in great enhancement of local macrophage activity and in complete prevention of Ni carcinogenesis. Studies on oxygen activation by Ni have continued to investigate possible mechanisms of tumor initiation by this metal. In addition to the inhibitory effects of Ni upon catalase and glutathione peroxidase discovered previously, Ni was found to inhibit glutathione reductase and enhance myeloperoxidase activity *in vitro*. Altogether, these effects may increase concentration of H₂O₂ and oxygen radicals in the target tissue. In a parallel *in vitro* study, Ni greatly enhanced hydroxylation of 2'-deoxyguanosine (dG) to 8-OH-dG and its deglycosylation as well as hydroxylation of the guanine residues in calf thymus DNA caused by active oxygen species. In preliminary tests, increased amounts of 8-OH-dG were found in renal DNA of rats treated with i.p. Ni. Also, Ni appeared to produce yet another potentially mutagenic effect, enhancement of deamination of 5-methyl-2'-deoxycytidine by the sulfite anion to form thymidine. The sulfite needed for this reaction can be derived by oxidation of the strongest Ni carcinogens, Ni₃S₂ and beta-NiS. Thus, DNA base oxidation and/or deamination constitute possible mechanisms by which Ni carcinogens may alter genetic material.

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

Mechanisms potentially responsible for formation, activation, and detoxication of carcinogenic *N*-nitroso compounds in the human body are under intense investigation. In the nitrosamine formation area, we have discovered an iron complex that catalyzes *N*-nitrosation of amines by nitrite ion according to a pathway whose rate is independent of nitrite concentration. This reaction appears to parallel the action of certain enzymes in several important respects and may serve as a useful model for the mechanism(s) by which at least some carcinogenic *N*-nitroso compounds form in the body or in the environment. The metabolic activation of nitrosamines is being pursued with a focus on *N*-nitrosoethylmethylamine (NEMA), β -deuteration which caused an organotropic shift from liver toward increasing esophageal tumorigenicity. Current work is aimed at elucidating the possible intermediacy of the 3-methyl-4,5-dihydro-1,2,3-oxadiazolium (MDO) ion in the activation of NEMA as a DNA methylating agent. An ion-pairing chromatography method has been developed for measuring MDO levels in solution, and the material has been prepared in radioactive form to facilitate its analysis in biological fluids following incubation of NEMA with subcellular fractions or its administration to rats. As to detoxication of these carcinogens, major emphasis is being placed on studying the denitrosation pathway of *N*-nitrosodimethylamine (NDMA) metabolism. This mechanism has now been shown by careful *in vivo* measurements to

account for 20% of total NDMA metabolism in the intact Fischer rat. Interestingly, fully deuterated NDMA appears to be denitrosated about 50% more extensively than NDMA under these conditions, suggesting that substrate deuteration may shift the metabolic balance toward increasing detoxication at the expense of the activation pathway. If alternate means (such as pretreatment with a chemopreventive regimen) can be designed to effect an even more extensive shift in this direction, it may be possible to protect an organism from the carcinogenic effects of NDMA exposure even when the NDMA is endogenously produced.

The Tumor Pathology and Pathogenesis Section (TPPS) (1) characterizes the biology and pathology of naturally occurring and experimentally induced preneoplastic and neoplastic lesions of laboratory animals; (2) uses morphologic, histochemical and ultrastructural methods to define the pathogenesis of experimental tumors; (3) develops animal models to aid in understanding causes, pathogenesis and pathology of human cancers; and (4) provides guidance, consultation and collaboration in tumor and laboratory animal pathology to investigators and scientists in the other Sections of the Laboratory.

The expression of the *ras* oncogene protein product, p21, was studied in pathology specimens of rats, mice and humans. Several different monoclonal or polyclonal antibodies were used to demonstrate that immunohistochemical reactivity depended on the tissue fixative, tissue and specific antibody. Some antibodies reacted with epitopes in p21 or other proteins in the cytoplasm of cells within normal tissues of all three species. Cell membrane immunoreactivity could only be found on tumor cells or transformed *in vitro* cell lines. A well-characterized anti-21 monoclonal antibody was found to react to a series of proteins in normal and transformed cells but never with cell membranes. We developed several control methods for proving specificity of the antibody-antigen reaction.

Urinary bladder tumors induced by a nitrosamine in rats were found to express high levels of *ras* p21. Transfection of NIH 3T3 cells with tumor DNA produced transformed colonies for only 2/11 tumors. After tumor DNA amplification by the polymerase chain reaction (PCR), still only 2/22 tumor DNAs were found to have mutations at codon 12 of *H-ras*. More than 60% (12/20) tumors were found to have mutations in codon 61 of *H-ras* by restriction fragment length polymorphism after PCR. Thus, for the first time we found, by immunohistochemistry, that increased expression of *ras* p21 was associated with a high rate of *ras* mutations in a chemically-induced rodent tumor.

A new method for detecting oncogene and retroviral proteins in fixed tissue sections was developed. *In vitro* cell lines with specific retroviral infections (e.g., HIV-IIIB and SIV-MnIV) or activated or amplified *ras* or *erbB-2* oncogenes were used to produce cell pellets. The pellets were used to prepare sections for immunohistochemical reactivity with monoclonal and polyclonal antibodies to oncogene or retroviral proteins. The use of cells with known expression of viruses or oncogenes proved extremely valuable in accessing protein expression in autopsy specimens of rodents and humans.

Rodent renal tumor promoters including barbital sodium and nitrilotriacetic acid were used to develop an *in vitro* rodent and human cellular assay system to study mechanisms of renal tumor promotion. Rat and human renal epithelial cells were cultivated *in vitro* and effects on colony formation, colony growth and cell-cell communication by the lucifer yellow dye transfer technique using the micro-injection technique were studied. Two rat renal cell lines, NRK and NK-4,

expressed differing levels of gap junctions and cell-cell communication. Despite this difference in cell surface physiology, the two cell lines responded similarly to the growth stimulating effects of the two tumor promoters. Attempts at immortalization of the human renal cells with SV40 T antigen proved difficult. Although cells appeared to survive for long periods of time, establishment of a permanent cell line was slow. Cell-cell communication in the human cells was variable between colonies. Tumor promoters had no obvious effect on cell survival or growth.

Relationships between cytochrome P-450IIB1 and tumor promotion continue to be studied. We have found that outbred Zucker rats (male or female) display a relatively normal induction (greater than 40x) of cytochrome P-450IIB1 following treatment with phenobarbital, but display minimal induction (less than 8x) following treatment with the phenobarbital analogs, barbital or ethylphenylhydantoin. In contrast, F-344 (either male or female) or DA rats (female) show high induction (greater than 40x) following treatment with phenobarbital, barbital, or ethylphenylhydantoin; and hepatocarcinogenesis in F344 rats can be strongly promoted by all three compounds. Promotion studies in Zucker rats are in progress. We have found that the benzodiazepine clonazepam is a specific and relatively potent inhibitor of cytochrome P450 both *in vitro* and *in vivo* in mice and does not promote hepatocarcinogenesis in that species. The cytochrome P450IIB activity we examined is presumably only one manifestation of a "pleiotropic" response which we have now shown includes epoxide hydrolase, UDP glucuronyl transferase and glutathione transferases. In addition to these mechanistic studies in rodents, we are examining the induction of these enzymes by phenobarbital in a variety of other species, including nonhuman primates, (*Erythrocebus patas*; *Macaca fascicularis*) to examine the relationship between induction of various enzymes, especially cytochrome P450IIB1, and liver tumor promotion. Finally, studies have been initiated to determine the mechanism by which potent inducers of cytochrome P450IIB1 promote carcinogenesis in the thyroid by examining their effects on follicular cell proliferation and levels of various thyroid hormones.

The pathogenesis of hepatic and renal tumors induced by nongenotoxic carcinogens or promoted by tumor promoters were studied in rats and mice. Levels of DNA synthesis in target hepatocytes or renal tubular cells were evaluated by BrdU immunohistochemistry or tritiated thymidine autoradiography.

While almost all renal tumor promoters are associated with increased levels of DNA synthesis (hyperplasia) in target cells for promotion in the renal tubules, some renal toxins, e.g., di(2-ethylhexyl)phthalate (DEHP), cause a marked hyperplasia without evidence of carcinogenesis or tumor promotion after initiation by *N*-nitrosoethylurea. Liver tumor promoters, however, often caused a transient increase in levels of hepatocyte DNA synthesis, while rarely causing chronic persistent increases.

Pulmonary carcinogenesis in mice was investigated using the transplacental *N*-nitrosoethylurea model. A quantitative method was developed for the first time to quantify the pathogenesis of pulmonary tumors of mice. A time-related and size-related sequence of histogenesis was found which helps account for the nature of the tumors seen. Mice receiving NEU on day 14 of gestation had fewer lung tumors which were more malignant than those in mice receiving NEU later in gestation. Thus, some evidence was found that the biology and pathology of pulmonary tumors in this model depended, in part, on the age of exposure to the carcinogen *in utero*.

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

PROJECT NUMBER

201CP04542-17 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

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

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

PI:	L. K. Keefer	Chief, Chemistry Section	LCC	NCI
Others:	Y.-H. Heur	Visiting Fellow	LCC	NCI
	A. J. Streeter	Visiting Associate	LCC	NCI
	R. Nims	Chemist	LCC	NCI
	T. Dunams	Intramural Research Training Awardee	LCC	NCI
	M. Ho	Intramural Research Training Awardee	LCC	NCI
	D. Wink	Staff Fellow	LCC	NCI
	L. M. Anderson	Research Biologist	LCC	NCI

COOPERATING UNITS (if any) PRI, Frederick, MD (J. Hrabie, K. Poff); U. of Zürich (P. Kleihues, E. von Hofe); BRI, Frederick, MD (R. Moschel); U. of Houston (E.O. Bennett); Thermedics, Inc. (U. Goff, J. Stevens); Hoffmann-La Roche (B. Mico); Am. Chem. Soc. (J. Malin); Rutgers U. (C.S. Yang); Smith Kline & French (C. Gombar)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701-1013

TOTAL MAN-YEARS

5.0

PROFESSIONAL:

4.5

OTHER:

0.5

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.)

Mechanisms potentially responsible for formation, activation, and detoxication of carcinogenic N-nitroso compounds in the human body are under intense investigation. In the nitrosamine formation area, we have discovered an iron complex that catalyzes N-nitrosation of amines by nitrite ion according to a pathway whose rate is independent of nitrite concentration. This reaction appears to parallel the action of certain enzymes in several important respects and may serve as a useful model for the mechanism(s) by which at least some carcinogenic N-nitroso compounds form in the body or in the environment. The metabolic activation of nitrosamines is being pursued with a focus on N-nitrosoethylmethylamine (NEMA), β -deuteration which caused an organotropic shift from liver toward increasing esophageal tumorigenicity. Current work is aimed at elucidating the possible intermediacy of the 3-methyl-4,5-dihydro-1,2,3-oxadiazolium (MDO) ion in the activation of NEMA as a DNA methylating agent. An ion-pairing chromatography method has been developed for measuring MDO levels in solution, and the material has been prepared in radioactive form to facilitate its analysis in biological fluids following incubation of NEMA with subcellular fractions or its administration to rats. As to detoxication of these carcinogens, major emphasis is being placed on studying the denitrosation pathway of N-nitrosodimethylamine (NDMA) metabolism. This mechanism has now been shown by careful in vivo measurements to account for 20% of total NDMA metabolism in the intact Fischer rat. Interestingly, fully deuterated NDMA appears to be denitrosated about 50% more extensively than NDMA under these conditions, suggesting that substrate deuteration may shift the metabolic balance toward increasing detoxication at the expense of the activation pathway. If alternate means (such as pretreatment with a chemopreventive regimen) can be designed to effect an even more extensive shift in this direction, it may be possible to protect an organism from the carcinogenic effects of NDMA exposure even when the NDMA is endogenously produced.

PROJECT DESCRIPTION

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

L. K. Keefer	Chief, Chemistry Section	LCC	NCI
Y.-H. Heur	Visiting Fellow	LCC	NCI
A. J. Streeter	Visiting Associate	LCC	NCI
R. Nims	Chemist	LCC	NCI
T. Dunams	Intramural Research Training Awardee	LCC	NCI
M. Ho	Intramural Research Training Awardee	LCC	NCI
D. Wink	Staff Fellow	LCC	NCI
L. M. Anderson	Research Biologist	LCC	NCI

Objectives:

Generally, to apply the methods and concepts of chemistry toward the solution of important problems in cancer research, especially by elucidating new mechanisms of formation, destruction, metabolism, and biological action of nitrosamines and related carcinogens. Specifically, (1) to establish mechanisms of nitrosamine formation so that strategies for preventing environmental contamination by these compounds can be developed; (2) to gather information on the chemistry of nitrosamine destruction so that procedures may be devised for intercepting these carcinogens before human exposure can occur; (3) to study the interactions between N-nitroso compounds and organisms exposed to them, with the aim of inferring ways of protecting victims of unavoidable nitrosamine exposure from their carcinogenic effects; (4) to characterize the fundamental physical and chemical properties of the carcinogenic N-nitroso compounds and other substances of interest in cancer research as a means of contributing to the general fund of knowledge about such materials.

Major Findings:

In our nitrosamine formation studies, we have discovered a mechanism in which nitrite is activated as an N-nitrosating agent by electrophiles capable of mobilizing it so efficiently that the rate of nitrosamine formation is independent of nitrite ion concentration. The mechanism of catalysis involves coordination of the nitrite nitrogen atom by the electrophilic center, following which an oxide ion is removed in an acid-base reaction to form an iron nitrosyl species as the active N-nitrosating agent. The reaction appears to parallel the action of nitrite reductase in several important respects and may serve as a useful model for elucidating the mechanistic origins of certain types of previously unexplained nitrosamine formation occurring in the body or in the environment (collaboration with K. Poff and J. Malin). A similar mechanism has now been utilized to effect deamination of 2'-deoxyguanosine in what we believe to be the first preparation of free 2'-deoxyxanthosine (collaboration with R. Moschel). Finally, we have found that commercial metal-working fluids still contain significant quantities of carcinogenic N-nitrosodiethanolamine. All six samples investigated were found to contain parts per million levels of this potent carcinogen. Though these concentrations are substantially smaller than the percent quantities found in previous studies by others over a decade ago, they may still pose a danger to the

machinists who work with such products (collaboration with E. Bennett, J. Stevens and U. Goff).

Metabolism studies have focused on the *in vivo* denitrosation of N-nitrosodimethylamine (NDMA). The extent of NDMA denitrosation (a presumably deactivating metabolic route) occurring in the intact Fischer rat has been found by careful measurements to be very close to 20% of the total metabolism in each of four animals studied. Repetition of these experiments with fully deuterated NDMA produced much more variable and extensive metabolism by this pathway, observed values ranging up to 50% or more. The mean for the rats receiving the deuterated substrate was 30%, suggesting that incorporation of the heavy isotope shifted the metabolism towards an increase in denitrosation at the expense of the normally predominant activation pathway (collaboration with B. Mico, C. Yang, C. Gombar, and J. Hrabie). We are currently testing certain pretreatment regimens to determine whether animals might be protected from the deleterious effects of NDMA exposure through the designed detoxication of the carcinogen. A detailed study of the low dose pharmacokinetic and deuterium isotope effect behavior of methylamine was also performed. This was necessary because methylamine, which is the diagnostic carbon-bearing product of the denitrosation pathway, is itself further metabolized upon metabolic formation from NDMA.

Another carcinogen of great current interest in our metabolism studies is N-nitrosoethylmethylamine (NEMA). It had been shown by others to undergo an organotropic shift in its carcinogenicity from liver towards increasing esophageal tumorigenicity and DNA alkylating ability when the β -methyl group is deuterium labeled. Since toxicokinetic studies showed that there was no difference in bioavailability between the normal NEMA and its deuterium-labeled analog, we are now searching for local effects that might explain the greater esophageal action for the latter compound. In one aspect of this study, we have developed an ion-pairing chromatography analytical method for the 3-methyl-4,5-dihydro-1,2,3-oxadiazolium (MDO) ion, which we suspect may be formed directly as a metabolite of NEMA in the liver and be delivered to the general circulation as a potential methylating agent. The chemical behavior of MDO is also being probed, with particular emphasis on its stability versus pH profile as well as the effects of various nucleophiles and media of different polarity on its stability. A tritium-labeled analog of MDO has also been prepared to facilitate the application of the analytical method in the biological milieu. *In vitro* kinetic studies are also being arranged using subcellular fractions from liver as well as, hopefully, the esophagus in an effort to determine how if at all the two organs differ in metabolic action on NEMA (collaboration with P. Kleihues, E. von Hofe, B. Mico, J. Hrabie, and L. Anderson).

Publications:

Heur Y-H, Streeter AJ, Nims RW, Keefer LK. The Fenton degradation as a non-enzymatic model for microsomal denitrosation of N-nitrosodimethylamine. *Chem Res Toxicol* (In Press).

Keefer LK, Hrabie JA, Hilton BD, Wilbur D. Nitrogen protonation of N-nitrosodimethylamine. *J Am Chem Soc* 1988;110:7459-62.

Keefer LK, Hrabie JA, Ohannesian L, Flippen-Anderson JL, George C. A symmetrically hydrogen-bonded "binitrosamine cation" produced on protonation of *N*-nitroso-pyrrolidine. *J Am Chem Soc* 1988;110:3701-2.

Keefer LK, Lunn G. Nickel-aluminum alloy as a reducing agent. *Chemical Rev* (In Press).

Keefer LK, Ohannesian L, Hrabie JA. Isolation of stable 1:1 and 2:1 salts of nitrosamines with protic acids. *J Org Chem* (In Press).

Keefer LK, Streeter AJ, Leung LY, Perry WC, Hu HS-W, Baillie TA. Isotope effects on formaldehyde oxidation: response. *Drug Metab Dispos* 1988;16:657.

Keefer LK, Wang S-M, Anjo T, Fanning JC, Day CS. Preparation of a thallium(I) diazotate. Structure, physicochemical characterization, and conversion to novel *N*-nitroso compounds. *J Am Chem Soc* 1988;110:2800-6.

Moschel RC, Keefer LK. Preparation of 2'-deoxyxanthosine by nitrosative deamination of 2'-deoxyguanosine under alkaline aqueous conditions. *Tetrahedron Lett* 1989;30:1467-8.

Ohannesian L, Keefer LK. Displacement of O- versus N-substituents from nitrosamine-derived diazenium ions by three divergent mechanisms. *Tetrahedron Lett* 1988;29:2903-6.

Stershic MT, Keefer LK, Sullivan BP, Meyer TJ. Formation of complexed nitrosamines by oxidation of coordinated ammonia in the presence of secondary amines. *J Am Chem Soc* 1988;110:6884-5.

Streeter AJ, Nims RW, Hrabie JA, Heur Y-H, Keefer LK. Sex differences in the single-dose toxicokinetics of *N*-nitrosomethyl(2-hydroxyethyl)amine in the rat. *Cancer Res* 1989;49:1783-9.

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

PROJECT NUMBER

Z01CP04582-14 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Mechanisms of Inorganic Carcinogenesis: Nickel

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

K.S. Kasprzak	Visiting Scientist	LCC	NCI
J.M. Rice	Chief, Laboratory of Comparative Carcinogenesis	LCC	NCI
J.M. Ward	Chief, Tumor Pathol. & Pathogen. Section	LCC	NCI
L.K. Keefer	Chief, Chemistry Section	LCC	NCI
R.E. Rodriguez	Intramural Research Training Awardee	LCC	NCI
M. Misra	Visiting Fellow	LCC	NCI
N. Konishi	Visiting Fellow	LCC	NCI

COOPERATING UNITS (if any)

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

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Inorganic Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21701-1013

TOTAL MAN-YEARS

4.5

PROFESSIONAL

3.5

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.)

Investigations of the effects of essential divalent metals on Ni carcinogenesis completed previously have led to two main conclusions: (1) The essential metals may inhibit Ni action at the tumor initiation stage; (2) The essential metals prevent Ni-caused inhibition of phagocytes and/or enhance cellular immunity; hence, inhibition of Ni carcinogenesis may be mediated by phagocytes. Subsequently, three bioassays have been undertaken to check the importance of the tumor-initiating, tumor-promoting, and immunotoxic properties of Ni to its carcinogenic action. A single i.p. injection of Ni resulted in the initiation in high incidence of renal tumors that were promoted with sodium barbital. In contrast, a bioassay to test promotional activity of oral Ni toward renal tumors initiated with methyl(methoxy-methyl)nitrosamine had negative results. In a third bioassay, addition of *M. bovis* antigen to i.m. Ni resulted in great enhancement of local macrophage activity and in complete prevention of Ni carcinogenesis. Studies on oxygen activation by Ni have continued to investigate possible mechanisms of tumor initiation by this metal. In addition to the inhibitory effects of Ni upon catalase and glutathione peroxidase discovered previously, Ni was found to inhibit glutathione reductase and enhance myeloperoxidase activity in vitro. Altogether, these effects may increase concentration of hydrogen peroxide and oxygen radicals in the target tissue. In a parallel in vitro study, Ni greatly enhanced hydroxylation of 2'-deoxyguanosine (dG) to 8-OH-dG as well as hydroxylation of the guanine residues in calf thymus DNA caused by active oxygen species. In preliminary tests, increased amounts of 8-OH-dG were found in renal DNA of rats treated with i.p. Ni. Also, Ni appeared to produce yet another potentially mutagenic effect, enhancement of deamination of 5-methyl-2'-deoxycytidine by the sulfite anion to form thymidine. The sulfite needed for this reaction can be derived by oxidation of the strongest Ni carcinogens, nickel subsulfide and beta-NiS. Thus, DNA base oxidation and/or deamination constitute possible mechanisms by which Ni carcinogens may alter genetic material.

PROJECT DESCRIPTION

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

K.S. Kasprzak	Visiting Scientist	LCC	NCI
J.M. Rice	Chief, Laboratory of Comparative Carcinogenesis	LCC	NCI
J.M. Ward	Chief, Tumor Pathol. & Pathogen. Section	LCC	NCI
L.K. Keefer	Chief, Chemistry Section	LCC	NCI
R.E. Rodriguez	Intramural Research Training Awardee	LCC	NCI
M. Misra	Visiting Fellow	LCC	NCI
N. Konishi	Visiting Fellow	LCC	NCI

Objectives:

To study mechanisms of nickel carcinogenesis by investigating a hypothesis that nickel and possibly other metal carcinogens act, at least in part, through interference with the functions of essential divalent metals, calcium, magnesium, zinc, iron, and others. An alternative hypothesis assuming that nickel may damage cellular genetic material through initiation of radical chain reactions with the participation of oxygen, some polypeptides and proteins, causing DNA-protein and protein-protein cross-linking, and modification of DNA bases, is also investigated.

Major Findings:

Previous findings that muscle carcinogenesis by nickel subsulfide, Ni₃S₂, at the site of injection can be strongly suppressed by local administration of essential metals, Mn, Mg, and/or Fe, and that this suppression is apparently mediated through activation of cellular immunity has been confirmed in an independent system using *M. bovis* (MB) antigen and anti-inflammatory drugs cortisol (CORT) and indomethacin (IND). Coinjection of MB with insoluble Ni₃S₂ into rat muscle completely prevented local tumor induction by Ni. The effect was strictly local and was accompanied by a vigorous infiltration of macrophages into the injection site.

It was shown for the first time that a single i.p. injection of a soluble nickel salt, Ni(II) acetate, to rats initiates renal epithelial tumors that were promotable by oral sodium barbital (NaBB). The same doses of Ni(II) acetate or NaBB administered separately did not produce renal or any other tumors. A concurrent bioassay in which rats were given i.p. methyl(methoxymethyl)-nitrosamine, a renal tumor-initiating agent, followed by Ni(II) acetate in the drinking water failed to prove suspected tumor-promoting properties of Ni.

The hypothesis that Ni-sustained oxidative damage to the genetic material is a possible molecular mechanism for Ni carcinogenicity, formulated by us recently, has been tested more extensively on 2'-deoxyguanosine (dG), 5-methyl-2'-deoxycytidine (5MedC), calf thymus DNA, and on rat renal DNA *in vivo*. The main findings of these studies include: (1) hydroxylation of dG at the C-8 position by Ni₃S₂ in the presence of oxygen; (2) enhancement by soluble Ni(II) salts of hydroxylation of either free or DNA-bound dG by physiological agents, ascorbic acid and H₂O₂; (3) much greater effect of Ni on dG hydroxylation in single-stranded

DNA than in double-stranded DNA; (4) enhancement by Ni(II) of ascorbic acid and H_2O_2 -caused deglycosylation of dG; and (5) presence of increased amounts of hydroxylated dG in DNA isolated from kidneys of rats treated with i.p. Ni(II) acetate. Soluble Ni(II) was also found to enhance another potentially mutagenic reaction, namely, the deamination of 5MeC with the sulfite anion to form thymidine. The reaction can also be completed with pure Ni(II) sulfite. The sulfite anion or Ni(II) sulfite may be derived by intracellular oxidation of the sulfur moiety of carcinogenic Ni_3S_2 which, for the first time, explains the exceptionally high carcinogenic activity of Ni sulfides compared to other Ni derivatives. Oxidation of Ni-sulfides to sulfites in turn, may be assisted by the Ni(II)-caused inhibition of H_2O_2 -controlling enzymes catalase, glutathione peroxidase, and glutathione reductase, as well as by Ni-caused enhancement of myeloperoxidase, as recently discovered by us. The latter enzyme generates hypohalites that with the H_2O_2 generate highly reactive singlet oxygen.

Thus, at least two new effects were found by which Ni_3S_2 can cause damage to genetic material: (1) oxidative modification of dG and (2) deamination of 5MeC.

Publications:

Kasprzak KS. Animal studies: an overview. In: Nieboer E, Aitio A, eds. Nickel in human health: current perspectives. New York: Wiley & Sons (In Press).

Kasprzak KS. Metal interactions in nickel, cadmium, and lead carcinogenesis. In: Foulkes EC, ed. Mechanisms of metal carcinogenesis. Boca Raton: CRC Press (In Press).

Kasprzak KS, Bare RM. In vitro polymerization of histones by carcinogenic nickel compounds. *Carcinogenesis* 1989;10:621-4.

Kasprzak KS, Kiser RF, Weislow OS. Magnesium counteracts nickel-induced suppression of T-lymphocyte response to concanavalin A. *Magnesium* 1988;7:166-72.

Kasprzak KS, Kovatch RM, Poirier LA. Inhibitory effect of zinc on nickel subsulfide carcinogenesis in Fischer rats. *Toxicology* 1988;52:253-62.

Kasprzak KS, Rodriguez RE. Inhibitory effects of zinc, magnesium, and iron on nickel subsulfide carcinogenesis in rat skeletal muscle. In: Nieboer E, Aitio A, eds. Nickel in human health: current perspectives. New York: Wiley & Sons (In Press).

Raos N, Kasprzak KS. Allosteric binding of nickel(II) to calmodulin. *Fund Appl Toxicol* (In Press).

Raos N, Kasprzak KS. Effect of nickel(II) acetate on distribution of calmodulin in the rat kidney. *Toxicol Lett* (In Press).

Rodriguez RE, Kasprzak KS. Effect of nickel on catalase and the glutathione peroxidase/reductase system in vitro. In: Nieboer E, Aitio A, eds. Nickel in human health: current perspectives. New York: Wiley & Sons (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05092-11 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Transplacental Carcinogenesis and Tumor Promotion in Nonhuman Primates

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

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

COOPERATING UNITS (if any)

SEMA, Inc., Rockville, MD (J. Phillips); Univ. of Texas, Galveston, TX (L. J. Lu); Oak Ridge Associated Universities, Oak Ridge, TN (N. Clapp)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Office of the Chief, Primate Research Working Group

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS

0.5

PROFESSIONAL

0.5

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.)

This project involves non-human primates of the species Erythrocebus patas, Macaca fascicularis, and, in collaboration with Oak Ridge Associated Universities, Saguinus oedipus. This project is evolving from its previous observation/description phase (induced tumors as the principal experimental endpoint) to an analytic phase in which biochemical measurements predominate. There are at present four active projects that involve such measurements: (1) pharmacokinetics of nitrosamine distribution and elimination in patas monkeys, and the effect on pharmacokinetic parameters of concomitant ingestion of ethanol; (2) quantification by ³²P-postlabeling of carcinogen-DNA adducts in placental, fetal, and maternal tissues, following single or multiple exposures to carcinogens known to be present in the human environment. Assays are performed at different stages of gestation and at different intervals between exposure and sampling; (3) quantification of the potency of various drugs that are known promoters of carcinogenesis in non-squamous epithelia in rodents, both as promoters of carcinogenesis and as inducers of transcription of mRNA leading to increased levels of specific enzymes. This finding may provide means of prediction of tumor promoting activity, and a way to extrapolate among species for at least one category of non-genotoxic carcinogens; (4) search for dominant oncogenes and recessive suppression genes in naturally-occurring and chemically-induced tumors in non-human primates.

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

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

Objectives:

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

Major Findings:

Studies on carcinogenesis by ethylnitrosourea (ENU) and diethylnitrosamine (DEN) in *Erythrocebus patas*, an Old World monkey, continue and have been expanded to include aflatoxin B1 (AFB1). Additional cases of mesenchymal and epithelial tumors were observed in the offspring of monkeys that received ENU intravenously during pregnancy, especially when exposure occurred during the first trimester of gestation. These additional findings support the tentative conclusions drawn previously that, like rodents, this species of nonhuman primate is quantitatively more susceptible to the direct-acting alkylating agent, ENU, during prenatal life, with animals exposed in utero exhibiting a higher incidence of tumors after a shorter latency than juvenile or adult animals that received a similar dose directly.

This project is evolving from its previous observation/description phase (induced tumors as the principal experimental endpoint) to an analytic phase in which biochemical measurements predominate. There are at present four active projects that involve such measurements: (1) pharmacokinetics of nitrosamine distribution and elimination in patas monkeys, and the effect on pharmacokinetic parameters of concomitant ingestion of ethanol; (2) quantification by ³²P-postlabeling of carcinogen-DNA adducts in placental, fetal, and maternal tissues, following single or multiple exposures to carcinogens known to be present in the human environment. Assays are performed at different stages of gestation and at different intervals between exposure and sampling; (3) quantification of the potency of various drugs that are known promoters of carcinogenesis in non-squamous epithelia in rodents, both as promoters of carcinogenesis and as inducers of transcription of mRNA leading to increased levels of specific enzymes. This finding may provide means of prediction of tumor promoting activity, and a way to extrapolate among species for at least one category of non-genotoxic carcinogens; and (4) search for dominant oncogenes and recessive suppression genes in naturally-occurring and chemically-induced tumors in non-human primates.

The formation and persistence of DNA adducts of the environmental carcinogen benzo[a]pyrene (BP) in fetal and placental tissues of patas monkeys has been studied by the ³²P-postlabeling technique, in collaboration with Dr. L.J. Lu (U. of Texas). The major adduct found was the BP-diolepoxide adduct of guanine, which has been implicated, in other systems, as the initiating event in BP tumorigenesis. This adduct was relatively abundant in placenta and fetal brain, skin, and kidney after treatment of the mother with 50 mg/kg at the end of the first trimester (gestation day 52) and had reached maximum levels after treatment on day 100, with amount of adduct in placenta, liver, and lung being particularly prominent. Labeling was lower but detectable after 5 mg/kg also. As this study proceeds, with BP and with other environmental carcinogens, it will shed light on (1) the occurrence and stability of promutagenic DNA adducts in the tissues of fetuses with a lengthy gestation period, of obvious relevance to human risk; and (2) the significance, by reference to an animal model, of ³²P-postlabeled adduct in human placenta, recently an important subject in metabolic epidemiology.

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

Publications:

Diwan BA, Rice JM. Organ and species specificity in chemical carcinogenesis and tumor promotion. In Sirica AE, ed. The Pathobiology of Neoplasia. New York: Plenum Publishing Corp 1989;149-71.

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

CONTRACT IN SUPPORT OF THIS PROJECT

SEMA, Inc., N01-CP-71079Title: Resources for Transplacental Carcinogenesis and Tumor Promotion in Old World MonkeysCurrent Annual Level: \$492,339Man Years: 0.5Objectives:

This contract provides animal care and technical support for colonies of 135 patas and 80 cynomolgus monkeys. The project is designed to demonstrate and characterize transplacental carcinogenesis and tumor promotion in the Old World monkey species patas (*Erythrocebus patas*) and cynomolgus (*Macaca fascicularis*). In addition, related phenomena are studied, including the increased risk of adult female patas exposed to chemicals during pregnancy, tumor promotion in both patas and cynomolgus monkeys, and metabolism and pharmacodynamics of systemically administered chemical carcinogens, including formation of carcinogen-DNA adducts in placental, fetal, and maternal tissues demonstrable by P-32 postlabeling.

Major Contributions:

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

The formation of carcinogen-DNA adducts in placental tissue allows a correlation with fetal tissue adduct levels and provides an approach to experimental validation of a promising method for biochemical epidemiology of human populations.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05093-11 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

In Vitro Studies on Organ Specificity in Transplacental Carcinogenesis

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

PI: J. M. Rice Chief LCC NCI

Others: P. Donovan Chemist LCC NCI
A. Perantoni Staff Fellow LCC NCI

COOPERATING UNITS (if any)

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

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section, Developmental Biology Working Group

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS

2.0

PROFESSIONAL

1.0

OTHER

1.0

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.)

This project consists of two parts: (1) application of *in vivo* somatic cell mutagenesis techniques to explore organ- and tissue-specificity in transplacental carcinogenesis; (2) definition of the role of morphogenic differentiation of the permanent kidney during prenatal and early postnatal life as a determinant of the origin and behavior of tumors induced by perinatal exposure to chemical carcinogens. Mutagenesis studies have been expanded to include (as a control) a factor that is coded by an autosomal recessive gene, diaminopurine resistance (DAPr). This parameter has reinforced the overall conclusion that rodent fetal cells in primary culture have spontaneous and induced mutation frequencies that are much lower than the frequencies observed in many established cell lines. Efforts to precisely define the nutritional requirements of renal blastemal and epithelial cells *in vitro* have shown that blastemal cells are supported by a pituitary growth factor that is acid-stable, heat-sensitive, and more than 3,000 molecular weight, presumably a polypeptide, the nature of which is under study.

PROJECT DESCRIPTION

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

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

Objectives:

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

Major Findings:

Transplacental mutagenesis. Somatic cells derived from single litters of day 13 Syrian hamster fetuses were measured for spontaneous mutation frequency using 6-thioguanine as well as diphtheria toxin selection. The mean TG-resistant mutant frequency was 1.85×10^{-7} for 24 litters, and 2.63×10^{-7} frequency for DT resistant mutants for 26 litters. As the statistical basis of comparison for transplacental experiments is the litter, this was important to determine and 90 and 95% confidence limits were calculated for comparison with induced frequencies. The spontaneous mutation frequency was determined for individual fetuses for DT as well as TG resistant mutants and has been reported.

Dose curves of various carcinogens have been determined using DT as a selective agent, including an extensive multipoint dose curve for ENU. We are continuing that work, also using TG with special attention to low doses to determine effective mutation doubling doses. Another study in progress and almost finished was to look at the possibility of a genetic or familial effect in susceptibility to mutant induction by ENU (0.5 mg/kg) using individual fetuses from known litters. We have also bred litters from known fathers and mothers, keeping the parental unit while testing the newborn males for spontaneous mutation to TG^r, keeping the remaining sibs and parents of high frequency males and continuing further breeding to detect if other family members were also high. That work is

continuing. Previously we have also reported that when treatment of the developing Syrian hamster is varied during gestation, maximum induction of DT^r and TG^r mutant frequency occurred when treatment was at day 9 with a lower frequency when treatment was either earlier or later. A study now in progress and almost finished is to determine the extent of mutation induction in individual fetuses after treatment at different days of gestation.

Lastly, we have started using a new selective system, that of DAP or diaminopurine resistance. This is coded for by an autosomal recessive gene. When using the same age fetus, its spontaneous frequency should be the square root of that for TG resistance, or 4×10^{-14} . Since that would be difficult to determine experimentally, we tried to determine DAP^r frequency of a positive control, a pool of N-nitrosoethylurea (1 mmole/kg) transplacentally-treated fetal hamster cells, ENU 1. From the ratio of ENU 1 to control TG^r mutation frequency ($2.41 \times 10^{-5}/1.85 \times 10^{-7}$), it could be estimated that this value should be about 4.8×10^{-12} . We could not find, experimentally, any DAP^r mutants after 1,537 plates, so the value is less than 5.26×10^{-9} . It would require at least 1.7 million plates to observe the first ENU 1-induced DAP^r mutant.

The conclusion, which is becoming clearer as each of all these studies is completed, is that the normal mammalian somatic cell in primary culture seems to exhibit an extremely low spontaneous and induced mutant frequency compared to that reported for cell lines *in vitro*. This might indicate differences in effectiveness of replication or repair. This mutant frequency could be used to make some estimates of spontaneous and induced mutants per day 13 fetus if all cells in the fetus were equally susceptible. The ratio of ENU 1-induced mutants to spontaneous for TG is 3181/fetus to 25/fetus. For the DAP^r system, the ENU 1-induced value would be 1 mutant per 1600 day 13 fetuses and for the spontaneous value, 1 mutant for 190,000 fetuses. Such estimates have implications for any one hit or two hit mutation theories of carcinogenesis or even a non-mutation "clonal-expansion-of-mutant" theory.

Renal differentiation. Previous studies indicated the feasibility of developing a culture medium that allows for maintenance and differentiation of renal blastema in the absence of an inductive tissue. Since this medium included pituitary extract, a preparation rich in a variety of growth-promoting substances, efforts have since been directed toward further definition of the factor(s) involved. Preliminary purification of the extract indicates that the factor is an acid-stable, heat-sensitive substance with a molecular weight greater than 3000, suggesting it is polypeptide in nature. Further purification will employ standard column chromatography techniques, including gel permeation chromatography and HPLC. In a simultaneous effort, isolated renal blastema have also been incubated in culture with a variety of commercially available growth factors, which were applied either individually or in combination. These included growth hormone, fibroblast growth factor, nerve growth factor, insulin-like growth factors, TGF- β , and platelet-derived growth factor. Only FGF enhanced blastemal cell growth, and none of the factors, whether individually or in concert, promoted tubulogenesis in the blastema. These observations suggest that a novel growth/differentiation factor may be responsible for renal tubule growth and development.

Similarly, preliminary efforts to define culture conditions for rat renal tumor growth and/or differentiation induction have involved application of a variety of

growth/differentiation factors to the media of primary cultures of dissociated renal tumor cells. While Type 1 collagen, pituitary extract, and epidermal growth factor initially improved the growth of renal mesenchymal tumor cells, epithelial-like populations eventually predominated and persisted in culture. This resulted either from selection of epithelial populations associated with these tumors or from the differentiation of these tumor cells when cultured in a milieu known to induce differentiation of normal renal blastema. Renal cortical epithelial tumors were also cultured in a similar series of growth factors and found to thrive in medium containing EGF and pituitary extract but lacking any collagen, which inhibited their growth. Attempts to replace the pituitary extract with commercial growth factors have, thus far, been unsuccessful as they were when we tried to replace pituitary extract in culture of normal blastema. Thus, the possibility remains that the same factor necessary for the conversion of renal mesenchyme to tubular epithelium may also enhance renal epithelial tumor cell growth.

Publications:

Diwan BA, Ohshima M, Rice JM. Effects of postnatal administration of tumour-promoting barbiturates on the development of tumours initiated by prenatal exposure of fetal rats and mice to N-alkylnitrosoureas. IARC Sci Publ (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05299-08 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Interspecies Differences in Transplacental Carcinogenesis and Tumor Promotion

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

PI:	R. A. Lubet	Expert	LCC	NCI
Others:	J. M. Rice	Chief	LCC	NCI
	L. K. Keefer	Chief, Chemistry Section	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology & Pathogenesis Sect.	LCC	NCI
	M. S. Miller	Sr. Staff Fellow	LCC	NCI
	R. W. Nims	Chemist	LCC	NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (B. Diwan); Univ. of Ohio Medical School, Toledo, OH (J. Klaunig); Vanderbilt University, Nashville, TN (F. P. Guengerich); Hoffmann LaRoche, Nutley, NJ (R. M. McClain)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

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)

Relationships between cytochrome P-450IIB1 and tumor promotion continue to be studied. We have found that outbred Zucker rats (male or female) display a relatively normal induction (greater than 40x) of cytochrome P-450IIB1 following treatment with phenobarbital, but display minimal induction (less than 8x) following treatment with the phenobarbital analogs, barbital or ethylphenylhydantoin. In contrast, F-344 (either male or female) or DA rats (female) show high induction (greater than 40x) following treatment with phenobarbital, barbital, or ethylphenylhydantoin; and hepatocarcinogenesis in F344 rats can be strongly promoted by all three compounds. Promotion studies in Zucker rats are in progress. We have found that the benzodiazepine clonazepam is a specific and relatively potent inhibitor of cytochrome P450 both in vitro and in vivo in mice and does not promote hepatocarcinogenesis in that species. The cytochrome P450IIB activity we examined is presumably only one manifestation of a "pleiotropic" response which we have now shown includes epoxide hydrolase, UDP glucuronyl transferase and glutathione transferases. In addition to these mechanistic studies in rodents, we are examining the induction of these enzymes by phenobarbital in a variety of other species, including nonhuman primates, (*Erythrocebus patas*; *Macaca fascicularis*) to examine the relationship between induction of various enzymes, especially cytochrome P450IIB1, and liver tumor promotion. Finally, studies have been initiated to determine the mechanism by which potent inducers of cytochrome P450IIB1 promote carcinogenesis in the thyroid by examining their effects on follicular cell proliferation and levels of various thyroid hormones.

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

R. A. Lubet	Expert	LCC	NCI
J. M. Rice	Chief	LCC	NCI
L. K. Keefer	Chief, Chemistry Section	LCC	NCI
J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
M. S. Miller	Sr. Staff Fellow	LCC	NCI
R. W. Nims	Chemist	LCC	NCI

Objectives:

To determine mechanisms by which a variety of compounds which induce cytochrome P450IIB1 promote tumorigenesis in liver, thyroid, and other tissues, employing biochemical, genetic, and chemical techniques. This includes characterization of a strain of rats which display an abnormally low response to the liver tumor promoters, barbital and ethylphenylhydantoin, greatly decreased induction of cytochrome P450IIB1; further characterization of the "pleiotropic response" induced by phenobarbital (PB)-like compounds in rodent and primate species which includes induction of epoxide hydrolase, specific forms of UDP-glucuronyl transferase, and glutathione transferases; and characterization of the benzodiazepine clonazepam, a relatively potent and specific inhibitor of cytochrome P-450 both *in vivo* and *in vitro*. To characterize cell proliferation and alterations in levels of various thyroid hormones following administration of compounds with different levels of promoting activity for thyroid tumorigenesis.

Major Findings:

Over the past few years we have characterized the tumor promoting activity of a variety of sedatives (barbiturates, hydantoins, alkylacetylureas, oxazolidinones) and found that there is a correlation between induction of cytochrome P450IIB1 and promotion of liver tumorigenesis. In an attempt to understand the mechanistic basis of the tumor promoting effects which we observed in rodents, we are pursuing four different avenues of investigation.

1) Genetic. We have characterized an outbred strain of rats (Zucker lean, both sexes) which show a relatively normal induction of cytochrome P450IIB1 following treatment with phenobarbital, but display more limited induction following treatment with barbital or ethylphenylhydantoin, two structural analogs of phenobarbital. We have observed this relative lack of induction in Zucker rats following barbital or ethylphenylhydantoin when using a variety of endpoints including induction of cytochromes P450IIB1 and IIB2, epoxide hydrolase, and glutathione transferases. These results support the hypothesis of a coordinately induced "pleiotropic" response to PB-type inducers (see below). In contrast, all three compounds are potent inducers in female DA or male or female Fischer 344 rats, and all three compounds are liver tumor promoters in F344 male rats. Therefore, we should be able to test in the Zucker lean rats whether the tumor promoting activities of phenobarbital and its structural analogs parallel the analog's ability to induce cytochrome P450IIB1. In addition, this rat strain may

prove particularly useful in understanding the nature of induction by phenobarbital-type compounds.

2) Pleiotropic Response. We have routinely characterized cytochrome P450IIB1 induction by measuring induction of benzyloxyresorufin O-dealkylase activities. However, PB-type inducers cause various hepatotoxic effects including hepatomegaly and induction of a variety of drug metabolizing enzymes. We have examined induction of cytochrome P450IIB1 (benzyloxyresorufin O-dealkylase activity), epoxide hydrolase (hydration of benzo(a)pyrene-4,5-oxides), UDP-glucuronyl transferase (glucuronidation of 3-OH-benzanthracene), glutathione transferases, (quantitated by hybridization of cellular RNA to specific plasmids), and cytochromes P450IIB1 and IIB2 (quantitated by hybridization to specific oligonucleotides). Using either various doses of PB or a variety of different inducers of cytochrome P450IIB1 (barbiturates, hydantoins), we have shown that all of these varied genes, and presumably others, are induced in a parallel, presumably coordinate, manner. These findings imply that there is some common step characteristic to induction by various inducers of cytochrome P450IIB1. However, the genes which we investigated are only a portion of the total "pleiotropic effect" which apparently includes other genes (e.g., α_2 globulin, aldehyde dehydrogenase), as well as still undefined processes. Induction of cytochrome P450IIB1 is associated with liver tumor promotion in many species.

Although the primary focus of this work on coordinate induction has involved the rat, for which we have various species-specific recombinant DNA probes, we are presently attempting to extend it to other rodents (mice, hamsters) and primate (*Erythrocebus patas*, *Macaca fascicularis*) species.

3) Development of specific chemical inhibitors. In view of the close association between P450 induction and promotion of liver tumorigenesis, it would appear that the actual expression of cytochrome P450IIB1, either directly or indirectly, may be involved in the promotion process. Therefore, it becomes of great importance to find potential specific inhibitors of cytochrome P450 which may be effective both *in vitro* and *in vivo*. We initially investigated clotrimazole, one of the most potent *in vitro* inhibitors of cytochrome P450 ($K_i < 10^{-7} M$) and a known antimycotic agent. This compound proved not to be an effective inhibitor *in vivo* based on zoxazolamine paralysis time of phenobarbital treated animals. We have recently shown clonazepam to have limited tumor promoting activity in mice, despite the fact that it is a good inducer of cytochrome P450IIB1, as are most inhibitors of this enzyme system. Clonazepam proved to be a moderate inhibitor *in vitro* ($K_{i2} \sim 30 \mu M$); however, it proved to be a significant inhibitor *in vivo* as well, greatly increasing the duration of paralysis induced by zoxazolamine. Therefore, it would appear to be of some interest to determine whether clonazepam itself is a tumor promoter in the rat where we have shown a close correlation between induction and promotion, and furthermore to determine whether or not clonazepam, acting as an inhibitor of P450, is able to inhibit the tumor-promoting effects of other chemicals.

4) Examination of structurally-unrelated compounds to further examine the relationship of P450IIB1 induction to liver tumor promotion. The primary focus of our studies has involved classes of compounds with sedative, anticonvulsant, or anxiolytic activity (barbiturates, hydantoins, dialkylacetylureas, oxazolidinediones, benzodiazepines). However, there are a variety of other

compounds, some of which are known tumor promoters, which apparently have the ability to induce cytochrome P450IIB1. Included among these are a variety of potential environmental contaminants, including DDT, α -hexachlorocyclohexane, certain isomers of the halogenated biphenyls (e.g., 2,4,5,2',4',5'-hexabromobiphenyl). These compounds, although structurally quite distinct from the sedative/anxiolytic series, all proved to be striking inducers of P450IIB1 and all have been shown to be liver tumor promoters. These results show that this relationship between P450 induction and tumor promotion is not strictly limited to the barbiturates and certain of their analogs.

TCPOBOP [1,4-bis[2,(3,5-dichloropyridyloxy)]-benzene] is perhaps the most potent inducer of cytochrome P450IIB1 in the mouse [ED₅₀~0.3mg/kg] but is a relatively weak inducer in the rat [ED₅₀>30mg/kg]. Our preliminary results with this compound in a tumor promotion assay show that TCPOBOP is a potent promoter in mice but appears to show little if any such activity in rats.

Another major focus of the laboratory has been to examine potential differences between various species and strains in their susceptibility to liver tumor promotion by CNS-active drugs and to examine for potential mechanisms underlying these differences. Our initial studies examined differences between strains or species of rodents, while our present focus has been on two species of nonhuman primates. The first, *Erythrocebus patas*, has been shown to be susceptible to phenobarbital promotion of hepatocarcinogenesis, while the second, *Macaca fascicularis*, apparently displays little or no sensitivity to promoting activity of PB. Interestingly, these results apparently correlate with the ability of phenobarbital to induce pentoxoresorufin O-dealkylase activity which is high in *patas* monkeys and low or negligible in *Macaca fascicularis*.

Promotion of thyroid tumorigenesis by potent inducers of cytochrome P450IIB1. We have previously observed that strong inducers of cytochrome P450IIB1 (e.g., phenobarbital, ethylphenylhydantoin, barbital, pentobarbital) all promoted the formation of thyroid tumors in DEN-initiated rats. In preliminary experiments, we found that a number of the thyroid tumor promoters (e.g., phenobarbital, barbital) caused increases in cell proliferation in the thyroid, while nonpromoting analogs did not. It has been demonstrated with the prototype inducer PB that the mechanism of thyroid tumor promotion probably entails increased metabolism of thyroxine (T4) which results in increased TSH release by the pituitary and increased cell proliferation in the target tissue. We are presently initiating a series of studies in collaboration with Dr. R. M. McClain (Hoffmann LaRoche) to determine whether such a mechanism is generally applicable to the compounds we have been examining. In this collaboration, blood levels of various thyroid hormones and TSH will be determined as well as proliferation in the thyroid itself, following short-term administration of various CNS-active agents.

The tumor promoting ability of clonazepam (CZP), a widely used benzodiazepine anticonvulsant, was investigated in the *in vivo* mouse liver tumor promotion assay and the *in vitro* mouse hepatocyte intercellular communication assay. The development of potentially preneoplastic hepatocellular foci of cellular alteration and hepatocellular neoplasms was studied in male B6C3F1 mice initiated, at 5 weeks of age, with a single i.p. injection of *N*-nitrosodiethylamine (NDEA; 90 mg/kg body weight) in tricapylin, followed by administration of either phenobarbital (PB; 0.05%) or CZP (0.068% or 0.14%) in diet beginning 2 weeks after carcinogen

injection and continuing to 60 weeks of age. Several mice from each group were killed at 16, 28, 40 or 60 weeks of age, and portions of liver and other organs were fixed in formalin and examined histologically. Unlike PB, CZP did not promote the development of preneoplastic hepatocellular foci or neoplasms (adenomas and carcinomas) in NDEA-initiated mice. Following limited (2 weeks) dietary exposure at 0.15%, CZP was a potent inducer of hepatic P450IIB1-mediated alkoxyresorufin *O*-dealkylase activities. In contrast, the degree of induction in hepatic tissue from mice fed 0.14% clonazepam for 53 weeks was markedly lower than that in mice fed 0.05% PB for 53 weeks. In the *in vitro* assay, the ability of two benzodiazepine derivatives, diazepam and CZP, to block intercellular communication between mouse primary hepatocytes was analyzed using a dye coupling assay. Diazepam, a strong tumor promoter in mouse liver, significantly inhibited mouse hepatocyte intercellular communication, while CZP had no significant effect.

In order to analyze the genetics of susceptibility to promotion of hepatocarcinogenesis in DBA/2Ncr and C57BL/6Ncr mice by phenobarbital (PB), reciprocal F₁ hybrid male mice were given 90 mg N-nitrosodiethylamine (NDEA)/kg body weight, i.p. at 5 weeks of age followed by 0.05% PB in drinking water. Hepatocellular adenomas and carcinomas were comparably increased in incidence and multiplicity in both reciprocal hybrids over mice given NDEA alone. Eight of 10 D2B6F1 progeny of DBA/2Ncr females mated with C57BL/6Ncr males, but only 1/10 B6D2F1 mice (progeny of C57BL/6Ncr females mated with DBA/2Ncr males) given PB after NDEA initiation developed single or multiple hepatoblastomas within 42 weeks. These small-celled, intensely basophilic tumors were characteristically hemorrhagic and highly malignant. Hepatoblastomas were mostly found within or adjacent to hepatocellular tumors. No hepatoblastomas were seen in either hybrid given NDEA alone. PB consistently enhanced development of malignant hepatoblastomas, as well as promoted hepatocarcinogenesis in D2B6F1 males, but did not elicit hepatoblastoma development in B6D2F1 males that were genetically identical to D2B6F1 males except for the reverse origin of their X and Y chromosomes.

Promoting effects of sodium salts of phenobarbital (NaPB) and barbital (NaBB) on the development of bladder tumors were investigated in F344 male rats initiated with *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) or *N*-nitrosobutyl-4-hydroxybutylamine (BBN). To initiate with FANFT, rats were fed 0.2% FANFT mixed in diet for either 2 or 6 weeks and 2 weeks later were offered diet containing 1000 ppm of NaPB or NaBB. Rats were killed either at 52 or 68 weeks of age. To initiate with BBN, rats were given 0.05% BBN in drinking water for 4 weeks and beginning one day later were fed NaBB mixed in diet at 1000 ppm for up to 52 weeks. NaBB promoted bladder carcinogenesis initiated by either FANFT or BBN; the incidence and average number of simple or preneoplastic nodular (PN) hyperplasias, papillomas and carcinomas per 10 cm of urothelium was significantly increased in the groups receiving NaBB following exposure to FANFT for 6 weeks ($p < 0.05$) or BBN for 4 weeks ($p < 0.01$). No such effect was seen in rats fed FANFT for only 2 weeks. NaPB also significantly increased ($p < 0.05$) the frequency of preneoplastic PN hyperplasias but not the average number of papillomas and carcinomas per 10 cm of urothelium in rats fed FANFT for 6 weeks. NaBB was an effective promoter of bladder carcinogenesis under these experimental conditions, as expected from its known promoting effect on transitional epithelium of the renal pelvis; but NaPB, in contrast, did not affect the incidence or multiplicity of bladder papillomas or carcinomas under these conditions. NaPB could be considered a promoter for bladder urothelium only by the less rigorous criterion that it increased the frequency of preneoplastic PN hyperplasia.

The ring hydrolysis products of the multi-tissue tumor promoting barbiturates, phenobarbital (PB) and barbital, were fed to F344/NCr male rats previously given a single initiating injection of *N*-nitrosodiethylamine. Ethylphenylacetylurea (EPAU) and diethylacetylurea (EEAU) derived respectively from PB and barbital both promoted development of hepatocellular adenomas, but were much weaker in this respect than PB. Both acetylureas were also selective inducers of P450IIB1-mediated benzyloxyresorufin *O*-dealkylase activity, but both were much less potent inducers than PB. Neither EPAU nor EEAU had an effect on tumor development in the thyroid, unlike both PB and, as shown previously, barbital. EEAU, but not EPAU, strongly promoted development of renal cortical epithelial tumors. The opening of the barbiturate heterocyclic ring and the subsequent decarboxylation to yield the dialkylacetylurea analogs thus resulted in compounds displaying a marked reduction in liver tumor promoting activity. EEAU appears to possess an ability to promote renal neoplasms similar to that of its parent compound, barbital. Ring opening appears to be accompanied by loss of promoting activity for thyroid follicular epithelium.

Publications:

Diwan BA, Hagiwara A, Ward JM, Rice JM. Effects of sodium salts of phenobarbital and barbital on development of bladder tumors in male F344/NCr rats pretreated with either *N*-[4-5(5-nitro-2-furyl)-2-thiazolyl]formamide or *N*-nitrosobutyl-4-hydroxybutylamine. *Toxicol Appl Pharmacol* 1989;98:269-77.

Diwan BA, Lubet RA, Nims RW, Klaunig JE, Weghorst CM, Henneman JR, Ward JM, Rice JM. Lack of promoting effect of clonazepam on the development of *N*-nitrosodiethylamine initiated hepatocellular tumors in mice is correlated with its inability to inhibit cell-to-cell communication in mouse hepatocytes. *Carcinogenesis* (In Press).

Diwan BA, Nims RW, Ward JM, Hu H, Lubet RA, Rice JM. Tumor promoting activities of ethylphenylacetylurea and diethylacetylurea, the ring hydrolysis products of barbiturate tumor promoters phenobarbital and barbital, in rat liver and kidney initiated by *N*-nitrosodiethylamine. *Carcinogenesis* 1989;10:189-94.

Diwan BA, Ohshima M, Rice JM. Promotion by sodium barbital of renal cortical and transitional cell tumors, but not intestinal tumors, in F344 rats given methyl(acetoxymethyl)nitrosamine, and lack of effect of phenobarbital, amobarbital, or barbituric acid on development of either renal or intestinal tumors. *Carcinogenesis* 1989;10:183-8.

Diwan BA, Ward JM, Rice JM. Promotion of malignant "embryonal" liver tumors by phenobarbital: increased incidence and shortened latency of hepatoblastomas in (DBA/2 x C57BL/6)F1 mice initiated with *N*-nitrosodiethylamine. *Carcinogenesis* (In Press).

Lubet RA, Nims RW, Ward JM, Rice JM, Diwan BA. Induction of cytochrome P-450b and its relationship to liver tumor promotion. *J Amer Coll Toxicol* 1989;8:253-62.

Lubet RA, Guengerich FP, Nims RW. Alkoxyresorufin *O*-dealkylase: potential indicator of exposure to environmental contaminants (e.g., DDT, α -hexachlorocyclohexane). *Arch Environ Contam Toxicol* (In Press).

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

PROJECT NUMBER

Z01CP05301-08 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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 Sect.	LCC	NCI
Others:	S. Rehm	Visiting Scientist	LCC	NCI
	R. Benveniste	Medical Officer	LVC	NCI
	P. Nara	Expert	LTCB	NCI

COOPERATING UNITS (if any)

Natl. Inst. of Allergy and Infectious Diseases, Bethesda, MD (E. Santos); Johns Hopkins University, Baltimore, MD (W. Gibson)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS

2.5

PROFESSIONAL

1.5

OTHER

1.0

CHECK APPROPRIATE BOX(ES)

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

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

A new method was developed to serve as a control for studies determining expression of oncogene and retroviral proteins in cells and tissues. Cell pellets were prepared from infected (HIV, SIV) cell lines or cell lines containing specific activating or amplified oncogenes. Localization of these antigens to cells and subcellular organelles was readily accomplished using the avidin biotin immunohistochemical technique. Expression of retroviral antigens in mice, humans, and nonhuman primates was found in unusual sites and cells including endothelium and epithelium. The major capsid protein of CMV was found to share an epitope with the HIV gag protein. The biology of pulmonary tumors induced in mice transplacentally by N-nitrosoethylurea were found to be dependent on time of target cell exposure in utero. A quantitative technique was developed to evaluate the pulmonary tumors.

PROJECT DESCRIPTION

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

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

Objectives:

To study and characterize the biology, pathology and pathogenesis of naturally-occurring and experimentally-induced tumors of laboratory animals and use this information to help elucidate the mechanisms of carcinogenesis in specific organs and by specific chemicals. To develop animal models for the study of human tumors and associated diseases. To develop methods especially immunohistochemistry, utilizing experimental animals to aid in understanding the nature, causes, natural history and prevention of human cancer and related diseases.

Major Findings:

To develop methods for detecting oncogenes, oncogene protein products, and retroviral expression in the various stages of carcinogenesis and retroviral infection as seen histologically and conceptually in laboratory animals and humans. A major new positive control technique was developed to insure accurate detection at the histopathological level of expression. In vitro monolayer cell culture, using cells with activated or amplified oncogenes (H-ras, K-ras or c-erbB-2), or lentiviral infections (HIV-RF or IIIB, SIV-MnIV or CAT), were prepared and cells were scraped from the flasks to produce a cell pellet by centrifugation (see also project Z01CP05399). The pellets were fixed in various fixatives including formalin and Bouin's fixative or frozen. Monoclonal or polyclonal antibodies to these antigens were used with avidin biotin peroxidase-complex (ABC) immunohistochemistry to localize the antigens in cell organelles by light microscopy. Antibodies were directed against protein peptides or the whole antigen. In all cases, protein expression could be demonstrated in the pellets derived from cell lines, usually in association with oncogene copy number and/or levels of protein expression as determined by Western immunoblots or mRNA levels (determined by others). Controls for these immunoreactive antibodies included absorption controls, i.e., absorption of the antibody with the antigen (peptide or protein) to which it was prepared, usually in protein excess. In several important cases the absorption inhibited or prevented immunoreactivity of the antibody with the antigen in fixed tissue sections. This pellet and absorption control provided us with important positive and negative controls for determination of protein expression in fixed tissue sections from animal and human biopsy and autopsy materials. This information was used in conjunction with determination of protein expression by others using Western blots. Most recently we attempted to determine levels of HIV-1 gene expression in H9 or Hut 78 lymphocytes infected in vitro and pelleted using an alkaline phosphatase-labelled HIV DNA probe.

Expression of retroviral antigens including those of the human and simian immunodeficiency viruses, mouse leukemia viruses and primate type D retroviruses were found in a variety of tissues of humans, monkeys and mice including some not usually considered sites of viral replication. *In situ* hybridization studies of some of the same tissues revealed HIV DNA expression. For mouse Moloney leukemia virus-infected mice, used as a model for tissue specific expression of retroviral antigens in formalin-fixed paraffin embedded tissues, many epithelial and lymphoid tissues were infected, as previously reported, using ultrastructural and immunofluorescent techniques. A major structural core protein of MoLV, p30, could be detected in salivary gland epithelium, pancreas acinar and endocrine cells, megakaryocytes, B lymphocytes in many lymphoid organs, and renal tubular epithelium. In parallel studies in Macaque monkeys with experimentally-induced SIV-induced immunodeficiency disease, SIV antigens were detected in brain macrophages and multinucleated giant cells, lymph node and splenic macrophages but not in lymphocytes in any stage of the disease. In humans with AIDS, cells expressing HIV antigens included lymph node follicular dendritic cells, brain macrophages and multinucleated giant cells, endothelium in brain and high endothelial venules of lymph nodes and rare node lymphocytes. In a few cases, HIV expression was seen in metastatic tumor cells in the heart, lung macrophages of children with pneumonia, intestinal epithelium and testes. *In situ* hybridization studies are in progress to determine HIV gene expression in the same tissues. These findings and those of other investigators have provided evidence that HIV infection of humans and monkeys may not only involve T cells and macrophages but also a variety of epithelial and mesenchymal cell types, which are host cells also of murine retroviruses. We also found that one rabbit polyclonal antiserum cross reacted to a cytomegalovirus (CMV) major capsid protein (MCP, 153kDa) and confirmed that the HIV gag protein was responsible for this cross reactivity. This finding may be indicative of a possible effect of HIV infection on CMV infection or vice versa. The immune response to one virus may affect the pathogenicity of the other virus in individuals.

Pulmonary alveolar tumors of mice were used as a model system to study effects of a transplacental carcinogen, N-nitrosoethylurea (NEU) on target cells and the biological and morphological nature and differentiation of tumors arising from these target cells. Pregnant C3H/HeNCr MTV⁻ mice were treated with a single intraperitoneal injection of 0.5 mmol NEU/kg on days 14, 16, or 18 of gestation. Six of the male offspring were studied at the ages of 2, 4, 8, 16, 32, and 52 weeks. The incidence and differentiation of lung tumors induced transplacentally in mice seemed to follow the development of fetal alveolar type II cells. Animals treated on day 14 of gestation had the lowest incidence (3.0 tumors/mouse); approximately 50% were papillary types at all ages. Tumor progression from solid to mixed types occurred at the earliest time (4 weeks), and most tumors of these mice were larger than those of mice treated later in gestation. Offspring of mice treated on day 16 of gestation had the highest lung tumor incidence (15.4 tumors/mouse). Mixed tumors occurred at 8 weeks, and tumor size and growth was intermediate between those of mice treated on days 14 and 18 of gestation. Mice treated on day 18 of gestation developed an average of 4.8 tumors/mouse. The incidence of papillary tumors varied but was usually above 50%; the first mixed tumors were observed late (at 32 weeks) and neoplasms were smaller than those of mice treated at days 14 or 16 of gestation. Only in animals treated on day 18 did tumors increase with age. The number of tumors detected grossly depended on size, and on average only 51% of neoplasms present were detected microscopically. Tumor

size can be determined from the number of sections per tumor since a mean number of histological sections correlated well with the perimeter length per tumor. These findings allowed us to develop a quantitative method for evaluation of pulmonary carcinogenesis in mice.

Publications:

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

Rehm S, Takahashi M, Ward JM, Singh G, Katyal SL, Henneman JR. Immunohistochemical demonstration of Clara cell antigen in lung tumors of bronchiolar origin induced by N-nitrosodiethylamine in Syrian golden hamsters. *Amer J Pathol* 1989;134:79-87.

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

Rhodes RH, Ward JM. Immunohistochemistry of human immunodeficiency virus in the central nervous system and an hypothesis concerning the pathogenesis of AIDS meningoencephalitis. In: Rotterdam H, Sommers SC, Pacz P, Meyer PR, eds. *Prog AIDS Pathol*. Philadelphia: Field & Wood (In Press).

Rhodes RH, Ward JM, Walker DL, Ross AA. Progressive multifocal leukoencephalopathy and retroviral encephalitis in acquired immunodeficiency syndrome. *Arch Pathol Lab Med* 1988;112:1207-13.

Ward JM. Classification of reactive lesions of lymph nodes. In: Jones TC, Ward JM, Mohr U, Hunt RD, eds. *Hemopoietic system monographs on pathology of laboratory animals*. New York: Springer-Verlag (In Press).

Ward JM. Classification of reactive lesions of spleen. In: Jones TC, Ward JM, Mohr U, Hunt RD, eds. *Hemopoietic system monographs on pathology of laboratory animals*. New York: Springer-Verlag (In Press).

Ward JM, Rehm S, Reynolds CW. Tumours of the hematopoietic system. In: Turusov VS, ed. *Pathology of tumours of the rat*. Second Edition. IARC Sci Publ (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05303-08 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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 & Pathogenesis Section	LCC	NCI
Others:	T. Enomoto	Visiting Fellow	LCC	NCI
	A. Perantoni	Staff Fellow	LCC	NCI
	N. Konishi	Visiting Fellow	LCC	NCI
	P. Donovan	Chemist	LCC	NCI

COOPERATING UNITS (if any)

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

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS

3.0

PROFESSIONAL

2.0

OTHER

1.0

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)

The possible contributions to carcinogenesis of activation of ras oncogenes by point mutation, transient or persistent enhanced levels of cell replication in target cells, and aging in mouse liver was investigated. In the liver of C3H mice, activation by point mutation of codon 61 of H-ras was found in much lower incidence than in B6C3F1 mice. Instead, the aging spontaneously initiated hepatocytes of these C3H mice were found to be highly susceptible to phenobarbital "carcinogenesis". BrdU immunohistochemistry was used for these and other strains of mice to study the rates of target cell replication in tumor promotion and carcinogenesis. Chronic increases in cell replication were not found to be associated with tumor promotion in mouse liver. In contrast, induced renal tubular hyperplasia did not play an important role in renal tumor promotion in mice exposed to a renal toxin, while in rats, hyperplasia was usually found associated with renal tumor promotion. We also found that aging nephropathy of F344 rats was associated with increased levels of DNA synthesis, a possible co-factor in spontaneous carcinogenesis or carcinogenesis by nongenotoxic carcinogens. In vitro models using renal epithelial cells of rats and humans and hepatocytes of two mouse strains were used to study cell survival and growth after exposure to the renal or hepatic in vivo tumor promoters of rats. In a comparison of rodent and human hepatocyte response in vivo, we found that autopsy specimens of humans exposed to phenobarbital for 10-49 years had hepatic lesions resembling those in rodents. Putative initiated cells were found in livers of aging F344/NCr rats. These cells were immunoreactive for glutathione S-transferase, placental form and may serve as targets of carcinogenesis by nongenotoxic tumor promoters, including phenobarbital.

PROJECT DESCRIPTION

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

J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
T. Enomoto	Visiting Fellow	LCC	NCI
A. Perantoni	Staff Fellow	LCC	NCI
N. Konishi	Visiting Fellow	LCC	NCI
P. Donovan	Chemist	LCC	NCI

Objectives:

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

Major Findings:

The primary causes and pathogenesis of naturally-occurring preneoplastic and neoplastic liver lesions and role of tumor promoters and chronic toxicity in selected strains of mice and F344/NCr rats was investigated. The role of oncogene activation and chronic toxicity with or without induced transient or persistent increased levels of hepatocyte DNA synthesis was studied in mice. In F344/NCr rats, we evaluated the phenotypic and biologic nature of naturally-occurring putative preneoplastic hepatocellular foci occurring in aging liver and the role of phenobarbital on focus and tumor progression. The role of renal toxicity and hyperplasia in renal carcinogenesis and tumor promotion was studied in select model systems in rats and mice. Finally, *in vitro* systems were developed to study the mechanisms of xenobiotic-induced endogenous carcinogenesis in mouse liver, rat urinary bladder and rat and human renal tubular epithelium using specific tumor promoters for these organs *in vivo*.

In natural liver tumors of C3H/HeNcr mice, *ras* proto-oncogenes were found to have a low rate of mutation as detected by the NIH 3T3 transformation assay and DNA amplification by the polymerase chain reaction. These findings are in great contrast to those in other mouse strains reported by other authors. The significance of this finding is under investigation. In order to understand the genetic susceptibility to tumor development and promotion, we found that C3H and DBA mice were highly susceptible to tumor promotion after carcinogen initiation, or phenobarbital "carcinogenesis" in aged mice, while C57BL mice were not susceptible. This difference appeared due to the response of the susceptible hepatocytes to phenobarbital or promotion of spontaneously or chemically-initiated hepatocytes rather than differences in metabolism of the xenobiotic. The aging liver of the C3H mouse (more than 52 weeks old) was particularly susceptible to phenobarbital (PB) "carcinogenesis," developing tumors in 12-24 weeks in contrast to more than 52 weeks for young mice. This phenomenon was not associated with persistent increases in levels of hepatocyte DNA synthesis. The same phenomenon occurred in mice given N-nitrosoethylurea, a liver and kidney carcinogen, transplacentally and di(2-ethylhexyl)phthalate (DEHP), a hepatocarcinogen and tumor promoter and renal toxin but noncarcinogen. Persistent increases in

hepatocyte labelling index were not found in mice given DEHP, although DEHP promoted the growth of hepatocellular neoplasms.

We applied Brdu (5-bromo-2'-deoxyuridine) immunohistochemistry for the first time to study chronic toxicity and its association with tumor promotion and carcinogenesis by nongenotoxic carcinogens and tumor promoters. Single pulse doses or long-term implantation of pellets containing Brdu were applied to mice or rats. Levels of DNA synthesis as measured by the Brdu labelling index (LI) were associated with organ-specific toxicity of the chemicals studied including the hepatic tumor promoters, PB, barbital sodium (BBS) and DEHP and the renal promoter BBS. Chronic toxins studied included the mouse hepatotoxin, acetaminophen, and mouse and rat renal toxin, DEHP. Surprisingly, although DEHP was a renal toxin in mice causing renal tubular hyperplasia and increases in levels of DNA synthesis, it was not a renal promoter and while it was a renal promoter in rats, it was not a renal toxin nor did it induce renal hyperplasia (increased levels of DNA synthesis). Aging nephropathy of F344 rats was found to be associated with significant increases of renal tubular levels of DNA synthesis. This hyperplastic state of the aging tubules may provide a basis for spontaneous renal carcinogenesis or as a cofactor in renal carcinogenesis by nongenotoxic carcinogens. Further development of the use of Brdu pellets for seven-day exposures to study the chronic hyperplastic effects of organ-specific toxins and promoters is in progress.

In an effort to provide significant comparative toxicology and carcinogenesis information for the assumption that rodent *in vivo* carcinogenesis and tumor promotion findings were relevant to the human experience, we studied the effects of PB in human autopsy liver specimens and developed *in vitro* cell culture systems to study the potential mechanisms of tumor promotion in rat, mouse and human cells *in vitro*. Human autopsy liver histopathology sections were reviewed from 35 neuropsychiatric patients from Denmark exposed to phenobarbital and other neuropsychiatric pharmaceuticals. In four patients exposed exclusively to phenobarbital for 10-49 years, adaptive hepatocellular changes were found which resembled those found in rodents exposed to PB. One patient also had hepatocellular foci similar to glycogen foci found in rats given carcinogens and tumor promoters.

We have established rat renal tubular epithelial-like cells in culture and exposed the cells to tumor promoters including BBS, nitrilotriacetic acid (NTA), and PB. We found that BBS and NTA inhibited cell-cell communication by a lucifer dye transfer assay, while PB did not. Also, the promoters had effects on colony size (growth). One of the two renal epithelial cell lines studied had no cell-cell communication as measured by the lucifer dye transfer method. These cells, however, responded to the hyperplastic effects of the renal tumor promoters. A comparative study in human renal cell cultures is in progress. Thus far, cell-cell communication has been found to vary greatly in the human cell cultures and methods for developing a uniform evaluation system are in development. After completion of this project, we will have a method for comparing the validity of *in vivo* rodent models for studying renal tumor promotion and *in vitro* findings with rat and human renal epithelial cells. This comparison should help us evaluate the comparative mechanisms of tumor promotion in rat and human kidney epithelium. A similar process is being developed for studying hepatocyte tumor promotion in

susceptible and resistant strains of mice or in susceptible aging C3H mice and the more resistant young C3H mice.

For several years we have been attempting to determine the mechanisms of "carcinogenesis" in aging rat liver by nongenotoxic chemicals. We have consistently demonstrated that the liver of the aging F344/NCr rat is highly susceptible to PB "carcinogenesis." Although we could not show that PB promoted the growth and development of the common basophilic foci found in aging F344 liver, we have recently identified a possible cell phenotype as a potential target of PB and other nongenotoxic carcinogens. Hepatocyte foci and individual hepatocytes containing glutathione S-transferase, ii (placental) form (GSTP) have been found in aging liver and progressively enlarge in size after exposure to PB. These foci are either amphophilic in phenotype or develop from a rare GSTP +/- basophilic focus which we can identify in formalin-fixed tissue sections with the use of ABC immunohistochemistry. These findings suggest a mechanism of "carcinogenesis" by nongenotoxic hepatocarcinogens; i.e., these chemicals promote the growth and development of naturally-occurring GSTP+ phenotypic amphophilic or basophilic hepatocellular foci. Procedures for the isolation from rat liver of these possibly spontaneously-initiated cells are under development.

Publications:

Diwan BA, Rice JM, Ward JM. Strain-dependent effects of phenobarbital on the development of liver tumors in inbred mice. In: Stevenson DE, Slaga TJ, eds. Mouse liver carcinogenesis: mechanisms and species comparisons. New York: Alan R. Liss (In Press).

Konishi N, Ward JM. Increased levels of DNA synthesis in hyperplastic renal tubules of aging nephropathy in female F344/NCr rats. *Vet Pathol* 1989;26:6-10.

Ward JM, Diwan BA, Lubet RA, Henneman JR, Devor DE. Liver tumor promoters and other mouse liver carcinogens. In: Stevenson DE, Slaga TJ, eds. Mouse liver carcinogenesis: mechanisms and species comparisons. New York: Alan R. Liss (In Press).

Ward JM, Hagiwara A, Anderson LM, Lindsey K, Diwan BA. The chronic hepatic or renal toxicity of di(2-ethylhexyl)phthalate, acetaminophen, sodium barbital, and phenobarbital in male B6C3F1 mice: autoradiographic, immunohistochemical, and biochemical evidence for levels of DNA synthesis not associated with carcinogenesis or tumor promotion. *Toxicol Appl Pharmacol* 1988;96:494-506.

Ward JM, Tsuda H, Tatematsu M, Hagiwara A, Ito N. Hepatotoxicity of agents that enhance formation of focal hepatocellular proliferative lesions (putative preneoplastic foci) in a rapid rat liver bioassay. *Fund Appl Toxicol* 1989;12:163-71.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05352-07 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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	Research Biologist	LCC	NCI
Others:	J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
	M. S. Miller	Senior Staff Fellow	LCC	NCI
	D. P. Chauhan	Visiting Fellow	LCC	NCI
	L. Beebe	Intramural Research Training Awardee	LCC	NCI
	I. S. Owens	Chief, Section of Drug Biotransformation	IRP	NICHD
	S. Park	Expert	LMC	NCI
	H. V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (H. Issaq); Pathology Associates (R. Kovatch); American Health Foundation, Valhalla, NY (S. Hecht and D. Hoffmann); University of Texas (L.J. Lu); Univ. of Chicago (R.G. Harvey)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS

2.50

PROFESSIONAL

1.75

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.)

In this work pharmacological, biochemical, and molecular approaches are used to study the factors that determine and modulate susceptibility to tumorigenesis during the perinatal period. Analysis of transplacental carcinogenesis and of imprinting of carcinogen metabolism by a polycyclic aromatic hydrocarbon, 3-methylcholanthrene (MC) continues with a murine model. Inducible fetal and maternal metabolism of MC influence these transplacental tumorigenic actions in complex ways, and the biochemistry and molecular biology of induction of activation of MC in maternal liver and in fetal liver and lung are being investigated in detail as related to tumor initiation. Phase II enzymes, resulting in solubilization, may also be important and several uridine diphosphoglucuronic acid transferases (UDGPT) have been found to be expressed or biochemically active in fetal tissue, though inducible only by repeated treatment with MC. Investigation of the DNA adducts of MC as related to tumorigenesis involves 32P-postlabeling; this work was done in collaboration with Dr. L.J. Lu. Adducts were found in the DNA of all fetal tissues examined (liver, thymus, lung, and skin) but were highest in the primary target organ, lung. Level of adducts in the maternal tissues was strain-dependent. Identification of the adducts is in progress. In another perinatal study, the transplacental effects of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK) were assessed in three strains of mice. The chemical was weakly positive in the fetuses, initiating tumors but only in the target organs of greatest susceptibility for the strain. Postnatal tumor promoters were employed in this investigation, and it was found that a single dose of a mixture of polychlorinated biphenyls (PCBs) to adult Swiss male mice, after transplacental NNK, resulted in promotion of liver tumors. The mechanism by which stored PCB congeners may influence tumor development is under investigation. A PCB-inducible enzyme, aminopyrine demethylase, was found to be elevated in mouse liver for nearly a year after a single treatment.

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

L. M. Anderson	Research Biologist	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
M. S. Miller	Senior Staff Fellow	LCC	NCI
D. P. Chauhan	Visiting Fellow	LCC	NCI
L. Beebe	Intramural Research Training Awardee	LCC	NCI
I. S. Owens	Chief, Section on Drug Biotransformation	IRP	NICHD
S. Park	Expert	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI

Objectives:

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

Major Findings:

Previous work in this section demonstrated, with a transplacental pharmacogenetic model, that the genetically-regulated capacity of both fetuses and mothers to respond to inducers of polycyclic aromatic hydrocarbon metabolism has an important determining role in susceptibility to tumorigenesis in lung and liver by 3-methylcholanthrene (MC). Inducibility in the fetus potentiates this process, but inducibility in the mother affords protection to the fetuses, and induction by the carcinogen itself or by preexposure to another active chemical can have these effects. Detailed analysis of the molecular, biochemical and pharmacokinetic events that underlie these important relationships is now in progress. Considerable progress has been made in delineating the relevant phase I enzyme activity in both maternal and fetal tissues (see Z01CP05524). Uridine diphosphoglucuronic acid transferase (UDGPT), a phase II enzyme, has also been studied, with dinitrophenol as substrate, and its ontogeny and induction characteristics examined. In addition, a cDNA probe for a mouse UDGPT has been employed in Northern and slot blots to demonstrate, for the first time, expression of this gene in mouse fetuses. It was not induced by MC or B-naphthoflavone. Another molecular aspect of this model for which investigation has started is DNA adduct formation, as indicated by ³²P-postlabeling, being carried out in collaboration with Dr. L.J. Lu. This is the first study of MC adducts by the postlabeling method and is revealing that there is strain dependence in total amount of adduct formed in various organs, with higher levels in lymph nodes and liver of pregnant DBA/2 mice vs the greater amounts in lung and placenta of C57BL/6 mice. In fetuses the level of adducts was particularly prominent in lung, the target tissue. Qualitative identification of the adducts is in progress.

Transplacental effects of N-nitroso compounds have also been investigated, since these have been implicated in childhood cancers. A study of N-nitrosodimethylamine's effects on mouse fetuses has been completed and published. Assay was also

completed of the transplacental effects of the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in mice. It caused a low but significant number of lung tumors in strain A mice and liver tumors in (C3H x C57BL)F1 and Swiss male offspring. Interestingly, a single 500 mg/kg dose of a mixture of polychlorinated biphenyls (PCBs) given at 8 weeks of age after transplacental NNK appeared to promote the development of liver tumors. A similar phenomenon had been observed earlier after neonatal N-nitrosodimethylamine. This finding seemed of potential significance in light of the common environmental occurrence of both nitrosamines and PCBs, the frequent presence of PCBs in breast milk, and the ubiquity of poorly metabolized congeners of PCBs in human body tissue. Investigation has started of the mechanism by which stored PCB congeners influence tumor development. In a preliminary experiment, a single high dose of PCBs resulted in persistent elevation of a PCB-inducible liver enzyme, aminopyrine demethylase, for nearly a year after treatment, to an extent that was directly correlated with body burden of PCBs. Continued biological effect of the retained congeners is thus confirmed and more detailed analysis is in progress.

Other perinatal experiments that are in progress or planned include (1) investigation of diethylstilbestrol as a perinatal carcinogen in the rat and modulation of this process by metabolism inducers; (2) assessment of other PAH carcinogens and modes of administration with the current pharmacogenetic mouse model, with an Ah congenic mouse, and with rats; (3) investigation of transplacental causation of ovarian tumors in C3H mice by 7,12-dimethylbenzanthracene; (4) effects of ethanol co-treatment on transplacental carcinogenic effects of nitrosamines (see project Z01CP05353).

Publications:

Anderson LM, Hecht SS, Dixon DE, Dove LF, Kovatch RM, Hoffmann D, Rice JM. Evaluation of the transplacental tumorigenicity of the tobacco-specific carcinogen 4-(methyl-nitrosamino)-1-butanone (NNK) in mice. *Cancer Res* (In Press).

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

Anderson LM, Jones AB, Riggs CW, Kovatch RM. Effect of pre-exposure of fetal mice to the enzyme inducer β -naphthoflavone on transplacental carcinogenesis by 3-methylcholanthrene. *Cancer Res* 1989;49:1676-81.

Anderson LM, Kovatch RM, Rehm S, Rice JM, Hagiwara A. Transplacental initiation of liver, lung, neurogenic, and connective tissue tumors by N-nitroso compounds in mice. *Fund Appl Toxicol* 1989;12:604-20.

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

PROJECT NUMBER

Z01CP05353-07 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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	Research Biologist	LCC	NCI
Others:	J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
	S. Rehm	Visiting Scientist	LCC	NCI
	S. S. Park	Expert	LMC	NCI
	H. V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)

Temple University, Philadelphia, PA (G. Harrington, P.N. Magee); Smith Kline Laboratories, King of Prussia, PA (C. Gombar); Univ. of South Florida, Tampa, FL (A. Giner-Sorolla); SEMA, Inc. (J. Phillips); Pathology Associates (R. Kovatch)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS

0.5

PROFESSIONAL

0.5

OTHER

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.)

This project focuses on metabolism of chemical carcinogens as a modulating factor in sensitivity to tumorigenesis in adult animals. A model for study of interaction of chemical carcinogens, physical factors, and genetics in causation of mesotheliomas has been established. Several studies on N-nitrosocimetidine as an initiator and enhancer of skin tumor development have been completed. A detailed review of immunohistochemical staining of cytochrome P450s is in press, and more work is planned in this area. Current new experimental work has addressed the mechanisms by which ethanol may modify tumorigenesis by the N-nitrosamines. This interaction is being studied in the mouse and in the patas monkey. In the mouse chronic co-treatment with 10-20% ethanol resulted in a three- to fivefold increase in numbers of lung tumors at doses of N-nitrosodimethylamine from 0.5-5 ppm. New results show a similar enhancing effect of ethanol on forestomach tumors caused by N-nitrosodiethylamine and on lymphomas caused by N-6-methylnitrosoadenosine. This effect was postulated to be due to competitive inhibition of nitrosamine metabolism in liver, a hypothesis that was confirmed by demonstration of dramatic increases in both maximum blood levels and duration in the blood. Under similar dosing conditions there was a three- to ninefold increase in amounts of the promutagenic adduct, O6-methylguanine in lung DNA. Interestingly, levels of adducts in liver DNA did not change. In the patas monkey, NDMA clearance was also greatly retarded by simultaneous exposure to ethanol, resulting in a 20-fold increase in area-under-the-clearance curve. In biochemical assays NDMA demethylase activity was considerably lower in patas liver vs mouse liver, but apparent bioavailability was high for both species; important extrahepatic clearance sites are suggested. This ongoing study may continue to yield important clues about the ways in which ethanol, and similar compounds, influence cancer etiology in humans.

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

L. M. Anderson	Research Biologist	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
S. Rehm	Visiting Scientist	LCC	NCI
S. S. Park	Expert	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI

Objectives:

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

Major Findings:

Humans are exposed to a variety of chemical agents, simultaneously or in sequence. The ways in which these may interact to affect cancer risk have received relatively little attention at either the organismal or cellular level. Ethanol contributes to risk of human cancer at a variety of sites, but the mechanism by which it does so is unclear, since it is not carcinogenic per se in any animal model. We are investigating the hypothesis that it acts by enhancing the tumorigenic effectiveness of genotoxic environmental carcinogens. In our model, ethanol co-administered orally to mice with N-nitrosamines results in increased numbers of tumors in several targets, including lung tumors caused by N-nitrosodimethylamine, forestomach tumors caused by N-nitrosodiethylamine, and lymphomas caused by N⁶-methylnitrosoadenosine. A likely component of the mechanism of this striking effect of ethanol on tumorigenesis is competitive inhibition of the cytochrome P450 isoform(s) that catalyzes nitrosamine oxidation in the liver, resulting in increased delivery to distal targets. To test this idea, we are carrying out detailed pharmacokinetic analysis of nitrosamine and ethanol clearance and the interaction between the two, in both mice and patas monkeys. In both mouse and monkey, cotreatment of ethanol with NDMA resulted in a dramatic increase in duration of high blood levels, and in some cases elevation in maximal levels as well. The effect was dependent on ethanol concentration. In the monkey, intragastric pretreatment with 20% ethanol before an i.v. dose of 1 mg/kg resulted in persistent circulating levels of NDMA for at least 6 hrs (vs 1.5 hrs. in the absence of ethanol) and a 20-fold increase in the area under the clearance curve. As this analysis continues, we are also testing the further hypothesis that the increased circulating levels may result in greater degrees of cellular change associated with tumorigenesis. In confirmation of this, when 20% ethanol was included in an intragastric NDMA dose of 5 mg/kg, levels of promutagenic O⁶-methylguanine DNA adducts in mouse lung were increased threefold at 2 hrs. and ninefold at 4 hrs., compared with mice treated with NDMA only. Further pursuit of this type of integrative analysis should eventually yield a full understanding of the interaction of these chemicals to modulate tumorigenesis.

Another component of this project, analysis of N-nitrosocimetidine as a tumor initiator and enhancer, has been completed and prepared for publication. Also, a detailed review of immunohistochemical staining of cytochrome P450s has been completed, and further work in this area is planned, related to both the above project and to other LCC projects.

Publications:

Anderson LM. Increased numbers of N-nitrosodimethylamine-initiated lung tumors in mice by chronic co-administration of ethanol. *Carcinogenesis* 1988;9:1717-9.

Anderson LM, Giner-Sorolla A, Hagiwara A, Perantoni A, Reed C, Enomoto T, Rice JM. Initiation of murine skin tumors and oncogene activation by N-nitrosocimetidine. *Carcinogenesis* (In Press).

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Anderson LM, Ward JM, Park SS, Rice JM. Immunohistochemical localization of cytochrome P450 with polyclonal and monoclonal antibodies. *Pathol Immunopathol Res* 1989;8:61-94.

Rice JM, Kovatch RM, Anderson LM. Intraperitoneal mesotheliomas induced in mice by a polycyclic aromatic hydrocarbon. *J Toxicol Environ Health* 1989;27:153-61.

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

PROJECT NUMBER

Z01CP05399-06 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Oncogene Expression in Chemically Induced Tumors

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

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

COOPERATING UNITS (if any)

Universitatsspital Zurich, Switzerland (O. Wiestler)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section, Developmental Biology Working Group

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS

5.0

PROFESSIONAL

3.5

OTHER

1.5

CHECK APPROPRIATE BOX(ES)

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

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

The expression of activated cellular oncogenes in chemically-induced tumors in rodents (rats, mice, and Syrian hamsters) and in selected homologous human neoplasms, and the relationship of oncogene expression to progression from the normal to a neoplastic phenotype are being studied by transfection of NIH 3T3 and SHOK cells and by hybridization techniques including oligonucleotide probing for specific point mutations. Polymerase chain reaction (PCR) techniques are being employed to amplify selected genomic segments in order to search for subpopulations of cells in a given tissue in which activating oncogene mutations may be present. Such subpopulations of cells seem to occur naturally, with a point mutation in the c-H-ras gene, in the skin of SENCAR mice that have been bred for high susceptibility to squamous papilloma and carcinoma induction. The c-neu (HER-2, c-erbB-2) gene is the only growth-factor receptor-protein kinase type oncogene known to be mutationally activated in an experimental tumor, the rat schwannoma. We are now able to confirm that activation of this gene also occurs in homologous tumors in the mouse and Syrian hamster as well, and gene cloning and sequencing studies are in progress to ascertain whether this also occurs in comparable human neoplasms. Oncogene protein expression was found to be associated with gene amplification or mutation and depended greatly on the antibody used, tissue fixative, and tissue examined. Chemically-induced bladder tumors with prominent H-ras p21 expression were found to have mutations in codon 61, only after utilizing the PCR and restriction fragment length polymorphism (RFLP) techniques.

PROJECT DESCRIPTION

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

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

Objectives:

To identify activated oncogene sequences in specific types of chemically induced neoplasms in rats, mice, and other species in comparison with normal, nonneoplastic tissues in the same animals. To isolate and characterize the oncogene sequences found as mutant or wild-type alleles.

Major Findings:

While previous investigation of rat renal mesenchymal tumors initiated by DMN-OMe showed the consistent activation of the K-ras oncogene by GGT → GAT transition mutation, further study of these tumors using polymerase chain reaction technology indicates that other mutations have also occurred within the same tumors. Single mutations, GGT to GAT or to GTT, were observed in some tumors with transforming K-ras sequences; however, these mutations in combination with GGT to GCT transversions were also frequently seen by dot blot analysis of PCR fragments. While we cannot distinguish whether mutations arose within different alleles in the same tumor cell or within different regions of the tumor, either possibility suggests that mutations in this region do not necessarily occur during the initiation phase, but instead may also occur during progression.

A significant number of the renal mesenchymal tumors with K-ras activation also contain no codon 12 mutations. To further characterize the transforming lesion, PCR-amplified sequences surrounding codon 12 and 13 have been synthesized asymmetrically and sequenced directly using Taq polymerase. Thus far, no additional mutations have been identified in this region, leaving codon 61 as the only other reported major site associated with *ras* transformation to be examined.

Efforts to expand these studies have included evaluations of similar tumors induced with other agents or renal tumors mimicking different stages of renal development. Six renal cortical epithelial tumors (adult-type), derived from the same protocol responsible for the tumors described above, contained no transforming sequences by NIH 3T3 transfection assay and no detectable codon 12 mutations in dot blot analyses of PCR amplified fragments, suggesting that these tumors progress via a different pathway than the mesenchymal tumors. Renal mesenchymal tumors induced by nickel subsulfide have all been negative in transfection assay despite their highly aggressive nature *in vivo*, and preliminary screening for codon 12 mutations in the K-ras oncogene has provided no indication for lesions at

this position. Most recently, evaluations of renal mesenchymal tumors or nephroblastomas induced in Noble rats by DMN-OMe or ENU, respectively, have identified transforming sequences in both tumor types.

Progress in the study of *neu* activation in chemically induced schwannomas includes the observation that *neu* is consistently activated in diverse species. Both hamster and mouse schwannomas contain transforming sequences associated with the *neu* oncogene. Attempts to identify the activating lesions have been thwarted by the considerable species sequence heterogeneity surrounding and including the transmembrane region responsible for activation in the rat. We are therefore in the process of cloning and sequencing this region for hamster and mouse.

To determine the general nature of *neu* activation in the rat despite different modes of tumor induction, spontaneous tumor DNAs from paraffin-embedded tissues have been extracted and PCR amplified. Previously we reported consistent detection of a specific activating point mutation, a T → A transversion, in the *neu* oncogene in ethylnitrosourea-induced rat schwannomas (PNAS 84:6317, 1987). Spontaneous schwannomas are very rare in rats, so evaluation of these tumors for a similar mutation requires the use of paraffin-embedded archival material and amplification of tumor DNA sequences by polymerase chain reaction (PCR). Paraffin sections (1 μm) of naturally-occurring tumors in BD VI rats were xylene- and ethanol-extracted, lyophilized, and rehydrated with PCR incubation mixture. Primer sequences (20mer) were selected from published cDNA *neu* sequences that surround the putative transmembrane domain of the protein product and have minimal homology with *erbB-1*. Use of such primers (1:1 ratio) was expected to generate a 144 bp fragment based upon cDNA sequences. PCR products were separated in 10% polyacrylamide gels and multiple bands were observed. Fragments were electroblotted to nylon filters and probed with oligonucleotides specific for the transmembrane region. Two distinct bands, 600 and 1000 bp in size, hybridized with the probe, indicating the presence of an intron(s) in this region. Efforts to detect mutations directly in PCR products by dot blot hybridization of heat-denatured samples were inconclusive. PCR products were then further amplified by asymmetric synthesis (100:1 primer ratio), which improved sensitivity for detection of *neu* sequences by more than 10-fold. As a result, the same T → A transversion as detected in at least 3 of 10 non-induced soft tissue tumors: one that was histologically a schwannoma and was positive for S-100 protein by immunohistochemistry, one (also positive for S-100) that contained predominantly the cystic component of Antoni type B tissue, and one that had histologic features of (neuro?)fibrosarcoma and was negative for S-100. These results indicate that the same T → A transversion observed in ENU-induced rat schwannomas also occurs in histogenetically identical naturally-occurring tumors. Thus, activation of *neu* by point mutation may represent a common determinant for neoplastic transformation in this specific cell type. Three of 10 neurogenic tumors (2 schwannomas and 1 (neuro?)fibrosarcoma contain the same T → A transversion found in ENU-induced F344 rat schwannomas. In addition, 2 of 6 DMBA-initiated schwannomas exhibited the same transversion. Despite the presence of transforming sequences in 3 of 4 schwannomas lacking the mutation, transformants from these tumors did not hybridize with a probe specific for the extracellular portion of the *neu* gene, suggesting that either the gene has suffered a significant deletion of the extracellular sequence or a different oncogene is responsible for the transforming activity. In any event, these results indicate that a different mechanism is involved in DMBA-induced schwannomas.

Finally, we have acquired a significant number of human tumors representing the variety of lesions that occur in neurofibromatosis patients, including schwannomas from patients with von Recklinghausen's disease and bilateral acoustic neurofibromatosis. Screening of these tumors by transfection assay resulted in the identification of an activated N-ras oncogene in a neurofibrosarcoma. Since the human *neu/erbB-2* oncogene is believed to be much larger in size than the limits placed on DNA fragmentation by purification procedures, it is not surprising that negative results were observed.

DNAs from human schwannomas were amplified by polymerase chain reaction using primers flanking the entire region encoding the putative transmembrane domain of the HER-2/*neu* oncogene. Analysis of the amplified DNA product by gel electrophoresis revealed multiple bands including the one with the expected size. This band, in addition to a larger one, hybridized with a synthetic oligonucleotide homologous to a portion of the putative transmembrane domain. The multiple bands might have arisen because of the sequence homology of the HER-2/*neu* gene with other receptor type proteins, e.g., EGF receptor. One of the five amplified DNAs tested failed to show any positive signal with the transmembrane specific oligonucleotide probe, indicating possible mutation in this tumor DNA.

Because of the amplification of multiple segments of DNA, it was necessary to size-fractionate and purify the desired fragment. In addition, partial degradation of genomic DNA during the amplification reaction resulted in undesirable contamination of the DNA even after purification of the fragments. We are attempting to eliminate this problem by cloning the DNA fragment into M13 mp 19 vector and repeatedly screening the plaques using c-DNA as probe. Some of the DNAs extracted from the phage containing inserts gave strong signals when probed with the c-DNA. These clones are currently being analyzed by nucleotide sequencing to detect any mutation. Application of this approach to detect subtle alteration in tumor DNA in other species is being considered.

It has been reported that about 50% of human colorectal carcinomas have *ras*-gene mutations (Vogelstein *et al.*, *New Eng. J. Med.* 319: 525, 1988) and most human carcinomas of exocrine pancreas contain K-*ras* mutations (Almoguera *et al.*, *Cell* 53: 549, 1988). To extend these observations to epithelial tumors of the human female reproductive tract, high-molecular-weight DNA was isolated from human ovarian, uterine, and cervical neoplasms and evaluated for oncogene activation by NIH 3T3 transfection assay. Two of 7 endometrial carcinomas showed transforming activity, while DNA from 4 of 4 ovarian tumors and 4 of 4 cervical carcinomas did not transform 3T3 cells. Southern blot analysis with probes specific for K-*ras* revealed that both transformants contained human-specific K-*ras* sequences. In order to evaluate NIH 3T3 transformants and primary tumors for specific mutations in K-*ras*, DNA sequences surrounding codon 12 were amplified by polymerase chain reaction. By dot blot hybridization with 20bp anti-sense oligonucleotides, GGT to GAT transitions in codon 12 were found in DNAs from both transformants and both primary endometrial carcinomas. One of the 4 ovarian tumors, an immature teratoma, contained a GGT → GAT transversion in codon 12. The 4 cervical carcinomas, which did not show transforming activity, contained no mutations in codon 12. Six additional endometrial carcinomas (2 primary tumors, 3 cell lines, and one tumor passaged in nude mice) yielded the same GGT → GAT mutation twice (1 cell line, 1 tumor passaged in nude mice) and a GGT → GTT transversion twice (1 cell line, 1 primary tumor), for a total of 6 K-*ras* codon 12 point mutations in 13

endometrial carcinomas. Eight additional ovarian tumors were also studied; 1 mucinous adenocarcinoma had a GGT → GAT mutation, for a total of 2 K-ras codon 12 mutations in 12 tumors. Point mutation of K-ras in codon 12 is comparably frequent in uterine endometrial carcinomas and in colorectal carcinomas and may have similar significance. Cervical and ovarian tumors do not appear to share this characteristic.

In order to develop assays for determining the pathogenesis of tumors and role of oncogene protein product expression in tumor development and progression, we studied the expression of ras p21, using monoclonal and polyclonal antibodies to ras p21 peptides or whole proteins, in human and rodent fixed tissue specimens. Generally, p21 expression is associated with gene amplification, increased level of gene expression, or gene mutation. In our most interesting finding, p21 was expressed on urinary bladder tumors induced by N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in Bouin's fixed tissue only. Only 10% of the tumors had transforming ability in the NIH 3T3 transfection assay. Application of the polymerase chain reaction (PCR), however, enabled detection of transforming sequences in a more sensitive assay. After enzymatic amplification of tumor DNA with PCR, 10% of tumors were demonstrated to contain a GGA → GAA transition mutation in codon 12 of H-ras by dot blot hybridization using mutation-specific probes, and more than 60% of the bladder tumors were found to have mutations in codon 61 of H-ras by restriction fragment length polymorphism using either XbaI or TaqI. Thus, we found that enhanced p21 expression in tumors by immunohistochemistry as a sensitive method for detecting oncogene activation.

Publications:

Rice JM, Ward JM. Cardiac neurilemma, rat. In: Jones TC, Mohr U, Hunt RD, eds. Nervous system. ILSI monographs on pathology of laboratory animals. Berlin, Heidelberg: Springer-Verlag 1988;165-9.

Rice JM, Ward JM. Schwannomas (induced), cranial, spinal, and peripheral nerves, rat. In: Jones TC, Mohr U, Hunt RD, eds. Nervous system. ILSI monographs on pathology of laboratory animals. Berlin, Heidelberg: Springer-Verlag 1988;154-60.

Ward JM, Perantoni AO, Santos E. Comparative immunohistochemical reactivity of monoclonal and polyclonal antibodies to H-ras p21 in normal and neoplastic tissues of rodents and humans. *Oncogene* 1989;4:203-13.

Watatani M, Perantoni AO, Reed CD, Enomoto T, Wenk ML, Rice JM. Infrequent activation of K-ras, H-ras, and other oncogenes in hepatocellular neoplasms initiated by methyl(acetoxy-methyl)nitrosamine, a methylating agent, and promoted by phenobarbital in F344 rats. *Cancer Res* 1989;49:1103-9.

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

PROJECT NUMBER

Z01CP05487-04 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Carcinogenesis and Mutagenesis by Fecapentaenes

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

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

COOPERATING UNITS (if any)

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

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS

0.75

PROFESSIONAL:

0.25

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.)

Fecapentaenes are potent human fecal mutagens derived from anaerobic bacteria and are therefore potential candidates as human colon carcinogens. Several in vitro studies by other investigators have demonstrated potent mutagenic effects of fecapentaene-12 (FP-12) in bacterial cells, low cell transforming activity in vitro in some cells and mammalian mutagenic activity. The chemical purity and stability of FP-12 was determined to develop effective handling procedures during rodent exposure. The diacetate of FP-12 was also synthesized. Rodent carcinogenesis experiments were carried out to determine potential carcinogenic activity. Skin painting studies in SENCAR mice showed lack of tumor-initiating activity, complete carcinogenesis or tumor-promoting activity in three separate experiments, some of which were repeated to conclusively demonstrate the negative findings. Intrarectal and subcutaneous administration to mice and rats have not provided convincing evidence of carcinogenesis by FP-12. Total doses of up to 16 mg of FP-12 were used. The limitation of these in vivo assays involved the potentially low total doses utilized. FP-12 was mutagenic in vivo in rats by the subcutaneous granuloma pouch assay, but no tumors developed at the injection site. Our studies provide no evidence for the carcinogenicity of FP-12, although a weak carcinogenic effect has not been eliminated. Recent epidemiological findings of fecapentaene levels in human stools have shown no increased FP levels in colon cancer patients. This project is being phased out at this time.

PROJECT DESCRIPTION

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

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

Objectives:

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

Major Findings:

Solutions of synthetic fecapentaene-12 (FP-12) intended for carcinogenicity studies were found to decompose extremely rapidly during customary dosage procedures. Apparent half-lives as short as 15 minutes were observed. While rates and even the qualitative course of decomposition were surprisingly variable in replicate experiments, high concentration and exposure to air were confirmed to be especially important destabilizing influences. The results suggested a primary role for a radical decomposition mechanism in the presence of atmospheric oxygen. Consistent with this hypothesis, FP-12 solutions were significantly stabilized by the radical chain-breaking antioxidant, vitamin E. On the other hand, dithiothreitol greatly destabilized FP-12, presumably because of its nucleophilicity. The diacetyl diester of FP-12 was more soluble than the parent diol, but its decomposition rates in the presence and absence of vitamin E were similar to those of unesterified FP-12. Ultraviolet irradiation of an all-*trans* FP-12 solution decreased its concentration by 70% in 0.5 minutes. The mutagenicities of the decomposition/isomerization products of FP-12, as studied in *Salmonella typhimurium* tester strain TA 100, ranged from negligible to comparable with all-*trans* FP-12 itself. It is concluded that unchecked decomposition of fecapentaene preparations can profoundly affect biological tests therewith. While this can be largely controlled through the use of rigorous precautions, including protection from air, light, nucleophiles, and acids as well as selection of the lowest concentration compatible with the application at hand, the data argue strongly for inclusion of appropriate quality control measures in all future dosing operations to prove that the biological activity reported is that of the fecapentaene itself rather than that of a decomposed dosing solution.

Publications:

Streeter AJ, Donovan PJ, Anjo T, Ohannesian L, Sheffels PR, Wu PP, Keefer LK, Andrews AW, Bradford WW, Reist EJ, Rice JM. Decomposition and quality control considerations in biological work with fecapentaene preparations. *Chem Res Toxicol* (In Press).

Ward JM, Anjo T, Ohannesian L, Keefer LK, Devor DE, Donovan PF, Smith GT, Henneman JR, Streeter AJ, Konishi N, Rehm S, Reist EJ, Bradford WW, Rice JM. Inactivity of fecapentaene-12 as a rodent carcinogen or tumor initiator. Cancer Lett 1988;42:49-59.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05488-04 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Mechanisms of Inorganic Carcinogenesis: Cadmium

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

PI:	M. P. Waalkes	Pharmacologist	LCC	NCI
Others:	M. S. Miller	Senior Staff Fellow	LCC	NCI
	A. O. Perantoni	Staff Fellow	LCC	NCI
	N. Konishi	Visiting Fellow	LCC	NCI
	S. Rehm	Visiting Scientist	LCC	NCI
	S. Rhodes	Intramural Research Training Awardee	LCC	NCI
	Z. Wahba	Intramural Research Training Awardee	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology & Pathogen. Sect.	LCC	NCI

COOPERATING UNITS (if any)

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

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Inorganic Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS

4.5

PROFESSIONAL

3.5

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.)

The mechanisms of cadmium carcinogenesis are under active investigation. At the organismal level, malignant testicular interstitial cell tumors were induced by repeated exposure to cadmium. Such exposure was also associated with a high incidence of hepatocytic metaplasia of the pancreas, a finding possibly related to the suppression of pancreatic carcinogenesis by cadmium in the rat. Likewise, cadmium was found to suppress liver tumor initiation and promotion in the B6C3F1 mouse. Cadmium feeding increased the incidence of prostatic tumors and leukemia in rats. Dietary zinc was found to have a variable effect on cadmium carcinogenesis. Deficiency in zinc decreased the incidence of prostatic lesions and leukemias in rats fed cadmium while increasing the number of neoplastic foci in the testes after cadmium injection. A suppression of cadmium-induced prostatic tumors by zinc deficiency was linked to increased incidence of prostatic atrophy in zinc-deficient animals probably due to reduced testicular function, further indicating the key role of androgen input for cadmium-induced prostatic tumorigenesis. In this regard it was shown that prostatic accumulation and retention of cadmium are dependent on circulating testosterone levels. Metallothionein, the low molecular weight protein thought to confer tolerance to cadmium, was found to be absent not only in the testes, but also in the rodent prostate and ovaries. The testicular cadmium-binding protein (TCBP) detected in place of metallothionein was found, unlike metallothionein, to be uninducible by pretreatments that result in tolerance to cadmium testicular carcinogenicity, such as high dose zinc or low dose cadmium. Such treatments, however, prevented cadmium-induced cytotoxicity in isolated testicular interstitial cells. TCBP also appeared to enhance nuclear disposition of cadmium in isolated interstitial cells. Hence, the absence of metallothionein in target tissues of cadmium carcinogenesis may contribute to the susceptibility of these tissues in several ways.

PROJECT DESCRIPTION

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

M. P. Waalkes	Pharmacologist	LCC	NCI
M. S. Miller	Senior Staff Fellow	LCC	NCI
A. O. Perantoni	Staff Fellow	LCC	NCI
N. Konishi	Visiting Fellow	LCC	NCI
S. Rehm	Visiting Scientist	LCC	NCI
S. Rhodes	Intramural Research Training Awardee	LCC	NCI
Z. Wahba	Intramural Research Training Awardee	LCC	NCI
J. M. Ward	Chief, Tumor Pathology & Pathogenesis Sect.	LCC	NCI

Objectives:

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

Major Findings:

The mechanisms of cadmium carcinogenesis are under active investigation at various levels of biological complexity. At the organismal level, malignant interstitial cell tumors of the testes, as assessed by metastases to the lung, were associated with repeated subcutaneous injections of cadmium in Wistar and Fischer rats. With a single injection, these tumors are always almost exclusively benign. Likewise, injection site tumors resulting from repeated exposure were much more highly malignant, as assessed by local and distant metastases, than those resulting from a single injection. Thus, it appears that the level of malignancy for cadmium-induced tumors of various sites is related to cadmium dose. Such exposure also induced a very high incidence (>90%) of hepatization of the rat pancreas, a finding possibly related to the suppression of spontaneous pancreatic islet or acinar cell carcinogenesis by cadmium injections in the rat. Likewise, cadmium was found to suppress liver tumor formation in the B6C3F1 mouse either when tumors resulted from initiation by diethylnitrosamine or when spontaneously occurring foci promoted by barbital treatment.

Dietary cadmium increased the incidence of both tumors of the ventral prostate and leukemias in rats. This is the first evidence of oral exposure to cadmium-inducing prostate tumors in animals and the first linkage between cadmium exposure and tumors of the lymph system. Dietary zinc was determined to have a variable, apparently tissue-specific effect, on cadmium carcinogenesis. Dietary deficiency of zinc was shown to decrease the incidence of neoplastic and preneoplastic prostatic lesions in rats receiving dietary cadmium and shifted the dose response curve for leukemias to the right such that higher doses were required for the same response. On the other hand, dietary zinc deficiency increased the number of neoplastic foci and the proportion of neoplasia in the combined total number of neoplastic and preneoplastic lesions in the rat testes following cadmium injection.

The suppression of cadmium-induced prostatic tumors by dietary zinc deficiency was linked to increased incidence of prostatic atrophy in zinc-deficient animals probably due to reduced testicular function, specifically reduced testosterone production, seen in zinc deficiency. This further indicates the fundamental role of androgen input for cadmium induction of prostatic tumorigenesis. In this regard, subchronic studies using constant release testosterone implants have clearly shown that both the accumulation of cadmium and the retention of cadmium by the rat prostate are dependent on circulating testosterone levels.

Metallothionein, the highly-inducible low-molecular-weight protein thought to confer tolerance to cadmium, was found to be absent not only in the testes, but also in the rodent prostate and ovaries. The cadmium-binding protein (TCBP) detected in place of metallothionein in the rodent testes was found, unlike metallothionein, to be uninducible by pretreatments that result in tolerance to cadmium testicular carcinogenicity, such as high dose zinc or low dose cadmium. Such treatments nonetheless prevented cadmium-induced cytotoxicity in isolated testicular interstitial cells, the target cell population of cadmium carcinogenesis in this tissue, indicating that other non-metallothionein based systems of cellular tolerance to cadmium carcinogenicity must be in effect. Cadmium in association with the TCBP resulted in a markedly higher disposition of cadmium in isolated interstitial cell nuclei than cadmium in association with metallothionein. Hence, the absence of metallothionein in target tissues of cadmium carcinogenesis may contribute to the susceptibility of these tissues in several ways including allowing an enhanced disposition of the toxic metal in the vicinity of the genetic material.

Publications:

Bhave MR, Wilson MJ, Waalkes MP. Methylation status and organization of the metallothionein-I gene in livers and testes of strains of mice resistant and susceptible to cadmium. *Toxicology* 1988;50:231-45.

Koizumi T, Waalkes MP. Cadmium toxicity and distribution in isolated interstitial cells in the rat testes: effect of zinc. *Toxicology* (In Press).

Koizumi T, Waalkes MP. Effect of zinc on the uptake and distribution of cadmium in rat testicular interstitial cell nuclei. *Toxicol In Vitro* (In Press).

Rehm S, Waalkes MP. Cadmium-induced ovarian toxicity in hamsters, mice, and rats. *Fund Appl Toxicol* 1988;10:635-47.

Rehm S, Waalkes MP, Ward JM. *Aspergillus* rhinitis in Wistar (Cr1:(WI)BR) rats. *Lab Anim Sci* 1988;38:162-6.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05524-03 LCC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Effects of Chemical Carcinogens on Gene Expression

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

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

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS

1.5

PROFESSIONAL

1.25

OTHER

0.25

CHECK APPROPRIATE BOXES)

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

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

This laboratory has been studying the role of drug metabolism in determining susceptibility to chemical carcinogens. Cytochrome P-450IA1 expression was previously implicated in playing an important role in determining the differential response of target organs of inducer responsive and nonresponsive offspring and mothers to tumor formation by methylcholanthrene (MC). These studies have been extended and it has now been shown that both B6D2F1 and D2B6F1 fetuses exhibit similar levels of aryl hydrocarbon hydroxylase (AHH) activity following transplacental exposure to MC. However, fetuses of non-responsive D2 mothers maintained induced levels of AHH activity over longer time periods, suggesting that pharmacokinetic parameters may account for the higher tumor incidence seen in D2 vs B6 offspring. While the incidence of MC-mediated lung tumors correlated with the inducibility phenotype of the transplacentally exposed offspring, adult exposure protocols exhibited similar tumor yields regardless of their phenotype. This was partly due to the higher induction ratio of P-450IA1 observed in fetal versus adult animals. Studies are still being carried out to determine if alterations of drug metabolic enzyme levels affect the toxicological and carcinogenic consequences of diethylstilbestrol exposure. The expanded 5-week toxicology study has been completed and the long-term carcinogenicity study is still in progress. Studies on the inhibition of steroid-inducible tyrosine aminotransferase expression by N-methyl-N'-nitro-N-nitrosoguanidine have also been extended. Using the isoschizomeric restriction enzyme pair HpaII/MspI, no evidence was discovered that methylation/demethylation events play a role in the inhibitory effects of this carcinogen. Work has also continued on the role of the polyoma virus middle-T (mT) gene in altering the levels of α -tubulin (AT) expression. Gene dosage experiments showed that AT RNA was maximally increased by mT antigen at the same levels causing morphological transformation of the cell lines used in these studies.

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

M. S. Miller	Senior Staff Fellow	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
L. M. Anderson	Research Biologist	LCC	NCI
D. P. Chauhan	Visiting Fellow	LCC	NCI

Objectives:

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

Major Findings:

Effects of carcinogens on steroid-inducible gene expression: We have been using steroid-inducible genes as a model system in which to study the mechanism(s) by which chemical carcinogens alter gene expression. Steroids are important regulatory hormones which act as enhancer elements to increase the transcription rate of genes located downstream from the hormone-receptor binding site(s) on DNA and are probably the best understood eukaryotic gene regulatory system, making this an ideal system in which to study the mechanism(s) by which carcinogens can alter the levels of expression of various genes. Previous studies have shown that treatment of the Fao clone of Reuber H35 hepatoma cells with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) caused concurrent decreases in the levels of steroid-inducible tyrosine aminotransferase (TAT) enzyme activity and total TAT RNA, suggesting that MNNG inhibited the accumulation of TAT RNA by acting at a pre-translational step. These studies have been extended to the genomic level and the effects of MNNG on the chromatin structure of the TAT gene are being assessed. We have examined the relative methylation status of the TAT gene by using the isoschizomeric pair of restriction enzymes HpaII/MspI. Both enzymes cut DNA at CCGG sequences; however, HpaII cannot cleave internally methylated cytosine residues. Digestion of untreated and hormone and/or MNNG treated samples have shown that there is a constitutively methylated CCGG residue in the upstream region of the TAT gene that is not affected by any of the reagents used. We intend to look with other methylation-sensitive restriction enzymes to examine the effects of MNNG on DNaseI hypersensitive sites and to delineate the molecular mechanism(s) mediating the inhibitory effect of MNNG on steroid-inducible TAT gene expression.

Polyoma virus middle T (mT) gene enhances expression of the α -tubulin gene: Two cell lines, mT-1 and gen-1, have been obtained which were derived from F111 rat fibroblasts transfected with recombinant DNA molecules, placing the mT gene of polyoma virus downstream of a steroid-inducible promoter. This construct renders the heterologous mT gene sensitive to induction by glucocorticoid hormones. These cell lines were employed in gene dosage experiments to determine the effects of the ras-like mT gene product on cytoskeletal proteins. We have previously shown that the mT-1 and gen-1 cell lines contain elevated levels of α -tubulin (AT) RNA transcripts relative to the untransfected parent F111 cells. Northern blot analysis has demonstrated the appearance of a second, higher molecular weight

transcript in mT-1 cells, although the levels of appearance of this transcript were somewhat variable. Gene dosage experiments utilizing various concentrations of DEX showed that, although mT antigen levels were induced in a concentration-dependent manner, AT levels were maximally induced at the lowest concentration employed (1 nM), in agreement with the high sensitivity of these cells to morphological transformation by very low levels of mT antigen expression. These results suggest that the transforming mT protein may mediate its effects on cell morphology by altering the levels of cytoskeletal gene expression.

Role of drug metabolism in determining susceptibility to chemical carcinogens: Previous studies conducted in the PCS by Dr. Anderson (see project Z01CP05352) have shown that the incidences of liver and lung tumors in mice exposed transplacentally to 3-methylcholanthrene (MC) were significantly influenced by the sensitivity of both mothers and fetuses to induction of cytochrome(s) P-450, as measured by the aryl hydrocarbon hydroxylase (AHH) assay. We have previously shown that these differences can be partly accounted for by the level of expression of the cytochrome P-450IA1 gene at both the biochemical and molecular level. A comparison of the induction kinetics in B6D2F₁ and D2B6F₁ fetuses from responsive and nonresponsive mothers, respectively, has shown that similar levels of AHH activity are attained following transplacental injection of MC regardless of the phenotype of the mother. However, fetuses from nonresponsive D2 mothers maintained their induced P-450IA1 levels, as measured enzymatically, for at least 48 hr whereas values for fetuses from responsive mothers had declined to control levels by this time. Thus, in both D2 and B6 mothers enough MC reaches the fetuses to cause a comparable induction of AHH activity, and it is likely that differences in clearance rates from the fetal compartment or some other metabolic parameter(s) must account for the differences in susceptibility to MC-initiated tumors in F₁ litters from B6 and D2 mice.

Unlike the fetus, treatment of adult mice with MC yields no differences in the incidence of lung tumor-bearing mice despite differences in their inducibility phenotype. Adults had much higher levels of constitutive lung AHH activity than did fetuses, while the maximally induced levels of AHH activity were similar in both adult and fetal animals. These results suggest that the correlation between susceptibility to MC-initiated lung tumors and P-450 inducibility may be a unique property of the fetus, due in part to the low basal levels of activating enzymes and their high induction ratio in the fetus.

Studies on the role of drug-metabolic enzymes on the toxic and carcinogenic effects of diethylstilbestrol exposure are still in progress. The expanded 5-week toxicology study has just been completed and necropsy and histological analysis of the rats is also near completion. The long-term carcinogenicity study is still in progress and will be completed by next year.

Publications:

Miller MS, Jones AB, Chauhan DP, Park SS, Anderson LM. Differential induction of fetal mouse liver and lung cytochromes P-450 by β -naphthoflavone and 3-methylcholanthrene. *Carcinogenesis* 1989;10:875-83.

ANNUAL REPORT OF

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

October 1, 1988 through September 30, 1989

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

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

Studies on Cellular and Molecular Aspects of Hepatocarcinogenesis

(1) Experimental hepatocarcinogenesis in the rat has been used as a model to study the cellular and molecular events during neoplastic development. The research is currently focused on defining the possible role of a stem cell compartment in the liver during oncogenesis as well as in the normal liver (see also Project No. Z01CP05262). We have shown that a facultative stem cell compartment exists in the liver, and these stem cells become a major source of new hepatocytes when the normal regenerative response of the liver is impaired. The liver-derived stem cells are multipotential and are capable of differentiating in vivo into hepatocytes, bile and intestinal epithelia as well as into pancreatic cell lineages. We have obtained data strongly indicating that transforming growth factor beta-1 (TGF- β 1) is a key determinant in differentiating the stem cells along the hepatocytic lineage in vivo as well as in vitro. However during hepatocarcinogenesis, TGF- β 1 may function as an endogenous promoter of the neoplastic process due to its strong growth inhibitory effects on normal hepatocytes in combination with a growth stimulatory effect on transformed liver cells. Moreover, we have shown that the major source of TGF- β 1 in the liver during later stages of tumorigenesis are the stromal cells and consequently the tumor promoting effect of TGF- β 1 may occur via a paracrine mechanism.

(2) The first regenerative response in the liver following partial hepatectomy of rats exposed to chemical carcinogens is seen in periductal cells that are the only cells labeled with radioactive thymidine 24 hours after the operation. Periductal cells similar to oval cells and preneoplastic lesions show expression

of glutathione-S-transferase P (GST-P), a widely used marker of neoplastic transformation in the liver. Alpha-fetoprotein (AFP)-positive cells first appear in the periductal area 32 to 56 hours after partial hepatectomy of carcinogen-exposed rats. From this location small AFP-positive cells infiltrate between hepatocytes. These cells are positive for OV-6 antibody that also recognizes oval cells and bile epithelium and show expression of GST-P and TGF- β 1 transcripts. However, these OV-6-positive cells are surrounded by cells strongly positive for both TGF- β 1 and GST-P but negative for OV-6. These satellite cells might play an important role in the differentiation of putative hepatic stem cells (periductal cells) along hepatocytic, bile ductal or intestinal pathways. Evidence for the stem-cell origin of cancer in the liver of rats treated with the Solt-Farber protocol is based on the following findings: (a) lack of pre-neoplastic lesions at the time of partial hepatectomy; (b) similar pattern of cell distribution in the early preneoplastic lesions as is characteristic for the regeneration of the liver via the stem cell compartment (see also Project No. Z01CP05453); (c) expression of GST-P in both periductal, oval and preneoplastic cells.

(3) Temporal and spacial distribution of TGF- β 1 and procollagen gene expressions was studied in carbon tetrachloride-induced liver fibrosis and in Solt-Farber's hepatocarcinogenesis models in rats. The studies were designed to clarify the involvement of different cell types in early events of liver fibrosis and the role of extracellular matrix (ECM) during the development of hepatocellular carcinoma. In the liver fibrosis, inflammatory cells (e.g., granulocytes and macrophages), Desmin-positive perisinusoidal cells and fibroblasts appeared in the necrotic area and expressed TGF- β 1 and procollagen genes which resulted in an excess accumulation of extracellular matrices. TGF- β 1 and procollagen genes were strongly expressed in mesenchymal cells along fibrous septa but not in hepatocytes. The expression of these genes increased with the progression of liver fibrosis. The simultaneous expression of TGF- β 1 and procollagen genes in mesenchymal cells during liver fibrosis, and the fact that TGF- β enhances type I collagen promoter suggest the possibility that TGF- β 1 may have an important role in the fibrogenesis of the liver. In the liver carcinogenesis, TGF- β 1 and procollagen genes were similarly and simultaneously expressed in stromal mesenchymal cells but not in cancer cells, preneoplastic cells or basophilic cells. The data suggest that ECM may indirectly stimulate growth of both preneoplastic and neoplastic liver cells by providing a supporting tumor stroma (see also Project No. Z01CP05454).

(4) In order to identify the changes in cellular protein expression and growth regulation that are associated with neoplastic development, we have developed an in vitro model of tumor progression using v-raf or v-raf/v-myc transformed rat liver epithelial (RLE) cells. A number of cell clones were generated from the parental transformed cells to produce a series of cell lines which displayed a range of morphological transformation and tumorigenicity. These cell lines were all shown to contain integrated DNA sequences corresponding to v-raf or v-raf/v-myc and also expressed mRNA for these oncogenes. Two-dimensional polyacrylamide gel electrophoretic (2D-PAGE) analysis demonstrated that the expression of a number of proteins correlated with the transformed phenotype and malignant potential of these cells. In particular the expression of three relatively high molecular weight proteins (RP1, RP2, RP3) were found to be reproducibly down-regulated in the most tumorigenic cell lines. The identification of these proteins is currently under investigation by both sequence analysis and the use of immunological techniques. A number of other alterations in the phenotype of

these cells in terms of their growth regulation and expression of extracellular matrix have also been defined. The transformed cells show aberrant growth control by TGF- β 1 and an analysis of TGF- β 1 receptor binding indicates that this is mediated by post-receptor events. Additional initial results indicate that the receptor complement of the transformed cells correlates with their in vitro morphology and the expression of certain extracellular matrix proteins.

(5) Isolation and characterization of a protein(s) from adult rat liver that produces a reversible inhibition of the proliferation of liver-derived cells continues. The purification procedure has recently been extensively modified in order to obtain high recovery and greater purification of the growth inhibitor. Following the initial anion exchange and gel filtration chromatographic procedures, two high resolution ion exchange chromatographic steps are utilized: anion exchange on a Mono Q column followed by cation exchange on a Mono S column. The resulting preparation which has an ID50 of about 1-5 ng/ml is then subjected to high-resolution hydrophobic interaction chromatography using propyl aspartamide HPLC. This produces a preparation with an ID50 of 150-500 pg/ml and two-dimensional polyacrylamide electrophoresis has revealed the presence of about 10-15 polypeptides. A further microscale purification procedure utilizing microbore reverse phase HPLC is currently being investigated. Chromatography of the propyl aspartamide purified samples indicates the presence of three major protein peaks. Although the conditions employed in this procedure cause a major loss of protein activity, it has been possible to tentatively narrow down the growth inhibitory activity to either one of these major proteins or a minor component of the preparation which coelutes with it at a 43.5% acetonitrile concentration. Current work is focused on determining which of these proteins is responsible for the inhibitory activity. This newly developed procedure should allow the purification of this inhibitor to the purity required for sequence analysis. Concomitantly the large-scale purification of this liver-derived growth inhibitor is being pursued with the aim of immunization in order to obtain neutralizing antibodies to its activity.

(6) The growth modulatory effects of a highly purified liver-derived growth inhibitor have been compared to those of two well-characterized growth regulators, TGF- β , and rTNF- α , in a variety of liver-derived and non-liver-derived normal and neoplastic cell culture systems. Normal rat liver epithelial cells were highly sensitive to the growth inhibitory effects of HPI/LDGI and TGF- β , but were less sensitive to rTNF- α . Aflatoxin B₁-transformed RLE cells showed some sensitivity to the cytostatic effects of HPI/LDGI but were completely resistant to the antiproliferative effects of TGF- β , and rTNF- α . In contrast rat hepatoma Reuber cells were extremely sensitive to the antiproliferative effects of rTNF- α and were completely resistant to TGF- β , and HPI/LDGI. Other liver-derived transformed cells also showed differences in sensitivity to these growth inhibitors. Among the non-liver-derived cells, human breast carcinoma (MCF-7) cells were extremely sensitive to rTNF- α , exhibited some sensitivity to HPI/LDGI, but were unaffected by TGF- β . In contrast to the epithelial cells examined, the growth of fibroblast cell lines were stimulated by all three growth regulators.

(7) It is well documented that chemical hepatocarcinogenesis results in tumors that frequently are resistant to the cytotoxic and growth inhibitory effects of carcinogens. The role of the multidrug resistant (MDR) gene family in the pleiotropic resistance that is observed in these tumors is at present unknown. The objective of this project is to define the role as well as the regulation of the MDR genes in hepatocarcinogenesis. Major findings include: (1) MDR-1 gene

is induced in rat liver following administration of various natural and synthetic xenobiotics, including agents that also induce a subfamily (P450IA2) of the cytochrome P450 supergene family. These data indicate that induction of selective members of the MDR and the cytochrome P450 gene families may depend on overlapping regulatory elements; (2) the relationship between transformation and multidrug resistance was examined by employing the v-H-ras or v-raf oncogenes to transform RLE cells in vitro. The data show that transformation of RLE cells with these oncogenes results in increased resistance to cytotoxins such as adriamycin, vinblastine and 2-acetylaminofluorene. This multidrug resistance is accompanied by increased expression of MDR-1 and glutathione-S transferase P. Thus, neoplastic transformation of RLE cells with v-H-ras and v-H-raf, independent of chemical exposure, results in multidrug resistance.

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

(1) The objective of this project is to study the mechanism of carcinogenesis using quantitative two-dimensional gel electrophoresis (2DG). This technique lets us examine both qualitative and quantitative changes in the synthesis of thousands of cellular polypeptides as the cell undergoes neoplastic transformation. Research is focused on (1) continued development of the computer system (dubbed ELSIE 4) used to automatically analyze gels and (2) use of ELSIE 4 to analyze experiments requiring computerized analysis of two-dimensional gels. In the past year, our laboratory has undergone a major change and consists now of four Sun Microsystems computer workstations, networked in a local area network. Major progress has been made in weaning the system from the use of specialized hardware. The system has been successfully ported to a number of different computers. Statistical tests designed to search for spots that may vary over the course of an experiment have been refined. Once interesting spots are flagged, they may be examined using standard image processing software developed in this laboratory. The ELSIE 4 system is being used in the laboratory to study the effects of different transforming oncogenes on the synthesis of proteins. RLE cells have been isolated and single-cell cloned. These cells have a normal diploid karyotype and represent a homogeneous, clonal system for the study of transformation. Different retroviruses containing transforming oncogenes, such as v-H-ras, v-raf, and v-myc have been used to transform these cells. All v-H-ras-transformed clones grow in soft agar and are highly tumorigenic. A number of significant variations in polypeptide synthesis have been noted between the 2DG patterns of transformed and non-transformed cells. Further studies are underway to determine if a common pattern exists for proteins whose synthesis are altered by the different oncogenes. A similar series of experiments, utilizing human fibroblasts, has been undertaken.

(2) The polypeptide patterns of MCF-7 human breast cancer cells (MCF-7gpt) and a stably v-H-ras-transfected subclone (MCF-7ras) have been analyzed following estradiol treatment. Since both estradiol and v-H-ras transfection increase tumorigenicity of MCF-7 cells, this study was designed to ascertain if specific changes in polypeptides were common in both treatments. Separation of cellular and secreted polypeptides was accomplished by 2-dimensional polyacrylamide gel electrophoresis, and the consequent patterns were analyzed with computer assistance. Estradiol treatment of the MCF-7gpt cells reduced the number of differences found in the polypeptide patterns between MCF-7gpt and MCF-7ras. Twelve cellular polypeptides were consistently modulated by either estradiol or v-H-ras, with four polypeptides clearly affected in the same way by both treat-

ments. Polypeptides Gchc-0845 (M, 54,000, pI 6.9) and Gchc-0902 (M, 52,000, pI 6.3) were suppressed by estradiol and v-H-ras, while Gchc-1240 (M, 34,000, pI 4.4) and Gchc-1396 (M, 23,000, pI 5.3) were induced by estradiol and v-H-ras. Sixteen secreted polypeptides were altered by at least twofold subsequent to estradiol treatment or v-H-ras transfection. Transfection with v-H-ras had a greater effect than estradiol, stimulating the secretion of eight polypeptides and suppressing the secretion of seven polypeptides compared to estradiol which increased secretion of five polypeptides and decreased secretion of an additional three polypeptides, respectively. Synergistic effects by estradiol and v-H-ras were noted for three polypeptides. The secretion of Gcls-175 (M, 50,000, pI 5.7) and Gcls-320 (M, < 14,000, pI 3.6, p-S2) was increased, while the secretion of Gcls-112 (M, 76,000, pI 6.9) was decreased. Opposing effects of estradiol and v-H-ras were seen for seven polypeptides including the M, 48,000 derivative of the M, 52,000 protein (cathepsin D). These studies support the possibility that an extremely few, but specific polypeptides are regulated in association with quite diverse tumorigenic stimuli in MCF-7 human breast cancer cells.

(3) The cellular and secreted polypeptide patterns of a control transfected clone of MCF-7 human breast cancer cells (MCF-7gpt) and a subclone of MCF-7 cells stably transfected with v-ras^H (MCF-7ras) have been analyzed following growth stimulation by estradiol (E2), IGF-I or TGF- α treatment. The goal of the current study was to determine if specific cellular-derived polypeptides were induced or suppressed in common with these treatments, specifically addressing the possibility that the tumorigenic effects of E2 and v-ras^H are mediated by the polypeptide growth factors. Cellular and secreted polypeptides were resolved by 2-dimensional polyacrylamide gel electrophoresis and the resultant polypeptide patterns analyzed with computer assistance. v-ras^H had the greatest effect on cellular polypeptide expression followed by IgF-I, E2 and with the least influence, TGF- α . The expression of 66 polypeptides were affected by v-ras^H, 40 were suppressed and 26 stimulated; IGF-I affected 37 polypeptides, 27 were suppressed and 10 stimulated; E2 affected 23 polypeptides, 14 were suppressed and 9 stimulated; and TGF- α affected 14 polypeptides, suppressing 9 and stimulating the expression of 5. In the control MCF-7gpt cells, only 3 polypeptides (M, 75,000, pI 4.0; M, 59,000, pI 6.2; M, 27,000, pI 4.8) were found common to E2, IgF-I and TGF- α modulation. Including v-ras^H in the comparison reduced this commonality to 1 polypeptide (M, 59,000, pI 6.2). IgF-I and E2 shared more common effects with each other than either with TGF- α . v-ras^H affected the secretion of more polypeptides than E2, IGF-I or TGF- α , stimulating the secretion of 7 polypeptides and suppressing the secretion of 7 polypeptides compared to the control transfect, MCF-7gpt. TGF- α only stimulated the secretion of 1 polypeptide. E2 and IgF-I had common effects on 4 secreted polypeptides (M, 76,000, pI 6.9; M, 62,000, pI 4.8; M, 55,000, pI 5.6; M, 36,000, pI 5.3) and showed no common effects with TGF- α . Additionally, E2 IGF-I appears capable of partially inducing its effect on polypeptide expression in a v-ras^H-transfected MCF-7 cell, while TGF- α could not. We conclude that the involvement of the growth factor TGF- α is unlikely in the context of either E2 or v-ras^H-associated tumorigenesis in the MCF-7 cells. However, the common effects observed between E2 and IGF-I would suggest a role for IGF-I in the tumorigenic progression of these cells.

Studies on Suppressor tRNAs

(1) Transfer RNA was isolated from HIV-1 (human immunodeficiency virus-1), HIV-2 (human immunodeficiency virus-2), HTLV-1 (human T-cell leukemia virus) and BLV (bovine leukemia virus)-infected and -uninfected cells and the elution profiles

of aminoacyl-tRNAs from infected and uninfected cells were compared by reverse phase chromatography. In each case examined, Asn-tRNA, which normally contains the highly modified Q base in the 5' position of its anticodon, was deficient in this base in infected cells. Phe-tRNA from HIV-1- and HIV-2-infected cells also lacked the highly modified Wye base on the 3' side of its anticodon. Interestingly, one or both of these tRNAs are required for translation within the ribosomal frameshift signal in expression of the gag-pol polyfusin protein of each retrovirus examined in this study and in each ribosomal frameshift signal sequenced to date. Addition of purified Phe-tRNA minus Wye base and Asn-tRNA minus Q base to rabbit reticulocyte lysates programmed with mRNA generated from the HIV gag-pol region gave varying results on their effects on ribosomal frameshifting. Our efforts to provide an assay for ribosomal frameshifting are now focusing on microinjection of purified tRNAs and HIV gag-pol mRNA into *Xenopus* oocytes.

(2) Two opal suppressor phosphoseryl-tRNAs which have been isolated and characterized in this laboratory have been shown to form selenocysteyl-tRNA and are now designated selenocysteyl-tRNA^{Ser}. The function of these isoacceptors is twofold: (1) they serve as a carrier molecule upon which selenocysteine is biosynthesized; and (2) they donate selenocysteine directly to protein in response to specific UGA codons. These isoacceptors have several unique features which set them apart from all other eucaryotic tRNAs: (1) they are 90 nucleotides in length and thus are the longest eucaryotic tRNAs sequenced to date; (2) they are phosphorylated on their serine moiety to form phosphoseryl-tRNA; (3) they have few modified bases compared to other tRNAs; (4) they are encoded by a single gene even though several pyrimidine transitions occur post-transcriptionally and one of the transitions occurs in the anticodon; and (5) the primary transcript arises, unlike any other known tRNA, without processing on the 5' side of the gene product. The genes encoding the selenocysteyl-tRNA^{Ser} isoacceptors which have been isolated and sequenced from human, rabbit, chicken, *Xenopus*, nematode and *Drosophila* genomes are transcribed *in vivo* in *Xenopus* oocytes and *in vitro* in HeLa cell extracts. Three upstream regulatory sites, a TATA box and a GC rich region near -30, and an AT rich region between nucleotides -62 and -76, regulate gene expression.

Protein Structure and Function

(1) Our previous investigations of glycoproteins isolated from the plasma membranes of normal and neoplastic rat livers revealed many qualitative and quantitative differences when analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The main goal of this project is to purify and characterize the specific glycoproteins whose expression is markedly altered during chemically induced hepatocarcinogenesis in order to understand their role either as markers or causal agents during cell transformation. Previous results established the N-terminal amino acid sequence for 4 of 9 glycoproteins purified and analyzed from a single 2D-PAGE experiment. The remaining 5 components of interest were not sequencable in this manner, presumably because of blocked N-termini. For this reason, extensive work was done to improve yields of starting materials and to develop procedures for obtaining amino acid sequence information from all of these proteins, whether blocked or unblocked. The purification of the glycoproteins was improved at the Concanavalin-A (Con A) affinity chromatography step by incorporating the use of fast protein liquid chromatography (FPLC) and adding detergents to the elution buffers. The use of PAGE as the final isolation step was evaluated by developing a radioactive

standard and determining the procedure for using it to quantify the recovery of proteins purified by gels and then transblotted to Immobilon-P membranes. Internal sequence results were obtained by determining procedures to cleave several standard proteins (which had been purified in PAGE experiments) with CNBr or formic acid. The results were as expected based on the size of the particular fragments selected. An assay was also developed which can be utilized to determine which steps or chemicals are responsible for the N-terminal blocking that occurs during the purification of proteins or peptides.

(2) It is now possible to design ex novo recognition peptides, called anti-sense peptides based on the anti-sense DNA sequence of a gene coding for a target protein. The main objective of this project is to devise a method to generate recognition peptides using only partial amino acid sequence information. Two peptides with hydrophobic complementarity to residues 356-375 of the c-raf protein were synthesized to determine if they recognize the raf-(356-375) peptide as well as the entire protein. One peptide was deduced from the complementary mRNA for the raf protein corresponding to residues 356-375, whereas the other was deduced solely from the amino acid sequence of the 20-mer segment using a computer program able to generate peptide sequences with hydrophobic complementarity to a given sequence. Specific binding of both peptides to the raf 20-mer segment was demonstrated when either the raf 20-mer peptide or the complementary peptides were immobilized on a column. Binding affinities were in the millimolar-micromolar range. Identical binding properties were observed with peptides synthesized with either all D- or all L-amino acids, suggesting a lack of conformational dependence. Binding was also unaffected by the presence of 8 M urea or detergents, was dependent on solvent characteristics of pH and ionic strength, and was abolished by the presence of competing peptides in the eluting buffer. Recognition between raf complementary peptides was accompanied by spectral changes in the far and near UV region, as monitored by circular dichroism. Proteolytic degradation was retarded by the binding of these peptides. Once immobilized on a column, these peptides proved useful for the isolation by affinity chromatography of a recombinant c-raf protein from an *Escherichia coli* crude cell extract.

(3) We have developed two methods for designing recognition anti-sense peptides without the use of DNA coding sequence information. One approach utilized the frequency usage patterns of codon for various amino acids, as compiled in the GenBank sequence data system. Another source was the data bank on the relative tRNA levels in mammalian cells for codons of interest. In a second approach a computer-assisted peptide design program was developed which determines the root-mean-square of the differences between the average hydrophobic score for a target peptide and all possible anti-sense sequences to it. Those sequences which gave the smallest differences in complementary scores at a given length of averaging were considered anti-sense peptides. To test these approaches, anti-sense peptides were designed to a rat liver glycoprotein N-terminal segment, FNLDAEAPAVLSG, and studied by analytical affinity chromatography to determine binding properties. The peptides derived using the codon frequency information showed dissociation constants ($M_{M/P}$) between $3.0 - 9.8 \times 10^{-4}$ M (100 mM NH_4OAc , pH = 5.7), while those peptides chosen by the hydrophobic minimization approach showed $K_{M/P}$ values between 3.2×10^{-7} M (same buffer). These affinity data indicate that the interactions increase as the hydrophobic complementarity score differences are minimized. Competitive elution studies showed that the recognition was specific and probably did not involve simple ionic interactions. Comparative binding constants with an all D-amino acid-containing analog

indicated that intermolecular binding may not be dependent on rigorous conformational specificity.

Studies on Chromatin Structure, Glucocorticoid Receptor and Gene Expression

(1) Activation of the MMTV LTR promoter by steroid hormones proceeds through the assembly of a transcription initiation complex composed of factors NF1/CTF and TFIID, both of which are present in unmodified form and concentration in the uninduced nucleus, at a region in the LTR covered by nucleosome B. Two processes are probably involved in the assembly of this complex: direct interaction of the receptor with some component of the initiation cascade and displacement of the nucleosome that inhibits binding of NF1/CTF. During activation of proto-oncogenes by MMTV proviral insertion, a second nucleosome (F) is displaced, exposing binding site(s) for proteins acting at an upstream activating element which is hormone-independent. Tissue-specific activation of reporter genes by this constitutive element has been observed, and protein(s) that bind to the region have been characterized. Cooperation between the HRE and a second transcriptional element are involved in efficient expression from the MMTV promoter, while hormone-independent activation of the proto-oncogene may require only the upstream element.

(2) A series of retroviral vectors have been constructed that incorporate dominant selectable markers in conjunction with oncogenes expressed from an inducible promoter. Previous work had shown that expression of an oncogene (v-rasH) under control of the steroid-regulated MMTV promoter could render NIH 3T3 cells conditionally transformed; i.e., cells were only transformed when the promoter was induced by steroid. These vectors have now been utilized to introduce the ras gene into normal epithelial liver cells. Infection with the retroviral vectors expressing the ras oncogene results in transformation of the liver cells, as monitored by a variety of parameters. Furthermore, some markers of the neoplastic state (growth kinetics and increased glucose uptake) were modulated by increasing the rate of ras gene expression by induction with glucocorticoids. Gross morphological parameters of transformation were not responsive to hormone treatment, although this quality of phenotype switching was also not seen in most of the cell lines generated earlier with the NIH 3T3 cells and may simply reflect the smaller population of transformants that was characterized in the liver cell experiments. These experiments further demonstrate the utility of vectors modelled on this series for the efficient convection of sequences into target cells of interest and subsequent regulated expression of the introduced oncogene.

(3) The sites to which steroid-receptors bind on the MMTV LTR are displayed on the surface of nucleosome B in a phased array. Hormone activation of the promoter leads to active displacement of this nucleosome in vivo. A disomic structure composed of the A and B nucleosomes can be reconstituted in vitro, with the octamer cores accurately positioned. Purified NF1/CTF, a requisite component of the MMTV initiation complex, is excluded from the disomic structure, in contrast to the glucocorticoid receptor. Thus, nucleosome displacement is necessary during transcription activation to permit binding of the initiation complex. The chromatin structure of this promoter represents a repressed state, which must be specifically modified to allow promoter activation. This process has also been shown to be independent of DNA replication. This is the first example of active nucleosome displacement initiated by a known regulatory protein, resulting in chromatin remodelling that provides altered access to a

second set of DNA-binding proteins. These results indicate that a chromatin template containing specifically positioned nucleosomes is an active participant in transcriptional activation, and that the complete regulatory process cannot be reconstituted *in vitro* with pure DNA and pure factors. A second region of hypersensitivity has been characterized that corresponds to nucleosome F, the last nucleosome in the phased array.

(4) The ability of steroids to regulate transcription from specific genes has been well established (1). In the case of glucocorticoids the hormone binds to a cytosolic cellular non-DNA binding form of its specific receptor which translocates into the nucleus where it is now able to bind to specific sites on chromatin and either enhance or repress transcription. We have examined the mechanism of DNA binding by individually synthesizing the putative "zinc finger peptides" from the rat glucocorticoid receptor. Atomic absorption studies show that the peptides will bind zinc on an equimolar basis. Circular dichroism experiments demonstrated a significant alteration in secondary structure in the presence of zinc. The results from DNA binding experiments establish that metal ion is required for binding to DNA and that amino terminal peptide shows a significantly greater affinity of GRE containing DNA over control DNA. The results suggest that a single synthetic "finger peptide" is able to bind to DNA in a sequence specific manner.

(5) The proopiomelanocortin (POMC) gene is expressed in a tissue-specific manner in the pituitary, hypothalamus and testes. The POMC gene is negatively regulated at the transcriptional level by glucocorticoids and positively regulated by ligands that stimulate the protein kinase C and C-AMP signal transduction pathways. It is therefore possible to examine tissue-specific, negative and positive transcriptional control mechanisms using the POMC gene as a paradigm. These studies initially focused on the negative regulation of transcription by glucocorticoids. Previous studies have demonstrated that a glucocorticoid receptor binding site centered at -63 may be involved in negative regulation. Although the mechanism of repression is unknown, one possibility is that receptor interaction with this sequence may displace a positive trans-acting factor that normally occupies this position. Using a combination of methods to detect protein-DNA interactions, we have defined 5 factors (PO-A,B,C,D, and E) that bind between -63 and the POMC cap site. To determine the functional significance of these sites and their possible involvement in negative regulation, we have made a series of deletion- and oligonucleotide-directed mutations in the POMC promoter linked to the firefly luciferase reporter gene. After transient transfection of these vectors into the ATt-20 pituitary tumor cell line, we have determined that a number of these mutations appear to decrease the basal transcription of the POMC gene. A particularly interesting mutation involves the PO-B site situated over the POMC CAP site that defines a novel trans-acting CAP binding factor distinct from classic TATA binding proteins. We have determined that the effects of these mutations can be mirrored in *in vitro* transcription assays. In future experiments this technique will enable more detailed analysis of the role of these trans-acting factors in transcription complex formation.

Studies on Food-Derived Mutagens and Cytochrome P-450

(1) 2-Amino-3-methyl-imidazo[4,5-f]quinoline (IQ) is a heterocyclic arylamine found in certain cooked foods such as beef and fish. IQ has been shown to be carcinogenic to both rodents and monkeys and to form DNA adducts in tissues of these animals fed IQ. It is well recognized that metabolism can play an important role in the carcinogenicity of chemicals. Therefore we have been examining the disposition of IQ in monkeys and identifying urinary and fecal metabolites of IQ which may be indicative of pathways of carcinogen activation or detoxification. Following administration of ^{14}C -IQ (2 $\mu\text{mol}/\text{kg}$) to cynomolgus monkeys by nasal-gastric intubation, blood levels declined rapidly from 1 to 8 hr following dosing. This was followed by a slow decline from 8 to 72 hr. Approximately 30-45% of the dose was excreted in the urine by 8 hr and by 72 hr 45-60% had been excreted. Through HPLC analysis we have observed that IQ is extensively metabolized in monkeys to at least 4 urinary metabolites. No IQ itself was found in the urine. We have found that one of the metabolites is IQ-N-sulfamate. In addition, we have tentatively identified two glucuronides of IQ by their sensitivity to β -glucuronidase. Structural identification of these metabolites is currently underway.

(2) The cytochrome P450s are a superfamily of enzymes which metabolize a wide array of compounds including xenobiotics such as drugs and carcinogens, and endobiotics such as steroids and prostaglandins. In animals, multiple forms of these enzymes are expressed simultaneously either constitutively or after administration of inducers. To define the contribution of a given cytochrome P450 to the metabolism of specific drugs and carcinogens, it is important to express these enzymes individually in cells which lack endogenous background activity for these enzymes. Toward this goal we have begun to develop expression systems in which individual cytochrome P450s are synthesized from their coding DNA sequences. Success in this effort will enable us to identify putative human mutagens and carcinogens as well as to define the contribution of each of these enzymes to toxicity, mutagenesis, and cell transformation by chemical carcinogens. To achieve this goal, we have constructed infectious recombinant vaccinia viruses and infectious recombinant retroviruses containing the full length coding cDNA sequences for mouse cytochrome P4501A1 and P4501A2. Human and rodent cells infected with the recombinant viruses expressed high levels of the authentic size proteins that were enzymatically active and displayed substrate specificities diagnostic of the respective enzymes. Employing the recombinant cytochromes we have demonstrated that the cytochrome P4501A2 selectively activates heterocyclic arylamines and cytochrome P4501A1 preferentially activates aromatic hydrocarbons; this preferential selectivity is mutually exclusive at limiting substrate concentrations. Furthermore, the cytochrome P4501A2 constitutively expressed in mammalian cells activated food-derived heterocyclic amine carcinogens and catalyzed the formation of specific DNA-carcinogen adducts. The DNA adducts detected were identical to those formed in mouse or rat liver after the *in vivo* administration of the food mutagens.

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

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

PROJECT NUMBER

Z01CP04986-12 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Molecular Basis of Steroid Hormone Action

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

PI: Gordon Hager Head, Hormone Action & LEC NCI
Oncogenesis Section

Others: Philippe Lefebvre Visiting Fellow LEC NCI
Charles Rories IRTA Fellow LEC NCI
Ronald Wolford Microbiologist LEC NCI
Diana Berard Microbiologist LEC NCI

COOPERATING UNITS (if any)

Metabolic Diseases Branch, NIDDKD (Cathy Smith, Steve Marx)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.4

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.)

Activation of the MMTV LTR promoter by steroid hormones proceeds through the assembly of a transcription initiation complex composed of factors NF1/CTF and TFIID, both of which are present in unmodified form and concentration in the uninduced nucleus, at a region in the LTR covered by nucleosome B. Two processes are probably involved in the assembly of this complex: direct interaction of the receptor with some component of the initiation cascade and displacement of the nucleosome that inhibits binding of NF1/CTF. During activation of proto-oncogenes by MMTV proviral insertion, a second nucleosome (F) is displaced, exposing binding site(s) for proteins acting at an upstream activating element which is hormone-independent. Tissue-specific activation of reporter genes by this constitutive element has been observed, and the protein(s) that binds to the region has been characterized. Cooperation between the hormone response element (HRE) and a second transcriptional element are involved in efficient expression from the MMTV promoter, while hormone-independent activation of the proto-oncogene may require only the upstream element.

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

Gordon Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Philippe Lefebvre	Visiting Fellow	LEC NCI
Charles Rories	IRTA	LEC NCI
Ronald Wolford	Microbiologist	LEC NCI
Diana Berard	Microbiologist	LEC NCI

Objectives:

- (1) Analysis of positive regulation of mouse mammary tumor virus (MMTV) transcription by steroid hormones; characterization of nuclear DNA-binding factors specific for MMTV regulatory sequences.
- (2) Determination of the molecular mechanism responsible for glucocorticoid receptor-mediated induction of transcription at hormonally regulated promoters.
- (3) Application of the methodologies developed for the study of hormone action to the study of other transcription regulatory systems important in cell growth.

Methods Employed:

- (1) DNA recognition elements involved in regulation are identified by deletion mutagenesis and oligonucleotide-directed site-specific mutagenesis, using transient expression or stable transfection assays to monitor the biological activity of mutant DNAs.
- (2) Proteins which interact at a given recognition element are characterized by the gel shift or gel retardation assay and a variety of footprinting techniques, including (in vitro) DNaseI footprinting, DMS methylation interference footprinting, and Exonuclease III footprinting; and (in vivo) DMS footprinting and ExoIII footprinting.
- (3) Proteins are purified by several chromatography technologies.
- (4) Potential sites of protein-DNA interaction are determined in vivo by the appearance of regions in chromatin that are hypersensitive to the action of DNaseI (HSR regions).
- (5) The impact of putative transcription factors is monitored in vitro by the reconstitution of specific transcription initiation in cell-free extracts.

Major Findings:

The mouse mammary tumor virus (MMTV) system has been studied for some time as a model for positive regulation of transcription by steroid hormones. Our approach has emphasized analysis of the interaction of transactivating factors with the nucleoprotein template found in the living cell, as opposed to naked DNA. This interest was stimulated by the finding that the MMTV LTR is reproducibly phased with regard to nucleosome positioning, indicating that DNA recognition sites for specific DNA-binding proteins are specifically organized in the nucleoprotein template.

The focus of this project is to explore the mechanisms involved in transcriptional activation by steroid hormones and to examine the interaction of the hormone-response pathway with promoters that are also regulated in a tissue-specific manner. The elucidation of these mechanisms requires not only the identification and characterization of the various transcription factors which act at a target promoter, but also an understanding of the extent to which the organization of promoters in cellular chromatin modulates the interaction of the soluble transcription apparatus with specific DNA recognition elements.

Using a series of cell lines in which MMTV LTR fusion genes are amplified on extrachromosomally replicating bovine papilloma virus (BPV) "minichromosomes," we previously demonstrated by an exonuclease protection assay that factors bind to the MMTV promoter *in vivo* in response to hormone stimulation. These factors were identified as NF1/CTF (-80 to -56 region) and TFIID (-42 to +1 region). Activation of transcription at the MMTV promoter therefore appears to result from recruitment of preformed transcription factors to the promoter by the steroid receptor.

Transcription *in vitro* shows that extracts containing TFIID and NF1/CTF are competent to efficiently initiate in the absence of steroid receptor, suggesting that *in vivo* regulation at this promoter may involve two separate processes: protein-protein contacts between the receptor and some target protein that is rate-limiting in the formation of the initiation complex, and displacement of a nucleosome that acts to block access of the initiation complex in chromatin (see project Z01CP5450-05). Reconstruction of receptor-mediated activation may therefore require the introduction of an accurately structured nucleoprotein template into cell-free systems.

We have also examined the mechanism of activation of the proto-oncogene *int-2* by MMTV during viral-induced mammary carcinogenesis. Accessibility of MMTV LTR chromatin was probed by digestion in whole nuclei with DNaseI and a variety of restriction endonucleases. We discovered a new region of hormone-independent increase in accessibility for a region of the LTR associated with nucleosome F (see project Z01CP5450-05). The hormone-independent status of this region correlates with the lack of hormone-response for the activated *int-2* promoter and suggests that an enhancer-like element is present in the 5' end of the LTR; it is this element (not the steroid responsive HRE) that is responsible for *int-2* locus activation during proviral insertion. Consistent with this view, transient expression analysis of the LTR indicates the presence of a region in the left end of the LTR that is required for efficient expression in mammary epithelial cells, and one DNA-specific binding protein has been characterized whose recognition

whose recognition site is located on the core region of nucleosome F. Thus, efficient expression of the MMTV promoter in mammary cells may involve the cooperation of two elements: one (F-region) which is tissue-specific and constitutively present on the chromatin, and a second (B-region or HRE) that is responsive to ligand-activated receptor.

Taken together, these findings have major implications for our understanding of steroid hormone-regulated promoters, tissue-specific regulated promoters, and particularly for the interaction between these two regulatory networks. A fundamental understanding of the mechanism by which binding of the steroid receptor molecule at its cis-regulatory sequence recruits transcription factors to form the active promoter complex may therefore be central to a future understanding of mechanisms of enhancer activation. Nucleoprotein structure and its maintenance, perhaps by specific negative modulatory factors, may be a critical determinant in regulation of gene expression.

Publications:

Archer TK, Cordingley MG, Marsaud V, Richard-Foy H, Hager GL. Steroid transactivation at a promoter organized in a specifically-positioned array of nucleosomes. In: Gustafsson JA, Eriksson H, Carlstedt-Duke J, eds. Proceedings of the second international CBT symposium on the steroid/thyroid receptor family and gene regulation. Berlin: Birkhauser Verlag AG (In Press).

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

PROJECT NUMBER

Z01CP05262-08 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Cellular Evolution of Chemically Induced Rat Hepatomas

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

PI:	Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC NCI
	Harushige Nakatsukasa	Expert	LEC NCI
	Elizabeth Marsden	Biologist	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.4

PROFESSIONAL:

2.4

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 first regenerative response in the liver following partial hepatectomy of rats exposed to chemical carcinogens is seen in periductal cells that are the only cells labeled with radioactive thymidine 24 hours after the operation. Periductal cells similar to oval cells and preneoplastic lesions show expression of glutathione-S-transferase P (GST-P), a widely used marker of neoplastic transformation in the liver. Alpha-fetoprotein (AFP)-positive cells first appear in the periductal area 32 to 56 hours after partial hepatectomy of carcinogen-exposed rats. From this location small AFP-positive cells infiltrate between hepatocytes. These cells are positive for OV-6 antibody that also recognizes oval cells and bile epithelium and show expression of GST-P and transforming growth factor- β 1 (TGF- β 1) transcripts. However, these OV-6-positive cells are surrounded by cells strongly positive for both TGF- β 1 and GST-P but negative for OV-6. These satellite cells might play an important role in the differentiation of putative hepatic stem cells (periductal cells) along hepatocytic, bile ductal or intestinal pathways. Evidence for the stem-cell origin of cancer in the liver of rats treated with the Solt-Farber protocol is based on the following findings: (a) lack of preneoplastic lesions at the time of partial hepatectomy; (b) similar pattern of cell distribution in the early preneoplastic lesions as is characteristic for the regeneration of the liver via the stem cell compartment (see also Project No. Z01CP05453); and (c) expression of GST-P in both periductal, oval and preneoplastic cells.

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

Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Harushige Nakatsukasa	Expert	LEC NCI
Elizabeth Marsden	Biologist	LEC NCI

Objectives:

The objective of this project is to characterize the cellular evolution of chemically-induced murine hepatomas and the stem cell population in the liver. The topics under investigation are: (1) the nature of the target cell in rat liver for chemical carcinogens; (2) appearance of putative stem cell population after partial hepatectomy and after carcinogen administration; (3) both temporal and cell-specific distribution of gene transcripts among fetal, normal, regenerating, preneoplastic and neoplastic liver; (4) contribution of growth factors and oncogenes to the proliferation and differentiation of putative stem cells in the liver.

Methods Employed:

(1) In situ hybridization for spatial localization of mRNA. (2) Immunocytochemistry for identification of cells positive for oval cell antibody. (3) Preparation of poly(A)RNA. (4) Techniques used in molecular biology, including Northern blotting and solution hybridization. (5) Quantitation of in situ hybridization data by using the Magiscan Image Analysis System.

Major Findings:

(1) A heterogenous population of small cells with oval or irregular nuclei is frequently induced in the rat liver after carcinogen treatment. It is composed of epithelial cells with different developmental potential ("oval cells") and other cell types of unknown development traits. An increase in the number of nondescript periductal cells is the first proliferative event after carcinogen administration, followed by the proliferation of the bile duct cells. Characterization of the gene expression in the periductal cells is of considerable importance in the elucidation of a specific role and function of these cells. We have demonstrated that oval cells show a prominent expression of hepatocyte-specific transcripts such as mRNAs for albumin and α -fetoprotein (AFP). Thus they have an important physiological function as a source of major serum proteins when the mechanism of protein synthesis in the liver is disturbed. Oval cells also provide functional hepatocytes when liver has to rely on a primitive cell population to replace the liver mass. An important question is whether periductal cells and oval cells belong to the same lineage that can differentiate along different pathways, or do they instead represent a specific cell entity of their own right and function? We have studied the occurrence of periductal cells after carcinogen administration using histochemical and immunohistochemical methods. Significantly increased cellularity of periductal cells were observed 56 hours after partial hepatectomy in Solt-Farber rats and in

rats treated with acetylaminofluorene. These cells were negative for oval cell- and bile duct cell-specific monoclonal antibody OV-6. Periductal cells were the only cells in these rats that became heavily labeled 24 hours after partial hepatectomy. Invasive behavior of the small cells from the periductal areas was evident 56 hours after partial hepatectomy, but became even more evident 3 days after the operation. These cells infiltrated between hepatocytes either as small groups or as longitudinal formations. OV-6-negative small cells trailed the OV-6-positive cells deep into the liver acini. γ -Glutamyltranspeptidase (GGT)-positive preneoplastic foci were present at the periphery of the oval cell compartment by day three after partial hepatectomy and were composed of similar cells as the oval cells or had small rounded nuclei. Their shape was irregular and it resembled the pattern of oval cell distribution.

(2) Expression of α -fetoprotein (AFP) was first observed 32 to 56 hours after partial hepatectomy as a few individual cells or as a small group of cells forming alveolar structures. Combination of immuno- and hybridization histochemistry revealed that OV-6-positive cells showed AFP transcripts. Invading oval cells were trailed by small cells that were negative for both OV-6 and AFP.

(3) Glutathione-S-transferase P (GST-P) is regarded as one of the earliest markers of initiated hepatocytes. It is thought to play an important role in the development of drug resistance to the cytotoxic effect of carcinogen in a rare initiated hepatocyte. No information as to the expression of this gene in the periductal cells or in the oval cells is presently available. In the normal liver GST-P is only expressed in the bile duct cells. As soon as the increase in the number of periductal cells was evident, all of these cells and the bile duct cells showed significant transcripts for GST-P. Similarly all the ductal formations at the periportal area and the infiltrating OV-6-positive or -negative cells showed GST-P transcripts as well as the preneoplastic lesions themselves.

(4) Transforming growth factor TGF- β is thought to play an important role in the control of growth and differentiation of a variety of cell types in vitro. It also may play an important role in the differentiation of primitive hepatic stem cells towards different cellular pathways. TGF- β transcripts were absent in the bile duct cells but were present in the thick layer of periductal cells surrounding the bile ducts and the vascular formations in the periportal area of the liver acini. Expression of the TGF- β was evident in the area of oval cell proliferation but did not necessarily coincide with the expression of AFP-positive oval cells, but rather was expressed in the small cells surrounding the oval cells. These TGF- β positive cells may be important for the differentiation of hepatic stem cells either towards hepatocytic, bile ductal, intestinal or neoplastic pathways after carcinogen administration (see project no. Z01CP05453).

Publications:

Nagy P, Evarts RP, Marsden E, Roach J, Thorgerirsson SS. Cellular distribution of c-myc transcripts during chemical hepatocarcinogenesis. Cancer Res 1988;48:5522-7.

Evarts RP, Nagy P, Nakatsukasa H, Marsden E, Thorgeirsson SS. In vivo differentiation of rat liver oval cells into hepatocytes. *Cancer Res* 1989;49:1541-7.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05263-08 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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	Research Chemist	LEC NCI
Others:	Arthur David Olson	Computer Programmer Analyst	LEC NCI
	Snorri S. Thorgeirsson	Chief	LEC NCI
	Kay Johnson	Biochemist	LEC NCI

COOPERATING UNITS (if any)

Michigan State University, East Lansing, MI (Dr. Justin McCormick)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.8

PROFESSIONAL:

1.1

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.)

The objective of this project is to study the mechanism of carcinogenesis using quantitative two-dimensional gel electrophoresis (2DG). This technique lets us examine both qualitative and quantitative changes in the synthesis of thousands of cellular polypeptides as the cell undergoes neoplastic transformation. Research is focused on (1) continued development of the computer system (dubbed ELSIE 4) used to automatically analyze gels and (2) use of ELSIE 4 to analyze experiments requiring computerized analysis of two-dimensional gels. In the past year, our laboratory has undergone a major change and consists now of four Sun Microsystems computer workstations, networked together in a local area network. Major progress has been made in weaning the system from the use of specialized hardware. The system has been successfully ported to a number of different computers. Statistical tests designed to search for spots that may vary over the course of an experiment have been refined. Once interesting spots are flagged, they may be examined using standard image processing software developed in this laboratory. The ELSIE 4 system is being used in the laboratory to study the effects of different transforming oncogenes on the synthesis of proteins. Rat liver epithelial (RLE) cells have been isolated and single-cell cloned. These cells have normal diploid karyotype and represent a homogeneous, clonal system for the study of transformation. Different retroviruses containing transforming oncogenes, such as v-H-ras, v-raf, and v-myc, have been used to transform these cells. All v-H-ras-transformed clones grow in soft agar and are highly tumorigenic. A number of significant variations in polypeptide synthesis have been noted between the 2DG patterns of transformed and non-transformed cells. Further studies are underway to determine if a common pattern exists for proteins whose synthesis are altered by the different oncogenes. A similar series of experiments, utilizing human fibroblasts, has been undertaken.

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

Mark J. Miller	Research Chemist	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Arthur David Olson	Computer Programmer	LEC NCI
Kay Johnson	Biologist	LEC NCI

Objectives:

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

Methods Employed:

The principal methods employed are: (1) two-dimensional gel electrophoresis, (2) tissue culture technique, (3) computer-based quantitation of autoradiograms, (4) radioisotope measurements, and (5) microsequencing techniques.

Major Findings:

From its inception, the major objective of the laboratory's computer facility has been to further expand and develop the two-dimensional gel analysis system in order to facilitate the use of this important research technique in the analysis of the neoplastic process. We have made major improvements in the portability of our computer system, making it available to other laboratories worldwide. Quantitative analysis of the two-dimensional gels patterns between transformed and nontransformed cells indicates there are very few, if any, qualitative changes in polypeptide synthesis. Most of the differences are quantitative in nature.

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

In the past year we have upgraded the hardware in our computer facility significantly. We now have four Sun Microsystems workstation computers connected by a local area network. This provides us with over ten times the computing

power of our old system. In converting the ELSIE 4 system to run on these new computers, significant care was taken to ensure the software was portable and adhered to emerging software standards.

Our former system made use of a sophisticated image-processing computer. This made ELSIE 4 very dependent on this hardware. The Sun computers are graphics workstations that are fully capable of displaying and manipulating the gel images. We have converted all the gel imaging programs to use the X-windows (TM- MIT) graphics-image-processing standard. This is a recently developed standard imaging system that will work across networks and is functional on a large number of different computers (and even different operating systems). Hence, all of the ELSIE programs are now capable of running on any computer capable of running X.

Efforts are continuing to develop techniques and tools that will allow us to automatically analyze experiments that use multiple two-dimensional gels. Recent modifications of the gel matching program have improved the robustness and accuracy of spot matching. Collaborators in Geneva have developed new techniques aimed at classifying sets of gels and identifying what spots are most characteristic of a particular sample. These techniques, heuristic clustering, and correspondence analysis, are under examination in this laboratory. Currently, the software can be run on any mid-sized computer system running under the UNIX (TM -- AT&T Bell Laboratories) operating system. We have brought the complete system up on computer systems made by SUN Microsystems, Digital Equipment Corporation, and IBM. We also brought a subset of the system up on a personal computer, the IBM PS/2 model 60, running under MS/DOS (Microsoft Corp.). This allows users to visualize and interact with their data from their desks.

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

Different retroviruses containing transforming oncogenes, such as v-ras, v-raf, and v-myc have been used to infect and transform these cells. Cells transformed by v-ras have been single-cell cloned and characterized. These cells grow at high efficiency in soft agar and are very tumorigenic in nude mice. Although more elongated than normal RLE cells, two different ras-transformed morphologies, one more flattened than the other, have been isolated. Karyotype analysis reveals an inversion in the q arm of chromosome #5 in all ras-transformed cells. One of the cell lines is also trisomic in chromosome #12. Normal RLE cells are inhibited by epidermal growth factor (EGF) at concentrations above 1 ng/ml, while the transformed cells are unaffected by EGF at concentrations up to 100 ng/ml. Two-dimensional gel electrophoresis show that there are a large number of quantitative, but few qualitative, differences between the normal and transformed RLE cells. Differences of tenfold, or more, are common. There is one, rather diffuse, high molecular weight spot that is consistently reduced in all the

transformed cells. There appears to be an increase in the synthesis of low molecular weight spots among the ras-transformed cells. We plan to compare these patterns with those generated by the other transforming oncogenes in hopes of identifying polypeptides whose expression is altered in a similar manner by the different modes of transformation. If such proteins are detected, it may be possible to characterize and identify them by microsequencing directly off the two-dimensional gels using peptide sequencing technology available in our laboratory.

(3) Analysis of Polypeptides in Transformed and Nontransformed Human Fibroblasts.

In collaboration with Prof. Justin McCormick at Michigan State University, we have undertaken a similar analysis of transformed human fibroblasts. Here an immortalized line of diploid human fibroblasts, MSU-1.1, has been transformed with different activated ras oncogenes, H-ras, K-ras, and N-ras. The parental cells are an immortal line that arose spontaneously from a myc transfection of the normal LG1-17, finite-lived human fibroblast line. Analysis of the gel patterns indicates that there are very few qualitative changes between the transformed and nontransformed cells. There appear to be only two spots in the ras-transformed cells that are consistently and significantly elevated (these may be the ras gene product). A high molecular weight, diffuse spot, similar in pI and molecular weight to that found in the RLE cells, is consistently reduced in the transformants. Qualitatively, the 2DG patterns of the different ras transformants are very similar to one another. These ras patterns differ significantly, but not overwhelmingly, from the nontransformed parental cell lines.

Publications:

Hochstrasser DF, Harrington MG, Hochstrasser A-C, Miller MJ, Merrill CR. Methods for increasing the resolution of two-dimensional protein electrophoresis. *Anal Biochem* 1988;173:424-35.

Miller MJ. Analysis of sets of two-dimensional gel electrophoretograms: Building a database, correction of errors, analysis of data. In: Schafer-Neilsen C, ed. *Electrophoresis '88*. Weinheim: VCH Verlagsgesellschaft, 1988;322-35.

Miller MJ. Computer analysis of two-dimensional gel electrophoretograms. In: Dunn MJ, ed. *Advances in electrophoresis*. Weinheim: VCH Verlagsgesellschaft (In Press).

Miller MJ, Merrill CR. Strategies and techniques for testing the precision, reliability and reproducibility of computerized two-dimensional gel electrophoresis analysis systems. *Appl Theor Electrophor* (In Press).

Miller MJ, Schwartz DM, Thorgeirsson SS. Inter- and intracell variability of polypeptides synthesized in a rat hepatoma cell line: quantitative two-dimensional gel analysis. *J Biol Chem* 1988;263:11227-36.

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

PROJECT NUMBER

Z01CP05283-07 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Conditional Expression of Mammalian Genes

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

PI: Gordon L. Hager Head, Hormone Action & Oncogenesis Section LEC NCI

Others: Diana Berard Microbiologist LEC NCI

COOPERATING UNITS (if any)

Virology Division, Merck, Sharpe & Dohme, Philadelphia, PA (Dr. Michael G. Cordingley); The Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, NC (Dr. Brian Huber)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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 un-reduced type. Do not exceed the space provided.)

A series of retroviral vectors have been constructed that incorporate dominant selectable markers in conjunction with oncogenes expressed from an inducible promoter. Previous work had shown that expression of an oncogene (v-rasH) under control of the steroid-regulated MMTV promoter could render NIH 3T3 cells conditionally transformed; i.e., cells were only transformed when the promoter was induced by steroid. These vectors have now been utilized to introduce the ras gene into normal epithelial liver cells. Infection with the retroviral vectors expressing the ras oncogene results in transformation of the liver cells, as monitored by a variety of parameters. Furthermore, some markers of the neoplastic state (growth kinetics and increased glucose uptake) were modulated by increasing the rate of ras gene expression by induction with glucocorticoids. Gross morphological parameters of transformation were not responsive to hormone treatment, although this quality of phenotype switching was also not seen in most of the cell lines generated earlier with the NIH 3T3 cells and may simply reflect the smaller population of transformants that was characterized in the liver cell experiments. These experiments further demonstrate the utility of vectors modelled on this series for the efficient convection of sequences into target cells of interest and subsequent regulated expression of the introduced oncogene.

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

Gordon Hager	Head, HAO Section	LEC NCI
Diana Berard	Microbiologist	LEC NCI

Objectives:

The primary objective of this project is to develop and evaluate methods for the controlled expression of genes in mammalian cells and to explore the application of these principles for the study of oncogenesis and gene therapy. Hormone-regulation of the glucocorticoid-inducible MMTV promoter is the model under investigation. Tester genes fused to this promoter are introduced into cells by retroviral transmission. Current efforts are directed at developing reagents that are efficient in terms of vector convection and predictable in terms of their subsequent regulatory behavior.

Methods Employed:

1. Promoter-enhancer-tester gene fusions are engineered in DNA chimeras containing a variety of retroviral expression vehicles.
2. DNA constructions are transfected into helper cells expressing the proteins necessary for retroviral genome packaging and virus production.
3. Defective virus stocks containing the engineered genomes are prepared.
4. Cells are infected with recombinant virus particles, and the consequences of tester gene expression are monitored. The effects of promoter induction by steroid are determined.

Major Findings:

The controlled expression of genetic information in cells in culture and in animals is a potentially valuable tool in the study of oncogene function, and eventually will prove central to the treatment of disease by introduced genetic material (gene therapy). We showed previously that conditional expression of the v-rasH oncogene from the glucocorticoid-responsive MMTV promoter could result in a regulated cell phenotype and demonstrated that regulated "phenotype-switching" can be employed to study the oncogenic process in whole animals. The metastatic potential of NIH 3T3 fibroblasts carrying the hormone-inducible v-rasH oncogene was markedly enhanced when expression of the oncogene was induced prior to inoculation of cells into the animal. These experiments underscored the potential applications of this technology in studying various processes in the intact animal.

The reagents produced in these studies have now been utilized to examine the consequences of oncogene expression in liver cells. A vector containing both the v-rasH oncogene driven from the MMTV promoter and the selectable neomycin

resistance marker was used to infect normal, anchorage-dependent rat liver epithelial cells. Infection produced G418 resistant, tumorigenic cell lines, which were subsequently characterized for acquisition of viral sequences and expression of the viral genes. The transformed lines were found to contain integrated copies, both full-length and truncated, of the retroviral vector. Expression of the v-rasH oncogene was responsive to regulation by dexamethasone, indicating hormone responsiveness remained a viable means of regulating introduced genes in these epithelial cell lines. Several phenotypic alterations were associated with ras expression. Contact inhibition and anchorage-dependence of cell growth was lost, morphological appearance was altered, and cells acquired the ability to induce tumors in nude mice. Differences in growth kinetics and glucose transport were altered in response to hormone stimulation, presumably as a result of altered levels of ras gene expression.

These experiments further support the usefulness of the retroviral vectors we have developed for oncogene transmission. Current and future efforts will focus on modification of vector structures to permit tighter control of expression; that is, to render transcription of target genes more dependent on hormone treatment. As with the earlier NIH 3T3 experiments, the liver epithelial results suggest that low constitutive expression in the absence of hormone is key to obtaining phenotype switching in vivo. A second goal is to render the MMTV promoter tissue-specific as well as hormone-responsive, thus conferring two levels of conditional expression on the vectors.

Publications:

Huber BE, Cordingley MG. Expression and phenotypic alterations caused by an inducible transforming ras oncogene introduced into rat liver epithelial cells. *Oncogene* 1988;3:245-56.

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

PROJECT NUMBER

Z01CP05317-06 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Opal Suppressor Phosphoserine and the 21st Naturally Occurring Amino Acid

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

PI:	Dolph L. Hatfield	Research Biologist	LEC NCI
Others:	Byeong Jae Lee	Visiting Fellow	LEC NCI
	Yeong S. Kim	Visiting Fellow	LEC NCI
	Sung-Goo Kang	Guest Researcher	LEC NCI
	O. Wesley McBride	Chief, Cellular Regulation Section	DCBD NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

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 un-reduced type. Do not exceed the space provided.)

Two opal suppressor phosphoseryl-tRNAs which have been isolated and characterized in this laboratory have been shown to form selenocysteyl-tRNA and are now designated selenocysteyl tRNA-Ser. The function of these isoacceptors is twofold: (1) they serve as a carrier molecule upon which selenocysteine is biosynthesized and (2) they donate selenocysteine directly to protein in response to specific UGA codons. These isoacceptors have several unique features which set them apart from all other eucaryotic tRNAs: (1) they are 90 nucleotides in length and thus are the longest eucaryotic tRNAs sequenced to date; (2) they are phosphorylated on their serine moiety to form phosphoseryl-tRNA; (3) they have few modified bases compared to other tRNAs; (4) they are encoded by a single gene even though several pyrimidine transitions occur post-transcriptionally and one of the transitions occurs in the anticodon; and (5) the primary transcript arises, unlike any other known tRNA, without processing on the 5' side of the gene product. The genes encoding the selenocysteyl-tRNA-Ser isoacceptors which have been isolated and sequenced from human, rabbit, chicken, Xenopus, nematode and Drosophila genomes are transcribed in vivo in Xenopus oocytes and in vitro in HeLa cell extracts. Three upstream regulatory sites, a TATA box and a GC rich region near -30, and an AT rich region between nucleotides -62 and -76, regulate gene expression.

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

Dolph L. Hatfield	Research Biologist	LEC NCI
Byeong Jae Lee	Visiting Fellow	LEC NCI
Yeong S. Kim	Visiting Fellow	LEC NCI
Sung G. Kang	Guest Researcher	LEC NCI
O. Wesley McBride	Chief, Cellular Regulation Section	DCBD NCI

Objectives:

The major goals of the project are to understand the structure, expression, function and evolutionary origin of the selenocysteyl tRNA^{Ser} genes and the role that the products of these genes have in protein synthesis.

Specific steps to achieve these goals are: (1) to isolate and characterize opal suppressor tRNA genes from genomes of a wide variety of organisms; (2) to sequence the genes and their flanking DNA segments; (3) to investigate the structure of the genomic regions that contain these genes with respect to transcription and evolutionary conservation; (4) to study the control of transcription by using in vivo and in vitro transcription systems; (5) to use in vivo transcription systems to study processing and localization of the tRNA product; (6) to make site-specific mutations in the internal control region and in the anticodon region of the tRNA genes and to replace the 5' flanking sequence with that of another tRNA gene in order to understand better the expression and cellular function of these genes; (7) to subclone the selenocysteine-tRNA^{Ser} gene which has the 5' flanking sequence replaced so that it will make a product in high yields and subclone the ochre suppressor tRNA gene which has been generated by site-specific mutagenesis into a mammalian cell line in order to determine the effects of these suppressors on cellular function (i.e., if the gene products are phosphorylated on the serine moiety and if phosphoserine is converted to selenocysteine which in turn is incorporated directly into protein); and (8) to investigate the distribution and evolutionary origin of this unique gene in nature.

Methods Employed:

Genomic DNAs from a wide variety of organisms were obtained from a number of laboratories or were isolated by standard techniques of preparing DNA. These DNAs were digested with restriction enzymes and electrophoresed on agarose gels, Southern blotted and hybridized with an appropriate probe under very stringent and under less stringent conditions of hybridization.

Transcription of the selenocysteine tRNA^{Ser} genes were carried out in the presence of HeLa cell extracts which were used as a source of RNA polymerase III. The selenocysteine tRNA^{Ser} genes were also injected into *Xenopus* oocytes, as well as

deletions of the flanking region; additional genomic libraries were screened; new selenocysteine tRNA^{Ser} genes were isolated; site-specific mutations were generated; and the deletions, site-specific mutations and new genes were sequenced by standard techniques.

Se-75 was added to rat mammary tumor cells in culture, Se-75-selenocysteine tRNA was isolated and the selenocysteine was characterized on an amino acid analyzer after stabilizing the deacylation product by derivatizing it to carboxymethyl and carboxyethylselenocysteine.

Major Findings:

Five selenocysteine tRNA^{Ser} genes and two pseudogenes have previously been sequenced from human, rabbit, chicken, *Xenopus* and *C. elegans* (a nematode) sources. The pseudogenes occur in mammalian genomes and the gene appears to occur in single gene copy in each of these organisms. This past year a selenocysteine tRNA^{Ser} gene was isolated and sequenced from *Drosophila*. The nematode and *Drosophila* genes have 79.3% and 74.7% homology, respectively, to the vertebrate genes and 77.0% homology to each other, demonstrating that the gene has undergone substantial evolutionary change. The gene is present in the genomic DNAs of representatives of many phyla of the Animal Kingdom. The gene occurs in the *Phyla Chordata* (tunicates, amphioxus, lamprey, hag fish, horned shark, winter flounder, *Xenopus*, chicken and bovine), *Arthropoda* (*Drosophila* and horse shoe crab), *Mollusca* (clam, oyster and snail), *Annelida* (oligochaets and polychaets), *Aschelminthes* (*Ascarus* and *C. elegans*) and *Porifera* (sponge). In most cases, only one or two DNA fragments from each organism hybridized to the probe, with the exception of polychaet DNA which contained several positive fragments. These data show that the gene is widespread in the Animal Kingdom and that it has undergone evolutionary change. In addition, we have detected the gene product(s) in the seryl-tRNA populations of fish, *Saccoglossus*, earthworms, snails, *Bugula* and *Cerebratulus*. Thus, the use of selenocysteine in protein is much more widespread in nature than previously thought.

Transcription of the human, rabbit, chicken and *Xenopus* genes was examined *in vivo* (in *Xenopus* oocytes) and *in vitro* (in HeLa cell extracts). They begin transcription, unlike any known tRNA, at the first nucleotide within the gene. They terminate transcription at a T cluster in the 3' flank. The trailer sequence is cleaved by a purified 3' processing enzyme. The mature tRNA is transported from the nucleus to the cytoplasm in *Xenopus* oocytes. The 5' triphosphate on the mature tRNA is transported from the nucleus and is preserved in the cytoplasm, suggesting that it may have a role in the function of this tRNA. The *Xenopus* gene is severalfold more efficiently transcribed both *in vivo* and *in vitro*, while the human and rabbit genes are transcribed about equally and more efficiently than the chicken gene. Since the *Xenopus* and chicken genes are identical in sequence and differ from those of human and rabbit by a single pyrimidine transition at position 11, the gene sequences do not account for variations in transcription levels. Exchange of the flanking regions between chicken and *Xenopus* genes and characterization of the transcription efficiencies of a number of deletions within the 5' flanking region demonstrate that all of the regulatory sites for expression of this gene occur upstream. There are three

separate sites in the 5' flank for transcription. One is a TATA box which is coupled with a second site that is an upstream GC rich region and the third occurs in an AT rich region which is present within nucleotides -62 to -76. The nature of transcription of the *Xenopus* gene *in vivo* suggests that the latter regulatory site may be developmentally expressed.

The bovine glutathione peroxidase (GPx) gene was obtained from Dr. G. Mullenbach (Chiron Corp) and subcloned into an expression vector for making GPx mRNA. Selenocysteine occurs at the active site of GPx and we are presently testing the incorporation of selenocysteine into GPx from selenocysteyl-tRNA^{Ser}.

The human GPx gene was also obtained from Dr. G. Mullenbach and used as a probe to hybridize to DNA isolated from human rodent somatic cell hybrids. The probe hybridized to chromosomes 3, 21 and X. Experiments using the 3' flanking region and a GPx gene intron as probes suggest that the gene is located in chromosome 21 and that the loci on chromosomes 21 and X are processed pseudogenes. Further mapping of the selenocysteine tRNA^{Ser} gene to chromosome 19 in the human genome (see McBride, Rajagopalan and Hatfield, *J. Biol. Chem* 262:11163,1987, for earlier mapping data) shows that the gene maps to 19q12.2. The latter studies were determined by RFLPs and by *in situ* hybridization.

The Se-75 labeled tRNA isolated from rat mammary tumor cells was identified as a selenocysteyl-tRNA^{Ser} which recognizes the termination codon, UGA. The intermediate in the pathway of selenocysteine biosynthesis, phosphoseryl-tRNA^{Ser}, was also isolated. These studies demonstrate that the function of selenocysteine tRNA^{Ser} is to serve both as a carrier molecule for the biosynthesis of selenocysteine and as a donor of selenocysteine directly to protein.

Publications:

Hatfield DL, Lee BJ, Smith DWE, Oroszlan S. Role of nonsense, frameshift and missense suppressor tRNAs in mammalian cells. In: Muller WEG, ed. *Progress in molecular and subcellular biology*. Berlin, Heidelberg: Springer-Verlag (In Press).

Hatfield D, Smith DWE, Lee BJ, Worland PJ, Oroszlan S. Structure and function of suppressor tRNAs in higher eucaryotes. *CRC Crit Rev Biochem* (In Press).

Lee BJ, Kang SK, Hatfield D. Transcription of *Xenopus* selenocysteine tRNA^{Ser} (formerly designated opal suppressor phosphoserine tRNA) is directed by multiple 5' extragenic regulatory elements. *J Biol Chem* 1989;264:9696-702.

Lee BJ, Worland PJ, Davis JN, Stadtman TC, Hatfield DL. Identification of a selenocysteyl-tRNA^{Ser} in mammalian cells which recognizes the nonsense codon, UGA. *J Biol Chem* 1989;264:9724-27.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05373-06 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Purification and Characterization of a Rat Hepatic Proliferation Inhibitor

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

PI:	Anthony C. Huggett	Visiting Associate	LEC NCI
Others:	Caroline P. Ford	Microbiologist	LEC NCI
	Betty Yu	DCBD Fellow	LEC NCI
	Snorri S. Thorgeirsson	Chief	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

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.)

The aim of this project is to isolate and characterize a protein from adult rat liver that produces a reversible inhibition of the proliferation of liver-derived cells. The purification procedure has recently been extensively modified in order to obtain high recovery and greater purification of the growth inhibitor. Following the initial anion exchange and gel filtration chromatographic procedures, two high resolution ion exchange chromatographic steps are utilized, anion exchange on a Mono Q column followed by cation exchange on a Mono S column. The resulting preparation which has an ID50 of about 1-5 ng/ml is then subjected to high-resolution hydrophobic interaction chromatography using an propyl aspartamide HPLC. This produces a preparation with an ID50 of 150-500 pg/ml and two-dimensional polyacrylamide electrophoresis has revealed the presence of about 10-15 polypeptides. A further microscale purification procedure utilizing microbore reverse phase HPLC is currently being investigated. Chromatography of the propyl aspartamide purified samples indicates the presence of three major protein peaks. Although the conditions employed in this procedure cause a major loss of protein activity, it has been possible to tentatively narrow down the growth inhibitory activity to either one of these major proteins or a minor component of the preparation which coelutes with it at a 43.5% acetonitrile concentration. Current work is focused on determining which of these proteins is responsible for the inhibitory activity. This newly developed procedure should allow the purification of this inhibitor to the purity required for sequence analysis. Concomitantly the large-scale purification of this liver-derived growth inhibitor is being pursued with the aim of immunization in order to obtain neutralizing antibodies to its activity.

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

Anthony C. Huggett	Visiting Associate	LEC	NCI
Caroline P. Ford	Microbiologist	LEC	NCI
Betty Yu	DCBD Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI

Objectives:

The purpose of this project is to isolate and characterize, both biologically and structurally, a growth inhibitory polypeptide from normal adult rat liver. The aim is to investigate the role of this factor in normal cell growth and also to determine its involvement in the neoplastic process.

Methods Employed:

The principal methods employed in these studies include: (1) use of tissue homogenization, protein precipitation, standard column chromatographies, fast protein liquid chromatography (FPLC), microbore high performance liquid chromatography (HPLC) and 1D- and 2D-sodium dodecyl sulfate (SDS)-PAGE to purify the growth inhibitor from the liver of adult rats; (2) use of an immortalized line of neonatal rat liver epithelial cells for the bioassay of growth inhibitors using analysis of DNA content and DNA synthesis; (3) culture of various cell types for the characterization of growth regulatory activity; (4) use of neutralizing antibodies directed against other growth inhibitors to discriminate inhibitory activities.

Major Findings:

Methodology for the analytical-scale isolation of a highly purified growth inhibitor (HPI) from 1000 adult rat livers has been developed. The resulting preparation (1 μ g total protein), although still not homogeneous, was highly active as an inhibitor of the proliferation of rat liver epithelial (RLE) cells and of mitogen-stimulated DNA synthesis in primary hepatocyte cultures (ID₅₀: 50 and 250 pg/ml, respectively). It is active as a growth inhibitor on RLE cells in their log-phase of growth and is effective in serum-free and serum-supplemented media. It is also effective in inhibiting transferrin-stimulated DNA synthesis in serum-starved density-arrested cultures of RLE cells. In addition it has been shown to be a growth regulator for other non-liver and non-epithelial cell types and has a growth inhibitory effect on some transformed cells such as the human breast cancer cell line, MCF-7. The factor responsible for these activities was shown to be an acid- and heat-labile polypeptide with a molecular weight in the range of 17-25 kD and a pI of 5.5. Physicochemical and biological characterization of the growth inhibitor demonstrated that it is distinct from any of the well-characterized growth inhibitory polypeptides previously reported including TGF- β s, tumor necrosis factor (TNFs), interleukins (ILs), interferon (IFNs) and mammary cell-derived growth inhibitor (MDGI). During the course of these studies

it was determined for the first time that interleukin-6 is a growth regulator for both normal primary hepatocytes liver epithelial cells and that analogous to other growth inhibitor polypeptides, such as TGF- β , and HPI/LDGI, it is less effective in transformed cells.

Most recent work has been focused on the large-scale purification of the liver-derived growth factor and on the development of microscale chromatographic procedures for the final purification of the inhibitor to allow amino acid sequence analysis. Livers from 12,000 adult rats (mixed sex and strain) have been used. Following homogenization and protein precipitation steps, the preparations were subjected to diethylaminoethyl (DEAE)-cellulose chromatography. The inhibitory activity eluted at about 0.05 M NaCl with an ID of about 500 ng/ml. FPLC-gel filtration was employed as the next step and the activity eluted at a MW of about 20 kD and with an ID₅₀ of about 50 ng/ml. Two new high resolution ion exchange chromatography procedures have been developed for the further purification of the inhibitor. Mono Q anion exchange followed by Mono S cation exchange chromatography proved to be the most suitable procedures for further purification at this stage. The resulting preparation which has an ID₅₀ of about 1-5 ng/ml is then subjected to hydrophobic interaction chromatography using propyl aspartamide HPLC to produce a highly purified and active preparation (ID₅₀ 150-500 pg/ml). However, the protein amount obtained at this stage is in the range 50-200 μ g necessitating the use of microscale procedures for the further purification of the growth inhibitory activity. Recently a microbore reverse phase HPLC procedure was developed which allows the analysis of microgram quantities of material and also allows recovery of the putative inhibitory polypeptide in an active form. Chromatography of the propyl aspartamide purified preparation using this new procedure indicates that this preparation contains three major polypeptides in addition to a number of minor polypeptide components. These results were confirmed by 2D-gel analysis. Initial results have indicated that the inhibitory activity reproducibly coelutes with the most hydrophobic of these polypeptides. Further work is aimed at determining whether this polypeptide or a minor coeluting protein is responsible for the inhibitory activity. If the major polypeptide is found to be the active growth inhibitor then it will be possible to utilize this technique as the final purification step prior to sequence analysis. Parallel studies are also currently being undertaken in order to purify more growth inhibitory material for use in immunization experiments in order to try and obtain a neutralizing antibody. This will be of considerable help in the final purification and characterization of the growth inhibitory polypeptide.

The growth modulatory effects of this highly purified liver-derived growth inhibitor have been compared to those of two well-characterized growth regulators, TGF- β , and rTNF- α , in a variety of liver-derived and non-liver-derived normal and neoplastic cell culture systems. Normal rat liver epithelial cells were highly sensitive to the growth inhibitory effects of HPI/LDGI and TGF- β , but were less sensitive to rTNF- α . Aflatoxin B₁-transformed RLE cells showed some sensitivity to the cytostatic effects of HPI/LDGI but were completely resistant to the antiproliferative effects of TGF- β , and rTNF- α . In contrast rat hepatoma Reuber cells were extremely sensitive to the antiproliferative effects of rTNF- α and were completely resistant to TGF- β , and HPI/LDGI. Other liver-derived transformed cells also showed differences in sensitivity to these growth

inhibitors. Among the non-liver-derived cells, human breast carcinoma (MCF-7) cells were extremely sensitive to rTNF- α , exhibited some sensitivity to HPI/LDGI, but were unaffected by TGF- β . In contrast to the epithelial cells examined, the growth of fibroblast cell lines were stimulated by all three growth regulators.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05450-05 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Chromatin Structure and Gene Expression

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

PI:	Gordon Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Others:	Philippe Lefebvre	Visiting Fellow	LEC NCI
	Trevor Archer	Visiting Fellow	LEC NCI
	Emery Bresnick	IRTA Fellow	LEC NCI
	Ronald Wolford	Microbiologist	LEC NCI
	Diana Berard	Microbiologist	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.4

PROFESSIONAL:

2.0

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 sites to which steroid-receptors bind on the MMTV LTR are displayed on the surface of nucleosome B in a phased array. Hormone activation of the promoter leads to active displacement of this nucleosome in vivo. A disomic structure composed of the A and B nucleosomes can be reconstituted in vitro, with the octamer cores accurately positioned. Purified NF1/CTF, a requisite component of the MMTV initiation complex, is excluded from the disomic structure, in contrast to the glucocorticoid receptor. Thus, nucleosome displacement is necessary during transcription activation to permit binding of the initiation complex. The chromatin structure of this promoter represents a repressed state, which must be specifically modified to allow promoter activation. This process has also been shown to be independent of DNA replication. This is the first example of active nucleosome displacement initiated by a known regulatory protein, resulting in chromatin remodelling that provides altered access to a second set of DNA-binding proteins. These results indicate that a chromatin template containing specifically positioned nucleosomes is an active participant in transcriptional activation and that the complete regulatory process cannot be reconstituted in vitro with pure DNA and pure factors. A second region of hypersensitivity has been characterized that corresponds to nucleosome F, the last nucleosome in the phased array.

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

Gordon Hager	Head, HAO Section	LEC NCI
Philippe Lefebvre	Visiting Fellow	LEC NCI
Trevor Archer	Visiting Fellow	LEC NCI
Emery Bresnick	IRTA Fellow	LEC NCI
Ronald Wolford	Microbiologist	LEC NCI
Diana Berard	Microbiologist	LEC NCI

Objectives:

The genetic information in mammalian cells is organized into a highly condensed nucleoprotein structure whose basic repeating subunit is the nucleosome. The primary objective of this project is to analyze this nucleoprotein structure of genes in mammalian cells subject to transcriptional control and to elucidate the role, if any, of chromatin organization in mechanisms of gene regulation.

Methods Employed:

1. Cell lines are isolated and characterized that contain regulated promoters of interest on episomal, bovine papilloma virus (BPV) vectors.
2. Chromatin of promoters and regulatory domains in the minichromosomes is examined for specific and reproducible nucleoprotein structures.
3. Using purified DNA, histones, and specific DNA-binding proteins, chromatin is reconstituted in vitro.
4. The in vitro reconstituted material is characterized to determine to what extent the structural features present in vivo can be duplicated, and what the effects of specific nucleoprotein organization are for the interaction with the soluble transcription apparatus.

Major Findings:

Previous efforts focused on characterization of cell lines with the steroid-inducible MMTV LTR promoter mobilized on BPV vectors, and the analysis of chromatin structure with micrococcal nuclease. The steroid transcriptional response was shown to take place in the episomal environment, validating the system as appropriate to study transcriptional regulation of the MMTV promoter with this system. Micrococcal nuclease and methidium propyl EDTA-FeII data indicated that nucleosomes were specifically positioned, or phased, over the MMTV promoter and associated regulatory regions in the episomal minichromosomes. We have now extended these experiments to single, integrated copies of the LTR, acquired either by DNA-mediated transfection after fusion to a selectable oncogene or present as an endogenous provirus in the mouse genome. In both cases, the normal phasing pattern is seen. Thus, MMTV DNA always acquires this specific nucleoprotein organization, irrespective of how the DNA is introduced into the cell.

When the MMTV promoter is induced, this highly structured nucleoprotein complex is specifically altered. A broad region of hypersensitivity develops across a region of the promoter associated with nucleosome B, suggesting that receptor binding and promoter activation is accompanied either by nucleosome displacement or by a major modification of nucleosome structure. We have now reconstituted the nucleosome structure *in vitro* and have found that the octamer cores for nucleosomes A and B will position correctly on a DNA fragment containing the A-B region. Thus, the information for positioning at least the A and B nucleosomes is present in the DNA itself.

We also have shown that NF1/CTF, one of the factors involved in the formation of the MMTV transcription initiation complex, is specifically excluded from its DNA recognition site when the A-B nucleosome is reconstituted. We conclude that one transcription factor, the glucocorticoid receptor, can bind to its recognition site organized on a nucleosome and actively displace the octamer core from the phased array. A second factor, NF1, is excluded from DNA in chromatin; the necessity to displace nucleosome B is to provide access for the NF1-TFIID initiation complex. Two models are consistent with these results. Synergistic interactions between soluble components of the initiation complex may provide sufficient energy to disrupt nucleosome structure and shift the equilibrium of the preinitiation complex to a promoter bound state. Alternatively, receptor-induced disruption of nucleosome structure could provide a modified template to which the other soluble components of the preinitiation complex could bind. Since correct positioning can be obtained *in vitro* with purified components, we can now address these models directly.

We have also discovered a second nucleosome displacement event in the LTR. In mammary tumor cells where a cellular protooncogene (*int-2*) was activated by proviral insertion, we have found a second region of hypersensitivity that corresponds to the position of nucleosome F. This region of chromatin is open constitutively, in contrast to the hormone-dependent B region, a finding that correlates with the transcription profile of the activated *int-2* oncogene. Thus, a second transcriptional regulatory element has been defined in the MMTV LTR. This element is a likely candidate for the *int*-locus activator. Consistent with this model, we have shown in transient expression experiments with a mammary cell line that maintains differentiated characteristics that the left end of the LTR is necessary for efficient expression. We have also characterized DNA-binding proteins that are specific to the F nucleosome core region.

The MMTV LTR thus becomes an interesting model not only for the interaction of transcription factors with specifically positioned nucleosomes, but also for the potential synergistic action of hormone-responsive and tissue-specific transcriptional regulatory elements.

Publications:

Hager GL. Chromatin template remodeling and steroid receptor transactivation of MMTV. In: Sund H, Gehring U, eds. Proceedings of the 40th Mosbach Colloquium; Molecular mechanisms of hormone action. Heidelberg: Springer-Verlag (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05453-05 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Cellular and Molecular Aspects of Hepatocarcinogenesis

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

PI:	Snorri S. Thorgeirsson	Chief	LEC NCI
Others:	Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
	Harushige Nakatsukasa	Expert	LEC NCI
	H. Cathrine Bisgaard	Guest Researcher	LEC NCI
	K-H. Lin	Visiting Fellow	LEC NCI
	Phuongnga Ton	Microbiologist	LEC NCI
	Nancy Sanderson	Chemist	LEC NCI

COOPERATING UNITS (if any)

The Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, NC (Dr. Brian E. Huber)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.7

PROFESSIONAL:

0.7

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Experimental hepatocarcinogenesis in the rat has been used as a model to study the cellular and molecular events during neoplastic development. The research is currently focused on defining the possible role of a stem cell compartment in the liver during oncogenesis as well as in the normal liver (see also Project No. Z01CP05262). We have shown that a facultative stem cell compartment exists in the liver, and these stem cells become a major source of new hepatocytes when the normal regenerative response of the liver is impaired. The liver-derived stem cells are multipotential and are capable of differentiating in vivo into hepatocytes, bile and intestinal epithelia as well as into pancreatic cell lineages. We have obtained data strongly indicating that transforming growth factor- β 1 (TGF- β 1) is a key determinant in differentiating the stem cells along the hepatocytic lineage in vivo as well as in vitro. However, during hepatocarcinogenesis, TGF- β 1 may function as an endogenous promoter of the neoplastic process due to its strong growth inhibitory effects on normal hepatocytes in combination with a growth stimulatory effect on transformed liver cells. Moreover, we have shown that the major source of TGF- β 1 in the liver during later stages of tumorigenesis are the stromal cells; consequently, the tumor promoting effect of TGF- β 1 may occur via a paracrine mechanism.

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

Snorri S. Thorgeirsson	Chief	LEC NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Harushige Nakatsukasa	Expert	LEC NCI
Hanne Cathrine Bisgaard	Guest Researcher	LEC NCI
K-H. Lin	Visiting Fellow	LEC NCI
Phuongnga Ton	Microbiologist	LEC NCI
Nancy Sanderson	Chemist	LEC NCI

Objectives:

- (1) Define the role of the stem cell compartment in rat and human liver in normal biology of the liver as well as in hepatocarcinogenesis and acute and chronic hepatitis.
- (2) Determine both humoral and genetic factors that determine differentiation of the liver stem cells into different cell lineages.
- (3) Establish epithelial cell lines from rat and human liver and pancreas.
- (4) Analyze the effects of transforming oncogenes on growth, differentiation and transformation of rat liver and pancreatic epithelial cells.
- (5) Use the transgenic mouse model to analyze tissue-specific and developmental stage-specific expression of transforming oncogenes in relation to the development of neoplasia.

Methods Employed:

Methods used in these studies include: tissue culture techniques, radioisotopic measurements, enzyme assays, histochemical and immunohistochemical methods, and recombinant and molecular technology including DNA and RNA preparation, Northern and Southern blotting, construction of cDNA, genome libraries and nucleic acid hybridization, construction of retroviral vector systems, and microinjection of cloned DNA fragments into the pronucleus of a fertilized egg.

Major Findings:

- (1) Transforming growth factor- β 1 (TGF- β 1) is capable of eliciting a myriad of biological responses associated with cellular proliferation as well as effects unrelated to the control of cell growth. We show that TGF- β 1 is a major regulator of both growth and differentiation in rat liver, and may function as an endogenous tumor promoter during the progression stage of chemical hepatocarcinogenesis. By using in situ hybridization and immunohistochemical techniques, we show that both TGF- β 1 transcripts and protein are localized in nonparenchymal cells in normal liver; are expressed in progenitor cells during early stages of hepatocytic differentiation in vivo; and are exclusively expressed in the non-tumorous mesenchymal cell compartment during hepatocarcinogenesis. Furthermore, we show that TGF- β 1 is capable of inducing

differentiation of rat liver-derived epithelial (RLE) cells in vitro consistent with early stages of hepatocytic lineage differentiation and indicate that the RLE cell may be an epithelial progenitor cell for hepatocytic cell lineage in adult mammalian liver.

(2) The development of chemically induced hepatocellular carcinoma in the rat proceeds through a series of premalignant changes that may ultimately progress to a primary malignant tumor. Using the selection technique based on diminished binding of preneoplastic hepatocytes to tissue culture plates precoated with asialofetuin, we have isolated poly(A⁺)RNA from early preneoplastic foci as well as preneoplastic persistent nodules and primary hepatocellular carcinoma induced by the Solt-Farber protocol in the Fischer rat. The steady-state poly(A⁺)RNA levels of genes traditionally associated with growth, differentiation and/or transformation were then determined to address the question of their temporal expression in the multistep nature of cancer development.

Ornithine decarboxylase- and P53-specific transcripts did not significantly change in preneoplastic foci but were increased in later-stage preneoplastic nodules and hepatocellular carcinoma. Albumin-specific transcripts were decreased in all hepatocellular carcinoma but there was no consistent coordinated increase in α -fetoprotein-specific transcripts. c-myc and raf transcripts increased at the very early preneoplastic foci stage and continued to increase throughout the neoplastic process. No L-myc or N-myc transcripts could be detected in any RNA sample. c-Ha-ras-specific transcripts were essentially unaltered in all RNA samples, whereas no c-Ki-ras or N-ras transcripts could be detected throughout the neoplastic process. In addition, no dominant-acting transforming mutations in the ras gene family were detected by DNA transfection experiments using NIH 3T3 cells.

(3) We have created transgenic mouse lines following microinjection of two recombinant DNA constructs into 1-cell stage embryos: (a) v-raf oncogenes and (b) hMT-v-H-ras. The genomic localization and expression of the introduced genes are being analyzed.

Publications:

Huber BE, Heilman CA, Thorgeirsson SS. Poly(A⁺)RNA levels of growth-, differentiation- and transformation-associated genes in the progressive development of hepatocellular carcinoma in the rat. *Hepatology* 1989;9:756-62.

Klinken SP, Holmes KL, Morse HC, Thorgeirsson SS. Transcriptional and post-transcriptional regulation of c-myc, c-myb, and p53 during proliferation and differentiation of murine erythroleukemia cells treated with DFMO and DMSO. *Exp Cell Res* 1988;178:185-98.

Thorgeirsson SS, Garfield SH, Huber BE, Burt RK. Acquisition of multidrug resistance in chemical hepatocarcinogenesis. In: Estabrook RW, Lindenlaub E, Oesch F, de Weck A], eds. Toxicological and immunological aspects of drug metabolism and environmental chemicals, Symposia Media Hoechst 22. Stuttgart, Germany: FK Schattauer Verlag, 1988;367-77.

Thorgeirsson SS, Nagy P, Everts RP. Cellular and molecular changes in early stages of hepatocarcinogenesis. In: Feo F, Pani P, Columbano A, Garcea R, eds. Chemical carcinogenesis: models and mechanisms. New York: Plenum Publishing. 1988;203-8.

Thorgeirsson SS, Wirth PJ. Biochemical marker alterations in hepatic preneoplasia and neoplasia. In: Sirica A, ed. The pathobiology of neoplasia. New York: Plenum Publishing. 1989;385-97.

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

PROJECT NUMBER

Z01CP05496-04 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Metabolism and Disposition of IQ in Monkeys

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

PI:	Snorri S. Thorgeirsson	Chief	LEC NCI
Others:	Elizabeth G. Snyderwine	Guest Researcher	LEC NCI
	Richard H. Adamson	Director	DCE NCI
	Maryellen Mazza	Biologist	LEC NCI

COOPERATING UNITS (if any)

National Cancer Center Research Institute, Tokyo, Japan (Drs. T. Sugimura, M. Nagao); Hazleton Laboratories, Vienna, VA (Dr. Daniel Dalgard)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

2.2

PROFESSIONAL:

1.2

OTHER.

1.0

CHECK APPROPRIATE BOX(ES)

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

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

2-Amino-3-methyl-imidazol(4,5-f)quinoline (IQ) is a heterocyclic arylamine found in certain cooked foods such as beef and fish. IQ has been shown to be carcinogenic to both rodents and monkeys and to form DNA adducts in tissues of these animals fed IQ. It is well recognized that metabolism can play an important role in the carcinogenicity of chemicals. Therefore we have been examining the disposition of IQ in monkeys and identifying urinary and fecal metabolites of IQ which may be indicative of pathways of carcinogen activation or detoxification. Following administration of ¹⁴C-IQ (2 μmol/kg) to cynomolgus monkeys by nasal-gastric intubation, blood levels declined rapidly from 1 to 8 hr following dosing. This was followed by a slow decline from 8 to 72 hr. Approximately 30-45% of the dose was excreted in the urine by 8 hr and by 72 hr 45-60% had been excreted. Through HPLC analysis we have observed that IQ is extensively metabolized in monkeys to at least 4 urinary metabolites. No IQ itself was found in the urine. We have found that one of the metabolites is IQ-N-sulfamate. In addition, we have tentatively identified two glucuronides of IQ by their sensitivity to β-glucuronidase. Structural identification of these metabolites is currently underway.

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

Snorri S. Thorgeirsson	Chief	LEC NCI
Elizabeth G. Snyderwine	Guest Researcher	LEC NCI
Richard H. Adamson	Director	DCE NCI
Maryellen Mazza	Biologist	LEC NCI

Objectives:

The objective of this study was to examine the metabolism and disposition of IQ in monkeys.

Methods Employed:

The principle methods employed are the following: (1) HPLC, (2) chemical synthesis, (3) nuclear mass resonance (NMR), (4) mass spectroscopy, and (5) liquid scintillation spectroscopy.

Major Findings:

Following administration of ^{14}C -IQ ($2 \mu\text{mol/kg}$) to cynomolgus monkeys by nasal-gastric intubation, blood levels declined rapidly from 1 to 8 hr following dosing. This was followed by a slow decline from 8 to 72 hr. Approximately 30-45% of the dose was excreted in the urine by 8 hr and by 72 hr 45-60% had been excreted. Through HPLC analysis we have observed that IQ is extensively metabolized in monkeys to at least 4 urinary metabolites. No IQ itself was found in the urine. We have found that one of the metabolites is IQ-N-sulfamate. In addition, we have tentatively identified two glucuronides of IQ by their sensitivity to β -glucuronidase. Structural identification of these metabolites is currently underway.

Publications:

Snyderwine EG, Yamashita K, Adamson RH, Sato S, Nagao M, Sugimura T, Thorgeirsson SS. Use of the ^{32}P -postlabeling method to detect DNA adducts of 2-amino-3-methylimidazol[4,5-f]quinoline (IQ) in monkeys fed IQ: Identification of the N-(deoxyguanosin-8-yl)-IQ adduct. Carcinogenesis (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05553-02 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Expression of Cytochrome P-450s and Their Role in Mutagenesis and Carcinogenesis

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

PI:	Narayana Battula	Expert	LEC NCI
Others:	Elizabeth G. Snyderwine	Guest Researcher	LEC NCI
	H. Cathrine Bisgaard	Guest Researcher	LEC NCI
	Snorri S. Thorgeirsson	Chief	LEC NCI

COOPERATING UNITS (if any)

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

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

2.0

PROFESSIONAL:

2.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

The cytochrome P450s are a superfamily of enzymes which metabolize a wide array of compounds including xenobiotics such as drugs and carcinogens, and endobiotics such as steroids and prostaglandins. In animals, multiple forms of these enzymes are expressed simultaneously either constitutively or after administration of inducers. To define the contribution of a given cytochrome P450 to the metabolism of specific drugs and carcinogens, it is important to express these enzymes individually in cells which lack endogenous background activity for these enzymes. Toward this goal we have begun to develop expression systems in which individual cytochrome P450s are synthesized from their coding DNA sequences. Success in this effort will enable us to identify putative human mutagens and carcinogens as well as to define the contribution of each of these enzymes to toxicity, mutagenesis, and cell transformation by chemical carcinogens. To achieve this goal, we have constructed infectious recombinant vaccinia viruses and infectious recombinant retroviruses containing the full length coding cDNA sequences for mouse cytochrome P4501A1 and P4501A2. Human and rodent cells infected with the recombinant viruses expressed high levels of the authentic size proteins that were enzymatically active and displayed substrate specificities diagnostic of the respective enzymes. Employing the recombinant cytochromes we have demonstrated that the cytochrome P4501A2 selectively activates heterocyclic arylamines and cytochrome P4501A1 preferentially activates aromatic hydrocarbons; this preferential selectivity is mutually exclusive at limiting substrate concentrations. Furthermore, the cytochrome P4501A2 constitutively expressed in mammalian cells activated food-derived heterocyclic amine carcinogens and catalyzed the formation of specific DNA-carcinogen adducts. The DNA adducts detected were identical to those formed in mouse or rat liver after the in vivo administration of the food mutagens.

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

N. Battula	Expert	LEC	NCI
E. G. Snyderwine	Guest Researcher	LEC	NCI
H. Bisgaard	Guest Researcher	LEC	NCI
S. S. Thorgeirsson	Chief	LEC	NCI

Objectives:

The overall goal of this project is to express individual cytochrome P450 enzymes in mammalian cells and define their role in toxicity, mutagenesis and chemical carcinogenesis. Specific aims are to (a) express P450s in using their coding DNA sequences as the source of the enzymes and recombinant vaccinia viruses and retroviruses as vectors; (b) analyze the expressed products for post-transcriptional processing, subcellular localization and enzymatic activities; (c) test the mutagenic activations of suspected mutagens and identify the damage to the cellular macromolecules; (d) test for mutations and cell transformation after exposure to carcinogens of cells expressing the P450 enzymes; and (e) characterize structure function relationships of P450s by site-directed mutagenesis.

Methods Employed:

Construction of recombinant viruses requires extensive use of recombinant DNA methodology, DNA separation procedures based on hydrodynamic properties and electrophoretic mobilities, DNA transfections, cell culture techniques, virological procedures and genetic selection of cells and viruses. Detection and isolation of expression products requires use of different blotting methods, subcellular fractionation procedures, protein separations by electrophoresis and column chromatography, immunological procedures employing monoclonal and polyclonal antibodies. Functional evaluation of the expressed proteins requires a variety of enzymatic assays such as aryl hydrocarbon hydroxylase, ethoxyresorufin O-deethylase, 7-ethoxycoumarin O-deethylase and acetanilide hydroxylase, spectrophotometry, spectrofluorimetry and high pressure liquid chromatography. Detection of DNA injury by the recently developed highly sensitive method of ³²P-postlabeling to identify and quantify DNA-carcinogen adducts.

Major Findings:

Infectious recombinant vaccinia viruses and retroviruses containing a selectable reporter gene marker and full length cDNAs for either mouse cytochromes P4501A1 or P4501A2 were constructed and characterized. These recombinant viruses have broad host range and can infect a variety of mammalian cells.

Mammalian cells, including human cells infected with the infectious recombinant vaccinia virus, efficiently expressed their respective proteins. Characterization of the newly synthesized proteins by immunochemical, spectral and enzymatic analysis indicates that the polypeptides expressed were authentic in size, have incorporated heme and the holoenzymes displayed catalytic activities characteristic of the two enzymes.

Mammalian cells infected with the cytochrome P4501A2 recombinant retrovirus have stably transduced the P4501A2 DNA sequences into the host cellular DNA. The transduced clones constitutively expressed new protein products which were indistinguishable from the cytochrome P4501A2 found in the mouse liver microsomes. Enzymatic analysis of cells in situ and of the cell homogenates in vitro showed catalytic properties diagnostic of cytochrome P4501A2.

We have examined the specificity of the recombinant enzymes expressed in human cells for the mutagenic activation of carcinogens. Cytochrome P4501A2 selectively catalyzed mutagenic activation of heterocyclic arylamines found in cooked foods. Cytochrome P4501A2 preferentially activated the aromatic hydrocarbon benzo(a)pyrene-7,8 diol. These enzymes thus are selective in the choice of their substrates and exhibit mutually exclusive activities at limiting substrate concentrations.

Clones of mammalian cells constitutively expressing cytochrome P4501A2 were challenged with the heterocyclic amine carcinogens AF, AAF and the food-derived carcinogens 2-amino-3-methyl-imidazo(4,5-f)quinoline (IQ), MeIQx and the specific DNA adducts formed were identified and quantified by ³²P-postlabeling assays. The fingerprints of these adducts formed in cells by the catalysis of cytochrome P4501A2 were indistinguishable from those formed in mouse or rat liver after the in vivo administration of these compounds. Thus, clones constitutively expressing cytochrome P450s provide an excellent model system to study the mechanism of activation of putative human mutagens and carcinogens.

Publications:

Battula N. Transduction of cytochrome P3-450 by retroviruses: constitutive expression of enzymatically active microsomal hemoprotein in animal cells. *J Biol Chem* 1989;264:2991-6.

Battula N, Schut HAJ, Snyderwine EG, Townsend GK, Thorgeirsson SS. Introduction of cytochrome P-450 genes into mammalian cells by recombinant viruses and analysis of the expressed P-450s. In: Schuster I, ed. *Cytochrome P-450: biochemistry and biophysics*. Vienna, Austria: Francis & Taylor, 1989;783-6.

Gelboin HV, Gonzalez FJ, Park SS, Sagara J, Battula N. Cytochrome P-450 function analysis with monoclonal antibodies and cDNA expression vectors. In Torino FB, ed. *Chemical carcinogenesis: models and mechanism*. Algero, Italy: Plenum Press, 1988;3-15.

Snyderwine EG, Battula N. Selective mutagenic activation by cytochrome P3-450 of carcinogenic arylamines found in foods. *JNCI* 1989;81:223-7.

Patents:

Battula N. US Patent (Pending): Transduction and Stable Expression of Enzymatically Active Cytochrome P-450 in Animal Cells.

Battula N, Gelboin HV, Gonzalez FJ, Moss B. US Patent (Pending): Construction of Infectious Recombinant Vaccinia Virus Containing DNA Coding Sequences for Cytochromes P-450 and Their Expression as Active Enzyme.

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

PROJECT NUMBER

Z01CP05555-02 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Aminoacyl-tRNAs in HIV and Other Retroviral Infected Cells

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

PI:	Dolph L. Hatfield	Research Biologist	LEC NCI
Others:	Byeong J. Lee	Visiting Fellow	LEC NCI
	Sung Goo Kang	Guest Researcher	LEC NCI

COOPERATING UNITS (if any)

Laboratory of Molecular Genetics, NICHD (Drs. X. F. Feng, Judith Levin);
 Bionetics Research, Inc. (Drs. Alan Rein and Stephen Oroszlan)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.5

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.)

Transfer RNA was isolated from HIV-1 (human immunodeficiency virus-1), HIV-2 (human immunodeficiency virus-2), HTLV-1 (human T-cell leukemia virus) and BLV (bovine leukemia virus)-infected and -uninfected cells and the elution profiles of aminoacyl-tRNAs from infected and uninfected cells were compared by reverse phase chromatography. In each case examined, Asn-tRNA, which normally contains the highly modified Q base in the 5' position of its anticodon, was deficient in this base in infected cells. Phe-tRNA from HIV-1 and HIV-2-infected cells also lacked the highly modified Wye base on the 3' side of its anticodon. Interestingly, one or both of these tRNAs are required for translation within the ribosomal frameshift signal in expression of the gag-pol polyfusion protein of each retrovirus examined in this study and in each ribosomal frameshift signal sequenced to date. Addition of purified Phe-tRNA minus Wye base and Asn-tRNA minus Q base to rabbit reticulocyte lysates programmed with mRNA generated from the HIV gag-pol region gave varying results on their effects on ribosomal frameshifting. Our efforts to provide an assay for ribosomal frameshifting are now focusing on microinjection of purified tRNAs and HIV gag-pol mRNA into *Xenopus* oocytes.

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

Dolph L. Hatfield	Research Biologist	LEC NCI
Byeong J. Lee	Visiting Fellow	LEC NCI
Sung G. Kang	Guest Researcher	LEC NCI

Objectives:

The major goals of this project are to determine if the aminoacyl-tRNAs in HIV and other retroviral-infected cells are altered and if such altered tRNAs have a role in the expression of the virus. Specific steps to achieve these goals are: 1) to isolate tRNA from HIV and other retroviral-infected cells and the corresponding uninfected cells; 2) to determine if differences in the tRNAs occur in infected and uninfected cells by comparing tRNA elution profiles on reverse phase chromatographic columns; 3) to subclone the gag-pol region of HIV into an expression vector to use as a model for examining the role of specific tRNAs in ribosomal frameshifting in in vitro and in vivo assays; and 4) to purify altered tRNAs from infected cells and determine if they may have a role in the expression of the virus.

Methods Employed:

HIV-1-, HIV-2-, HTLV-1- and BLV-infected cells and a control set of uninfected cells were grown under identical conditions. A matched set of uninfected cells corresponding to HTLV-1-infected cells were not available. Transfer RNA was extracted from cells, aminoacylated with radioactive amino acids by standard techniques and their elution profiles compared by reverse phase chromatography. Those tRNAs which were altered were purified by standard techniques.

A portion of the HIV genome encoding the gag-pol region was subcloned into an expression vector in order to prepare mRNA. The mRNA was then used to program rabbit reticulocyte lysates for investigating the role of the purified, hypomodified tRNAs in ribosomal frameshifting in in vitro assays.

Purified tRNAs were injected into *Xenopus* oocytes along with HIV gag-pol mRNAs to provide an in vivo assay for determining the role of hypomodified tRNAs on ribosomal frameshifting.

Major Findings:

Asn-tRNA and other tRNAs which normally contain Q base in the 5' position of their anticodon (i.e., Asp-, His- and Tyr-tRNAs) were deficient in this base in each of the infected cells. Asn-tRNA occurs just before the upstream ribosomal frameshift sites in HTLV-1 and BLV and occurs one amino acid upstream of the ribosomal frameshift site in HIV-1. In addition, Phe-tRNA lacked the highly modified Wye base in HIV-1 and HIV-2. Wye base normally occupies the position immediately 3' to the anticodon. Phe-tRNA occurs just before the ribosomal frameshift site in HIV-1 and HIV-2.

Phe-tRNA with and without Wye base and Asn-tRNA without Q base were purified to homogeneity and used in rabbit reticulocyte lysates programmed with mRNA generated from the HIV gag-pol region. Purified hypomodified tRNAs gave varying results which in some assays appeared to have no effect. Thus, the in vitro assay apparently cannot be used reliably to investigate ribosomal frameshifting. We have now turned our attention to in vivo assays using Xenopus oocytes and these studies are now in progress.

Publications:

Feng Y-X, Hatfield DL, Rein A, Levin JG. Translational readthrough of the murine leukemia virus gag gene amber codon does not require virus-induced alteration of tRNA. *J Virol* 1989;63:2405-10.

Feng Y-X, Levin JG, Hatfield DL, Schaefer TS, Gorelick RJ, Rein A. Suppression of UAA and UGA termination codons in mutant murine leukemia viruses. *J Virol* 1989;63:2870-3.

Hatfield DL, Lee BJ, Feng Y-X, Levin JG, Rein A, Oroszlan S. Mechanisms of ribosomal frameshifting for synthesis of the protease in HIV and other retroviruses and the possible use of hypomodified tRNAs in the frameshift event. In: Kostka V, ed. Colloquium of the 14th international congress of biochemistry, Heidelberg: Walter De Gruyter & Co (In Press).

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

PROJECT NUMBER

Z01CP05556-02 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Computer-Assisted Design of Recognition Peptides

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

PI:	Snorri S. Thorgeirsson	Chief	LEC NCI
Others:	James G. Omichinski	IRTA Fellow	LEC NCI
	Arthur D. Olson	Computer Programmer Analyst	LEC NCI
	Chien-Hua Niu	Special Volunteer	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.2

PROFESSIONAL:

2.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

It is now possible to design ex novo recognition peptides, called anti-sense peptides, based on anti-sense DNA sequence of a gene coding for a target protein. The main objective of this project is to devise a method to generate recognition peptides using only partial amino acid sequence information. Two peptides with hydrophobic complementarity to residues 356-375 of the c-raf protein were synthesized to determine if they recognize the raf-(356-375) peptide as well as the entire protein. One peptide was deduced from the complementary mRNA for the raf protein corresponding to residues 356-375, whereas the other was deduced solely from the amino acid sequence of the 20-mer segment using a computer program able to generate peptide sequences with hydrophobic complementarity to a given sequence. Specific binding of both peptides to the raf 20-mer segment was demonstrated when either the raf 20-mer peptide or the complementary peptides were immobilized on a column. Binding affinities were in the millimolar-micromolar range. Identical binding properties were observed with peptides synthesized with either all D- or all L-amino acids, suggesting a lack of conformational dependence. Binding was also unaffected by the presence of 8 M urea or detergents, was dependent on solvent characteristics of pH and ionic strength, and was abolished by the presence of competing peptides in the eluting buffer. Recognition between raf complementary peptides was accompanied by spectral changes in the far and near UV region, as monitored by circular dichroism. Proteolytic degradation was retarded by the binding of these peptides. Once immobilized on a column, these peptides proved useful for the isolation by affinity chromatography of a recombinant c-raf protein from an Escherichia coli crude cell extract.

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

Snorri S. Thorgeirsson	Chief	LEC	NCI
James G. Omichinski	IRTA Fellow	LEC	NCI
Arthur D. Olson	Computer Programmer Analyst	LEC	NCI
Chien-hua Niu	Special Volunteer	LEC	NCI

Objectives:

The main objectives of this project are 1) to devise a method to generate ex novo recognition peptides from amino acid sequence information of the target polypeptide, 2) to use such recognition peptides immobilized on a solid support to isolate and purify novel polypeptides associated with biological relevant processes, and 3) to investigate their applicability as potential structure-function modifiers.

Methods Employed:

The principal methods employed are (1) computer-assisted modeling of peptide hydrophobic plots, (2) peptide synthesis and purification, (3) analytical high performance affinity chromatography, (4) circular dichroism, (5) enzymatic assays, (6) sequence analysis, and (7) one- and two-dimensional polyacrylamide gel electrophoresis (PAGE).

Major Findings:

Using values of individual amino acid hydrophobicity, it was possible to develop a computer-assisted method to generate hydrophobic complementary peptide. In the several systems tested so far, specific interactions were observed between target peptides and computer-designed recognition peptides. Once immobilized on a solid support, they maintained specific binding properties, indicating great potential as purification tools.

Two peptides with hydrophobic complementarity to residues 356-375 of the c-raf protein were synthesized to determine if they recognize the raf-(356-375) peptide as well as the entire protein. One peptide was deduced from the complementary mRNA for the raf protein corresponding to residues 356-375, whereas the other was deduced solely from the amino acid sequence of the 20-mer segment using a computer program able to generate peptide sequences with hydrophobic complementarity to a given sequence. Specific binding of both peptides to the raf 20-mer segment was demonstrated when either the raf 20-mer peptide or the complementary peptides were immobilized on a column. Binding affinities were in the millimolar-micromolar range. Identical binding properties were observed with peptides synthesized with either all D- or all L-amino acids, suggesting a lack of conformational dependence. Binding was also unaffected by the presence of 8 M urea or detergents, was dependent on solvent characteristics of pH and ionic strength, and was abolished by the presence of competing peptides in the eluting buffer. Recognition between raf complementary peptides was accompanied by

spectral changes in the far and near UV region, as monitored by circular dichroism. Proteolytic degradation was retarded by the binding of these peptides. Once immobilized on a column, these peptides proved useful for the isolation by affinity chromatography of a recombinant c-raf protein from an *Escherichia coli* crude cell extract.

We have developed two methods for designing recognition anti-sense peptides without the use of DNA coding sequence information. One approach utilized the frequency usage patterns of codon for various amino acids, as compiled in the GenBank sequence data system. Another source was the data bank on the relative tRNA levels in mammalian cells for codons of interest. In a second approach a computer-assisted peptide design program was developed which determines the root-mean-square of the differences between the average hydrophobic score for a target peptide and all possible anti-sense sequences to it. Those sequences which gave the smallest differences in complementary scores at a given length of averaging were considered optimal anti-sense peptides. To test these approaches, anti-sense peptides were designed to a rat liver glycoprotein N-terminal segment, FNLDAEAPAVLSG, and studied by analytical affinity chromatography to determine binding properties. The peptides derived using the codon frequency information showed dissociation constants (M_{MP}) between $3.0 - 9.8 \times 10^{-4}$ M (100 mM NH_4OAc , pH = 5.7), while those peptides chosen by the hydrophobic minimization approach showed K_{MP} values between 3.2×10^{-7} M (same buffer). These affinity data indicate that the interactions increase as the hydrophobic complementarity score differences are minimized. Competitive elution studies showed that the recognition was specific and probably did not involve simple ionic interactions. Comparative binding constants with an all d-amino acid containing analog indicated that intermolecular binding may not be dependent on rigorous conformational specificity.

Publications:

Fassina G, Roller PP, Olson AD, Thorgeirsson SS, Omichinski JG. Recognition properties of peptides hydrophobically complementary to residues 356-375 of the c-raf protein. *J Biol Chem* (In Press).

Fassina G, Thorgeirsson SS, Omichinski JG. Sequence directed design of recognition peptides. In Wittman-Liebold B, ed. *Methods in protein sequence analysis*. Berlin: Springer-Verlag, 1989;431-438.

Omichinski JG, Olson AD, Thorgeirsson SS, Fassina G. Computer assisted design of recognition peptides. In Hugli T, ed. *Techniques in protein chemistry*. New York: Academic Press, 1989;430-438.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05558-02 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Cellular Proteins in Oncogene Transformed Rat Liver Epithelial Cells

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

PI:	Anthony C. Huggett	Visiting Associate	LEC NCI
Others:	Lori Hampton	Biologist	LEC NCI
	Betty R. Yu	Biologist	LEC NCI
	Snorri S. Thorgeirsson	Chief	LEC NCI
	Peter J. Wirth	Chemist	LEC NCI

COOPERATING UNITS (if any)

None

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.6

PROFESSIONAL:

0.4

OTHER:

1.2

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.)

In order to identify the changes in cellular protein expression and growth regulation that are associated with neoplastic development, we have developed an in vitro model of tumor progression using v-raf or v-raf/v-myc-transformed rat liver epithelial (RLE) cells. A number of cell clones were generated from the parental transformed cells to produce a series of cell lines which displayed a range of morphological transformation and tumorigenicity. These cell lines were all shown to contain integrated DNA sequences corresponding to v-raf or v-raf/v-myc and also expressed mRNA for these oncogenes. Two-dimensional polyacrylamide gel electrophoretic (2D-PAGE) analysis demonstrated that the expression of a number of proteins correlated with the transformed phenotype and malignant potential of these cells. In particular the expression of three relatively high molecular weight proteins (RP1, RP2, RP3) were found to be reproducibly down-regulated in the most tumorigenic cell lines. The identification of these proteins is currently under investigation by both sequence analysis and the use of immunological techniques. A number of other alterations in the phenotype of these cells in terms of their growth regulation and expression of extracellular matrix have also been defined. The transformed cells show aberrant growth control by transforming growth factor- β 1 (TGF- β 1) and an analysis of TGF- β 1 receptor binding indicates that this is mediated by post-receptor events. Additional initial results indicate that the receptor complement of the transformed cells correlates with their in vitro morphology and the expression of certain extracellular matrix proteins.

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

Anthony C. Huggett	Visiting Associate	LEC NCI
Lori Hampton	Biologist	LEC NCI
Betty R. Yu	Biologist	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Peter J. Wirth	Chemist	LEC NCI

Objectives:

The search for proteins associated with the development of cancer requires comparison between normal and tumorigenic cells. In this laboratory, we have developed a series of *v-raf* and *v-raf/v-myc*-transformed RLE cells that represent a model of tumor progression in vitro. This system now allows us to identify changes in polypeptide expression that occur during tumor progression. In addition the alterations in growth control that occur during neoplastic transformation in the liver can be closely studied at a mechanistic level using this in vitro model system.

Methods Employed:

The principle methods employed are: (1) tissue culture techniques, (2) animal husbandry, (3) histochemical staining, (4) 2-dimensional polyacrylamide gel electrophoresis, (5) autoradiography, (6) Southern analysis of DNA, (7) Northern analysis of RNA, (8) immunohistochemistry, (9) receptor binding analysis.

Major Findings:

A series of clonal cell lines were derived from rat liver epithelial cells following their infection with a defective retrovirus containing either *v-raf* (3611-MSV) or *v-raf/v-myc* (J2) as well as a helper virus. These clones (R3611-3, R3611T-2, RJ2-14) exhibited distinct morphologies from the regular cuboidal shape of the control cells (RLEC-2) infected only with the helper virus. The R3611-3 cell line had the most normal morphology and the RJ2-14 clone was most abnormal, being poorly attached and having an elongated spindle shape. All of the infected cell lines contained at least one full-length copy of appropriate proviral DNA and expressed comparable levels of *v-raf* mRNA. Only the RJ2-14 cells were capable of anchorage-independent growth in soft agar. All of the clones except the RLEC-2 cells formed tumors in nude mice, but with markedly different latency periods and growth rates. The RJ2-14 cells were the most tumorigenic and the R3611-3 cells the least. Thus, these cell lines represent an in vitro model for tumor progression.

Two-dimensional polyacrylamide electrophoresis was used to investigate changes in cellular protein expression related to malignant conversion in these cells. The expression of three proteins of pI/M, 5.9-6.2/205 (RP1), 6.5-7.5/160 (RP2) and 4.0/85 (RP3) was consistently shown to match the transformed phenotype. In particular the expression of RP1 and RP2 correlated with the relative tumorigenicity of the cell lines in that they were present in the control RLEC-2

cells, expressed at a lower level in the R3611-3 cells and barely detectable in the R3611T-2 and RJ2-14 cells. Crude fractionation studies determined RP1 to be soluble and RP2 and RP3 to be membrane-associated. RP2 was shown to be a glycoprotein containing mannose and galactose residues. These three proteins are consistent markers for the tumorigenic potential of rat liver epithelial cells.

The identity of these three proteins is under investigation. Transblotting and direct sequence analysis of RP2 has revealed it to be N-terminally blocked, so this protein is being purified by gel filtration chromatography and SDS-PAGE prior to digestion with cyanogen bromide. This will allow the generation of peptides for use in N-terminal sequence analysis. Polypeptides RP1 and RP3, in addition to RP2, have recently been found to be TGF- β_1 -inducible proteins. RP1 has the characteristics of an extracellular protein, and Western blotting with a specific antibody has led to its tentative identification as fibronectin.

Parallel studies have been aimed at determining alterations in responsiveness of these cells to the multifunctional growth inhibitor polypeptide TGF- β_1 . The more tumorigenic clones had increased expression mRNA for this factor and also for TGF- α , but had a decreased expression of the extracellular matrix proteins fibronectin and collagen $\alpha 1(I)$. All of the oncogene-transformed cells were resistant to the growth inhibitory effects of TGF- β_1 , compared to RLE or control cells. Scatchard analysis of ^{125}I -TGF- β_1 binding data showed that RLE and RLEC-2 cells had about 10000 receptors/cell and an affinity of approximately 30 pM. R3611-3, R3611T-1,4 and 5 had normal TGF- β_1 binding in contrast to R3611T-2,3,7 and RJ2-14 which bound negligible amounts of TGF- β_1 . These findings suggest that the phenotypic heterogeneity of these transformed cells may be modulated by TGF- β_1 -mediated events.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05559-02 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Plasma Membrane Proteins in Normal and Neoplastic Rat Hepatocytes

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

PI:	David C. Parmelee	Expert	LEC NCI
Others:	Timothy Benjamin	Chemist	LEC NCI
	Tanya Hoang	Chemist	LEC NCI
	Chien-Hua Niu	Volunteer Scientist	LEC NCI
	Snorri S. Thorgeirsson	Chief	LEC NCI
	Amir Venegas	Laboratory Worker	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.3

PROFESSIONAL:

1.3

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

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

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

Our previous investigations of glycoproteins isolated from the plasma membranes of normal and neoplastic rat livers revealed many qualitative and quantitative differences when analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The main goal of this project is to purify and characterize the specific glycoproteins whose expression is markedly altered during chemically induced hepatocarcinogenesis in order to understand their role either as markers or causal agents during cell transformation. Previous results established the N-terminal amino acid sequence for 4 of 9 glycoproteins purified and analyzed from a single 2D PAGE experiment. The remaining 5 components of interest were not sequencable in this manner, presumably because of blocked N-termini. For this reason, extensive work was done to improve yields of starting materials and to develop procedures for obtaining amino acid sequence information from all of these proteins, whether blocked or unblocked. The purification of the glycoproteins was improved at the Concanavalin-A (Con A) affinity chromatography step by incorporating the use of fast protein liquid chromatography (FPLC) and adding detergents to the elution buffers. The use of PAGE as the final isolation step was evaluated by developing a radioactive standard and determining the procedure for using it to quantify the recovery of proteins purified by gels and then transblotted to Immobilon-P membranes. Internal sequence results were obtained by determining procedures to cleave several standard proteins (which had been purified in PAGE experiments) with CNBr or formic acid. The results were as expected based on the size of the particular fragments selected. An assay was also developed which can be utilized to determine which steps or chemicals are responsible for the N-terminal blocking that occurs during the purification of proteins or peptides.

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

David C. Parmelee	Expert	LEC NCI
Timothy Benjamin	Chemist	LEC NCI
Tanya Hoang	Chemist	LEC NCI
Chien-Hua Niu	Special Volunteer	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Amir Venegas	Laboratory Worker	LEC NCI

Objectives:

The main objective of this project is to isolate, purify, and structurally characterize the specific glycoproteins whose expression is qualitatively and quantitatively altered during chemically induced hepatocarcinogenesis in order to understand their biological functions during cell transformation.

Methods Employed:

The principle methods employed are (1) affinity chromatography utilizing Con-A, (2) fast protein liquid chromatography (FPLC), (3) two dimensional polyacrylamide gel electrophoresis (2D-PAGE), (4) electroblotting, (5) gas-phase protein sequencing, (6) high performance liquid chromatography (HPLC), and (6) peptide synthesis.

Major Findings:

(1) The purification of glycoproteins by Con-A was tested using a FPLC system and buffers containing different detergents. This procedure greatly simplifies this step compared to the method used previously and improves the overall yield of the proteins being isolated.

(2) A procedure was developed to iodinate beta-lactoglobulin (the Applied Biosystems, Inc. protein sequencing standard). This provides a means for determining the recovery of proteins isolated and transblotted during PAGE experiments. Assays were developed (using FPLC and PAGE) to determine the quality of the iodinated protein and to assess the best storage method.

(3) The conditions were optimized for eluting proteins from gels after treatment with 4M sodium acetate, dansylchloride, or Coomassie Blue. This was necessary for purifying proteins for subsequent cleavage reactions

(4) A gradient field transblotting apparatus was tested and compared with a normal system which used a single voltage. The voltage gradient improved the transblotting recovery of peptides that would be generated by internal cleavages.

(5) Procedures were developed for obtaining internal sequence information from proteins isolated from polyacrylamide gels. This involved selecting the best cleavage method for several protein standards having known amino acid sequences. The best results were obtained using cyanogen bromide or acid cleavage. The mixture of peptide fragments from the various standards had approximately the correct molecular weights when analyzed by PAGE. Specific peptides chosen for analysis had the expected amino acid sequence.

(6) An assay was developed for determining which chemicals or steps might be responsible for N-terminal blockage during the purification of proteins or peptides by PAGE. Of those tested thus far, no specific one was responsible for the total amount of blockage observed in the entire procedure.

Publications:

Benjamin T, Niu C, Parmelee DC, Huggett AC, Yu B, Roller PP, Thorgeirsson SS. Direct N-terminal sequence analysis of rat liver plasma membrane glycoproteins separated by two dimensional polyacrylamide gel electrophoresis. Electrophoresis (In Press).

Parmelee DC, Benjamin T, Niu C, Thorgeirsson SS. Direct sequence analysis of rat liver plasma membrane glycoproteins separated by 2D-PAGE. In: Hugli TE, ed. Techniques in protein chemistry. New York: Academic Press 1989;42-50.

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

PROJECT NUMBER

Z01CP05599-01 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Mechanism of Fibrogenesis and Cirrhosis in Rat Liver

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

PI:	Harushige Nakatsukasa	Expert	LEC NCI
Others:	Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
	Snorri S. Thorgeirsson	Chief	LEC NCI
	Elizabeth Marsden	Biologist	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.3

PROFESSIONAL:

1.2

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.)

Temporal and spacial distribution of transforming growth factor β -1 (TGF- β 1) and procollagen gene expressions were studied in carbon tetrachloride-induced liver fibrosis and in Solt-Farber's hepatocarcinogenesis models in rats. The studies were designed to clarify the involvement of different cell types in early events of liver fibrosis and the role of extracellular matrix (ECM) during the development of hepatocellular carcinoma. In the liver fibrosis, inflammatory cells (e.g., granulocytes and macrophages), Desmin-positive perisinusoidal cells and fibroblasts appeared in the necrotic area and expressed TGF β -1 and procollagen genes which resulted in an excess accumulation of extracellular matrices. TGF- β 1 and procollagen genes were strongly expressed in mesenchymal cells along fibrous septa but not in hepatocytes. The expression of these genes increased with the progression of liver fibrosis. The simultaneous expression of TGF- β 1 and procollagen genes in mesenchymal cells during liver fibrosis and the fact that TGF- β enhances type I collagen promotor suggest the possibility that TGF- β 1 may have an important role in the fibrogenesis of the liver. In the liver carcinogenesis, TGF- β 1 and procollagen genes were similarly and simultaneously expressed in stromal mesenchymal cells but not in cancer cells, preneoplastic cells or basophilic cells. The data suggest that ECM may indirectly stimulate growth of both preneoplastic and neoplastic liver cells by providing a supporting tumor stroma (see also Project No. Z01CP05454).

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

Harushige Nakatsukasa	Expert	LEC NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Elizabeth Marsden	Biologist	LEC NCI

Objectives:

The objective of this project is to elucidate (1) the involvement of different cell types in the early events of liver cirrhosis (fibrosis), which is the most common terminal feature of chronic liver diseases and is also considered as a precancerous state; and (2) the role of extracellular matrices during the development of hepatocellular carcinoma (HCC) by looking at the expressions of several different procollagen genes as well as TGF- β 1 gene, which has been reported to be implicated in wound healing and carcinogenesis through its multifunctional effects on both mesenchymal cells and epithelial cells.

Methods Employed:

(1) In situ hybridization for spacial localization of mRNA. (2) Immunohistochemistry for identification of cells positive for Desmin, vimentin and cytokeratins as well as for identification of special proteins such as TGF- β , Laminin and type IV collagen. (3) Techniques used in molecular biology including Northern blot analysis and isolation of mRNA. (4) Quantitation of in situ hybridization data by using Magiscan Image Analysis System.

Major Findings:

1) Liver fibrosis was produced in rats by administering 0.33 ml/kg body weight of carbon tetrachloride through a gastric tube once a week for 1 to 12 weeks. In the very early stages following CCl₄ administration, inflammatory cells (e.g. granulocytes and macrophages) infiltrated into the necrotic area and expressed TGF- β 1 and procollagen al(I), al(III) and al(IV) genes. Within a short time (approximately 0.5 to 1 day) Desmin-positive perisinusoidal cells (e.g., fat-storing cells and myofibroblasts) and fibroblasts at the same necrotic area proliferated and started to express all these genes. In the later stages, TGF- β 1 and procollagen al(I), al(III) and al(IV) genes were all strongly expressed in the mesenchymal cells, mainly Desmin-positive perisinusoidal cells and fibroblasts along the fibrous septa, as well as in inflammatory cells; and their expression increased with the progression of liver fibrosis. Although collagens are thought to be produced in hepatocytes, these genes were not expressed in hepatocytes throughout the process of liver fibrosis. We have found that inflammatory cells, then Desmin-positive perisinusoidal cells and fibroblasts are the particular cell types which produce collagens in CCl₄-induced liver fibrosis. Furthermore, the simultaneous expression of TGF- β 1 and procollagen genes in mesenchymal cells during liver fibrosis suggests the possibility that TGF- β 1 may have an important role in the fibrogenesis of the liver.

(2) We have used Solt-Farber's protocol, which utilizes diethylnitrosamine for the initiation, and 2-acetylaminofluorene and partial hepatectomy for promotion to obtain basophilic foci, preneoplastic nodules and finally hepatocellular carcinoma (HCC) in rat liver. TGF- β 1 and procollagen genes were not expressed in basophilic hepatocytes, preneoplastic cells, or cancer cells. TGF- β 1 and procollagen gene expressions were similarly and simultaneously observed in certain types of mesenchymal cells and oval cells. These results suggest that TGF- β 1, as produced by non-tumor cells, may have an indirect stimulative effect for growth of tumor cells via its effects on formation of supporting tumor stroma (see also Project No. Z01CP05453).

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

PROJECT NUMBER

Z01CP05600-01 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Role of Multidrug Resistance Genes in Hepatocarcinogenesis

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

PI:	Snorri S. Thorgeirsson	Chief	LEC NCI
Others:	Jeffrey A. Silverman	NRC Fellow	LEC NCI
	Richard K. Burt	Biotechnology Fellow	LEC NCI
	Timothy W. Gant	Visiting Fellow	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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Chemical Carcinogenesis Section

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.2

PROFESSIONAL:

3.2

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.)

It is well documented that chemical hepatocarcinogenesis results in tumors that frequently are resistant to the cytotoxic and growth inhibitory effects of carcinogens. The role of the multidrug resistant (MDR) gene family in the pleiotropic resistance that is observed in these tumors is at present unknown. The objective of this project is to define the role as well as the regulation of the MDR genes in hepatocarcinogenesis. Major findings include: (1) the MDR-1 gene is induced in rat liver following administration of various natural and synthetic xenobiotics including agents that also induce a subfamily (P450IA2) of the cytochrome P450 supergene family. These data indicate that induction of selective members of the MDR and the cytochrome P450 gene families may depend on overlapping regulatory elements; (2) the relationship between transformation and multidrug resistance was examined by employing the *v-H-ras* or *v-raf* oncogenes to transform rat liver epithelial (RLE) cells in vitro. The data show that transformation of RLE cells with these oncogenes results in increased resistance to cytotoxins such as adriamycin, vinblastine and 2-acetylaminofluorene. This multidrug resistance is accompanied by increased expression of MDR-1 and glutathione-S transferase P. Thus, neoplastic transformation of RLE cells with *v-H-ras* and *v-H-raf*, independent of chemical exposure, results in multidrug resistance.

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

Snorri S. Thorgeirsson	Chief	LEC	NCI
Jeffrey A. Silverman	NRC Fellow	LEC	NCI
Richard K. Burt	Biotechnology Fellow	LEC	NCI
Timothy W. Gant	Visiting Fellow	LEC	NCI

Objectives:

(1) Clone and characterize multidrug resistant (MDR) genes in rat liver; (2) determine the molecular mechanism responsible for induction of MDR genes by xenobiotics and characterize the genetic element(s) responsible for the coinduction of MDR and cytochrome P450IA2 genes; and (3) define the role of MDR genes in normal cellular physiology as well as their importance in the transformed phenotype.

Methods Employed:

(1) Mechanism of MDR Induction by Xenobiotics. We have examined the relationship between MDR and cytochrome P450 induction by xenobiotics. The levels of mRNA for MDR-1 and selective cytochrome P450 genes were determined in adult rat liver following administration of various natural and synthetic xenobiotics. MDR-1 (also known as PGY1) was induced following administration of aflatoxin B₁, 2-acetylaminofluorene (AAF), N-hydroxy-2-acetylaminofluorene, isosafrole, phenothiazine, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), but not after phenobarbital or 7-hydroxy-2-acetylaminofluorene treatment. Cytochrome P450 isoform d was induced by TCDD, isosafrole, phenothiazine, and AAF, while cytochrome P450 isoform b was induced by phenobarbital, and to a lesser extent by isosafrole. These observations suggest that both MDR and cytochrome P450 gene families are evolutionarily selected by the capacity of various xenobiotics to induce their own detoxification, either through metabolism to hydrophilic derivatives by the cytochrome P450 system or direct excretion from the cell by the MDR gene family. Furthermore, the data indicate that induction of selective members of the MDR and the cytochrome P450 gene families may depend on overlapping regulatory elements.

(2) MDR and Oncogene-induced Transformation in Rat Liver Epithelial (RLE) Cells. It is well documented that chemical carcinogenesis results in tumors that are resistant to cytotoxic and growth inhibitory effects of carcinogens. Whether the multidrug resistance of chemically induced tumors is an integral part of the transformed phenotype or reflects a selection by chemical exposure and is unrelated to the mechanism involved in transformation is unclear. We have examined the relationship between transformation and multidrug resistance by employing the *v-H-ras* or *v-raf* oncogenes to transform RLE cells in vitro. The data indicate that transformation of RLE cells with *v-H-ras* or *v-raf* results in increased resistance to the cytotoxins adriamycin, vinblastine and 2-acetylaminofluorene. This multidrug resistance is accompanied by increased expression of P-glycoprotein (MDR-1) and glutathione-S-transferase P (GST-P). Thus, neoplastic transformation of RLE cells with *v-raf* or *v-H-ras*, independently of chemical exposure, results in multidrug resistance.

Publications:

Burt RK, Garfield SH, Johnson K, Thorgeirsson SS. Transformation of rat liver epithelial cells with v-H-ras or v-raf causes expression of MDR-1, glutathione-S-transferase P and increased resistance to cytotoxic chemicals. Carcinogenesis 1988;9:2329-32.

Burt RK, Thorgeirsson SS. Coinduction of multidrug-resistance (MDR-1) and cytochrome P-450 genes in rat liver xenobiotics. JNCI 1988;80:1383-6.

Thorgeirsson SS, Garfield SH, Huber BE, Burt RK. Acquisition of multidrug resistance in chemical hepatocarcinogenesis. In Estabrook RW, Lindenlaub E, Oesch F, de Weck AL, eds. Toxicological and immunological aspects of drug metabolism and environmental chemicals, Symposia Media Hoechst 22. Stuggart, Germany: FK Schattauer Verlag. 1988;367-77.

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

PROJECT NUMBER

Z01CP05601-01 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Analysis of POMC Tissue-Specific Expression and Glucocorticoid Repression

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

PI:	Anna Riegel	Visiting Associate	LEC NCI
Others:	Gordon Hager	Head, HAO Section	LEC NCI
	Ronald Wolford	Microbiologist	LEC NCI
	Diana Berard	Microbiologist	LEC NCI
	Jim Reminick	Guest Researcher	LMV NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.3

PROFESSIONAL:

1.3

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

The proopiomelanocortin (POMC) gene is expressed in a tissue-specific manner in the pituitary, hypothalamus and testes. The POMC gene is negatively regulated at the transcriptional level by glucocorticoids and positively regulated by ligands that stimulate the protein kinase C and C-AMP signal transduction pathways. It is therefore possible to examine tissue-specific, negative and positive transcriptional control mechanisms using the POMC gene as a paradigm. These studies initially focused on the negative regulation of transcription by glucocorticoids. Previous studies have demonstrated that a glucocorticoid receptor binding site centered at -63 may be involved in negative regulation. Although the mechanism of repression is unknown, one possibility is that receptor interaction with this sequence may displace a positive trans-acting factor that normally occupies this position. Using a combination of methods to detect protein-DNA interactions we have defined 5 factors (PO-A,B,C,D, and E) that bind between -63 and the POMC CAP site. To determine the functional significance of these sites and their possible involvement in negative regulation, we have made a series of deletion and oligonucleotide-directed mutations in the POMC promoter linked to the firefly luciferase reporter gene. After transient transfection of these vectors into the ATt-20 pituitary tumor cell line, we have determined that a number of these mutations appear to decrease the basal transcription of the POMC gene. A particularly interesting mutation involves the PO-B site situated over the POMC CAP site that defines a novel trans-acting CAP binding factor distinct from classic TATA binding proteins. We have determined that the effects of these mutations can be mirrored in in vitro transcription assays. In future experiments this technique will enable more detailed analysis of the role of these trans-acting factors in transcription complex formation.

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

Aron Riegel	Visiting Associate	LEC NCI
Gordon Hager	Head, HAO Section	LEC NCI
Ronald Wolford	Microbiologist	LEC NCI
Diana Berard	Microbiologist	LEC NCI
Jim Reminick	Guest Researcher	LMV NCI

Objectives:

- (1) To determine areas of the POMC promoter that mediate negative regulation of transcription by glucocorticoids.
- (2) To determine by in vitro protein-DNA binding techniques the sequence-specific binding sites of trans-acting factors within areas responsible for negative regulation.
- (3) To determine the role of trans-acting factors in constitutive and negative regulation of the POMC promoter using oligonucleotide-directed mutational analysis. The effects of site-specific mutations will be determined in vivo with transient transfection assays.
- (4) To determine if negative regulation of transcription can be mimicked in vitro by addition of purified glucocorticoid receptor to in vitro transcription extracts.
- (5) Purification of transcription factors demonstrated to have an effect on POMC gene expression by mutational analysis. Initial experiments will focus on determining the effects of purified PO-B on transcription complex formation in vitro.

Methods Employed:

- (1) To detect specific interactions of transcription factors with DNA: gel retardation assay, exonuclease III footprinting, DNase I footprinting, and methylation interference assay.
- (2) Oligo-directed mutagenesis, deletion mutational analysis.
- (3) Liposome-mediated transient transfection.
- (4) In vitro transcription assay. Primer extension analysis of transcription products.

Major Findings:

Previous preliminary reports from other laboratories had indicated that the glucocorticoid receptor binding site centered at -63 might be involved in negative regulation of the POMC promoter. The mechanism of repression is

unknown, although it has been suggested for other negatively regulated genes that activator displacement or repressor recruitment may play a role. This would suggest that factors binding in the vicinity of the -63 site could be involved in negative regulation. Initial experiments with the gel retardation assay demonstrated that two distinct factors (PO-A and PO-B) were present in crude nuclear extracts of C127 mammary tumor cells that bound between -2 and -60 relative to the POMC transcription start site. These factors could be separated by fractionation on phosphocellulose. Further characterization of the PO-A site by exonuclease III footprinting demonstrated this protein bound to a region centered at -44 whose sequence has strong homology to that of the CACCC box binding protein which is known to play a role in globin gene expression. In contrast the PO-B binding site was shown by methylation interference and DNase I footprinting to be downstream of the TATA box between -3 and -15 relative to the POMC CAP site. Three other factors were identified by exonuclease III footprinting of nuclear extract PO-C,D, and E. The 5' boundaries of these factors lay at -65, -54 and -51 respectively.

To determine the functional significance of these binding sites, we constructed oligonucleotide-directed mutations of the POMC promoter at the PO binding sites. These promoters were cloned into vectors harboring the firefly luciferase gene and transfected into AtT-20 mouse pituitary tumor cell lines. We are currently assessing the effects of these mutations on constitutive and glucocorticoid repression of transcription. A particularly interesting result is the effect of a mutation of the PO-B site that prevents binding of the factor *in vitro* and causes a significant (4- to 6-fold) down regulation of transcription *in vivo*. This implies that PO-B is a novel transcriptional activator factor situated at the POMC transcription initiation site and clearly distinct from the upstream TATA binding site. The effects of this mutation can also be observed in *in vitro* transcription assays in nuclear extracts prepared from the C127, HeLa and AtT-20 cell lines. Future experiments will focus on the mechanism of transcriptional stimulation by PO-B *in vitro* using purified PO-B factor.

It is currently unclear whether the other PO factors are involved in glucocorticoid-mediated transcriptional repression. Preliminary evidence from transient transfection experiments using luciferase vectors harboring various deletions of the POMC promoter suggests that negative regulation may be mediated by an element in the region upstream of -300 relative to the transcription start site. We are currently repeating these experiments since this data conflicts with earlier reports of the negative element being at -60. We are also subcloning various fragments of the POMC promoter into vectors that contain the thymidine kinase promoter to examine if these fragments can confer hormone-mediated repression to this promoter.

Publications:

Charron J, Richard-Foy H, Berard DS, Hager DL, Drouin H. Persistent gene-specific glucocorticoid inhibition of transcription in the presence of a contiguous glucocorticoid-inducible gene. *Mol Cell Biol* (In Press).

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

PROJECT NUMBER

Z01CP05602-01 LEC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Analysis of Zn Finger DNA Binding Domains

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

PI:	Gordon Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Others:	Trevor Archer	Visiting Fellow	LEC NCI
	Jim Omichinski	IRTA Fellow	LEC NCI
	Ronald Wolford	Microbiologist	LEC NCI

COOPERATING UNITS (# any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

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 ability of steroids to regulate transcription from specific genes has been well established. In the case of glucocorticoids the hormone binds to a cytosolic cellular non-DNA binding form of its specific receptor which translocates into the nucleus where it is now able to bind to specific sites on chromatin and either enhance or repress transcription. We have examined the mechanism of DNA binding by individually synthesizing the putative "zinc finger peptides" from the rat glucocorticoid receptor. Atomic absorption studies show that the peptides will bind Zinc on an equimolar basis. Circular dichroism experiments demonstrated a significant alteration in secondary structure in the presence of zinc. The results from DNA binding experiments establish that metal ion is required for binding to DNA and that amino terminal peptide shows a significantly greater affinity of glucocorticoid response element (GRE) containing DNA over control DNA. The results suggest that a single synthetic "finger peptide" is able to bind to DNA in a sequence-specific manner.

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

Gordon Hager	Head, HAO Section	LEC NCI
Trevor Archer	Visiting Fellow	LEC NCI
Jim Omichinski	IRTA Fellow	LEC NCI
Ronald Wolford	Microbiologist	LEC NCI

Objectives:

The primary objective of this project is to examine, in detail, the mechanisms by which steroid hormone receptors are able to distinguish their cognate binding sites. To achieve this end we have synthesized peptides corresponding to the proposed "zinc finger" domain of the glucocorticoid receptor. Our initial experiments are geared to establish that these structures do form, *i.e.*, the peptides bind zinc, and to determine what, if any, structural features of the peptide are metal dependent. Subsequent studies will examine the question of DNA binding and determine if such binding by the isolated peptides is sequence-specific.

Methods Employed:

Peptides were prepared by solid-phase synthesis on an Applied Biosystems 430A automated peptide synthesizer. Completed peptides were released from the resin and deprotected using hydrogen fluoride cleavage methods. The crude peptides were purified by gel filtration and by semi-preparative reverse phase liquid chromatography. Identity of the synthetic peptides were confirmed by amino acid analysis and the number of cysteine residues present in the peptides was confirmed with DTNB (Ellman's Reagent).

Oligonucleotides were prepared on an Applied Biosystems 380A DNA synthesizer and purified as per manufacturers instructions. Completed peptides were deblocked, then passed over an oligonucleotide purification cartridge (ABI); evaporated, then resuspended in water.

The ability of the peptides to bind zinc was measured by atomic absorption spectrophotometry using a Perkin-Elmer Atomic Absorption Spectrophotometer model 4000.

Secondary structure of the peptides was analyzed by circular dichroism studies. Spectra were recorded from 260 to 190 nm on a Jasco J500 spectropolarimeter for the native apo-peptide, the peptide complexed with zinc and for the peptide from which zinc was stripped using EDTA.

A nitrocellulose filter binding assay was utilized to monitor the ability of the peptides to bind DNA in a sequence-specific manner. ³²P-labeled oligonucleotides were incubated with various peptide preparations prior to filtration and the quantity of DNA bound determined by liquid scintillation counting.

Major Findings:

Steroid hormone receptors can activate or repress transcription from responsive loci by binding to DNA. With the isolation and sequencing of cDNA clones for the various steroid receptors, it has become apparent that they form a superfamily of transcription factors. The receptors share a common organizational plan with the most conserved region of the molecules being a cysteine rich DNA binding domain. Analysis of the predicted protein sequence of this region revealed an intriguing homology with the DNA binding transcription factor IIA from *xenopus laevis* (TFIIIA). These findings led to the proposal that the receptor folds to form two "zinc fingers" which interact with the DNA in a manner that is similar to the well-established binding of TFIIIA. Our experiments have explicitly tested this proposal by synthesizing the putative "fingers" independently and examining their interaction with DNA.

The synthesis of peptides corresponding to amino acid sequences 438-464 and 474-504 of the rat glucocorticoid receptor represent the amino terminal "CI finger" and carboxyl terminal "CII finger," respectively. The successful synthesis of these peptides represents the initial synthesis of putative Cys-Cys fingers from a known transcriptional activator.

The first series of experiments were designed to determine if an isolated "finger peptide" could bind zinc and to determine what, if any, effect this would have on the conformation of the peptide. The quantity of bound zinc was ascertained by atomic absorption and the results of this analysis indicated that ~1 mol of zinc was bound by each mole of peptide. Subsequent dialysis of the peptide zinc complex against a buffer containing the metal chelator, EDTA, demonstrated that more than 90% of the metal associated with the peptide could be removed by the EDTA. This result is consistent with the suggestion that four cysteines are complexed to a single metal ion. Previous studies have failed to distinguish the possibilities that metal ion may be shared between the two proposed "finger" motifs rather than a single ion per "finger." The data presented here demonstrate that the proposed "zinc fingers" can act as independent units in the chelation of zinc.

Subsequent studies have utilized circular dichroism (CD) spectroscopy to monitor changes in secondary structure of the peptides that may accompany metal binding. Our initial studies have focused on the CII peptide for which we have been able to obtain a set of preliminary spectra. A comparison of the CD spectra obtained in the absence and presence of exogenous zinc suggests that the peptide:zinc complex has a different secondary structure relative to the apo-peptide. The CD spectra of the peptide:zinc complex, when compared to free or apo-peptide, shows an increase in molar ellipticity at 190 nm with a decrease at 222 nm. This metal-dependent change in conformation is reversed upon the addition of the chelating agent EDTA, producing a spectrum that is identical to that seen with the apo-peptide alone. The spectra we have determined are similar to those obtained recently for a biologically produced "finger peptide" from TFIIIA of *xenopus laevis* and a synthetic "finger peptide" derived from the yeast transcription activator (ADR1) of the alcohol dehydrogenase gene. In all cases the spectra suggest that the peptide moves from a relatively random structure to a more ordered state when metal is complexed. The data obtained here do not unequivocally indicate an increase in the α -helical content of the peptide upon zinc binding, as reported for the ADR1 peptide, but would be consistent with that interpretation.

Given that the peptides were able to bind zinc and that binding altered the conformation of the peptide, experiments utilizing the nitrocellulose filter binding assay were performed to examine whether the peptides were able to bind selectively to specific DNA sequences. In the case of peptide CI the data demonstrate that in the absence of exogenous zinc, the peptide will bind a synthetic DNA oligonucleotide containing a consensus GRE at levels at or only slightly above background. This binding is reduced to background levels at low concentrations of peptide. The addition of zinc leads to a significant increase in the quantity of DNA complexed with the peptide as monitored by this assay. The metal-dependent binding in this experiment shows a greater than 30-fold elevation in binding relative to that seen in the absence of zinc. DNA binding studies with peptide CII yield results that were similar to that seen with peptide CI. The addition of zinc leads to a 10-fold elevation in binding of the peptide to DNA relative to that in the absence of the metal.

To address the question of the specificity of the binding by the peptide, we have carried out similar studies with DNA binding sites for Nuclear Factor 1 (NF1), and the Estrogen Receptor (ER). The results demonstrated that peptide CI binds poorly to both sites for NF1 and ERE in both the presence and absence of zinc. When the level of binding by the peptide for the NF1 and ERE oligonucleotides are compared to that of the GRE, they represent less than 10% of the metal-dependent binding of the peptide for its cognate binding site. This data is of significance as the GRE and ERE differ by only four nucleotides, yet the peptide is able to discriminate between the two binding sites by 10-fold. Thus, these data are consistent with previous experiments in that they support the idea that the amino terminal "finger" provides the specificity for the selection of receptor sites.

In contrast to the above results with peptide CI, the data with peptide CII demonstrate a significant metal-dependent binding to an ERE site that rivals the binding to the GRE oligonucleotide. A comparison of binding of the GRE and ERE for this CII "finger peptide" reveals that the peptide will bind to either site with near equal affinity. Thus, the finding that the CII finger did not significantly distinguish between an ERE and a GRE by the intact chimeric receptor was maintained with the isolated peptides. In addition neither peptide CII nor peptide CI demonstrated a significant level of binding with respect to the unrelated NF1 oligonucleotide.

To extend these investigations a "mutation" of the CI "finger peptide" (CI_m) was synthesized in which the cysteine at 440 of the GR is replaced by alanine, with all other sequences being identical to the wild-type peptides. Atomic absorption studies demonstrated that the CI_m peptide was able to bind zinc at roughly equivalent levels as the wild-type CI and CII peptides. DNA binding experiments with the CI_m and CI peptides revealed that substitution of cysteine by alanine resulted in a significant reduction in binding a GRE oligonucleotide. The levels of binding this "mutated finger peptide" never exceeded 10% of the wild-type "finger peptide." Thus, alterations in the isolated finger behave as do mutations in the intact receptor in that mutation of a single cysteine abolishes DNA binding. In addition the data may be interpreted to suggest that binding zinc per se is necessary but not sufficient to allow binding to DNA.

We have synthesized peptides corresponding to the putative "zinc fingers" of the glucocorticoid receptor protein. The peptides were able to bind zinc and able to interact with a GRE oligonucleotide only when the metal is present. In addition the CI peptide displayed significant interaction with GRE sequences only while

the CII peptide was more promiscuous and was able to bind an ERE sequence as well as a GRE sequence, a property shared by the parent molecule. Finally, a mutation which in the native receptor results in loss of function, produces the same effect when introduced into the synthetic peptide.

ANNUAL REPORT OF

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

October 1, 1988 through September 30, 1989

The Laboratory of Experimental Pathology (LEP) plans, develops and implements research on the experimental pathology of carcinogenesis, especially concerned with the induction of neoplasia by chemical and physical factors in epithelial tissues, including: (1) development, characterization and evaluation of experimental pathology models of human cancer, such as cancers of the respiratory tract, by in vivo and in vitro carcinogenesis methods; (2) development and characterization of tissue culture systems for quantitative study of the effects of carcinogens alone or in combination; and (3) research on mechanisms of carcinogenesis correlating different levels of biological organization, from whole organisms (human and animal), organs and tissues, to the cellular, subcellular and molecular levels.

General Research Objectives

Present studies in the LEP are concerned with two correlated problems: (1) the pathogenesis of neoplastic disease, induced by chemical and physical factors, in epithelial cells studied in selected biological models in vivo and in vitro, particularly in the respiratory tract and the epidermis; and (2) the interactions of different factors in multifactorial carcinogenesis mechanisms, including the role of carcinogens, promoters, oncogenes, growth factors, cellular mediators and certain types of tissue injury.

In order to correlate mechanisms of carcinogenesis investigated at the cellular and molecular levels with the corresponding events in animal and human tissues and organs, it is important to connect these different levels of observation and to study the mechanisms of action of carcinogens in relevant biological systems. Such an approach requires the development of a range of biological models related to human cancer pathology and particularly to those epithelial target tissues from which most of the major forms of human cancer originate. A great deal of progress has occurred in this direction in the past two decades and experimental animal models have been established, by chemical induction, for most of the major types of human cancer. For many of these models adequate culture systems have been developed for the target tissues and cells, including both animal and human target cells. Pathogenetic mechanisms have been clarified through major advances in experimental pathology, cell biology, molecular biology and biochemistry. Work in the LEP has contributed substantially to this progress. The current LEP studies continue this approach.

Work in this fiscal year has been primarily devoted to the following activities: extensive histopathological evaluation and data analysis of animal experiments on respiratory carcinogenesis by crystalline silica in different species and by multifactorial treatments; experimental cell biology studies on the response of

recently established mouse epidermal cell lines to growth factors and carcinogens in serum-free media and now in chemically defined media; pilot studies on toxicity and transformation silica particles, asbestos fibers and other materials in BALB/3T3 cells; and completion of a study on lack of metastatic activity in nude mice of cell lines transformed by metals and other carcinogens.

Results Obtained in the Current Year

(1) Respiratory carcinogenesis in vivo. The relationship between chronic pulmonary granulomatosis/fibrosis and the induction of cancer of the lung was further investigated by the recently developed experimental model of lung carcinogenesis induced by crystalline silica particles. Crystalline silica, in the form of quartz, is the second most common mineral in the earth's crust. In fine particulate form of respirable size (about 1-5 μm) it is known to induce a progressive obstructive pulmonary disease, silicosis, characterized by granulomatous and fibrogenic reactions. Recent findings of lung cancer induction in rats by silica have been consistent with reports of increased human lung cancer risk in silicotic subjects. It was hypothesized (Saffiotti, In Goldsmith, D.F., Winn, D.M., and Shy, C.M.(Eds.): Silica, Silicosis and Cancer. Praeger Publishers, Philadelphia, 1986, pp. 287-307) that the induction of carcinomas of the lung by silica may be mediated by the action of the silicotic granuloma on the adjacent epithelia of the peripheral airways. Extensive serial sacrifice studies in rats, hamsters and mice, following single intratracheal instillations of silica particles in the lung, have now demonstrated marked species differences in the structure and cellular composition of the granulomatous reaction, accompanied by fundamental species differences in the reaction of the epithelium of the bronchioles and alveoli. The rat shows an intense hyperplasia of the bronchiolar and alveolar (type II) epithelium, adjacent to silicotic lesions, starting within days of silica exposure. Subsequent diffuse epithelial hyperplasias and bronchiolar/alveolar adenomatoid lesions are followed by the appearance of carcinomas, beginning in less than one year. About 70% of the lung tumors are adenocarcinomas, the others include a few epidermoid carcinomas, undifferentiated carcinomas, mixed carcinomas and adenomas. About 30% of the adenocarcinomas develop around fibrotic areas and are comparable to the pulmonary "scar cancers" of human pathology, of which they represent a new, reproducible experimental model. The female rats showed earlier onset and higher multiplicity of lung cancers than male rats. The high response in rat lungs was closely reproduced in three separate experiments. Immunohistochemical studies showed that the rat epithelial hyperplasias and tumors are derived from alveolar type II epithelial cells and not from bronchial or bronchiolar cells. In contrast, the epithelial reaction to silica in the lung is very mild and transitory in mice with no tumor induction in this species (including mice of strain A, otherwise prone to lung adenoma induction by carcinogens). In the hamster, silica was phagocytized by macrophages, but induced no apparent toxicity, no silicotic fibrosis and no epithelial reaction nor tumors. The three strikingly different response patterns in these three species are investigated as different models of susceptibility. The pathogenesis of the epithelial proliferation is being further studied in relation to the adjacent inflammatory granulomatous reaction. The present results support the view that the cellular mediators of inflammation, extensively secreted in the proximity of the peripheral airways, are likely to stimulate epithelial proliferation and neoplastic growth.

These and other previously reported experiments in respiratory carcinogenesis are in the process of being completely analyzed and prepared for publication.

A multifactorial study on respiratory carcinogenesis in hamsters, of which the complex mechanisms of induction of respiratory tumors were previously reported, was further analyzed to demonstrate the effects of a single low dose of N-methyl-N-nitrosourea (MNU), instilled at the level of the larynx in five-week-old hamsters, in the induction of significant carcinogenic responses at multiple target sites (oropharynx, esophagus, forestomach, pancreas, biliary system, small and large intestine, blood vessels and lymphoid tissue). Some of these responses are selectively enhanced by subsequent administrations of benzo[a]pyrene. The single dose of MNU at a young age not only acts as a full carcinogenic treatment at several sites, but it further "initiates" several tissues for subsequent response to other factors, a potentially useful model for multistage mechanisms of carcinogenesis in this species.

(2) Studies on growth and transformation of cells in culture. Newly established cell lines of mouse epidermal keratinocytes in serum-free media were characterized. These lines, even at advanced passage levels, retain epithelial morphology and markers, respond to the induction of terminal differentiation by calcium and by serum, and remain non-tumorigenic. It was found that increased amounts of bovine serum albumin in the medium support cell growth in the absence of bovine pituitary extract; therefore, a new chemically defined medium (LEP/MK4) was formulated and used for further studies on cell differentiation and transformation, currently under way. The BALB/3T3 clone A31-1-1, previously extensively characterized in this laboratory and found susceptible to transformation by a broad spectrum of soluble organic and inorganic carcinogens, was used for pilot studies on penetration, toxicity and transformation by particulate and fibrous materials, including various forms of crystalline silica and asbestos fibers.

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

PROJECT NUMBER

Z01CP04491-13 LEP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Quantitative Studies on Concurrent Factors in Neoplastic Transformation

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

PI: U. Saffiotti Chief LEP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.2

PROFESSIONAL:

0.2

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.)

Studies were continued on mechanisms of transformation in the mouse embryo cell line BALB/3T3 clone A31-1-1. This cell line was previously characterized in this laboratory and shown to be susceptible to transformation by a broad range of organic and inorganic carcinogens. Pilot studies were conducted on the penetration, toxicity and transformation by particulate materials, including various forms of crystalline silica, and by fibrous materials such as asbestos.

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

U. Saffiotti	Chief	LEP	NCI
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Objectives:

(a) To study mammalian cell culture systems quantitatively for induction kinetics and mechanisms of cytotoxicity, neoplastic transformation, mutagenicity and DNA damage and repair in response to different exposures to carcinogens.

(b) To investigate the induction of invasiveness and metastatic activity as a marker of advanced transformation.

Methods Employed:

The BALB/3T3 clone A31-1-1 mouse embryo cell line was used under test conditions previously standardized in this laboratory for transformation assays with different carcinogens. Additional assays, previously established for these cells, include induction of ouabain resistance, alkaline elution analysis for DNA damage and repair and HPLC analysis for removal of specific alkylated DNA adducts. Arsenic metabolism studies were conducted with ^{73}As sodium arsenite and arsenate, gamma-counting and paper electrophoresis. Degradation of labelled type IV collagen substrate was measured in control and chemically transformed BALB/3T3 cell lines, and the collagen degradation pattern was examined by gel electrophoresis. Assays for tumorigenic and metastatic activity were conducted in nude mice.

Major Findings:

Pilot studies showed dose-dependent uptake and toxicity of various types of crystalline silica; the optimal assay conditions are currently being developed, both for silica particles and for asbestos fibers.

Publications:

Garbisa S, Negro A, Kalebic T, Pozzatti R, Muschel RJ, Saffiotti U, Liotta LA. Type IV collagenolytic activity. Linkage with the metastatic phenotype induced by ras transfection. In: Prodi G, Liotta LA, Lollini P-L, Garbisa, S, Gorini S, Hellmann K eds. Cancer metastasis. Biological and biochemical mechanisms and clinical aspects, Advances in Experimental Medicine and Biology, New York: Plenum Press, 1988;233:179-186.

Saffiotti U, Bertolero F. Neoplastic transformation of BALB/3T3 cells by metals and the quest for induction of a metastatic phenotype. Biol Trace Element Res (In Press).

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

PROJECT NUMBER

Z01CP05274-08 LEP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Respiratory Carcinogenesis by Chemical and Physical Factors

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

PI:	U. Saffiotti	Chief	LEP	NCI
Others:	S. F. Stinson	Biologist	DTP	NCI

COOPERATING UNITS (if any)

Department of Pathology, University of Maryland, School of Medicine, Baltimore, MD
 (E. M. McDowell, K. P. Keenan)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

1.1

PROFESSIONAL

0.6

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 role of chronic pulmonary granulomatosis/fibrosis in the induction of lung cancer is being studied in a new experimental model, i.e., carcinogenesis by silica. Crystalline silica forms (quartz, hydrofluoric acid-etched quartz, cristobalite and tridymite) in fine particles were instilled intratracheally in inbred rats, hamsters and mice. Long-term studies with quartz and HF-etched quartz showed that rats developed high incidences of pulmonary carcinomas (mostly adenocarcinomas) with prevalence in females. Serial sacrifice studies showed that this carcinogenic response of the rat is preceded by an intense hyperplasia of the bronchiolar and alveolar type II epithelia, in areas adjacent to silicotic granulomatous lesions. Mice developed silicosis, but no carcinogenic response, even in the adenoma-prone strain A. Hamsters failed to respond to silica and showed no silicosis and no tumors. The correlation of epithelial proliferation with the adjacent inflammatory cells and their mediators is being investigated. In a multifactorial hamster carcinogenesis study, a single intralaryngeal instillation of 2 mg methyl nitrosourea at a young age induced a significant carcinogenic response in several target tissues and organs, selectively enhanced by subsequent doses of benzo[a]pyrene.

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

U. Saffiotti	Chief	LEP	NCI
S. F. Stinson	Biologist	DTP,DCE	NCI

Objectives:

The objective of this project is the elucidation of the pathogenetic mechanisms by which respiratory tract cancers, representing major forms of human cancer, are induced by chemical and physical factors, alone or in various combinations.

Animal models for the induction of respiratory carcinogenesis are being characterized, especially in relation to their human counterpart. Current studies have the objective of elucidating the relationship between chronic pulmonary granulomatosis/fibrosis and the induction of cancers of the lung in the recently developed experimental model of lung carcinogenesis by crystalline silica particles.

Methods Employed:

Lifetime and serial sacrifice studies, with histopathological investigation of respiratory tract pathology in inbred rats, hamsters, and mice. Intratracheal or intralaryngeal instillations of particulate suspensions are used to study respiratory tissue reactions by histologic, histochemical, autoradiographic and immunochemical methods and by scanning and transmission electron microscopy. Particulate materials currently investigated include crystalline silica (quartz, hydrofluoric acid-etched quartz, cristobalite, tridymite) and ferric oxide. Multifactorial effects of N-methyl-N-nitrosourea and benzo[a]pyrene (BP) are investigated in respiratory and other organs of hamsters.

Major Findings:

(1) Pulmonary carcinogenesis by crystalline silica. Extensive pathogenetic studies are conducted on the relationship between granulomatous/fibrogenic responses induced by crystalline silica particles in the lung and the induction of epithelial hyperplasia and epithelial cancers. Serial sacrifice studies have provided the histopathogenetic basis for the evaluation of the granulomatous and epithelial lesions induced by single intratracheal instillations of silica particles in inbred animals of three species: Fischer 344 rats, 15.16/EHS:CR hamsters, and mice of three strains: A/JCr, BALB/cAnNCr and NCr:NU (athymic nude). In the current year, further study of the resulting histopathology has identified details of the cellular reaction and the types of induced tumors.

Marked species differences were found by serial sacrifice following single intratracheal instillation of quartz (Min-U-Sil), HF-etched quartz, tridymite and cristobalite. Hamsters showed extensive and persistent macrophagic storage, minimal necrosis, minimal granulomatous reaction, and no epithelial proliferation. Mice showed marked granulomatous nodular reactions with extensive

necrosis and fibrosis, alveolar lipoproteinosis, early transient epithelial hyperplasia in smaller bronchi and bronchioles, but no adenomatous reaction. In contrast, rats showed early, intense and complex granulomatous reactions characterized by macrophages (phagocytosis, rapid necrosis and further recruitment), fibroblasts, lymphocytes, some plasma cells and granulocytes, many mast cells, progressive fibrosis and areas of alveolar lipoproteinosis. Epithelial hyperplasia of the alveolar type II cells and of some bronchioles develops early and persists for the lifetime of the rats, adjacent to and around granulomatous reactions, evolving into adenomatoid lesions and lung tumors. The rat lung tumors retain immunohistochemical markers of alveolar type II origin (surfactant apoprotein), but no markers of bronchiolar or bronchial cells. In 53 male and 49 female rats, 12 mg of quartz induced lung tumors in 17% males and 42% females killed at 11 months, in 32% males and 59% females at 17 months, and in 86% males and 89% females that died at 17-26 months. Tumor multiplicity reached 2.2 in males and 3.4 in females. Tumors were 74% adenocarcinomas; 6% epidermoid, 4% undifferentiated and 7% mixed carcinomas; and 9% adenomas. About 31% of adenocarcinomas arose from silicotic fibrous cores, suggesting a model for human lung scar cancer. These results were found to be closely reproduced in three separate experiments. Epithelial-mesenchymal relations, cell mediators, species-specific factors and mechanisms of in vitro toxicity and transformation by silica particles are under further study. The interaction of the cellular components of the granulomatous reaction (macrophages, polymorphonuclear monocytes, lymphocytes, fibroblasts and mast cells) with the adjacent epithelial cells of the peripheral airways is under further study by immunohistochemical methods. The present findings confirm the interest of the silica model for the study of the role of cellular mediators of inflammation in respiratory carcinogenesis. The markedly different reactions to silica in the three tested species provide a biological model for studies of susceptibility factors.

(2) Multifactorial carcinogenesis mechanisms. A large experiment on 14 groups of 40 hamsters each, designed to investigate the interaction of MNU, BP and mucosal injury in the induction of respiratory carcinogenesis, was previously analyzed for the complex response in the respiratory tract. Currently, the histological and statistical data are analyzed for evidence of carcinogenic effects on organs and tissues outside the respiratory tract. A single intralaryngeal instillation of MNU to five-week-old male hamsters was found to be subeffective per se in the respiratory tract, but it was sufficient to induce significant carcinogenic effects at several other target sites (oropharynx, esophagus, forestomach, pancreas, biliary system, small and large intestine, blood vessels and lymphoid tissue). Some of these responses to MNU were selectively enhanced by subsequent instillations of BP. Thus, a single dose of MNU acts as a full carcinogen and /or initiator on multiple target sites, a potentially useful model for multistage mechanisms of carcinogenesis in the hamster.

Publications:

Saffiotti U, Stinson SF. Lung cancer induction by crystalline silica: relationships to granulomatous reactions and host factors. J. Envir. Sci. Hlth, Part C: Envir. Carcino. Revs, 1988;6:197-222.

Keenan KP, Saffiotti U, Stinson SF, Riggs CW, McDowell EM. Morphological and cytokinetic responses of hamster airways to intralaryngeal or intratracheal cannulation with instillation of saline or ferric oxide particles in saline. Cancer Res 1989;49:1521-7.

Keenan KP, Saffiotti U, Stinson SF, Riggs VE, McDowell EM. Multifactorial hamster respiratory carcinogenesis with interdependent effects of cannula-induced mucosal wounding, saline, ferric oxide, benzo[a]pyrene and N-methyl-N-nitrosourea. Cancer Res 1989;49:1528-40.

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

PROJECT NUMBER

Z01CP05276-08 LEP

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Growth Control in Epithelial Cells and its Alteration in Carcinogenesis

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

PI: U. Saffiotti Chief LEP NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

0.4

PROFESSIONAL

0.2

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.)

Mouse keratinocyte cell lines were obtained without initial crisis by use of a serum-free medium, LEP/MK2 (low calcium MEM with non-essential amino acids supplemented with eight factors). The lines are subtetraploid with random gain and loss of otherwise normal chromosomes. At higher passage number, the lines acquire greater independence from growth factors and become less sensitive to growth inhibitors; the requirement for bovine pituitary extract becomes replaceable with bovine serum albumin or with somatomedin C. A new, chemically defined medium (LEP/MK4) was developed from LEP/MK2 by omitting the bovine pituitary extract and raising the level of bovine serum albumin from 0.1 to 1.5 mg/ml. The effects of carcinogens and growth factors are studied in these cell lines.

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

U. Saffiotti	Chief	LEP	NCI
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Objectives:

The overall objectives are to develop and characterize epidermal cell culture systems suitable for investigating the sequence of changes produced by carcinogens and growth factors in the control of growth and differentiation of epithelial cells. Mouse epidermal keratinocytes (MK) are currently used in these studies. Specific objectives include: (a) establishment of cell lines in serum-free media, (b) investigation of altered growth properties acquired by these cells with continued growth in culture and by exposure to growth factors, and (c) development of selective conditions for cells transformed by oncogenes or chemical carcinogens.

Methods Employed:

BALB/c newborn mouse epidermal cells, isolated by cold trypsinization as previously described, are cultured in serum-free media developed in this laboratory. The growth medium, LEP/MK2, consists of Eagles's MEM with nonessential amino acids but without added calcium, supplemented with epidermal growth factor (5.0 ng/ml); insulin (5.0 µg/ml); transferrin (5.0 µg/ml); hydrocortisone (0.5 µM); ethanolamine and phosphoethanolamine (0.5 µM each); bovine serum albumin (100 µg/ml); and bovine pituitary extract (0.5%). In the new medium, LEP/MK4, bovine pituitary extract is omitted and bovine serum albumin is raised to 1.5 mg/ml. Clonal growth is measured by colony-forming efficiency (CFE) and by average colony size after 7-10 days. The clonal growth rate is defined as population doublings per day (PD/d). For transformation experiments, toxicity is determined by clonal survival assays. The appearance of altered growth properties, ultrastructure (using both SEM and TEM) and karyological changes are studied by standard methods. Growth in soft agar and tumorigenicity in nude mice are used to demonstrate acquired neoplastic properties.

Major Findings:

A new serum-free growth medium was developed for the culture of mouse epidermal keratinocyte cell lines. This new medium, LEP/MK4, is entirely chemically defined and was derived from the previously reported LEP/MK2 medium by eliminating bovine pituitary extract (the only non-defined component of LEP/MK2) and replacing it with an increased level (from 0.1 to 1.5 mg/ml) of bovine serum albumin (BSA, essentially globulin-free). The new medium supports growth of MK4 cells and is currently being used for studies of toxicity, transformation and growth control. Additional characterization studies on the establishment of continuous MK cell lines were completed and published.

Publications:

Kaighn ME, Camalier RF, Bertolero F, Saffiotti U. Spontaneous establishment and characterization of mouse keratinocyte cell lines in serum-free medium. *In Vitro Cell Dev. Biol* 1988;24:845-54.

Kaighn ME, Reddel RR, Lechner JF, Camalier RF, Brash DE, Saffiotti U, Harris CC. Transformation of human neonatal prostate epithelial cells by strontium phosphate transfection with a plasmid containing SV40 early region genes. *Cancer Res* (In Press).

ANNUAL REPORT OF
THE LABORATORY OF HUMAN CARCINOGENESIS
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

The Laboratory of Human Carcinogenesis (LHC) conducts investigations to assess (1) mechanisms of carcinogenesis in epithelial cells from humans and experimental animals, (2) experimental approaches in biological systems for the extrapolation of carcinogenesis data and mechanisms from experimental animals to the human situation, and (3) host factors that determine differences in carcinogenic susceptibility among individuals.

The scientific and managerial strategy of the Laboratory is reflected in its organization into three sections, i.e., In Vitro Carcinogenesis Section (IVCS), Molecular Genetics and Carcinogenesis Section (MGCS), and Biochemical Epidemiology Section (BES). Scientifically, the emphasis is on the role of inherited or acquired host factors as important determinants in an individual's susceptibility to carcinogens, cocarcinogens and tumor promoters. Our investigations of host factors involve interspecies studies among experimental animals and humans, cover the spectrum of biological organization ranging from molecular and cellular biology, pathology, epidemiology, and clinical investigations. Two sections (IVCS and MGCS) devote their major efforts to more fundamental and mechanistic studies. The scientific findings, techniques, and concepts developed by these two sections and, of course, the scientific community at large, are utilized by the BES in selected and more applied studies of carcinogenesis and cancer prevention. The laboratory-epidemiology studies in this section require the expertise found in the IVCS and MGCS, and in the NCI Epidemiology Program. The Laboratory requires unique and complex resources. For example, collection of viable normal and neoplastic epithelial tissues and cells--well characterized by morphological and biochemical methods from donors with an epidemiological profile--requires continued cooperation among donors and their families, primary care physicians (internists, surgeons, house staff), surgical pathologists, nurses, epidemiologists, and laboratory scientists.

Since its establishment, the LHC has been fortunate to have the constructive criticism of a group of colleagues who are recognized experts in molecular biology (Carlo Croce, M.D., Fels Research Institute, Philadelphia, PA; Lennart Philipson, M.D., Ph.D., Director, European Molecular Biology Laboratory, Heidelberg, FRG), cell biology (Ted Puck, Ph.D., Director, Eleanor Roosevelt Center for Cancer Research, Denver, CO; David Prescott, Ph.D., Distinguished Professor, Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO), and biochemistry (Allan Conney, Ph.D., State of New Jersey Professor of Pharmacology & Chairman, Department of Chemical Biology and Pharmacognosy, College of Pharmacology, Rutgers The State University of New Jersey, Piscataway, NJ). These colleagues visit the LHC on an individual

basis at least once a year and review ongoing research projects with LHC staff. The exchange of ideas and their continuing advice have made these visits invaluable.

In addition to the frequent and informal exchanges of information among LHC staff, the Laboratory and each section have monthly scientific and administrative meetings. We also sponsor, with the Laboratory of Cellular Carcinogenesis and Tumor Promotion (LCCTP), a weekly Journal Club. A monthly joint rotating seminar series is presented by LHC, LCCTP, and the Laboratory of Experimental Carcinogenesis. The LHC sponsors seminars with extramural speakers at monthly intervals.

The LHC also organizes meetings of the Human Studies Collaborative Group, which take place in Bethesda and include participants from the NIH scientific community, extramural experts and collaborators, and staff from LHC resource contracts. These biannual meetings provide a forum for the survey of an ongoing research area, e.g., respiratory carcinogenesis, and for informal discussions.

RESEARCH STRATEGY

A central problem in carcinogenesis research is the extrapolation of data and knowledge of mechanisms from experimental animals to the human population, and within this heterogeneous population, extrapolation among individuals. A subset of this problem is the difficulty associated with extrapolation from one level of biological organization to another, i.e., molecules to macromolecules to organelles to cells to tissues to organs to intact organisms.

The strategy currently employed by the LHC is a refinement of the one we formulated a decade ago. Epidemiologic and clinical observations provide clues for generating hypotheses. In many cases, clinical investigations and studies using animal models can be used to test hypotheses. In other cases, *in vitro* models are more suitable. These models utilize human tissues and cells collected at the time of immediate autopsy (i.e., from organ donors) and surgery. Remarkable progress has been made during the last several years in the development of methodology to culture normal human tissues and their epithelial cells from most major sites of human cancer. Therefore, the mechanisms of action of carcinogens, tumor promoters, growth factors, differentiation inducers, etc., can be investigated at the tissue, cellular, and subcellular levels of biological organization. An integral facet of this strategy is that the same types of tissues and cells from experimental animals can be maintained in identical, controlled *in vitro* experimental settings so that comparative studies using human and experimental animal material can be conducted.

After developing *in vitro* models, we are systematically investigating several research areas, i.e., (I) *in vitro* model development; (II) carcinogen metabolism, DNA damage, and DNA repair; (III) cellular and molecular biology of normal and neoplastic cells; (IV) carcinogenesis and related studies (Table 1); and (V) biochemical and molecular epidemiology of human cancer. In recent years, the majority of the research projects have progressively shifted from areas I and II to areas III-V. Although we have developed *in vitro* models for several types of human tissues, the emphasis has been on lung, esophagus, liver and colon. Summaries of research projects in each of these integrated areas of our research program are listed in the following sections.

TABLE 1. STRATEGY FOR STUDYING HUMAN CARCINOGENESIS

- I. In Vitro Model Development
 - A. Collection of Human Tissues and Cells
 - B. Explant and Cell Cultures
 - C. Serum-Free Culture Conditions

- II. Carcinogen Metabolism, DNA Damage and DNA Repair
 - A. Interspecies Comparisons
 - B. Interindividual Comparisons
 - C. Intercellular Comparisons
 - D. Cell-Mediated Responses
 - E. Others

- III. Cellular and Molecular Biology of Normal and Neoplastic Cells
 - A. Growth Factors
 - B. Differentiation Factors
 - C. Cytoskeleton
 - D. Others

- IV. Carcinogenesis and Tumor Suppression
 - A. Oncogenes and Tumor Suppressor Genes
 - B. Chemical Carcinogenesis
 - C. Physical Carcinogenesis
 - D. Tumor Promoters, Aldehydes and Peroxides
 - E. Others

- V. Biochemical and Molecular Epidemiology
 - A. Lung Cancer
 - B. Others

I. IN VITRO MODEL DEVELOPMENT

Remarkable progress has been made during the last few years by this and other laboratories in establishing conditions for culturing human epithelial tissues and cells. Normal tissues from most of the major human cancer sites can be successfully maintained in culture for periods of weeks to months. We have developed chemically-defined media for long-term culture of human bronchus, colon, esophagus, liver and pancreatic duct. Primary cell cultures of human epithelial outgrowths have been obtained from many different types of human tissues. Defined culture conditions for normal human epithelial cells from the

bronchus, esophagus, pleural mesothelioma and liver have been established in the LHC. For example, methods have also been developed to culture pleural mesothelial cells obtained from non-cancerous donors. Pure cultures are initiated by pelleting the mesothelial cells from pleural effusion fluid and inoculating the resuspended cells into FN/C/BSA-coated dishes containing LHC basal nutrient medium supplemented with growth factors. Using this protocol, mesothelial cell cultures have been established from more than 200 donors. The cells can be subcultured at clonal density with a colony-forming efficiency of more than 10% and high density cultures can be subcultured four to six times before senescence.

These culture systems are now sufficiently established to permit; 1) pathobiologic investigations of normal human bronchial epithelial (NHBE) cells, e.g., regulation of growth and differentiation pathways and their dysregulation during carcinogenesis; 2) short- and long-term asbestos carcinogenesis investigations of mesothelial cells; and 3) studies of putative synergistic effects of hepatitis B virus and chemical carcinogens in hepatocellular carcinogenesis.

A. Model Systems for Studying Physical Carcinogens in Normal Human Mesothelial (NHM) Cells

These studies include the following: (1) development of defined media for replicative mesothelial cell cultures; (2) evaluation of cytotoxicity of asbestos fibers and synthetic nonmineral fibers in mesothelial and bronchial epithelial cells; (3) evaluation of the effects of asbestos fibers on progression of chromosome rearrangements in mesothelial cells; and (4) evaluation of the putative role of oxygen radicals in the mode of action of asbestos-caused carcinogenesis.

Methods to culture NHM cells have been defined. Cultures are initiated by pelleting the mesothelial cells from pleural effusion fluid and inoculating the resuspended cells into dishes containing LHC basal nutrient medium supplemented with serum (3%), hydrocortisone (0.5 micromoles), insulin (5 micrograms/ml), epidermal growth factor (EGF) (5 ng/ml), transferrin (10 micrograms/ml), trace elements, and 2% chemically-reduced (factor-free) serum (FFS). Using this protocol, mesothelial cell cultures have been established from more than 200 donors. The cells can be subcultured at clonal density with a colony-forming efficiency of more than 10% and high density cultures can be subcultured four to six times before senescence. We have now established that many factors will induce serum-starved cells to undergo one round of DNA synthesis in the absence of serum. These factors include EGF, transforming growth factor β_1 (TGF- β_1), TGF- β_2 , interleukin 1- α (IL-1- α), IL-1- β , transforming growth factor α (TGF- α), human platelet-derived growth factor (hPDGF), porcine PDGF (pPDGF), fibroblast growth factor-acidic (FGF-a), FGF-basic (FGF-b), β -interferon (Inf- β), γ -interferon (Inf- γ), and cholera toxin. However, for sustained growth, the medium must also be supplemented with high density lipids (HDL). Since human mesothelial cells have a peculiarly plastic cytoskeleton, we have characterized the effects of amosite fibers and code 100 glass fibers on the fidelity of division in these cells in an effort to understand the mechanism by which asbestiform fibers induce transformation and mesothelioma. Our results indicate that both amosite and code 100 glass fibers disturb the fidelity of cell division

leading to the induction of aneuploid daughter cells. However, the mechanism of action of these two agents appears to be different. Amosite fibers induce chromosome clustering, suggesting an inhibition of mitotic tubulin formation and/or centriole separation. In contrast, code 100 glass fibers cause chromosome dislocation from the spindle at metaphase, suggesting an effect on centromere/kinetochore function and/or mitotic spindle function.

B. Growth and SV40 Transformation of Human Hepatocytes

Hepatocellular carcinoma is one of the most frequent worldwide causes of cancer mortality. Hepatitis B virus and certain chemical carcinogens have been implicated as etiological agents. An in vitro model system of replicative normal human hepatocytes is needed to better define the mechanistic roles of these etiological agents. Serum-free medium composed of a modified Ham's F-12 medium was found to support the long-term multiplication of human liver epithelial cells. These epithelial cells were positive for general cytokeratin expression as well as positive for cytokeratin 18 and albumin expression through four passages. In addition, human hepatocytes in primary culture transfected with the SV40 large T antigen gene formed foci within 6-8 weeks that were positive for both keratin and large T antigen expression. Conditioned medium from cultures of the transfected human liver cells was shown to cause a 30% increase in DNA synthesis of a malignant human liver cell line (HepG2). These cells should prove to be useful in studying the molecular mechanisms of hepatocellular carcinogenesis.

C. Three Dimensional Tissue Models

A three dimensional (tissue equivalent) culture model is being developed that will recapitulate more faithfully the in vivo nature of normal airway epithelium. Specifically, mesenchymal cells are being combined with collagen. The metabolic activities of the mesenchyme cells cause changes in the structure of the collagen fibrils resulting in their contraction and producing a mesenchyme-like tissue structure. Subsequently, NHBE cells are inoculated on the surface of the pseudo-mesenchyme to allow for their differentiation into a muco-ciliary epithelium. A similar three dimensional culture model is being developed that recapitulates more faithfully the in vivo nature of normal liver tissue. These airway epithelium and hepatocellular models are also being developed to study the actions of pre-malignant and malignant epithelial cells when they are in association with normal epithelial cells and a mesenchyme.

II. CARCINOGEN METABOLISM, DNA DAMAGE AND DNA REPAIR

The earliest events in the multistage process of chemical carcinogenesis are thought to include (1) exposure to the carcinogen; (2) transport of the carcinogen to the target cell; (3) activation to its ultimate carcinogenic metabolite, if the agent is a procarcinogen; and (4) DNA damage leading to an inherited change. Therefore, one important use of cultured human tissues has been in the investigation of the metabolism of chemical carcinogens because many environmental carcinogens require metabolic activation to exert their oncogenic effects; the metabolic balance between carcinogen activation and deactivation

may, in part, determine a person's oncogenic susceptibility. Knowledge of the comparative metabolism of chemical carcinogens among animal species will aid efforts to extrapolate data on carcinogenesis from experimental animals to humans. We and our coworkers have systematically examined the metabolism of procarcinogens of several chemical classes which are considered to be important in the etiology of human cancer. Procarcinogens of several chemical classes can be activated enzymatically to electrophilic reactants that bind covalently to DNA in cultured human tissues. The studies of activation and deactivation of representative procarcinogens have revealed that the metabolic pathways and the predominant adducts formed with DNA are generally similar between humans and experimental animals. Wide quantitative interindividual differences (50- to 150-fold) are found in humans and other outbred animal species. When the metabolic capabilities of specimens from different levels of biological organization are compared, the profile of benzo(a)pyrene metabolites is similar in cultured tissues and cells, but subcellular fractions, e.g., microsomes, produce a qualitative and quantitative aberrant pattern.

Although DNA repair has been studied extensively in human fibroblasts, lymphoid cells, and neoplastic cells, little information is available concerning DNA repair in normal human epithelial cells. Using the methodology to culture human bronchial epithelial and fibroblastic cells developed in our laboratory, we have initiated studies to investigate DNA damage and repair caused by chemical and physical carcinogens as examined by alkaline elution methodology, BND cellulose chromatography, unscheduled DNA synthesis, and high pressure liquid chromatographic analysis of the formation and removal of carcinogen-DNA adducts. As we reported last year, human bronchial epithelial cells repair single-strand breaks in DNA damaged by X-irradiation, ultraviolet (UV)-radiation, chromate, polynuclear aromatic hydrocarbons, formaldehyde, or N-nitrosamines at rates similar to bronchial fibroblasts.

A. DNA Damage and DNA Repair

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. Alkyltransferase activity varies in the different human tissues tested in the decreasing order of liver, colon, esophagus, peripheral lung and brain. Formaldehyde inhibits repair of O⁶-methylguanine and potentiates the mutagenicity of an alkylating agent, N-methyl-N-nitrosourea, in normal human fibroblasts. In some experimental studies, repeated exposure to alkylating agents has led to an increase in O⁶-methylguanine-DNA alkyltransferase activity, i.e., an adaptive response. We have shown that human bronchial epithelial cells do not adapt and increase their DNA repair capability. This finding has important implications in carcinogenesis caused by low doses of N-nitrosamines. The effects of cigarette smoke condensate (CSC), catechol and smoke "conditioned" media on the activity of O⁶-methylguanine-DNA alkyltransferase (O⁶-MT) and the effects of uracil-DNA glycosylase (UDG) on cultured human bronchial epithelial cells, HuT-292 cells and BEAS-2B cells, is currently under investigation. The activity of these two DNA repair enzymes is also being measured in the alveolar macrophages and peripheral blood lymphocytes of smokers and nonsmokers. Inter- and intra-individual variation in these activities is up to 100-fold and sixfold, respectively. O⁶-MT

activity is lower in macrophages of smokers which is consistent with the hypothesis that tobacco smoke inhibits repair of alkyl-DNA adducts.

B. Assessment of Tobacco Smoke Genotoxicity

Puck and coworkers have developed a highly sensitive assay for measuring mutations. The marker genes used in this system are present on a human chromosome that has been transferred to form a Chinese hamster ovary (CHO) hybrid cell. Because the CHO cell does not require the human marker chromosome for normal cell division, mutations that might not be detectable in other systems due to lethality, such as large deletions or rearrangements, will be scored using this system.

Cigarette smoke is carcinogenic in animals, and most of this carcinogenic property is recovered in nonpolar subfractions of the neutral fraction. Although cigarette smoke condensate is mutagenic following activation by metabolic enzymes found in microsomes or the S9 fraction, it has not been reported to act as a direct mutagen in systems used thus far. In addition, the fractions determined to be most carcinogenic have not been found to be the most mutagenic in the Ames assay. We have employed Chinese hamster ovary (CHO) cells containing a human chromosome 11 (termed AL hybrid cells) as a highly sensitive detection system for mutagenesis in an effort to evaluate possible direct mutagenic effects of CSC and its fractions. Cytotoxicity induced by N-methyl-N'-nitro-N-Nitrosoguanidine (MNNG) in AL cells increased with time of incubation. The 50% inhibitory concentration of MNNG after 1, 3, 6 and 20 hr of incubation was 0.8, 0.4, 0.2 and 0.1 micromoles, respectively. Mutagenicity increased with dose and time reaching a maximum of 1,250 mutants/100,000 survivors (800% above background) after 3 hr of incubation with 2 micromolar MNNG and a maximum of 1,700 mutants/100,000 survivors (1,100 % above background) after 20 hr of incubation with 0.2 micromolar MNNG. The cytotoxicity of CSC increased with increasing incubation time with 50% inhibitory concentrations of 100, 80, 50 and 30 micrograms/ml after 1, 3, 6 and 20 hr of incubation, respectively. CSC mutagenicity increased with time of incubation up to 3 hr with a maximum of 300 mutants/100,000 survivors (250% above control) after incubation with 100 micrograms/ml CSC (p value less than 0.0005, Student's t-test). Cytotoxicity and mutagenicity of CSC were inversely proportional to cell density, while cytotoxicity and mutagenicity of MNNG were unaffected by cell density. The 3 hr incubation time, 50% inhibitory concentration of the acidic fraction of CSC (30 micrograms/ml) induced 350 mutants/100,000 survivors (a 230% increase above background, p value less than 0.0005). The basic and neutral fractions caused a much lower increase at the 50% inhibitory concentrations (80 and 200 micrograms/ml, respectively). The possible role of oxy-radicals generated by tobacco smoke condensate and its fractions in mutagenesis of AL hybrid cells is being investigated.

III. CELLULAR AND MOLECULAR BIOLOGY OF NORMAL AND NEOPLASTIC CELLS

In vitro models provide an opportunity to investigate the cellular and molecular mechanisms controlling growth and differentiation of normal human epithelial cells. Aberrations in these normal mechanisms can be studied in carcinoma cell lines. The comparison between normal versus neoplastic cells generates hypotheses that can be tested in carcinogenesis studies. In addition, cellular

and molecular markers of normal versus neoplastic cells and selective conditions for growth have pragmatic value in carcinogenesis studies and in cancer diagnosis.

A. Growth and Differentiation of Human Bronchial Epithelial Cells

Defined methods to grow replicative cultures of NHBE cells without serum have been developed. These cells can be subcultured several times, will undergo 35 population doublings, and have expected epithelial cell characteristics of keratin, desmosomes and cell surface antigens. NHBE cells inoculated at clonal density will multiply with an average generation time of 28 hr; the majority of the cells are small and migratory and have few tonofilaments. Adding human whole blood-derived serum (BDS) depresses the clonal growth rate of NHBE cells in a dose-dependent fashion. In contrast, human lung carcinomas either replicate poorly or fail to grow at all when inoculated at clonal density in serum-free medium. Their rates of multiplication increase in direct proportion to the amount of BDS added to the optimized medium. TGF- β_1 was found to be the primary differentiation-inducing factor in serum for NHBE cells, while TGF- β_1 was not growth inhibitory for malignant cells. These differential effects of TGF- β_1 on normal versus malignant cells are not because of lack of TGF- β_1 -specific receptors on malignant cells. Epinephrine antagonized the effect of TGF- β_1 without binding characteristics of TGF- β_1 -specific receptors.

We previously reported that sparse cell density cultures of NHBE cells uniformly undergo terminal squamous differentiation, e.g., their morphology becomes squamous, they irreversibly cease synthesizing DNA and subsequently, they form cross-linked envelopes when incubated in media containing fetal bovine serum (FBS) or TGF- β_1 . We have now found that high cell density modulates the efficiency of these differentiation inducing agents. Thus, whereas tritiated-thymidine incorporation into acid-precipitable material stops permanently in low density cultures within 24 hr after exposure, only a transient depression in DNA synthetic activity was observed in high density cultures. In addition, although phase microscopic image analysis revealed that nearly all of the cells displayed the squamous morphology within 1 hr after exposure to FBS, only a portion of the cells became terminally differentiated. Morphological observations of high density cultures 24 to 72 hr after exposure to FBS or TGF- β_1 showed the presence of two types of cells, i.e., clusters of small prolate spheroid-shaped cells surrounded by squamous cells. Only the small cells were capable of DNA synthesis and cell division as determined by autoradiography and time-lapse photomicrographic images. These dividing cells did differentiate if they were subcultured at low cell density and incubated in FBS- or TGF- β_1 -containing medium. Our studies suggest that as cultures of NHBE cells expand from clonal to high cell density, a transient phenotype arises that does not undergo terminal squamous differentiation when exposed to FBS or TGF- β_1 .

B. Cell Surface Antigen Expression - Normal and Neoplastic Cells

The expression of a variety of cell surface antigens was studied on normal bronchial epithelial cells, small cell lung carcinomas, mesothelial cells, mesotheliomas, as well as fibroblasts and mesothelial cells transfected with SV40 T antigen gene. The monoclonal antibodies used to study these cell surface

antigens defined determinants normally expressed on a variety of different cell types, mainly those cells of hematopoietic and lymphoid origin. Small cell lung carcinoma cell lines and freshly explanted tumor expressed antigens defined by monoclonal antibodies MY4, MY7 and MY9 (myeloid differentiation antigens), as well as certain monoclonal antibodies that detect antigens usually associated with the B cells or, in some cases, epithelial cells. Cultured normal bronchial epithelial cells did not express these myeloid differentiation antigens (MY4, MY7, MY9), but these antigens are expressed when bronchial epithelial cells were immortalized by infection with adeno-12 SV40 hybrid virus. Higher levels of these antigens were also found when these cells were transfected with and expressed raf and myc oncogenes. In addition, cells expressing both raf and myc had transient expression of cell surface major histocompatibility complex (MHC) class II antigens. Normal mesothelial cells did not express these myeloid antigens. MY4 and MY7 but not MY9 were expressed when these cells were transfected and immortalized with the SV40 T antigen gene.

These findings indicate that bronchial epithelial cells as well as mesothelial cells, both normal and malignant, may express cell surface antigens that are commonly found on other cell types and that expression of certain surface antigens do not necessarily indicate the origin of a malignant cell and appear to reflect markers of differentiation.

C. Cytosine Methylation and Cellular Physiology and Pathology

The role DNA methylation plays in the promoter regions of two separate gene systems has been clarified. Demethylation of at least one Hpa II restriction site upstream from the structural coding sequences of human gamma globin or the hepatitis B core antigen gene is necessary but not sufficient for the initiation of transcription. The final conversion of a quiescent, demethylated gene (gamma globin or hepatitis B core antigen) to an active state requires some endogenous or exogenous inducing agent, which may be highly specific for any given gene or gene complex. Clonal selection is not responsible for observed changes in gene expression, since we have clearly shown that cells remethylate DNA that has been demethylated by 5-azacytidine treatment.

New micro techniques have been developed which enable the analytical quantitation of 5-methylcytosine in less than one microgram of DNA isolated from any source. Thus, the genomic 5-methylcytosine content of NHBE and pulmonary mesothelial cells has been measured for the first time. These techniques have enabled the determination of changes in the genomic content of 5-methylcytosine during normal physiological processes. The genomic content of 5-methylcytosine in NHBE cells and in rodent tissues decreases with increasing *in vivo* age. Significant decreases in DNA 5-methylcytosine occur concomitantly with the induction of squamous differentiation in NHBE cell cultures. These techniques have also provided for the demonstration that chemical carcinogens can induce decreases in DNA 5-methylcytosine levels in dividing NHBE cells.

D. Molecular Analysis of Gene Regulation and Proliferation Control in Human Lung Cells

The goal of this project is to understand, at the molecular level, regulatory interactions of human cells with growth factors, metabolites, and foreign chemicals implicated in carcinogenesis. These substances govern or affect the growth potential and differentiation status of the cells. The topics of present interest are: induction of plasminogen activator inhibitor (PAI-1), interleukin 1 (IL-1) and urokinase in NHBE cells, T antigen transformed NHBE cells, and lung tumors; development of model systems for the study of differentiation of NHBE cells and for fiber sensitivity and growth factor regulation in NHM cells; and responsiveness to growth factors and auto-production of growth factors by NHM cells, T antigen transformed NHM cells, and mesotheliomas.

NHBE cells respond to signals initiated consequent to the binding of TGF- β_1 by activating pathways that will result in terminal squamous differentiation. Utilizing both normal and SV40 T antigen immortalized bronchial epithelial cells, we have found that treatment with TGF- β_1 transiently increases steady state mRNA levels for urokinase (uPA) and PAI-1 about 5- and 50-fold, respectively, at 4 hr. The optimal inducing dose (4pM TGF- β_1) has been shown previously to be the most effective dose for initiation of squamous differentiation. PAI-1 protein is increased in TGF- β_1 -treated cells in both extracellular matrix and conditioned medium. The net effect of TGF- β_1 induction on plasminogen activator activity in the medium is a 50% reduction as measured by a caseinolytic assay. A T antigen gene immortalized cell line that does not undergo squamous differentiation in response to TGF- β_1 also does not respond to TGF- β_1 by modulation of either uPA or PAI-1 expression. Comparison of human bronchial epithelial, pleural mesothelial, and lung fibroblastic cell strains indicates that the bronchial epithelial cells have a constitutively higher ratio of uPA to PAI-1 expression than the other cell types. These data suggest that modulation of pericellular proteolysis in bronchial epithelial cells in response to TGF- β_1 represents a major biological change in their pericellular environment. Thus, differential induction of uPA and PAI-1 in response to TGF- β_1 may be involved in the squamous differentiation of human bronchial epithelial cells.

E. Proto-oncogene Expression in Growing or Differentiating Human Bronchial Epithelial Cells

TGF- β_1 and epinephrine have opposing effects on NHBE cells. While TGF- β_1 induces squamous differentiation in NHBE cells, epinephrine promotes their growth and neutralizes the effect of TGF- β_1 . To investigate mechanisms which might be involved in their antagonism, we examined steady state expression levels of proto-oncogenes c-myc, c-fos, and c-Ha-ras in response to treatment with these two agents. Expression of specific mRNA was detected by Northern blotting and normalized by the constitutive probe glyceraldehyde-3-phosphate-dehydrogenase. Expression of c-myc was transiently inhibited by TGF- β_1 up to 40% at 1 hr.

Epinephrine induced the expression of c-myc about two- to threefold and neutralized the effect of TGF- β_1 on c-myc mRNA level. Expression of c-fos was transiently induced by either TGF- β_1 or epinephrine up to 1.5 to two- to threefold at 1 hr, respectively. These two agents synergistically induced c-fos up to

fivefold. The c-Ha-ras mRNA level was not altered by TGF- β_1 or epinephrine treatments. While the initial changes in c-myc expression correlated with the proliferative activity of the cells, the steady state level of c-fos mRNA did not.

IV. CARCINOGENESIS AND TUMOR SUPPRESSION

A central problem of cancer research is the extrapolation of carcinogenesis data and knowledge of carcinogenesis mechanisms from laboratory animals to humans and, within this heterogeneous population, extrapolation among individuals. An aspect of this problem is the difficulty associated with extrapolating from one level of biological organization to another, i.e., from molecules to macromolecules to organelles to cells to tissues to intact organisms. Multiple experimental systems are needed to help investigators find solutions to these and other problems in carcinogenesis research. Animal models are obviously required for experimental *in vivo* carcinogenesis studies. They are also essential because the integral multisystemic interactions of the organism remain intact and because laboratory animals can be environmentally and genetically controlled. *In vitro* models using tissues, cells, and subcellular fractions are also useful. This approach can aid in the resolution of the central problem of extrapolation in that one can conduct comparative studies with tissues and cells from experimental animals and humans that are maintained in the same controlled *in vitro* experimental setting. Carcinogenesis studies using human tissues and cells offer unique opportunities. For example, some rare forms of human cancer reflect inherited, predisposing conditions and their genetic basis, and perhaps common pathways of carcinogenesis may be understood through the study of cells from individuals with these specific types of cancer. In addition, because human cells *in vitro* are apparently genetically more stable and undergo less "spontaneous" neoplastic transformation than most rodent cells, they may be especially suitable for studying the multistage process of carcinogenesis.

Epithelial cells are of particular interest because most adult human cancers are carcinomas. As noted above, significant progress has been made in the past decade in developing methods for culturing human epithelial tissues and cells. Chemically defined media have been developed for culturing normal human tissues and cells from organs with a high rate of cancer in humans. Serum-free media have several advantages in studies of cultured human cells, including: (a) less experimental variability compared to serum-containing media; (b) selective growth conditions of either normal cells of different types (e.g., epithelial versus fibroblastic cells) or normal versus malignant cells; (c) identification of growth factors, inhibitors of growth, and inducers of differentiation; and (d) ease of isolating and analyzing secreted cellular products. Advances in cell biology, including the delineation of biochemical and morphological markers of specific cell types, have also facilitated the identification of cells *in vitro* (e.g., keratins as markers for epithelial cells and collagen types I and III for identifying fibroblasts).

Carcinogenesis is a multistage process that can be operationally divided into tumor initiation, promotion, conversion, and progression. Genetic changes, perhaps mutations, are considered to be responsible for tumor initiation and malignant conversion. As was noted earlier, metabolism of carcinogens, DNA

damage, and DNA repair are considered to be important factors in these stages of carcinogenesis.

A. Characterization and Mode of Action of the raf and myc Families of Oncogenes

An amplification of the c-myc, N-myc or L-myc gene and overexpression of the c-raf sequence are common features of small cell lung carcinomas. The major objective of this project is to determine the role of these genes in lung carcinogenesis by a) overexpressing c-raf and/or c-myc genes in human lung cells, and b) reversion of the malignant phenotype by plasmids capable of anti-sense RNA transcription.

Retroviral recombinants pZip-raf and pZip-myc were constructed to examine the role of the c-raf-1 and c-myc proto-oncogene in lung carcinogenesis. Immortalized human bronchial epithelial cells (BEAS-2B) transfected with pZip-raf DNA and pZip-myc DNA gave rise to undifferentiated carcinomas (raf/myc tumors) when tested in athymic nude mice, whereas c-myc or c-raf transfected cells are non-tumorigenic. The raf/myc tumors expressed markers of small cell lung carcinomas, i.e., neuron-specific enolase and neurosecretory granules. In addition, BEAS-2B cells transformed with the c-raf and c-myc proto-oncogenes, and derived tumor cell lines acquired HLA class II antigen expression.

The c-raf-1 gene has been identified as the predominant transforming gene of three radiation-resistant head and neck cancer cell lines in the NIH 3T3 transfection assay (SQ-20B, JSQ-3, SCC-35). NIH 3T3 cells transformed with SQ-20B DNA also became radiation-resistant, suggesting a correlation between the presence of c-raf sequences and the radiation-resistant phenotype. Inhibition of the c-raf function by introduction of anti-sense raf transcribing plasmids into the SQ-20B cell line reverted not only the tumorigenic phenotype but also reduced the radiation resistance. As a consequence of these experiments, the construction of an inducible promoter system for anti-sense sequences in human cells was undertaken.

NHBE cells transformed with an Ad 12-SV40 hybrid virus (BEAS-2B) were transfected or infected with pZip-c-raf, pZip-c-myc or pZip-v-Ha-ras. BEAS-2B cells containing both c-raf and c-myc, or the oncogene v-Ha-ras, gave rise to "small cell" or "undifferentiated" carcinoma, respectively. To determine the role of the c-raf gene for transformation in conjunction with c-myc and to study the activation of c-raf in the pathway of ras transformation in BEAS-2B cells, we examined the autophosphorylation and the serine/threonine-specific kinase activity of c-raf in various BEAS-2B transfectants compared to mouse 3T3 cells. Immune-complex kinase assays were performed applying a new peptide antibody directed against the amino-terminal end of the c-raf sequence which specifically detects the 74KD c-raf protein. Compared to mouse 3T3 cells, autophosphorylation and serine/kinase activity were significantly higher in the immortalized BEAS-2B cells. Both activities were further increased in transfectants containing c-raf and c-myc or the v-Ha-ras oncogene. The correlation between p21 expression, c-raf kinase activity and tumorigenicity of various ras-transfected BEAS-2B cell lines is under investigation. Ongoing studies question whether the relatively greater efficiency shown by BEAS-2B cells as compared to NHBE cells for

transformation to tumorigenicity is associated with the relative levels of c-raf kinase activity in these cell types.

B. In Vitro Transformation of Human Lung Cells by SV40 and ras Oncogenes

Five human bronchial epithelial cell lines have been established from NHBE cells by SV40 early region gene transfer. One additional cell line has been established following transfection of SV40 early region genes into bronchial epithelial cells known to contain an abnormality of the short arm of chromosome 11 from an individual who was cancer-free; the resulting cell line is tumorigenic in athymic nude mice. At least one other cell line with a chromosome 3p deletion has developed weak tumorigenicity. Several different mutant ras oncogenes have resulted in malignant transformation. These cell lines, and tumor cell lines established from the nude mouse tumors, are being utilized to study aspects of multistage carcinogenesis, including chromosomal changes, progressive changes in response to inducers of squamous differentiation and the development of invasiveness.

C. Tumor Suppression and Somatic Cell Genetics

The goal of this project is to determine whether normal cells contain genes that have the ability to suppress the tumorigenicity of human lung carcinoma cell lines. Hybrid cell lines between HuT-292DM, a human lung carcinoma line, and either NHBE cells or an SV40 "immortalized" but non-tumorigenic derivative thereof (BEAS-2B) have been isolated by double selection. Hybrids of NHBE and HuT-292DM cells had an extended culture life span, but senesced after approximately 60 population doublings (PDs) as compared to the 30-35 PDs seen with NHBE cells. In contrast, hybrids of BEAS-2B and HuT-292DM showed no sign of a culture "crisis" and have an indefinite life span. HuT-292DM cells produced tumors in 100% of the nude mice with a latency of 27 days, whereas tumorigenicity was suppressed 80% in BEAS-2B x HuT-292DM hybrids, with a two- to threefold increased latency for the remaining 20%. When serum responsiveness was examined, HuT-292DM cells were stimulated, while NHBE and BEAS-2B cells were inhibited by serum. The BEAS-2B x HuT-292DM hybrids' growth response to serum was similar to that of HuT-292DM cells. Thus, tumorigenicity and culture longevity are dominantly controlled by the nontumorigenic parent, while serum responsiveness resembles that of the tumorigenic parent.

D. Tumor Suppression Activities of Specific Human Chromosomes

Genetic changes related to carcinogenesis are being studied using hybrids from fusion of human lung carcinoma cells with NHBE cells and of microcells of individual marked human chromosomes with human lung tumor cells. Initial studies suggest that a limited PD potential (mortality) is the dominant genetic trait in hybrid cells. Other hybrid cell lines have been isolated and are being characterized for doubling potential, karyotype and tumorigenicity in athymic nude mice. When specific human chromosomes have been transferred by microcell methodology into HuT-292 cells, chromosome 11 has decreased the tumorigenicity of the HuT-292. The location of the putative tumor suppressor gene on chromosome 11 will be determined.

E. Tumor Suppressor Genes: Isolation of Terminal Differentiation Genes by Subtraction Libraries

The cancer cell is the product of at least two events. First, the cell loses control of its proliferative pathways, resulting in continual cell growth. Second, those signals which normally induce terminal differentiation are not processed correctly by the cancer cell, resulting in cell immortality. Although numerous proto-oncogenes/oncogenes have been implicated in the first event, little is known about the genes involved in the second event. The objective of this project is to identify genes which are essential for the process of (terminal) differentiation of human epithelial cells with the goal of finding and bypassing the defects in human lung carcinomas.

In order to investigate genes associated with terminal differentiation of epithelial cells, cDNA libraries are being constructed from a variety of epithelial cell types in various stages of differentiation. By using the method of subtraction cloning, messages expressed preferentially in one cell type or in a certain stage of differentiation may be examined. Furthermore, effects of inducers of differentiation upon gene expression can be examined as epithelial cells progress from an undifferentiated state to a terminally differentiated state.

NHBE cells can be induced to terminal differentiation *in vitro* by treatment with a variety of agents such as TGF- β_1 , 5-azacytidine, calcium, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and FBS. To investigate differences in gene expression of undifferentiated NHBE cells and differentiated NHBE, messenger RNA is isolated from both cell types and used to produce two cDNA libraries. From these two libraries, RNA is synthesized *in vitro* making sense strand RNA from one type and anti-sense strand RNA from the other type. The sense and anti-sense RNAs are then hybridized, and the unique single-stranded sequences are removed from the common double-stranded sequences. By this subtraction protocol, unique messages expressed in differentiating cells can be used as probes to investigate the clones from which they were synthesized.

F. Prostatic Carcinogenesis

Cultures of neonatal human prostatic epithelial cells (NP-2S) near the end of their life span were transfected by strontium phosphate coprecipitation with a plasmid (pRSV-T) containing the SV40 early region genes. Colonies of transformed cells, isolated from a background of senescing normal cells yielded cell lines with growth potential well beyond that of the normal cells. In all, 28 cell lines have been isolated in three separate experiments. Four lines, 267B1, 272E1, 272E4, and 272A9 which have undergone 96.3, 92.3, 30.8, and 64.7 PDs, respectively, have been studied further and appear to be "immortalized" without having passed through a crisis. These lines contain cytokeratins and SV40 T antigen by immunofluorescence and have ultrastructural features of epithelial cells. All lines consist of mixtures of normal and aneuploid karyotypes with modal chromosome numbers in the near-diploid range ($2n=46$) resulting from random loss, gain and rearrangement of chromosomes. None produced tumors within one year after subcutaneous (sc) injection in nude mice. The transformed lines, as well as the parental NP-2S, are stimulated by TGF- β_1 in clonal growth assays.

These lines should be useful in investigating prostatic carcinogenesis and progression.

V. BIOCHEMICAL AND MOLECULAR EPIDEMIOLOGY

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

Currently available techniques exist that would allow the utilization of biochemical and molecular measures to better characterize exposure to carcinogens, to serve as intermediate endpoints on the path to malignancy, to identify measures which halt or reverse this process, and to investigate the mechanisms of human carcinogenesis. Included in these investigations would be the following: (1) efforts to evaluate body burden of chemical carcinogens in studies of occupational and general environmental cancer risk factors; (2) sophisticated analyses of air, water, and biological specimens for carcinogenic and mutagenic substances in conjunction with specific analytical studies; (3) search for evidence of viral infection including viral segments or oncogenes in the DNA of individuals at high risk of cancers that may be associated with infectious agents or heritable states; (4) evaluation of disturbances in immune function as they may relate to malignancies, particularly those of the hematopoietic system; (5) investigation of the relationship between micronutrients and a variety of epithelial cancers; and (6) determinations of the relationship between macronutrients, including dietary fat and subsequent hormonal changes, to subsequent risk of breast, endometrial, and perhaps colon cancer.

A. Development of Techniques for the Measurement of Carcinogen-Adducts in Humans

Classical epidemiology and xenobiochemical studies have revealed questions relating to the genotoxic effects of environmental contaminants in humans. Development of assays for carcinogen exposure at the molecular level in humans has therefore been a major focus of research and from the battery of assays that are currently available, both immunological and physico-chemical approaches are being further developed. Since each type of assay system clearly has its own advantages and disadvantages, the use of more than one corroborative assay has been of importance. The problems that appear to confront the development of assay systems for human biomonitoring are essentially twofold: the levels of material present in macromolecular complexes challenge the detection limits of conventional assay systems, and complex mixtures of adducted materials confound simple assay systems. Following recognition and definition of cross-reactivity profiles for antibodies raised against aromatic-DNA adducts, protocols that combine immunoaffinity chromatography, high performance liquid chromatography (HPLC), fluorescence spectroscopy, gas chromatography/mass spectroscopy and

32P-postlabeling are being developed. The development of HPLC-32P-postlabeling techniques for the measurement of either hydroxylated residues in DNA (for example, 8-hydroxydeoxyguanosine) or alkylated residues in DNA (for example, O6-methyl- or N7-methyldeoxyguanosine) are also in progress. With regard to oxidative damage, 8-hydroxydeoxyguanosine in particular, electrochemical detection is being explored.

B. Analyses of Genetic Polymorphisms and Allelic Deletions in Human Lung Cancer

There is a clear association between smoking and lung cancer, but it is still not known why some individuals, who are heavily exposed to large concentrations of chemical carcinogens, do not develop tumors, whereas others do. These observations provide circumstantial evidence for the involvement of a genetic factor that predisposes for tumor formation. Recent restriction fragment length polymorphism (RFLP) studies have shown that the loss of normal cellular sequences from chromosome 13 (in the case of retinoblastoma, osteosarcoma, breast cancer and small cell lung cancer), chromosome 11 (in the case of Wilms' tumor, bladder cancer and breast cancer), chromosome 1 (in the case of melanoma), chromosome 22 (in the case of acoustic neuroma), chromosome 3 (in the case of renal cancer and small cell carcinoma of the lung) and chromosome 17 (in the case of colorectal carcinoma) have been associated with malignancy. It appears that this method might be generally applied to the analysis of inherited susceptibility to cancer and may therefore be informative in risk assessment for lung cancer. High molecular weight DNA samples from tumor and normal tissue have been collected from more than 60 cancer patients for restriction enzyme digestion and Southern analysis. Initial experiments centered on examination of genes located on the short-arm of chromosome 11; loss of allelic fragments during tumorigenesis was detected at the cellular Harvey ras locus, the insulin locus, the calcitonin locus, the beta-globin locus, the catalase locus and the Int-2 locus (homologous to the MMTV locus). These observations have been extended to define commonly deleted regions by the shortest overlapping region of deletion analysis. In addition, polymorphic genetic loci on five other chromosomes (3, 13, 17, 18 and 20) have been studied. Examination of "rare" allelomorphs at the Harvey ras locus has been made in a case control study to determine the use of this marker as a lung cancer risk factor. The inheritance of Ha-ras rare alleles and the extensive debrisoquine metabolic phenotype increase the relative risk of lung cancer (squamous cell carcinoma and small cell carcinoma) in an independent manner.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05192-09 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Repair of Carcinogen-Induced DNA Damage in Human Cells

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

P.I.:	Glennwood E. Trivers	Biologist	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Ainsley Weston	Visiting Associate	LHC	NCI
	Bonita G. Taffe	IRTA Fellow	LHC	NCI
	Richard B. Hayes	Epidemiologist	EEB	NCI

COOPERATING UNITS (if any)

Department of Pharmacology, Oulu, Finland (K. Vahakangas); Georgetown University Hospital, Washington, DC (H. Yeager); LA State University, Baton Rouge, LA (M. Newman)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.0	PROFESSIONAL	1.5	OTHER	0.5
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CHECK APPROPRIATE BOX(ES)

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

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

Certain carcinogens damage cells by binding to DNA. Enzymatic removal of the promutagenic alkylation lesion O6-methylguanine from DNA has been achieved in vitro in normal human tissues, cultured normal human bronchial epithelial cells (NHBE) and fibroblasts (HBF). This demonstrates the catalytic activity of O6-alkylguanine-DNA alkyltransferase (O6-MT). Alkyltransferase activity has been detected in all human tissues tested. Repair of the spontaneous uracil-DNA lesion, which is associated with Bloom's syndrome and repaired by uracil-DNA glycosylase (UDG), has also been demonstrated in vitro in human tissues and cells. Repeated exposure to alkylating agents induces higher levels of O6-MT, i.e., adaptive responses, in some cells. In our previous studies using NHBE cells, O6-MT was not induced by N-methyl-N-nitrosourea (MNNU); aldehydes inhibited DNA repair by both O6-MT and enhanced MNNU mutagenicity in HBF. To resolve the indications of these results, we are studying; a) in vitro measure of O6-MT and UDG in broncho-alveolar lavage cells (BALCs) and peripheral blood mononuclear (PBM) cells from smokers and nonsmokers, and b) the in vitro effects of cigarette smoke condensate (CSC) on cultured NHBE cells, lung tumor cells (HuT-292) and DNA repair enzymes. Preliminary results show: large inter- and intra-individual variations in enzyme levels that are smaller in smokers than in nonsmokers; smokers and cotinine-positive donors with overall lower enzyme levels and higher adduct levels in PBMs (but not in BALCs) than nonsmokers and cotinine-negative donors; higher O6-MT levels in adduct-containing PBMs and BALCs than in those without adducts, but lower increases in cotinine-positive donors as compared to cotinine-negative donors.

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

Glennwood E. Trivers	Biologist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Ainsley Weston	Visiting Associate	LHC	NCI
Bonita G. Taffe	IRTA Fellow	LHC	NCI
Richard B. Hayes	Epidemiologist	EEB	NCI

Objectives:

To study the effects of tobacco smoke and related compounds on the mechanisms of enzymatic repair of DNA damaged by environmental agents, using normal, premalignant, and malignant human epithelial tissues and cells.

Methods Employed:

Measurement of O⁶-MT activity in extracts of human tissues by the following methods: (1) measuring the loss of labeled O⁶-methylguanine (O⁶-MeGua) from a ³H-methylated DNA substrate; (2) measuring the production of protein containing S-[³H]-methylcysteine during protein reaction with this DNA substrate; (3) measuring the formation of [8-³H]-guanine in DNA when the extracts were incubated with a synthetic DNA substrate containing O⁶-MeGua labeled in the 8-position; (4) measuring the removal of ³H-deoxyuracil monophosphate from DNA following incubation of ³H-deoxyuridinemonophosphate-DNA with cellular supernatants.

Collection of BALCs by saline bronchial lavage; separation of PBMs (phycol-hypaque) and serum (coagulation) from whole blood; O⁶-MT and UDG measurements (as above) in the supernatants from homogenized PBMs and BALCs; purification of cellular DNA; detection and measure of polycyclic aromatic hydrocarbons (PAH)-DNA adducts using ultrasensitive radioimmunoassay (USERIA) and/or HPLC and synchronous scanning fluorescence spectrophotometry; detection of serum antibodies (Ab) to DNA adducts using immunoassay; detection of cotinine and tetrahydrocannabinol (Δ -9 THC) in serum using immunoassay and gas chromatography; and detection of 8-OH-deoxyguanosine phosphate (8-OH-dGp)-adducts with HPLC and electron capture.

Major Findings:

We have investigated O⁶-MT and UDG activity in human tissues and cultured NHBE cells and HBF. O⁶-MT activity in colon, esophagus and lung was lower than reported for liver, the highest of human tissues studied. Both O⁶-MT and UDG repair of DNA caused mutations. Cultures of NHBE cells in single or repeated low doses of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were not induced to higher O⁶-MT activity.

Formaldehyde (HCHO), a respiratory carcinogen in rats and a potential carcinogen in humans, is found in tobacco smoke and as a metabolite of demethylation of drugs and carcinogenic N-nitrosamines. In cultured human cells, DNA-protein cross-links (DPC) and single strand breaks (SSB) were induced and removed in all HCHO-treated cells. HCHO also enhanced the cytotoxicity of ionizing radiation and MNNU. Further, HCHO caused DNA DPC directly and SSB indirectly during ultraviolet-type excision repair. Since HCHO is formed during the metabolic activation of N-nitrosodimethylamine, we examined the repair of the promutagenic lesion O⁶-MeGua formed following N-nitrosodimethylamine metabolism. HCHO decreased O⁶-MT activity, inhibited removal of O⁶-MeGua, and synergistically enhanced cytotoxicity and mutagenicity of MNNU. Micromolar concentrations of the highly cytotoxic, tobacco-smoke related α,β -unsaturated aldehyde, acrolein, reduced intracellular thiols in cultured HBF. The less toxic formaldehyde and acetaldehyde also inhibited O⁶-MT (known to have a cysteine residue in its active site), but had no effect on UDG activity. In high doses HCHO is also mutagenic. Accordingly, HCHO and other reactive aldehydes in cigarette smoke may render cells vulnerable to carcinogenic clonal expansion and other toxic chemicals by directly damaging DNA, simultaneously inhibiting the DNA repair activity of sensitive enzymes, and impairing detoxification by systems requiring glutathione.

More recently, O⁶-MT and UDG activities were measured in BALCs and PBMs from 25 smokers and 32 nonsmokers. All donors had detectable levels of both enzymes. The donors were 50%, 19 to 24 years; 50%, 25 to 39 years; 80% male; 84% white; 44% smokers; 23/39 tested cotinine-positive [COTN(+)] of which 84% were confessed smokers and 38% were nonsmokers (COTN(+)) nonsmokers were probably unconfessed smokers). However, three donors smoked materials other than tobacco, raising the potential for enzyme inhibiting aldehydes from sources other than tobacco.

Large inter- and intra-individual variations were observed (up to 387-fold and 25-fold, respectively), similar to reports for other tissues and cells. These were large inter- and intra-individual variations for enzymes, cells and smoking categories. Variations were lower for O⁶-MT, PBMs, smokers, and COTN(+) cases (22- to 178-fold) than for UDG, BALCs, nonsmokers, and cotinine-negative [COTN(-)] cases (36- to 378-fold). Intra-individual variations were much lower and without patterns within the groups (7 smokers; 3 nonsmokers). Intra-individual variations were lowest for O⁶-MT in PBMs of smokers (1.2 to 3.4), and highest for O⁶-MT in BALCs of smokers (1.5 to 25).

Smokers had lower levels of O⁶-MT and UDG in PBMs compared to nonsmokers, but both groups had similar levels of both enzymes in BALCs. The results were similar for COTN(+) and COTN(-) cases. However, the differences observed were not statistically significant ($P > .05$), due probably to the large inter-individual variations. There was no correlation between the two enzyme activities in BALCs ($r = 0.02$), but significant correlation in PBMs ($r = 0.79$). However, UDG was significantly elevated ($P < .05$) in the BALCs of smokers compared to nonsmokers.

Eight (24%) of 33 (13 smokers; 20 nonsmokers) donors were positive in the USERIA for PAH-DNA adducts, and among 20 cases tested for adducts and cotinine. COTN(-) cases with adducts had threefold increases in O⁶-MT levels and little affect on UDG (0.8-fold) in PBMs; both activities increased in BALCs (1.3- and 1.7-fold, respectively). COTN(+) cases with adducts had a lower increase (1.9-fold) in O⁶-MT and a decrease in UDG (0.34-fold), but major increases in the both enzymes (3- and 7.5-fold, respectively) in BALCs. Compared to COTN(-) cases with adducts, COTN(+) cases with adducts had lower levels of both enzymes (0.7- and 0.6-fold, respectively) and twofold higher adduct levels in PBMs. However, they had 2.7-fold higher levels of both activities and lower adduct levels (0.42-fold) in BALCs.

To date, 17 sera (11 smokers, 6 nonsmokers) have been assayed for Ab to PAH-DNA adducts, using calf thymus DNA modified in vitro with benzo(a)pyrene diol epoxide (BPDE), chrysene diol epoxide (CRDE), benzanthracene diol epoxide (BADE), and benzo(k)furanthrene (BFDE). Antibodies were detected for 3 of the 5 carcinogens: 2 smokers and 5 nonsmokers had anti-BPDE; 2 smokers had anti-BADE; and 4 nonsmokers had anti-BADE. Four smokers without Ab's had lower enzymes than nonsmokers without Ab's. However, more cases are required to study Ab and enzyme relationships.

Publications:

Curren RD, Yang LL, Conklin PM, Grafstrom RC, Harris CC. Mutagenesis of xeroderma pigmentosum fibroblasts by acrolein. *Mutat Res Let* 1988;209:17-22.

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

PROJECT NUMBER

Z01CP05409-06 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Control of Growth and Differentiation of Human Bronchial Epithelial Cells

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

P.I.:	John F. Lechner	Section Chief	LHC	NCI
Others:	Brenda I. Gerwin	Research Chemist	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI
	Yang Ke	Visiting Fellow	LHC	NCI

COOPERATING UNITS (if any)

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 20892

TOTAL MAN-YEARS

4.0	PROFESSIONAL	2.0	OTHER	2.0
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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)

We previously reported that sparse cell density cultures of normal human bronchial epithelial (NHBE) cells uniformly undergo terminal squamous differentiation, e.g., their morphology becomes squamous, they irreversibly cease synthesizing DNA and subsequently, they form cross-linked envelopes when incubated in media containing fetal bovine serum (FBS) or transforming growth factor (TGF) type beta-1. We have now found that high cell density modulates the efficiency of these differentiation inducing agents. Thus, whereas tritiated-thymidine incorporation into acid-precipitable material stops permanently in low density cultures within 24 hr after exposure, only a transient depression in DNA synthetic activity was observed in high density cultures. In addition, although phase microscopic image analysis revealed that nearly all of the cells displayed the squamous morphology within 1 hr after exposure to FBS, only a portion of the cells became terminally differentiated. Morphological observations of high density cultures 24 to 72 hr after exposure to FBS or TGF-beta-1 showed the presence of two types of cells, i.e., clusters of small prolate spheroid-shaped cells surrounded by squamous cells. Only the small cells were capable of DNA synthesis and cell division as determined by autoradiography and time-lapse photomicrographic images. These dividing cells did differentiate if they were subcultured at low cell density and incubated in FBS- or TGF-beta-1-containing medium. Our studies suggest that as cultures of NHBE cells expand from clonal to high cell density, a transient phenotype arises that does not undergo terminal squamous differentiation when exposed to FBS or TGF-beta-1.

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

John F. Lechner	Section Chief	LHC	NCI
Brenda I. Gerwin	Res. Chemist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Yang Ke	Visiting Fellow	LHC	NCI

Objectives:

To study mechanisms controlling growth and differentiation of human bronchial epithelial cells. These studies include the following: (1) development of efficient assays to quantify the various squamous differentiation-inducing factors; (2) identification and characterization of an autocrine growth factor; (3) identification and characterization of an autocrine squamous differentiation-inducing factor; (4) elucidation of the pathways of squamous differentiation and determination of 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 NHBE cells are used in growth and differentiation studies or are cryopreserved for future use.

Major Findings:

We have previously shown that FBS will induce sparse cell density cultures of normal but not tumorigenic human bronchial epithelial cells to undergo squamous differentiation. We have also reported the isolation of isogenic serum-sensitive (S.6) and serum-resistant (R.1) sub-clones of a premalignant SV40 T antigen gene immortalized bronchial epithelial cell line that recapitulate these particular differentiation characteristics of the normal and malignant cells. We have also shown that a potent FBS constituent that causes both the NHBE and S.6 cells to undergo squamous differentiation is TGF- β_1 . However, NHBE, S.6, R.1 and lung carcinoma cells all have similar numbers and affinities of TGF- β_1 receptors. Thus, the molecular mechanism controlling whether a human lung epithelial cell will or will not undergo squamous differentiation is distal to the binding of the ligand to its receptor. Unexpectedly, the high cell density cultures of normal and S.6 continued to display mitotic cells. Thus, before we could initiate experiments to dissect the squamous differentiation pathways, we

had to clarify the influence that cell density has on the response of normal and S.6 cells to FBS and TGF- β_1 .

Cultures were initiated at cell densities varying from 50 to 25,000 cells/cm² to ascertain the density at which FBS failed to stop mitotic activity. Those initiated at cell densities less than 3,000 cells/cm² incorporated very little thymidine relative to control (serum-free) cultures, whereas cultures inoculated with more than 10,000 cells/cm² were not significantly different from their controls.

High cell density cultures of R.1 cells showed no depression of DNA synthesis after treatment with FBS. On the other hand, an immediate inhibition of DNA synthesis was seen for NHBE and S.6 cells. However, DNA synthesis returned to control values after 36-40 hr of incubation. The period of time before NHBE cultures re-exhibited the normal rate of DNA synthesis was unaffected by replacing the medium with fresh FBS-supplemented medium at 6 hr intervals. Further, experiments using higher concentrations of FBS (20%) gave identical results. Thus, the transient inhibition of DNA synthesis observed in high cell density cultures is not due to a simple shift in the dose-response kinetics. The effect of FBS on protein and RNA synthesis was also measured; no depressions were found during the time period (72 hr) of the experiment.

One explanation for these observations could be an autogenous "anti-differentiation" factor. This was tested using double cell density cultures where the central area was high and the outer ring was sparse cell density, respectively. Serum (8%) was added to the medium after one day of incubation and the cultures were gently rocked during the ensuing 4 days. Tritiated thymidine was then added and the cultures were analyzed by autoradiography. No colonies and no nuclei with silver grains above them were found in the clonal density portion, whereas numerous foci of labeled cells were present in the high cell density area. Thus, no cell elaborated anti-differentiation factor was detected.

The foci of DNA synthesizing cells that were observed in the high cell density portion of the double cell density cultures suggested the presence of a sub-population of differentiation resistant cells. Thus, phase microscopic images were collected at 10 min intervals. Analysis showed that more than 95% of the cells exhibited a flat (squamous) morphology with a 250% increase in surface area within 60 min after the addition of FBS. Subsequently, three classes of cells were discernable. Class 1 cells showed no mitotic activity. Class 2 cells were squamous-appearing cells that underwent cytokinesis. However, the daughter cells did not divide again. In contrast, the morphology of the class 3 cells returned to prolate spheroid 1-3 hr prior to undergoing division and gave rise to daughter cells.

The autoradiographic results could be explained by the presence of a few contaminating cells that were inherently unable to differentiate. This possibility was tested by incubating high cell density cultures for one week in medium with FBS (8%). Control high cell density cultures, i.e., without FBS,

were incubated in parallel. These three sets of cultures were then subcultured and the cells were reinoculated at sparse cell density. The colony forming efficiencies of the control culture cells and the cells that had been incubated with FBS were 13% and 13.5%, respectively, when assayed in the absence of FBS. However, no colonies were found if the medium contained FBS, irrespective of whether the dishes had been inoculated with cells from the original control cultures or with cells that had been exposed for 7 days to FBS. Thus, the differentiation refractive cells in the high cell density cultures are not due to cells that were inherently resistant to these agents.

The differentiation characteristics of the S.6 T antigen gene transformed cells and the NHBE cells are comparable. However, whereas no immutable [R] variants arise in NHBE cell cultures, these deviants rapidly emerge in clonally-derived lines of [S] cultures. Specifically, after the progenitor S.6 cell had divided 20 times to become a colony, 5% of the cells were found to be [R] variants. The percent of these [R] variants increased with subculturing until an equilibrium of 30% to 50% was established. Identical results were found for other [S] clones (213). In contrast, genetic (hypoxanthine phosphoribosyl transferase) mutants arise in S.6 cultures at a rate of 1 mutant/five million cells. Since NHBE cells at high cell density contain [R] phenotype cells, we reasoned that [R] clones might arise as a consequence of a dysfunction of the mechanism that causes conversion of [R]-type NHBE cells back to the [S] phenotype. If so, the rate of generation of SV40 T antigen [R] variants should be affected by the cell density of the S.6 culture. This prediction was borne out experimentally; the percent of [R] variants in the cultures continually maintained at low cell density averaged 5.5% over 60 population doublings, whereas an equilibrium of 51% [R] cells was found in the high cell density cultures.

Publications:

Bonnard C, Lechner JF, Gerwin BI, Fujiki H, Harris CC. Effects of palytoxin or ouabain on growth and squamous differentiation of human bronchial epithelial cells in vitro. *Carcinogenesis* 1988;9:2245-9.

Ke Y, Reddel RR, Gerwin BI, Miyashita M, McMenamin M, Lechner JF, Harris CC. Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. *Differentiation* 1988;38:60-6.

LaVeck MA, Lechner JF. Isolation and culture of normal human bronchial epithelial cells from autopsy tissue. In: Freshney RE, ed. *Culture of animal cells*, 2nd ed. New York: Alan R Liss, 1987;191-202.

Lechner JF, Gerwin BI, Ke Y, Reddel RR, Pfeifer AMA, Masui T, Miyashita M, Wilson VL, Harris CC. Regulation of differentiation in human bronchial epithelial cells. In: Harris CC, Liotta L, eds. *Genetic mechanisms in carcinogenesis and tumor progression*. New York: Alan R Liss (In Press).

Lechner JF, Ke Y, Masui T, Reddel RR, Pfeifer AMA, Gerwin BI, Harris CC. Growth, differentiation, and transformation studies of human bronchial epithelial cells. In: Nettesheim P, Thomassen D, eds. *Biology, toxicology and carcinogenesis of respiratory epithelium*. New York: Hemisphere Publishing Co (In Press).

Lechner JF, Masui T, Miyashita M, Willey JC, Reddel R, LaVeck MA, Ke Y, Yoakum GH, Amstad P, Gerwin BI, Harris CC. Human lung cells: In vitro models for studying carcinogens. In: Langenbach R, Elmore E, Barrett JC, eds. *Tumor promoters: biological approaches for mechanistic studies and assay systems*. New York: Plenum Press, 1988;275-88.

Lechner JF, Stoner GD, Haugen A, Autrup H, LaVeck MA, Trump BF, Harris CC. In vitro human bronchial model systems for carcinogenesis studies. In: Webber MM, Sekely L, eds. *In vitro models for cancer research*. Boca Raton: CRC Press, 1988;3-17.

Miyashita M, Smith MW, Willey JC, Lechner JF, Trump BF, Harris CC. Effects of serum, transforming growth factor type β , or 12-O-tetradecanoylphorbol-13-acetate on ionized cytosolic calcium concentration in normal and transformed human bronchial epithelial cells. *Cancer Res* 1989;49:63-7.

Pfeifer AMA, Lechner JF, Masui T, Reddel RR, Mark GE, Harris CC. Control of growth and squamous differentiation in normal human bronchial epithelial cells by chemical and biological modifiers and transferred genes. *Environ Health Perspect* 1989;80:209-20.

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

PROJECT NUMBER

Z01CP05426-05 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Characterization and Mode of Action of the raf Subfamily of Oncogenes

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

P.I.: Curtis C. Harris Chief LHC NCI
 Others: Andrea Pfeifer Special Volunteer LHC NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

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NCI, NIH, Bethesda, Maryland 20892

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.)

Immortalized human bronchial epithelial cells (BEAS-2B) expressing the c-raf-1 and c-myc genes induced large cell carcinomas in athymic nude mice with neuro-endocrine features. Both BEAS-2B cells transfected with either c-raf-1 (2B-raf) or c-myc (2B-myc) showed elevated mRNA levels of neuron-specific enolase (NSE) consistent with earlier findings of neuro-endocrine markers in nude mice tumors induced by c-raf-1 and c-myc. This effect was additive in BEAS-2B cells carrying c-raf-1 and c-myc (2B-raf/myc), and derived human tumor cell lines. Applying c-raf-1 or murine c-myc-specific antibodies the expression of proto-oncogenes was specifically demonstrated in immunoprecipitations of transfected BEAS-2B lines. Using a highly efficient retrovirus isolation/infection protocol, the reproducible induction of tumors with c-raf-1 in combination with c-myc was confirmed by infecting 2B-raf or 2B-myc cells with amphotropic c-raf-1 or c-myc recombinant virus. Experiments analyzing the function of the c-raf-1 kinase in malignant transformation showed the highest activity in 2B-raf and 2B-raf/myc cells followed by 2B-myc, BEAS-2B and normal human bronchial epithelial (NHBE) cells. Tumorigenic BEAS-2B cells transfected with various mutant ras genes expressed elevated c-raf kinase in a p21 protein-dependent manner.

The association of oncogenes, radiation resistance, and tumorigenicity was tested in the tumorigenic human laryngeal carcinoma, SQ-20B, by introduction of sense (raf) and anti-sense (far) human c-raf-1 cDNA sequences. The malignant potential of SQ-20B and the radiation-resistant phenotype was significantly increased in the presence of raf and decreased in the presence of far sequences. S1 nuclease protection assays revealed a specific reduction of steady-state levels of the endogenous c-raf-1 sense transcript in SQ-20B cells transfected with far DNA. In accordance, transfection of BEAS-2B cells with c-raf-1 or v-Ha-ras but not c-myc induced the expression of a radiation-resistant phenotype.

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

Andrea Pfeifer	Special Volunteer	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

Overexpression of members of the myc family of oncogenes and the c-raf-1 proto-oncogene is commonly found in small cell lung carcinomas, whereas abnormalities in the ras family of oncogenes are associated with non-small cell lung tumors. The objectives of this project are: (1) to determine the role of c-raf-1 and c-myc in lung carcinogenesis, (2) to define the molecular basis of c-raf-1 and c-myc cooperation, and (3) to examine the role of c-raf-1 as a target in the pathway of transformation by ras oncogenes.

Several oncogenes have been linked to radiation resistance. In the head and neck tumor line, SQ20-B, as well as in other radiation-resistant head and neck cancers, the c-raf-1 gene was identified as the predominant transforming gene. Further objectives are to analyze the molecular linkage between the malignant and radiation-resistant phenotype of SQ-20B and to determine the functional role of c-raf-1 for the induction of radiation resistance.

Methods Employed:

DNAs were transfected into recipient mammalian cells employing calcium or strontium phosphate co-precipitation. High titer epitropic or amphotropic retroviruses containing oncogenes/proto-oncogenes cloned into pZipSV(x) vector were obtained by isolating recombinant viruses in serum-free medium from confluent cultures of Y2 (epitropic) or YAM and PA317 (amphotropic) packaging lines. RNAs and DNAs were isolated from cells in culture by the guanidinium isothiocyanate-cesium chloride (CsCl) method and tested by Southern and Northern blot analysis. Proteins for the c-raf and c-myc immunoprecipitation were isolated based on a protocol for nuclear proteins in the presence of 0.5% sodium dodecyl sulfate (SDS) and 0.5% Nonidet P-40 (NP-40) immunoprecipitated with antibodies specific for the human c-raf-1, R-2 or the mouse c-myc gene and analyzed on 12% denaturing polyacrylamide gels. The c-raf-1 kinase activity was determined in immune complexes with the R-2 antibody assaying the phosphorylation capacity of the complexes on histone V-S as a substrate. The radiation resistance of cell lines was determined as described.

Major Findings:

1. Characterization of BEAS-2B cells malignantly transformed with c-raf-1 and c-myc. BEAS-2B cells transfected with the c-raf-1 and c-myc proto-oncogenes (2B-raf/myc) were tumorigenic in irradiated, athymic nude mice, whereas neither

c-raf-1 (2B-raf) or c-myc (2B-myc) alone induced tumors. Southern blot analysis showed the presence of transfected c-raf-1 and c-myc sequences. 2B-raf cells contain 2-3 copies/cell of c-raf-1, 2B-myc cells; 4-5 copies of c-myc; and 1 copy of each cDNA of 2B-raf/myc cells. The expression of the murine c-myc gene product was specifically detected by using a peptide c-myc antiserum directed against the 12 carboxyl-terminal amino acids in immunoprecipitation reactions. The specificity of the assay was demonstrated by the absence of an immunoprecipitate in human HL60 cells which is characterized by an amplified c-myc gene. The polyvalent peptide anti-serum, R-2 (see below), directed against the amino-end of the c-raf-1 gene specifically detected the 74 kD c-raf-1 protein in immunoprecipitations of BEAS-2B and transfected cell lines. The careful quantitation of the c-raf-1 protein revealed an increase in c-raf-1 expression of sevenfold (2B-raf) or one and one half-fold (2B-raf/myc) compared to the levels of BEAS-2B cells. Since the raf/myc tumors (RMT) showed certain neuro-secretory granules, the transfected cell lines were tested for the expression of NSE. NSE mRNA steady state levels are increased in 2B-raf, 2B-myc, and 2B-raf/myc by 300, 290, and 440%, respectively, suggesting an additive effect of c-raf-1 and c-myc on the induction of NSE expression. In RMT 1, 2, or 3 tumor cell lines, NSE mRNA is elevated by 450 to 720%.

2. Development of a highly efficient retroviral gene transfer system. The more efficient gene introduction by retroviral recombinants combined with the simple application suggested the development of such a system. The induction of differentiation of our cell lines by serum required serum-free conditions. High titer viruses (2×10^5 to 10^6 cfu/ml) of several oncogenes were achieved by a) isolation of virus in PC-1 medium (Vendrex) from nearly confluent cultures, b) storage of small aliquots of virus at -70°C which avoids thawing and refreezing, and c) infection of the recipient cell line with virus in PC-1 medium in the presence of polybrene. Amphotropic viruses with titers above 2×10^5 cfu/ml were generated from c-raf-1, c-myc, v-Ha-ras, Ki-ras, N-ras, N-ras mutant, Rb, SV40 T antigen, Zip-neo and others. 2B-raf or 2B-myc cells infected with 2B-myc or 2B-raf virus, respectively, developed tumors of 0.2 to 1 cm in size in nude mice within 14 weeks and confirmed the induction of a tumorigenic phenotype in BEAS-2B cells by the combination of c-raf-1 and c-myc genes.

3. Development of c-raf-1-specific antibodies. Five different peptides corresponding to the c-raf-1 sequences were employed for the generation of polyvalent antibodies in rabbits. Only the anti-peptide serum directed against the amine-terminal amino acids 37-55 showed specificity for the product of the c-raf-1 proto-oncogene. The antibody was successfully used in immunoprecipitations, Western blot analyses, and c-raf-1 kinase assays. The development of a monoclonal antibody directed against the same peptide sequence is in progress.

4. Analysis of c-raf-1 kinase activity in NHBE, BEAS-2B, and oncogene expressing derivatives. Primary cultures of human bronchial epithelial cells showed either the same or higher levels of c-raf-1 kinase, and the more efficient transformation of BEAS-2B cells cannot be explained by a constitutive higher activity of the c-raf-1 kinase. The kinase activity was significantly elevated

in 2B-raf-transfected or -infected cells, slightly increased in 2B-myc/raf-infected and unaltered in 2B-myc-infected cells, suggesting that constitutive expression rather than overexpression of c-raf-1 and c-myc are crucial for transformation by these two oncogenes. BEAS-2B cells expressing v-Ha-ras or v-K1-ras showed an increase in c-raf-1 kinase activity which was dependent on the mutant p21 expression. This identifies c-raf-1 as a possible target in the pathway of transformation by ras oncogenes.

5. The functional role of c-raf-1 for the induction of radiation resistance. To determine the molecular linkage between the malignant and radiation-resistant phenotype, sense or anti-sense human c-raf-1 cDNA were introduced into the radiation-resistant human laryngeal carcinoma cells, SQ-20B. The additional expression of raf sequences in sense orientation further increased tumorigenicity and radiation resistance of SQ-20B. The presence of anti-sense DNA delayed the tumor growth of SQ-20B and enhanced radiation sensitivity paralleled by a ten-fold reduction of steady-state levels of the endogenous c-raf-1 gene in SQ-20B cells. These data indicate that the reduced expression of c-raf-1 is sufficient to modulate the tumorigenicity and the radiation-resistant phenotype of SQ-20B cells, implicating c-raf-1 in a pathway important to the genesis of this type of cancer.

A molecular linkage between c-raf-1 and radiation resistance was further examined by transfection/infection of a radiation-sensitive cell line BEAS-2B with c-raf-1, c-myc, and various mutant ras genes. 2B-raf cells and 2B-raf/myc expressed a radiation-resistant phenotype, whereas 2B-myc remained radiation-sensitive. The similar increase in radiation resistance of v-Ha-ras transfected BEAS-2B cells again presented evidence that the ras genes act "upstream" of c-raf-1 and might use this gene as a target to induce their biological effects. Experiments are in progress to determine if c-raf-1 induces enzymes involved in repair mechanism or inactivation of radicals, e.g., superoxide dismutase, catalase, glutathione peroxidase, and others.

Publications:

Kasid U, Pfeifer A, Brennan T, Beckett M, Weichselbaum RR, Dritschilo A, Mark GE. Effect of antisense c-raf-1 on tumorigenicity and radiation sensitivity of a human squamous carcinoma. *Science* 1989;243:1354-6.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05435-05 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Development of Techniques for the Measurement of Carcinogen-Adducts in Humans

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

P.I.:	Ainsley Weston	Visiting Associate	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Robert Metcalf	Medical Staff Fellow	LHC	NCI
	Miriam C. Poirier	Research Chemist	CCTP	NCI
	Andrew C. Povey	Visiting Fellow	LHC	NCI
	Peter G. Shields	IRTA Fellow	LHC	NCI
	Bonita G. Taffe	IRTA Fellow	LHC	NCI
	Glennwood E. Trivers	Research Biologist	LHC	NCI

COOPERATING UNITS (if any)

Louisiana State Univ, Baton Rouge, LA (M. Newman); M.R.C., Carshalton, England (P. Farmer); MIT, Boston, MA (S. Tannenbaum, J. Essigmann); Am Hlth Fdn, Valhalla, NY (S. Hecht, P. Foiles); IARC, Lyon, France (C. Wild, R. Montesano).

LAB/BRANCH

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)

Classical epidemiology and xenobiochemical studies have revealed questions relating to the genotoxic effects of environmental contaminants in humans. Development of assays for carcinogen exposure at the molecular level in humans has therefore been a major focus of research and from the battery of assays that are currently available, both immunological and physico-chemical approaches are being further developed. Since each type of assay system clearly has its own advantages and disadvantages, the use of more than one corroborative assay has been of importance. The problems that appear to confront the development of assay systems for human biomonitoring are essentially twofold: the levels of material present in macromolecular complexes challenge the detection limits of conventional assay systems, and complex mixtures of adducted materials confound simple assay systems. Following recognition and definition of cross-reactivity profiles for antibodies raised against aromatic-DNA adducts, protocols that combine immunoaffinity chromatography, high performance liquid chromatography (HPLC), fluorescence spectroscopy, gas chromatography/mass spectroscopy and ³²P-postlabeling are being developed. The development of HPLC-³²P-postlabeling techniques for the measurement of either hydroxylated residues in DNA (for example, 8-hydroxydeoxyguanosine) or alkylated residues in DNA (for example, O-6-methyl- or N-7-methyldeoxyguanosine) are also in progress. With regard to oxidative damage, 8-hydroxydeoxyguanosine in particular, electrochemical detection is being explored.

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

Ainsley Weston	Visiting Associate	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Robert Metcalf	Medical Staff Fellow	LHC	NCI
Miriam C. Poirier	Research Chemist	CCTP	NCI
Andrew C. Povey	Visiting Fellow	LHC	NCI
Peter G. Shields	IRTA Fellow	LHC	NCI
Bonita G. Taffe	IRTA Fellow	LHC	NCI
Glennwood E. Trivers	Research Biologist	LHC	NCI

Objectives:

To develop and apply sensitive techniques for the quantitation of adducts in humans; these include bulky adducts formed from polycyclic aromatic hydrocarbons and aromatic amines as well as small adducts formed by DNA alkylation or hydroxylation.

Methods Employed:

Competitive enzyme immunoassays are performed on test DNA using rabbit anti-carcinogen (PAH or aromatic amines)-DNA antibodies. Polyvinyl 96-well microtitre plates are coated with control DNA and carcinogen-modified DNA (0.2 - 1.0 µg/well). Standard competitive inhibition curves are obtained by mixing serial dilutions of known standard materials with rabbit antisera. Percentage inhibition of the test samples are determined from the standard curves. All tests and assays are performed in triplicate.

Immunoaffinity columns are prepared with rabbit polyclonal anticarcinogen-DNA antibodies. DNA samples are hydrolyzed enzymatically and up to 2 mg of digested DNA is applied to a single column at one time. Materials that are specifically bound to the columns are eluted in either NaOH (50 mM) or methanol (HPLC grade) prior to their characterization and measurement by either ³²P-postlabeling, HPLC and fluorometry, gas chromatography/mass spectroscopy (GC/MS) or enzyme immunoassay.

HPLC, using reverse-phase C-18 octadecasilane (ODS) columns (4.6 x 250 mm) that are eluted with linear gradients of methanol in water, is used to separate complex mixtures of hydrolyzed adducted materials.

Mixtures of the 3'-nucleotide monophosphates are first separated by HPLC, then small amounts of either deoxyguanosine or deoxyadenosine or deoxycytidine are added to HPLC fractions expected to contain the alkylated or hydroxylated residues. The samples are then postlabeled with ³²P using T₄ polynucleotide kinase and the radiolabeled materials are resolved by 2-dimensional thin layer

chromatography (TLC) and detected by autoradiography. A collaboration has been established to utilize immunoaffinity chromatography for separation of normal from adducted nucleotides prior to HPLC to further aid sensitivity and specificity. The combination of HPLC separation with ^{32}P -postlabeling provides a highly specific and sensitive assay for detection of lesions in DNA (e.g., at deoxyguanosine residues) that results from the action of alkylating agents and oxidizing agents.

Synchronous fluorescence spectra (SFS), for the detection of aromatic-DNA adducts, are acquired by driving the excitation and emission monochromators or a fluorescence spectrophotometer simultaneously with a fixed wavelength difference.

Major Findings:

Antibodies directed against BPDE-DNA adducts have been shown to recognize a variety of PAH-DNA adducts. Similarly, antibodies directed against 2-acetylaminofluorene deoxyguanosine were found to recognize a variety of aromatic amine-DNA adducts, notably, 4-aminobiphenyl (4-ABP). However, antibodies directed against PAH-adducts did not recognize aromatic amine adducts, and anti-aromatic amine adduct antibodies were found not to recognize PAH-DNA adducts. These data indicate that the antibodies are adduct-class specific and will probably be of most value as preparative tools, for example, immunochromatography.

Initial experiments have focused on the isolation of PAH-DNA adducts from human placental DNA and their characterization by fluorescence spectroscopy and GC/MS. These experiments corroborate previous immunoassay data generated in a number of laboratories that have measured adduct levels as high as 1 in 5×10^6 unmodified bases for benzo[a]pyrene-DNA adducts. Immunoaffinity chromatography is now being developed for the isolation of 4-ABP-DNA adducts. This method is currently being interfaced with HPLC and ^{32}P -nucleotide-postlabeling. Preliminary results indicate that human placental DNA and human lung DNA contain aromatic amine DNA adducts at levels of greater than 1 adduct in 10^8 nucleotides.

Attempts to measure oxidative damage and DNA alkylation by the combination of HPLC, ^{32}P -nucleotide-postlabeling, TLC and autoradiography have resulted in the development of these methods capable of measuring 1 adduct in 10^6 for N^7 -methyldeoxyguanosine, 1 adduct in 10^7 for O^6 -methyldeoxyguanosine, 1 adduct in 10^7 for O^6 -ethyldeoxyguanosine and 1 adduct in 10^6 for 8-hydroxydeoxyguanosine. These assays are being applied to human lung tissues and peripheral blood DNA samples from people exposed to a number of alkylating and oxidizing agents, for example, tobacco smoke, dietary carcinogens and chemotherapeutic agents. Since an aliquot of one of the unmodified bases is reintroduced into the reaction mixture as an internal standard, each assay can be quantitated accurately.

A combination of these and other methods has been used to measure a range of carcinogen-DNA adducts in 17 human lung DNA samples. A spectrum of different

carcinogen-DNA adducts were detected using a variety of sensitive techniques. HPLC-linked SFS and an ultrasensitive enzyme radioimmunoassay detected adducts derived from benzo[a]pyrene diol epoxide and other apparent polycyclic aromatic hydrocarbons. An amplified enzyme-linked immunosorbent assay demonstrated the presence of 4-ABP-DNA adducts in many of these samples. A number of these specimens also contained O⁶-alkyldeoxyguanosine as measured by ³²P-postlabeling techniques. This pilot study indicates not only that human lung contains a spectrum of carcinogen-DNA adducts, but also that a full-scale molecular dosimetry study of human exposure to both aryl and alkyl chemical carcinogens is warranted.

Publications:

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Weston A, Rowe M, Poirier M, Trivers G, Vahakangas K, Newman M, Haugen A, Manchester D, Mann D, Harris CC. The application of immunoassays and fluorometry to the detection of polycyclic hydrocarbon-macromolecular adducts and anti-adduct antibodies in humans. Int Arch Occup Environ Health 1988;60:157-62.

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

PROJECT NUMBER

Z01CP05480-04 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Genetic Polymorphisms and Allelic Sequence Deletions in Human Lung Cancer

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

P.I.:	Ainsley Weston	Visiting Associate	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Haruhiko Sugimura	Visiting Fellow	LHC	NCI
	Neil Caporaso	Medical Staff Fellow	EEB	NCI
	Robert Hoover	Chief	EEB	NCI

COOPERATING UNITS (if any)

New England Medical Center, Boston, MA (T. Krontiris); Children's Hospital of LA, Los Angeles, CA (W. Benedict); Johns Hopkins University, Baltimore, MD (B. Vogelstein); University of Maryland, Baltimore, MD (B. F. Trump)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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 unexpanded type. Do not exceed the space provided.)

There is a clear association between smoking and lung cancer, but it is still not known why some individuals, who are heavily exposed to large concentrations of chemical carcinogens, do not develop tumors, whereas others do. These observations provide circumstantial evidence for the involvement of a genetic factor that predisposes for tumor formation. Recent restriction fragment length polymorphism (RFLP) studies have shown that the loss of normal cellular sequences from chromosome 13 (in the case of retinoblastoma, osteosarcoma, breast cancer and small cell lung cancer), chromosome 11 (in the case of Wilms' tumor and bladder cancer and breast cancer), chromosome 1 (in the case of melanoma), chromosome 22 (in the case of acoustic neuroma), chromosome 3 (in the case of renal cancer and small cell carcinoma of the lung) and chromosome 17 (in the case of colorectal carcinoma) have been associated with malignancy. It appears that this method might be generally applied to the analysis of inherited susceptibility to cancer and may therefore be informative in risk assessment for lung cancer. High molecular weight DNA samples from tumor and normal tissue have been collected from more than 60 cancer patients for restriction enzyme digestion and Southern analysis. Initial experiments centered on examination of genes located on the short-arm of chromosome 11; loss of allelic fragments during tumorigenesis was detected at the cellular Harvey ras locus, the insulin locus, the calcitonin locus, the beta-globin locus, the catalase locus and the Int-2 locus (homologous to the MMTV locus). These observations have been extended to define commonly deleted regions by the shortest overlapping region of deletion analysis. In addition, polymorphic genetic loci on five other chromosomes (3, 13, 17, 18 and 20) have been studied. Examinations of "rare" allelomorphs at the Harvey ras locus and the debrisoquine metabolizer phenotype have been made in a case control study to determine the use of these polymorphisms as a lung cancer risk factor. Rare Ha-ras DNA allelomorphs and the extensive debrisoquine phenotype substantially increase the relative risk of lung cancer in an independent manner.

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

Ainsley Weston	Visiting Associate	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Haruhiko Sugimura	Visiting Fellow	LHC	NCI
Neil Caporaso	Medical Staff Fellow	EEB	NCI
Robert Hoover	Chief	EEB	NCI

Objectives:

To examine the DNA restriction patterns of normal and lung tumor tissues with human genomic and cDNA probes. Initial studies focused on genetic loci assigned to the short (p) arm of chromosome 11, since deletions in this region of the genome have been shown to be associated with a variety of malignant conditions. Polymorphic loci throughout the human genome have also been examined to determine whether specific genetic polymorphisms are associated with carcinogenesis per se or whether deletion of genetic loci in carcinogenesis is part of a more general mechanism.

Methods Employed:

High molecular weight DNA was extracted from fresh or frozen tissues by gentle mechanical disruption of the tissue; enzyme digestion to degrade protein and RNA; phenol, phenol:chloroform extractions; alcohol precipitation and spooling high molecular weight materials.

Restriction analysis was performed by the method of Southern blotting. DNA was digested to completion with appropriate restriction enzymes, phenol extracted and subjected to agarose gel electrophoresis. The DNA was transferred to nitrocellulose filters that were baked to immobilize the DNA. The filters were hybridized to cloned human genomic or cDNA fragments of known genetic loci, which were previously radiolabeled to high specific activity with ³²P according to the random primer method or Nick-translation. X-ray films were exposed to the filters in light-proof cassettes at -70°C for periods of between 4 hr and 6 days.

Major Findings:

The hypothesis that "rare" variable nucleotide tandem repeat alleles of the Ha-ras-1 polymorphism are an inherited predisposing factor in human lung carcinogenesis has been evaluated in an age, race and tobacco smoking matched case-control study. Twenty-three different alleles were identified by their restriction fragment length in DNA isolated from peripheral blood lymphocytes and were categorized into three groups; common, intermediate and rare. The relative risk of lung cancer for individuals with one rare allele was found to

be 1.9 - 2.0 (for individuals with two rare alleles this value rose to 3.6 - 6.1), depending on histological classification. The frequencies of rare alleles in patients with either squamous cell carcinoma or small cell carcinoma were found to be significantly higher than those patients with either other types of lung cancer or two control groups comprised of chronic obstructive pulmonary disease patients and non-lung cancer patients.

We tested the hypothesis that the genetically determined ability to metabolize debrisoquine is related to lung cancer risk in a case-control study. Overall, black (odds ratio = 4.6 [95% confidence interval 1.3 - 16.9]) and white (odds ratio = 8.1 [95% confidence interval 1.9 - 34.1]) extensive metabolizers are at significantly increased risk. Considered separately, black and white extensive metabolizers were at significantly increased risk after adjustment for age and smoking (odds ratio = 6.1 [95% confidence interval 1.2 - 29.8] and 12.3 [95% confidence interval 2.2 - 67.6], respectively). A significant association is present when either subjects with chronic obstructive pulmonary disease or a group of subjects with a variety of other cancers were considered the controls. The classification into three metabolic phenotypes based on the metabolic ratio (MR) is controversial; a ranking statistic and stepwise regression technique are presented as alternate approaches to demonstrate a significant association of the MR with lung cancer risk. The determination of the debrisoquine MR is unrelated to age, anthropomorphic measures, recent diet, or clinical laboratory determinations or renal or liver function in the controls in our study. In particular, questionnaire and biochemical measures of smoking are unrelated to the debrisoquine metabolic ratio.

Analysis by shortest region of overlapping deletion has been used to identify two commonly deleted regions of chromosome 11 in non-small cell lung cancer (11q13-11p13 and 11p15.5). Analysis of polymorphic loci on chromosomes 3, 13, 17, 18 and 20 showed constitutional loss of heterozygosity for chromosome 17p in 8/9 squamous cell carcinomas but only 2/11 adenocarcinomas and 1/3 large cell carcinomas. Coincident loss of heterozygosity was observed in 7/8 squamous cell carcinomas for chromosomes 11 and 17; in addition, chromosome 3 deletions were accompanied by chromosome 11 or 17 deletions in more than 50% of squamous cell carcinoma cases that were informative for both chromosomes. Mitotic recombination was a relatively infrequent mechanism for loss of heterozygosity, although evidence of mitotic recombination was found in two cases of adenocarcinoma at 13qter-13q33, distal to the retinoblastoma locus. The int-2 locus was amplified in 1 of 54 cases (exactly the same number of cases that has been found in stomach cancer). Since in a number of tumor types consistent loss of specific genes suggests the existence of putative tumor suppressor genes and since in the lung tumors examined here loss of heterozygosity was seen for many of the previously identified candidate tumor suppressor gene regions (for example, 3p, 11p13, 11p15.5, 13q and the region of 17p that is proximal to the p53 locus), it may be that a more general mechanism of carcinogenesis, involving loss of tumor suppressor genes and activation and amplification of proto-oncogenes, operates in human non-small cell carcinoma of the lung.

A portion (8 cases examined for loss of heterozygosity at the 17p locus) of these data have been independently confirmed at the oncology center, Johns Hopkins University School of Medicine, Baltimore, by Dr. B. Vogelstein and his co-workers.

Publications:

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

PROJECT NUMBER

Z01CP05505-05 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

In Vitro Transformation of Human Bronchial Epithelial Cells

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

P.I.:	Curtis C. Harris	Chief	LHC	NCI
Others:	Ke Yang	Visiting Fellow	LHC	NCI
	Brenda I. Gerwin	Research Chemist	LHC	NCI
	Andrea Pfeifer	Special Volunteer	LHC	NCI

COOPERATING UNITS (if any)

Fox Chase Cancer Center, Philadelphia, PA (A. Klein-Szanto); Medical College of Ohio, Toledo, OH (G. Stoner); Children's Medical Research Foundation, Camperdown, Australia (R. Reddel)

LAB/BRANCH

Laboratory of Human Carcinogenesis

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Molecular Genetics and Carcinogenesis Section

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NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

1.5

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.)

Six families of proto-oncogenes, ras, raf, fur, jun, neu and myc have so far been associated with human lung cancer. Human bronchial epithelial cells in vitro are being used to investigate the functional role of these specific oncogenes and growth regulatory genes in carcinogenesis and tumor progression. When transferred into normal human bronchial epithelial cells (NHBE) by the highly efficient protoplast fusion method, the v-Ha-ras oncogene initiates a cascade of events in the normal human bronchial cells leading to their decreased responsiveness to inducers of squamous differentiation, aneuploidy, and less frequently, "immortality" and tumorigenicity with metastasis in athymic nude mice. Transfection of the SV40 T antigen gene leads to nontumorigenic cell lines that have a nearly normal pathway of terminal squamous differentiation and can be readily transformed to malignant cells by transfected Ha-ras, N-ras or Ki-ras. The combination of transfected c-myc and c-raf-1 will also cause transformation of human bronchial epithelial cells to neoplastic cells that exhibit some phenotypic traits found in small cell carcinomas. These and other results indicate that proto-oncogenes dysregulate pathways of growth and differentiation of human bronchial epithelial cells and play an important role in human lung carcinogenesis.

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

Ke Yang	Visiting Fellow	LHC	NCI
Brenda I. Gerwin	Research Chemist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Andrea Pfeifer	Special Volunteer	LHC	NCI

Objectives:

To establish cell culture models of the development of human lung cancer. In particular, the objective of this ongoing project is to develop nontumorigenic human bronchial epithelial (BE) cell lines for the systematic investigation of the relationship between differentiation and carcinogenesis and of the effects of chemical carcinogens, cloned oncogenes, and tumor oncogenes.

Methods Employed:

This project utilizes techniques developed in this laboratory, including culture of human BE cells in serum-free media and transfection of these cells with plasmid DNA via strontium phosphate coprecipitation. Cell lines have been characterized by Southern analysis, Northern analysis, indirect immunofluorescence, Western blotting, light and electron microscopy, and isoenzyme and karyotype analysis. In vivo growth potential has been tested by subcutaneous (sc) injection into athymic nude mice and an assay of ability to re-epithelialize rat tracheas implanted in nude mice. Colony forming efficiency has been examined in the presence of agents that induce squamous differentiation in NHBE cells. In addition to measuring metastases in athymic nude mice, in vitro invasion and collagenase IV activity have been determined.

Major Findings:

Activated ras oncogenes have previously been implicated in the pathogenesis of human lung carcinomas. A v-Ha-ras-containing retrovirus, Zip-ras, was generated by inserting the coding region of the v-Ha-ras oncogene into the ZipNeoSV(X) retroviral vector. Amphotrophic Zip-ras retrovirus was used to infect an SV40 large T antigen-positive immortalized cell line, BEAS-2B, derived from normal bronchial epithelial cells, the predominant progenitor cells of human lung carcinomas. Zip-ras-infected BEAS-2B cells selected for G418 resistance formed anaplastic carcinomas in 12/15 athymic nude mice with a latency of 3 weeks, whereas ZipNeoSV(X)-infected BEAS-2B control cultures inoculated into 12 nude mice have formed no tumors after a minimum of 7 months. Tumor cell lines were established and demonstrated to be of human epithelial origin and to express v-Ha-ras p21 protein. A common feature of the tumor cell lines was an increase in ploidy. The increased efficiency of neoplastic transformation by v-Ha-ras of cell lines as compared to our previous results with normal bronchial epithelial

cells is consistent with the hypothesis that the "immortalization" step is rate-limiting in in vitro human epithelial cell carcinogenesis.

Many human bronchial adenocarcinomas have been shown to contain an activated Ki-ras oncogene. To test the hypothesis that activated Ki-ras may be causally related to human bronchial carcinogenesis, v-Ki-ras oncogene was transferred into an established human bronchial epithelial cell line, BEAS-2B, by infection with Kirsten murine sarcoma virus (Ki-MSV) or by transfection with a plasmid containing the transformed region of Ki-MSV. These cells formed poorly differentiated adenocarcinomas in athymic nude mice. Cell lines established from these tumors expressed v-Ki-ras p21 protein and were highly tumorigenic. Whereas serum or transforming growth factor β_1 (TGF- β_1) induced the BEAS-2B cells at clonal density to undergo growth arrest and squamous differentiation, BEAS-2B cells containing activated ras genes were unaffected by TGF- β_1 and were mitogenically stimulated by serum.

The in vivo growth behavior and invasive potential of normal and "immortalized" human bronchial epithelial cells were studied by xenotransplantation procedures, an in vitro assay of invasiveness, and determinations of type IV collagenase activity and mRNA expression. BEAS-2B cells, immortalized after hybrid virus infection (adenovirus 12-simian virus 40), reconstituted a columnar epithelium when xenotransplanted into de-epithelialized rat tracheas transplanted sc into athymic BALB/c mice. A few adenomatous growths could be seen 16 weeks after transplantation. BZR cells, obtained by transfer of the v-Ha-ras oncogene into BEAS-2B cells, were tumorigenic in this xenotransplantation model. BZR-T33 cells, obtained from a tumor produced after injection of BZR cells, were also tumorigenic; however, they exhibited a shorter latent period. When these same cell lines were injected sc and intravenously into athymic BALB/c mice, BEAS-2B cells were not tumorigenic, and the BZR-T33 cells were more tumorigenic than the BZR cells. The incidence of spontaneous metastases after sc inoculation was zero for BEAS-2B cells, 33% for BZR cells, and 100% for BZR-T33 cells. Similar increasing values that correlated well with the data on in vivo growth were noted in the in vitro invasion assay, the collagenolytic ability, and the mRNA expression of type IV collagenase. NHBE cells showed the lowest values in all the assays. These progressive changes occurring in cells derived from the same parental line indicate that the presence of the v-Ha-ras oncogene in immortalized bronchial cells is associated with a full-fledged malignant phenotype, which is further enhanced by in vivo passaging.

The immortalized human bronchial epithelial cell line BEAS-2B was used to test the effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on the invasive ability of non-malignant cells. These cells are characterized by slow in vivo growth and a very low ability to traverse matrigel-coated filters. Treatment of tracheal transplants repopulated with BEAS-2B cells in nude mice showed an enhanced proliferation of epithelial cells after TPA treatment. In addition, a statistically significant number of epithelial downgrowths were found in the reconstituted epithelium after 1 or 2 weeks of in vivo TPA treatment. An enhancement of the invasive capacity 20- to 25-fold was observed in TPA-treated BEAS-2B cells. A similar result was obtained after in vitro treatment with

diacylglycerol. Furthermore, the protein kinase C inhibitor H-7 reverted the effect of TPA, pointing to a possible role of protein kinase C activation in the phorbol-induced invasion enhancement.

Overexpression of *c-raf-1* and the *myc* family of proto-oncogenes is primarily associated with small cell carcinoma which accounts for about 25% of human lung cancer. To determine the functional significance of the *c-raf-1* and/or *c-myc* gene expression in lung carcinogenesis and to delineate the relation between proto-oncogene expression and tumor phenotype we introduced both proto-oncogenes, alone or in combination, into human bronchial epithelial cells. Two retroviral recombinants, pZip-*raf* and pZip-*myc*, containing the complete coding sequences of the human *c-raf-1* and murine *c-myc* genes, respectively, were constructed and transfected into SV40 T antigen immortalized bronchial epithelial cells (BEAS-2B) followed by the selection for G418 resistance. BEAS-2B cells expressing both the transfected *c-raf-1* and *c-myc* sequences formed large cell carcinomas in athymic nude mice with a latency of 4-21 weeks, whereas either pZip-*raf*- or pZip-*myc*-transfected cells were nontumorigenic after 12 months. Cell lines established from tumors (designated RMT) revealed the presence of the co-transfected *c-raf-1* and *c-myc* sequences and expressed morphological, chromosomal and isoenzyme markers which identified BEAS-2B cells as the progenitor line of the tumors. A significant increase in the mRNA levels of neuron-specific enolase was detected in BEAS-2B cells containing both the *c-raf-1* and *c-myc* genes and derived tumor cell lines. The data demonstrate that the concomitant expression of the *c-raf* and *c-myc* proto-oncogenes causes neoplastic transformation of human bronchial epithelial cells resulting in large cell carcinomas with certain neuro-endocrine markers. The presented model system should be useful in studies of molecular events involved in multistage lung carcinogenesis.

Publications:

Amstad P, Reddel RR, Pfeifer A, Malan-Shibley L, Mark GE, Harris CC. Neoplastic transformation of a human bronchial epithelial cell line by a recombinant retrovirus encoding viral Harvey *ras*. *Mol Carcinogenesis* 1988;1:151-60.

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Harris CC, Gerwin B, Ke Y, Masui T, Miyashita M, Pfeifer A, Reddel R, Wilson VL, Lechner JF. Growth, differentiation, and neoplastic transformation of human bronchial epithelial cells. In: Moses HL, Lengyel P, Stiles CC, eds. Growth factors and their receptors: genetic control and rational application. New York: Alan R Liss (In Press).

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Harris CC, Reddel R, Pfeifer A, Amstad P, Mark G, Weston A, Modali R, Iman D, McMenamin M, Kaighn E, Gabrielson E, Jones R, Trump BF. Oncogenes and tumor suppressor genes in human lung carcinogenesis. In: Harris CC, Liotta L, eds. Genetic mechanisms in carcinogenesis and tumor progression. New York: Alan R Liss (In Press).

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

PROJECT NUMBER

201CP05508-05 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Isolation of Tumor Suppressor Genes by Subtraction Libraries

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

P.I.:	Curtis C. Harris	Chief	LHC	NCI
Others:	Chad Giri	Guest Researcher	LHC	NCI
	Deborah S. Iman	IRTA Fellow	LHC	NCI
	John Lechner	Section Chief	LHC	NCI
	Hiroyasu Ogawa	Visiting Fellow	LHC	NCI

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Laboratory of Human Carcinogenesis

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NCI, NIH, Bethesda, Maryland 20892

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.)

Genes controlling terminal differentiation are one class of putative tumor suppressor genes. In order to identify genes associated with the terminal differentiation of epithelial cells, two cDNA libraries are being constructed. The first library is from R.1 cells which do not differentiate in response to serum or transforming growth factor (TGF)-beta. The second is from S.6 cells treated with TGF-beta, which induces their differentiation. To investigate differences in gene expression in these cells, a subtractive hybridization approach will be taken. Briefly, in vitro synthesized RNA from one library and cDNA from the other library will be hybridized to subtract out common messages, and unique single-stranded messages will be subcloned to make a third, "subtracted" library. The clones from the subtracted library can then be tested for their ability to induce differentiation and/or to suppress tumorigenicity in normal or neoplastic human bronchial epithelial cell lines.

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

Deborah S. Iman	IRTA Fellow	LHC	NCI
Hiroyasu Ogawa	Visiting Fellow	LHC	NCI
John Lechner	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Chad Giri	Guest Researcher	LHC	NCI

Objectives:

The specific goal of this project is to identify genes involved in terminal differentiation of human bronchial epithelial cells. If tumorigenesis is due to a defect in the differentiation pathway, then introducing a gene necessary for differentiation into a tumor cell may serve to suppress the tumorigenic phenotype. In order to identify these genes, a subtraction hybridization approach will be taken using a cDNA library from differentiation defective cells and a library from responsive cells induced to differentiate.

Methods Employed:

The BEAS-2B cell line was developed by immortalizing normal human bronchial epithelial (NHBE) cells with SV40 virus. Subclones of this line were isolated which were either resistant (R.1) or sensitive (S.6) to inducers of differentiation such as serum or TGF- β . Libraries of S.6 cells treated for 4 hr with TGF- β and of R.1 cells will be constructed by vector-primed cDNA synthesis based on the Okayama-Berg protocol using the vector described below.

Major Findings:

A vector has been developed with several important characteristics. First, it permits expression of cDNA inserts in mammalian cells. Second, it contains both an SP6 polymerase promoter and a T7 RNA polymerase promoter oriented in opposite directions and separated by a multiple cloning site for insertion of the cDNA. This allows RNA to be made in vitro for the subtraction. Third, the vector contains the Epstein-Barr virus origin of replication and a gene coding for hygromycin resistance so that the plasmid can be maintained stably and episomally in mammalian as well as in bacterial cells. Having these features will allow us to directly select for the expression of even rare cDNA clones in mammalian cells and, more importantly, to recover these episomes for further characterization.

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

PROJECT NUMBER

Z01CP05541-02 LHC

PERIOD COVERED

October 1, 1988 to September 1989

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

Growth of Human Hepatocytes

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

P.I.:	John F. Lechner	Section Chief	LHC	NCI
Others:	Katherine E. Cole	Special Volunteer	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI
	Andrea M. Pfeifer	Special Volunteer	LHC	NCI

COOPERATING UNITS (if any)

Children's Medical Research Foundation, Camperdown, Australia (R. Reddel)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

2.0	1.5	0.5
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CHECK APPROPRIATE BOX(ES)

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

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

Serum-free PFMR-4 medium was found to support the long-term multiplication of normal human liver epithelial cells. Human liver epithelial cells from three separate autopsy cases were induced to divide for up to 12 divisions (4 passages). At passage 4 these cells were positive for cytokeratin 18 and a small proportion was also positive for albumin.

Additionally, human hepatocytes in primary culture were transfected with SV40 large T antigen gene. The life span of these cells was extended to 40 population doublings at which time they became quiescent. These transfected cells were also positive for cytokeratin 18, T antigen and a small number were positive for albumin.

Infection with a zip-neo virus containing the SV40 large T antigen gene has yielded a cell line that has also shown an extended doubling potential of greater than 40 population doublings and has to date shown no indications of senescence. These cells are positive for cytokeratin 18 and T antigen.

Differentiation studies with the normal and SV40 transformed human liver epithelial cells using intrasplenic nude mouse injections and in vitro 3-dimensional collagen matrices are being employed in order to induce the liver epithelial cells to re-express a more differentiated phenotype.

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

Katherine E. Cole	Special Volunteer	LHC	NCI
John F. Lechner	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Andrea M. Pfeifer	Special Volunteer	LHC	NCI

Objectives:

To establish replicative cultures of human hepatocytes through the development of optimal medium formulations. To characterize the proliferating cells for specific markers of differentiated hepatocytes. Additionally, studies are directed towards the development and subsequent characterization of a transformed human liver epithelial cell line through transfection or infection of human hepatocytes in primary culture with SV40 T antigen DNA. The normal and transformed cell lines will be used to study metabolism and possible sensitivity to known carcinogens.

Methods Employed:

In order to optimize factors responsible for human liver cell proliferation, studies using a malignant human liver cell line (HEPG2) and human hepatocytes transformed with SV40 large T antigen (THLE) as models have been employed. Human hepatocytes in primary culture are transfected with recombinant plasmids containing the SV40 large T antigen gene linked to the Rous sarcoma virus long terminal repeat (THLE-1). Additionally, human hepatocytes are infected with a zip-neo virus containing the SV40 large T antigen gene (THLE-2). Known growth factors, as well as unknown growth factors, contained in conditioned medium from normal, transformed and malignant liver cells are evaluated using an assay for DNA synthesis of HEPG2 and THLE cells. Morphology of the normal and transformed replicating liver cells is evaluated using light and electron microscopy. Markers for epithelial cells are identified using fluorescent staining techniques. Markers specific for differentiated hepatocyte phenotype are examined using fluorescent staining, in situ hybridization techniques and Northern blotting. In order to induce the normal replicating liver cells to express a more differentiated phenotype, HEPG2 cells and THLE cells are used as models and are injected into the spleen of nude mice and evaluated for hepatocyte-specific markers using alkaline phosphatase immunocytochemical staining and in situ hybridization. In vitro differentiation models using the HEPG2 cells and THLE cells seeded onto collagen/fibroblast 3-dimensional matrices are also evaluated for hepatocyte-specific markers using immunocytochemical staining and in situ hybridization.

Major Findings:

This project has three major findings: (1) development and partial optimization of a serum-free medium that supports long-term multiplication of human liver epithelial cells. This medium is a modification of Ham's F-12 medium supplemented with fatty acids, insulin, hydrocortisone, epidermal growth factor, cholera toxin, 0.25% pituitary extract, 10% chemically denatured serum and 35% conditioned medium from HEPG2 cells. The human liver cells cultured in this medium have been shown to express general cytokeratins, cytokeratin 18 and albumin; (2) development of a transformed human liver epithelial cell using both transfection (THLE-1) and infection (THLE-2) of human hepatocytes with the SV40 large T antigen gene. These two cell lines have been shown to express general cytokeratins, cytokeratin 18, cytokeratin 19, T antigen and albumin (THLE-1 only; THLE-2 not tested); (3) since normal and transformed human liver cells induced to divide in culture have shown a progressively differentiating phenotype, we have developed a differentiation model using THLE-2 and HEPG2 cells by injecting them into the spleen of a nude mouse or placing them in a 3-dimensional collagen/fibroblast matrix. Both cell lines will divide and grow in the collagen matrices, and the HEPG2 cells are able to grow within the spleen of the nude mouse maintaining a hepatocyte-like appearance.

Publications:

Lechner JF, Cole KE, Reddel RR, Anderson LJ, Harris CC. Replicative cultures of adult human and Rhesus monkey liver epithelial cells. *Cancer Detect Prevent* (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05542-02 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Assessment of Tobacco Smoke Genotoxicity

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

P.I.:	Curtis C. Harris	Chief	LHC	NCI
Others:	James C. Willey	Expert	LHC	NCI
	Norio Matsukura	Expert	LHC	NCI
	Bonita G. Taffe	IRTA Fellow	LHC	NCI

COOPERATING UNITS (if any)

Eleanor Roosevelt Research Institute, Denver, CO (C. Waldren and T. Puck)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

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

We have employed Chinese hamster ovary (CHO) cells containing a human chromosome 11 (termed AL hybrid cells) as a highly sensitive detection system for mutagenesis in an effort to evaluate possible direct mutagenic effects of cigarette smoke condensate (CSC) and its fractions. Cytotoxicity induced by N-methyl-N'-nitro-N-Nitrosoguanidine (MNNG) in AL cells increased with time of incubation. The 50% inhibitory concentration of MNNG after 1, 3, 6 and 20 hr of incubation was 0.8, 0.4, 0.2 and 0.1 micromoles, respectively. Mutagenicity increased with dose and time reaching a maximum of 1,250 mutants/100,000 survivors (800% above background) after 3 hr of incubation with 2 micromolar MNNG and a maximum of 1,700 mutants/100,000 survivors (1,100 % above background) after 20 hr of incubation with 0.2 micromolar MNNG. The cytotoxicity of CSC increased with increasing incubation time with 50% inhibitory concentrations of 100, 80, 50 and 30 micrograms/ml after 1, 3, 6 and 20 hr of incubation, respectively. CSC mutagenicity increased with time of incubation up to 3 hr with a maximum of 300 mutants/100,000 survivors (250% above control) after incubation with 100 micrograms/ml CSC (p value less than 0.0005, Student's t-test). Cytotoxicity and mutagenicity of CSC were inversely proportional to cell density, while cytotoxicity and mutagenicity of MNNG were unaffected by cell density. The 3 hr incubation time and 50% inhibitory concentration of the acidic fraction of CSC (30 micrograms/ml) induced 350 mutants/100,000 survivors (a 230% increase above background, p value less than 0.0005). The basic and neutral fractions caused a much lower increase at the 50% inhibitory concentration (80 and 200 micrograms/ml, respectively). The possible role of oxy-radicals generated by tobacco smoke condensate and its fractions in mutagenesis of AL hybrid cells is being investigated.

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

Curtis C. Harris	Chief	LHC	NCI
James C. Willey	Expert	LHC	NCI
Norio Matsukura	Expert	LHC	NCI
Bonita G. Taffe	IRTA Fellow	LHC	NCI

Objectives:

To study effects of cigarette smoke and cigarette smoke fractions on human chromosomal genes. The marker genes used in this system are present on human chromosome 11 in a CHO hybrid cell. Because the CHO cell does not require the human marker chromosome for normal cell division, mutations that might not be detectable in other systems due to lethality, such as large deletions or rearrangements, are efficiently scored using this system.

To evaluate the significance of direct mutagenicity resulting from CSC. Due to the increased sensitivity of this assay, it may be possible to detect direct mutagenicity that is not detectable by other assays.

To determine what types of mutations are occurring most commonly in response to CSC, i.e., deletions, rearrangements, or point mutations.

To investigate the lack of quantitative correlation between mutagenicity (tested by the Ames Salmonella assay) and carcinogenicity (animal studies) caused by cigarette smoke condensate and its fractions. For instance, while the neutral fraction of CSC is the most carcinogenic, it is poorly mutagenic, even after activation; and while the weakly acidic fraction is the most mutagenic, it is weakly carcinogenic.

Methods Employed:

The mutagenesis assay employs Chinese hamster ovary cells that have acquired a human chromosome 11 through somatic cell hybridization. There are three surface antigen genes on chromosome 11, termed a1, a2, and a3. Cells that acquire a mutation in one of these genes loses the corresponding cell surface marker. If a population of wild-type and mutated cells is then exposed to antibodies to the surface marker in the presence of complement, only those cells that possess a mutated gene and are unable to produce the surface antigen will survive. The hybrid cells are routinely cultured in medium F-12 with 8% FCS. Cells are seeded at 1×10^5 cells/60 mm dish; two days later, cells are incubated with medium containing the test compound with or without 8% FCS for 3 hr. When the cells are pre-confluent, they are subcultured as necessary for 7-14 days for expression of cell surface antigen gene mutational events; then they are dissociated and seeded at 5×10^4 cells/dish. Cells are then incubated in

medium containing antibody and complement. Six to seven days later the cells are fixed and stained.

Major Findings:

1. CSC direct mutagenicity increases with time and concentration of exposure.
2. Most of the direct mutagenicity of CSC is in the acidic fraction.
3. The cytotoxicity and mutagenicity of CSC decrease with increasing cell density.

Publications:

Miyashita M, Willey JC, Sasajima K, Lechner JF, LaVoie LJ, Hoffmann D, Smith M, Trump BF, Harris CC. Differential effects of cigarette smoke condensate and its fractions on cultured normal and malignant human bronchial epithelial cells. Exp Pathol (In Press).

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

PROJECT NUMBER

Z01CP05543-02 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Tumor Suppression and Somatic Cell Genetics

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

PI:	Curtis C. Harris	Chief	LHC	NCI
Others:	Deborah S. Iman	IRTA Fellow	LHC	NCI
	Edward Kaighn	Expert	LHC	NCI

COOPERATING UNITS (if any)

Francis Scott Key Medical Center, Baltimore, MD (E. Gabrielson)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

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.)

The goal of this project is to determine whether normal cells contain genes that have the ability to suppress the tumorigenicity of human lung carcinoma cell lines. Hybrid cell lines between HuT-292DM, a human lung carcinoma line, and either normal human bronchial epithelial cells (NHBE) or an SV40 "immortalized" but non-tumorigenic derivative thereof (BEAS-2B) have been isolated by double selection. Hybrids of NHBE and HuT-292DM cells had an extended culture life span, but senesced after approximately 60 population doublings (PDs) as compared to the 30-35 PDs seen with NHBE cells. In contrast, hybrids of BEAS-2B and HuT-292DM showed no sign of a culture "crisis" and have an indefinite life span. HuT-292DM cells produced tumors in 100% of the nude mice with a latency of 27 days, whereas tumorigenicity was suppressed 80% in BEAS-2B x HuT-292DM hybrids, with a two- to threefold increased latency for the remaining 20%. When serum responsiveness was examined, HuT-292DM cells were stimulated, while NHBE and BEAS-2B cells were inhibited by serum. The BEAS-2B x HuT-292DM hybrids' growth response to serum was similar to that of HuT-292DM cells. Thus, tumorigenicity and culture longevity are dominantly controlled by the nontumorigenic parent, while serum responsiveness resembles that of the tumorigenic parent.

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

Deborah S. Iman	IRTA Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Edward Kaighn	Expert	LHC	NCI

Objectives:

The overall goal is to determine whether there are genes present in NHBE cells which are able to suppress the tumorigenicity of the human mucoepidermoid carcinoma cell line HuT-292DM. To achieve this, somatic cell hybrids are generated by fusing this lung carcinoma line with normal or immortalized bronchial epithelial lines. The tumorigenic phenotype of the hybrids is then assessed by injection into nude mice. Other characteristics such as culture life span, response to serum, and karyotype are also being studied to see whether there is a feature which correlates with tumorigenicity.

Methods Employed:

Media and methodology for the culture of both normal and neoplastic bronchial epithelial cell lines were previously developed in this laboratory. Fusions have been performed between 1) NHBE cells and HuT-292DM (HPRT-, ouabain-resistant); 2) BEAS-2B, a non-tumorigenic, immortalized bronchial epithelial cell line and HuT-292DM; and 3) B39-TL, a mouse tumor line resulting from injection of late passage BEAS-2B and HuT-292DM. Fusions were carried out both in monolayer and in suspension by treatment with polyethylene glycol. One day later, selective medium (containing hypoxanthine/aminopterin/thymidine and ouabain) was added. The hybrid clones were expanded and culture life span, tumorigenicity, response to serum, and karyotypes were analyzed.

Major Findings:

Evidence that the cells isolated after polyethylene glycol fusion are hybrids includes 1) growth in double selective medium, 2) increased chromosome number, and 3) the presence of marker chromosomes of both parents. Hybrids between normal and neoplastic cells senesced, while those formed between immortalized and neoplastic cells showed no signs of senescence. Tumorigenicity was suppressed in hybrids between HuT-292DM and BEAS-2B (77%) and in hybrids of HuT-292DM and B39-TL (52%). Tumor latency in the hybrids that formed tumors was 3 times longer than in the tumorigenic parent. The nontumorigenic hybrids were shown to undergo squamous differentiation in nude mice. Serum, which stimulates HuT-292DM and inhibits BEAS-2B, was found to either not affect or stimulate the hybrid lines. This serum response did not correlate with tumorigenic potential.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05610-01 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Oxy-radicals and Aldehydes in Carcinogenesis

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

P.I.:	Curtis C. Harris	Chief	LHC	NCI
Others:	Bonita G. Taffe	IRTA Fellow	LHC	NCI
	Andrew C. Povey	Visiting Fellow	LHC	NCI
	James C. Willey	Expert	LHC	NCI
	Norio Matsukura	Expert	LHC	NCI

COOPERATING UNITS (if any)

Karolinska Inst., Stockholm, Sweden (R. Grafstrom); Microbiol. Assoc. Inc., Rockville, MD (R. Curren); Am. Hlth. Fdn., Valhalla, NY (D. Hoffmann); Nippon Med. Sch., Tokyo, Japan (M. Miyashita); Eleanor Roosevelt Inst. (C. Waldren, T. Puck)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

3.0

2.0

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Oxy-radicals damage DNA and may be involved in lung carcinogenesis. Highly sensitive and specific methods are needed for molecular dosimetry of this damage in epidemiological studies. A new method is in development for detection of oxidative modification to DNA, using the 32-P-postlabeling technique to detect 8-OH-deoxyguanosine phosphate. This method will detect one modification in 10 x 6 nucleotides.

Aldehydes are present in tobacco smoke and are metabolites of carcinogenic N-nitrosamines. Formaldehyde, acetaldehyde and acrolein have each been shown to cause DNA damage and mutations in human lung cells in vitro. Therefore, aldehydes may contribute to the multistage process of lung carcinogenesis.

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

Curtis C. Harris	Chief	LHC	NCI
Bonita G. Taffe	IRTA Fellow	LHC	NCI
Andrew C. Povey	Visiting Fellow	LHC	NCI
James C. Willey	Expert	LHC	NCI
Norio Matsukura	Expert	LHC	NCI

Objectives:

To examine the relationship between oxidative modification to DNA and the process of carcinogenesis. Oxidant exposure has been shown to result in a variety of cellular responses including mutation, promotion and progression of carcinogenesis, cell death, and cellular differentiation. Although a variety of oxidative DNA adducts have been identified, the oxidative lesions responsible for mutation or other phenotypic alterations have not been characterized (in mammalian systems). Modification of DNA as well as phenotypic response to cigarette smoke condensate (CSC) (a complex mixture of oxidants and mutagens), aldehydes, and reactive alkenes (such as fecapentaene), are under investigation in human cells in vitro. Characterization of the mutations produced by oxidants will be studied in the AL cell line. Examination of DNA from oxidant-exposed human populations is planned to correlate exposure with risk for acquiring cancer.

Methods Employed:

The human bronchial epithelial cell culture system developed in this laboratory has been utilized to study the effects of aldehydes and CSC on cellular phenotype. Human fibroblasts from xeroderma pigmentosum patients have also been used. The phenotypic changes which have been studied include cytotoxicity, terminal differentiation and mutation, all contributing events in the process of carcinogenesis. Parameters which have been assessed to measure phenotypic changes include reduction of colony forming efficiency (a measure of cytotoxicity), DNA strand breakage, DNA-protein cross-linking, 6-thioguanine resistance (a measure of mutation), thiol depletion and/or oxidation, and cross-linking of cellular envelopes (a measure of differentiation of epithelium). Mutation by oxidants will be assessed using the AL cell system, a Chinese hamster ovary cell which contains a human chromosome 11. Mutants which have lost human cell surface antigens coded by chromosome 11 are selected by antibody-complement lysis of non-mutated cells (which express the human cell surface antigens).

Methods for detecting oxidative modification to DNA are in progress and include ^{32}P -postlabelling of 8-OH-deoxyguanosine-3'-phosphate, and high performance

liquid chromatography with electron capture detection for 8-OH-deoxyguanosine-3'-phosphate. The ^{32}P -postlabelling technique is a new method under development in which sensitivity in detection is 1 modified nucleotide per 10^6 , at least 10-fold greater than for electron capture detection. The levels of modification to DNA which are found in vivo will determine which method will be used.

Major Findings:

Acrolein is an oxidizing aldehyde formed during the incomplete combustion of natural products and is contained in the gaseous phase of cigarette smoke, auto emissions and other environmental combustion exposures. Acrolein exposure at micromolar concentrations resulted in cytotoxicity, squamous differentiation, depletion of cellular thiols through conjugation, DNA single strand breaks and DNA-protein cross-links in bronchial epithelium. Mutation of xeroderma pigmentosum fibroblasts occurred following exposure to acrolein, although normal human fibroblasts were not sensitive to the mutagenic effects of acrolein as assessed by 6-thioguanine resistance. The differential sensitivity of normal versus xeroderma pigmentosum fibroblasts may be due to differences in both the cell survival rates and the DNA repair rates following exposure to acrolein.

Cigarette smoke has been shown to contain promoters of carcinogenesis. The effects of the non-gaseous components of cigarette smoke can be studied using CSC. CSC is composed of the residue trapped by bubbling cigarette smoke through phosphate buffered saline. This complex mixture has been further fractionated into neutral, acidic and basic fractions and the effects of these fractions on human bronchial epithelium, the target tissue in bronchogenic cancer versus the effects on lung carcinoma cell lines has been studied. As previously demonstrated by this laboratory, CSC and the neutral and acidic fractions induced terminal squamous differentiation in normal human bronchial epithelium, while the carcinoma cell lines were more resistant to these agents, suggesting a growth advantage for tumor cells. The induction of squamous differentiation by CSC does not appear to be mediated through changes in cytosolic calcium. 12-O-tetradecanoylphorbol-13-acetate (TPA)-like activity is associated with the neutral fraction since this fraction inhibited specific binding to the cellular receptor for TPA. Induction of squamous differentiation by TPA does not appear to involve a free radical mechanism since electron paramagnetic resonance did not detect oxygen radicals in bronchial epithelial cells following exposure to TPA.

Fecapentaene is a fecal mutagen in the Ames mutagenesis assay and was previously shown by this laboratory to be mutagenic in human xeroderma pigmentosum fibroblasts. Micromolar concentrations of fecapentaene decreased levels of low molecular weight thiols, including glutathione, by both direct alkylation as well as oxidation reactions in human fibroblasts. Electron microscopic examination of fecapentaene-plasmid DNA interactions demonstrated both cross-links and strand breaks.

Publications:

Dypbukt JM, Edman CC, Sundqvist K, Kakefuda T, Plummer SM, Harris CC, Grafstrom RC. Reactivity of fecapentaene-12 towards thiols, DNA and these constituents in human fibroblasts. *Cancer Res* (In Press).

Gabrielson EW, Rosen GM, Grafstrom RC, Strauss KE, Miyashita M, Harris CC. Role of oxygen radicals in 12-O-tetradecanoylphorbol-13-acetate-induced squamous differentiation of cultured normal human bronchial epithelial cells. *Cancer Res* 1988;48:822-5.

Grafstrom RC, Dypbukt JM, Willey JC, Sundqvist K, Edman C, Atzori L. Pathobiological effects of acrolein in cultured human bronchial epithelial cells. *Cancer Res* 1988;48:1717-21.

Harris CC, Grafstrom R, Willey JC, Gabrielson E, Lechner J, Somers A, Miyashita M, Sundqvist K, Dypbukt JM, Matsukura N, Trump BF. Pathobiological effects of tobacco smoke, tumor promoters, and asbestos in human lung cells in vitro. In: Cerutti PA, Fridovich I, McCord JM, eds. *Oxy-radicals in molecular biology and pathology*. New York: Alan R Liss, 1988;405-23.

Harris CC, Willey JC, Matsukura N, Lechner J, Miyashita M, Grafstrom RC, Trump BF. Pathobiological effects of fibers and tobacco-related chemicals in human lung cells in vitro. In: Mohr U, Dungworth D, Kimmerle G, Lewkowski J, McClellan R, Stober W, eds. *Assessment of inhalation hazards: integration and extrapolation using diverse data*. New York: Springer-Verlag (In Press).

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

PROJECT NUMBER

Z01CP05611-01 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

In Vitro Studies of Human Mesothelioma

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

P.I.:	Brenda I. Gerwin	Research Chemist	LHC	NCI
Others:	John F. Lechner	Section Chief	LHC	NCI
	Anne Van der Meeren	Visiting Fellow	LHC	NCI
	Teresa Lehman	IRTA Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

Inst. of Occupational Health, Helsinki, Finland (K. Linnainmaa); University of Uppsala, Uppsala, Sweden (C. Betsholtz); Key Med. Ctr., Baltimore, MD (E. Gabrielson); Children's Med. Res. Fdn., Camperdown, NSW, Australia (R. Reddel)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.5	PROFESSIONAL	1.5	OTHER	1.0
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CHECK APPROPRIATE BOX(ES):

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

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

Human mesothelial cells respond to mitogenic signals from a wide range of peptide growth factors. Mesothelial cells from different donors show surprisingly wide interindividual variations in the pattern of their growth factor responsiveness. These differences do not appear to be the result of selection due to growth conditions. Twelve human mesothelioma cell lines have been established. Eight of 12 are tumorigenic in athymic nude mice. Tumors generated by these cell lines have a very long latency (about 8 months) and grow slowly. Although it has been demonstrated that mesothelioma cell lines, but not normal mesothelial cells, produce platelet-derived growth factor (PDGF) AA homodimer, it can be demonstrated that mesothelioma cell lines have lost the mitogenic response to PDGF which is characteristic of normal mesothelial cells. A nontumorigenic, immortalized human mesothelial cell line has been established by transfection of normal human mesothelial cells with an SV40 T antigen construct. Attempts to transform this cell line to tumorigenicity by treatment with asbestos fibers have been unsuccessful, but transfection of the cells with EJ-ras resulted in a cell line that produced rapidly growing tumors in athymic nude mice.

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

Brenda I. Gerwin	Research Chemist	LHC	NCI
John F. Lechner	Section Chief	LHC	NCI
Anne Van der Meeren	Visiting Fellow	LHC	NCI
Teresa Lehman	IRTA Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

- 1) Analysis of the growth factor responsiveness of normal human mesothelial cells and human mesothelioma cell lines.
- 2) Development of additional mesothelioma cell lines and analysis of their tumorigenicity in athymic nude mice.
- 3) Development of a tissue culture model for the generation of tumorigenicity in human mesothelial cells.

Methods Employed:

Normal human mesothelial (NHM) cells and mesotheliomas are grown in culture according to protocols established in this lab. Cells are tested for mitogenic effects by determinations of colony forming efficiency, population doubling time or thymidine incorporation as compared to controls. Gene expression is determined by RNA purification and Northern blotting. Protein expression is determined by immunoprecipitation, PAGE analysis and Western blotting. Tumorigenicity of cell lines is determined by subcutaneous inoculation of viable cells into athymic nude mice.

Major Findings:

Growth Control Studies: It has been shown that NHM cells show a mitogenic response to a wide variety of peptide growth factors. Furthermore, there is a wide interindividual variation in the pattern of response to the same growth factors. These interindividual differences do not result from conditioning of the cells due to exposure to any specific factors. When cells were first tested for their mitogenic response to a wide spectrum of growth factors, then grown for several population doublings in one specific subset of growth factors and retested, the cells exhibited their original growth factor responses. These experiments demonstrated not only that the individual cultures tested displayed varying growth factor responses but that the responsiveness was a characteristic of the cell which was not alterable by growth conditions. Since it had been shown that mesothelioma cells exhibited a mitogenic response to their own conditioned medium and that mesotheliomas produced PDGF-A chain, the mitogenic

effects of PDGF were tested on several mesothelioma cell lines. It was demonstrated that these tumor cell lines did not show a mitogenic response to PDGF in minimal medium or in the presence of half-maximal serum or fibroblast growth factor (FGF). It was shown, however, that both NHM cells and mesotheliomas respond to mitogenic stimulation by FGF. Non-tumorigenic human mesothelial cells immortalized by transfection with SV40 T antigen have been further transfected with constructs of human PDGF A or B chain and have been shown to overexpress the appropriate mRNA. The production of urokinase plasminogen activator (uPA) and plasminogen activator type 1 (PAI-1) has been examined in media with and without transforming growth factor (TGF)- β_1 which has been shown to induce expression of these genes. The constitutive level of expression was not altered by the continued presence of TGF- β_1 . NHM cells showed a high level of PAI-1 expression relative to the level of uPA mRNA and would be expected to show a low level of plasminogen activator activity.

Tumorigenicity Studies:

Mesothelioma cell lines have been established in culture and tested for tumorigenicity in nude mice. Interestingly, not all cell lines were tumorigenic. Of 12 tested, 8 (75%) were tumorigenic. These tumors grew very slowly with a latency of approximately 8 months. However, when tumors arose they were transplantable, and karyotype analysis indicated that they were derived from the inoculated human cell line. NHM cells have not been converted to a tumorigenic phenotype by immortalization with SV40 T antigen or by treatment with asbestos which produced chromosomal aberrations and an extended life span. In addition, asbestos treatment of the immortalized cells also failed to produce tumorigenic variants. It is therefore of interest that transfection of the T antigen-immortalized cells with a construct expressing EJ-*ras* did result in a tumorigenic cell line. This cell line, however, showed a much shorter latency and more rapid growth rate than tumors derived from human mesothelioma specimens.

Tumor Suppressor Studies:

We have obtained probes from the retinoblastoma cDNA clone and a monoclonal antibody for the Rb protein and are proceeding with an analysis of whether loss of normal expression of this gene may be implicated in the development of mesothelioma as it has been in small cell lung cancer, osteosarcoma, and breast carcinoma, as well as in retinoblastoma itself.

Publications:

Gerwin BI, Betsholtz C, Linnainmaa K, Pelin K, Reddel RR, Gabrielson EW, Seddon M, Greenwald R, Harris CC, Lechner JF. In vitro studies of human mesothelioma. In: Nettekheim P, Thomassen D, eds. Biology, toxicology and carcinogenesis of respiratory epithelium. New York: Hemisphere Publishing Co (In Press).

Ke Y, Reddel RR, Gerwin BI, Reddel HK, McMenamin MG, Somers ANA, LaVeck MA, Stahel RA, Lechner JF, Harris CC. Establishment of a human in vitro mesothelial cell model system for investigating mechanisms of asbestos-induced mesothelioma. Am J Pathol (In Press).

LaVeck MA, Somers ANA, Moore LL, Gerwin BI, Lechner JF. Dissimilar peptide growth factors can induce normal human mesothelial cell multiplication. In Vitro Cell Dev Biol 1988;24:1077-84.

Lechner JF, LaVeck MA, Gerwin BI, Matis EA. Differential responses to growth factors by normal human mesothelial cultures from individual donors. J Cell Physiol (In Press).

Reddel RR, Malan-Shibley L, Gerwin BI, Metcalf RA, Harris CC. Tumorigenicity of a human mesothelial cell line transfected with EJ-ras oncogene. JNCI (In Press).

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

PROJECT NUMBER

Z01CP05612-01 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Tumor Suppression in Monochromosome Transfer Hybrids

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

PI:	Curtis C. Harris	Chief	LHC	NCI
Others:	Deborah S. Iman	IRTA Fellow	LHC	NCI
	Teresa A. Lehman	IRTA Fellow	LHC	NCI
	Yuan Su	Visiting Fellow	LHC	NCI
	Ainsley Weston	Visiting Associate	LHC	NCI

COOPERATING UNITS (if any)

Children's Hospital of Michigan, Detroit, MI (W. Peterson)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.5	PROFESSIONAL	1.5	OTHER	1.0
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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.)

The specific goal of this project is to determine whether normal human chromosomes have the ability to suppress the tumorigenicity of a human lung carcinoma cell line. The technique of microcell transfer was used to introduce the human chromosome 11 (with a translocation to the hypoxanthine guanine phosphoribosyl transferase (HGPRT) region of the X chromosome) of a donor cell line into HGPRT-, ouabain-resistant HuT-292DM mucoepidermoid carcinoma cells. Colonies were isolated which grew in selective medium containing hypoxanthine/aminopterin/thymidine (HAT) and ouabain, and tumorigenicity was assayed by injecting these colonies into nude mice. Most microcell hybrids were suppressed in that there was a decreased probability of tumor formation and the tumors that did arise had an increased latency. The 11/X chromosome was shown by restriction fragment length polymorphism (RFLP) analysis and by karyotype analysis to be present in the colonies injected as well as in the tumors which developed. Thus, the portions of chromosome 11 transferred in these experiments were able to partially suppress the tumorigenic phenotype of HuT-292DM lung carcinoma cells.

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

Deborah S. Iman	IRTA Fellow	LHC	NCI
Teresa A. Lehman	IRTA Fellow	LHC	NCI
Yuan Su	Visiting Fellow	LHC	NCI
Ainsley Weston	Visiting Associate	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The goal of this project is to identify which chromosomes, or combinations of chromosomes, contain genes capable of suppressing the tumorigenicity of a human lung carcinoma cell line. To accomplish this, single chromosomes are being introduced into the recipient tumor line HuT-292DM by the technique of microcell transfer. The microcell hybrids are then examined for their tumor phenotype. These experiments will generate suppressed cell lines which will be used to create cDNA libraries. Either a subtraction hybridization or insertional mutagenesis approach will then be taken to determine which messages are expressed in the suppressed cells that are not present in the tumorigenic cells.

Methods Employed:

The technique of microcell transfer was used. Two donor cells were used, MCH110.1 with a human (11q23-11pter::Xq26-Xqter) chromosome and XER-7 with a human (11p13-11qter::Xqter-Xq13) chromosome. Donor mouse cells containing the genetically selectable human chromosome 11/X were treated with colcemid and cytochalasin B. This creates "microcells" containing one or a few chromosomes. The microcells were fused to the recipient tumor cell (HGPRT-, ouabain-resistant) with polyethylene glycol, and selective medium containing HAT and ouabain was used to select the hybrids. The colonies were injected into irradiated nude mice for tumorigenicity assays. Karyotype analyses were done by Ward Peterson at Children's Hospital of Michigan.

Major Findings:

When either of the 11/X donors were used in microcell transfers, most microcell hybrids were suppressed in that there was a decreased probability of tumor formation, and the tumors that did arise had an increased latency. All of the hybrids but one were shown by karyotype analysis to contain the donor 11/X chromosome. The one exception had only the X region and was as tumorigenic as the HuT-292DM recipient. The MCH110.1 - HuT-292DM hybrids were examined by RFLP analysis using a probe for insulin (at 11p15) and found to contain the donor locus in every case except the tumorigenic hybrid which had lost most of the donated chromosome 11. The tumor DNAs were also analyzed and the insulin

banding pattern was found to be identical in the tumor to that of the colony injected. The probe PYGM (11q12-13) was informative for the XER-7 - HuT-292DM hybrids. Again, the donor PYGM locus was present in every hybrid and in the tumors that developed. Since the donated chromosome 11/X has not been lost in the tumors, this chromosome alone is not able to completely suppress the tumorigenic phenotype. It is possible that chromosome 11 as well as a separate chromosome (3, 13, or 17 are possibilities) are needed. Another possibility is that more than one normal chromosome 11 is required for complete suppression (gene dosage theory). These questions are being investigated by further microcell transfer experiments with neomycin-marked chromosomes 3, 13, and 17.

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

PROJECT NUMBER

Z01CP05613-01 LHC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Tissue Equivalent Models for Studying Cellular Interactions and Carcinogenesis

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

PI: John F. Lechner Section Chief LHC NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

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.)

A three dimensional (tissue equivalent) culture model is being developed that will recapitulate more faithfully the in vivo nature of normal airway epithelium. Specifically, mesenchymal cells are being combined with collagen. The metabolic activities of the mesenchyme cells cause changes in the structure of the collagen fibrils resulting in their contraction and producing a mesenchyme-like tissue structure. Subsequently, normal human bronchial epithelial cells are inoculated on the surface of the pseudo-mesenchyme to allow for their differentiation into a muco-ciliary epithelium. A similar three dimensional culture model is being developed that recapitulates more faithfully the in vivo nature of normal liver tissue. These airway epithelium and hepatocellular models are also being developed to study the actions of pre-malignant and malignant epithelial cells when they are in association with normal epithelial cells and a mesenchyme.

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

John F. Lechner	Section Chief	LHC	NCI
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Objectives:

To determine 1) optimal conditions for contraction of collagen gels; 2) the optimal conditions that promote muco-ciliary epithelium differentiation of normal human bronchial epithelial cells in a stable and reproducible manner when they are inoculated onto a contracted collagen gel surface; 3) to create a system to allow liver epithelial cells to interact with one another and organize into their appropriate higher levels of differentiation; and 4) to provide a three dimensional substrate that is better suited for growing, isolating, and purifying subpopulations of cells that are more highly metastatic or have been induced to express cancer suppressor genes.

Methods Employed:

Our basic system uses type IV collagen dissolved in Laboratory of Human Carcinogenesis (LHC)-MM medium made without epidermal growth factor or serum, but with the additions of chemically-denatured serum and transforming growth factor-beta-1 (TGF- β_1). To this mixture is added 0.25 million/ml human lung or foreskin fibroblastic cells. For the airway epithelium investigations, bronchial epithelial cells are inoculated onto the surface of the collagen matrix once it has contracted into a pseudo-mesenchyme. For the liver tissue equivalents investigations, the liver epithelial cells are either inoculated into the gel concurrently with the fibroblastic cells or subsequently onto the surface of the collagen matrix once it has contracted into a pseudo-mesenchyme.

Major Findings:

In order to understand the system and subsequent behavior of the fibroblasts in the gel and to clearly identify the medium constituents that can affect the bronchial epithelial cells, we are evaluating the effects of calcium ions, TGF- β_1 , chemically-denatured serum, fibronectin, epidermal growth factor, fetuin, chondroitin sulfate and collagen types on the degree of gel contraction. Preliminary results indicate that type IV collagen, chemically-denatured serum and high concentrations of calciums are critical for maximal contraction. We have also begun explorations on the role of the fibroblast in gel contraction. Again, preliminary observations suggest that normal skin and lung fibroblasts and normal mesothelial cells induce contraction. On the other hand, bronchial epithelial cells and SV40 T antigen gene-transformed cells cause minimal contraction.

In assessing the efficacy of the tissue equivalent model as a tumor suppression gene assay, in very preliminary experiments, we have found that various neoplastic cell lines grow primarily as "metastatic" colonies, whereas benign cells develop tight, presumably organized structures and normal cells do not produce colonies. Our intent is to isolate individual clones of specific phenotypes and presumed genotype.

Regarding the evolution of a muco-ciliary epithelium, initial experiments revealed that normal human bronchial epithelial cells inoculated on the surface of contracted gels become squamous metaplastic unless the level of retinoic acid exceeds 1 mM. When this was incorporated into the medium, the cells were not squamous and had evidence of acid and neutral mucins.

We have just begun examining conditions necessary for allowing human hepatoblastoma and SV40 T antigen gene-transformed liver epithelial cells to organize into tissue structures. Surprisingly, if these cells are inoculated onto the surface of the contracted pseudo-mesenchymes they appear more liver-like in structure than if they are incorporated into the gel at the time it is cast.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05192-09 LHC, Z01CP05324-07 LHC, Z01CP05409-06 LHC, Z01CP05426-05 LHC, Z01CP05435-05 LHC, Z01CP05480-04 LHC, Z01CP00NEW-BIG LHC, Z01CP00NEW-DSI, Z01CP00NEW-JFL and Z01CP00NEW-BGT

UNIVERSITY OF MARYLAND (N01-CP-95624, REPLACES N01-CP-51000)

Title: Collection and Evaluation of Human Tissues and Cells from Patients with an Epidemiological Profile

Current Annual Level: \$527,710

Man Years: 5.54

Objectives: To provide a resource to the LHC for the procurement, transport, and characterization of normal, preneoplastic, and neoplastic human bronchus, lung, colon, liver and other human tissues (pancreatic duct, placenta, etc.), as requested from surgery and autopsy patients for whom an epidemiological profile can be obtained.

Major Contributions:

1. Collection of Tissues

Tissue specimens (747) were collected from a total of 431 cases involving surgery patients (77) with and without cancer [including cardiothoracic (36) and colonic (41) cases]; preoperative workups for lung surgery (case-studies) (33); at parturition (placentas) in the maternity wards (29); and at the time of autopsy (114). Autopsy specimens were collected from non-cancer patients subjected to immediate autopsy (6) (i.e., within 60 minutes after death) or routine autopsy (108) (i.e., between 2 and 12 hours after death).

Blood and urine samples were acquired from patients qualifying for case studies of metabolic markers associated with lung cancer, and placentas were obtained to develop cultures for pilot studies.

Tissues received at the NIH were residuals of materials taken for diagnostic and corrective purposes and not for research per se. Routinely excluded from the collection procedure were AIDS patients, known drug overdose victims, intravenous drug users, and HTLV-III-profiled individuals.

A. Surgical Specimens

A total of 77 surgeries resulted in tissue donations.

Bronchus: Tumor and noninvolved tissues were collected from 36 cases of lung carcinoma. Bronchial specimens uninvolved with tumor were provided from all lung surgery cases and transported to the NIH. The tumors and normal tissues were defined and classified as described below.

Colon: Tumor tissues were collected from 41 cases of colon carcinoma. Colonic tissue uninvolved with tumor was available from 36 of these. All of the tumors were defined and classified as described below.

B. Autopsy Specimens

Immediate Autopsy (IA): There were six IA cases. One specimen each of colon, bronchus with lung attached, and liver (2 for liver cell collections) was obtained from each case.

Tissues required for pathological examination and assessment of viability were retained by the contractor. The major portions of the specimens were received at the NIH.

Routine Autopsy: The specimens collected from 108 routine autopsies and other procedures (pleural fluids for mesothelial cells to study asbestos) are as listed below:

<u>Organ</u>	<u>Number of Specimens</u>
Liver	37
Bronchus	86
Pleural Fluids	223
Pulmonary arteries	35

2. Viability Evaluation

Bronchus: Pieces of nontumorous bronchial epithelium were successfully grown in explant cultures from intermediate autopsy cases. Surgical and immediate autopsy cases have been shown to be routinely viable and are not culturable only in septasemic cases.

Colon: Previous failures to culture using fetal calf serum with neoplastic colon were reversed in this period by using serum free medium and normal human large intestine epithelium (NHLIE). One culture has been carried for up to 5 months and 2 passages to date.

Liver: Isolation of viable liver cells from IA cases were performed using a two step profusion with a) Hank's solution and b) L-15 medium with collagenase, producing cells with good viability. Two livers cultured from immediate autopsies had viabilities of 66% and 68%. One culture (HL-88-6) attached well to plastic and formed expanding colonies. Fifteen vials of these cells were frozen and delivered to the LHC (now stored in liquid N₂) for use in ongoing studies. In addition, liver tissue obtained at increasing times after death showed decreasing ability to survive in culture. Tissues taken within 90 minutes, 2-2.5 hours, 3-3.5 hours, 4-5 hours, and 5-7 hours had viabilities of 82%, 74%, 60%, 32%, and 4%, respectively. Typically, monolayer cultures could be established from tissue taken up to 3 hours after death.

Pancreas: Pancreatic epithelia from the IA cases and four intermediate autopsy cases (within 6 hours) were tested in explant organ cultures as described for bronchus. All IA cases were viable by morphology and cellular outgrowth criteria. Outgrowth cells were collected and frozen for use at the LHC, and human pancreatic cells are currently being carried in cultered by the contractor.

3. Definition and Classification of Non-neoplastic and Neoplastic Tissue

Bronchus: Morphological and histochemical characterization of human primary lung carcinomas are routine. Tissues are examined for beta human chorionic gonadotropin (HCG-beta), calcitonin, adrenocorticotrophic hormone (ACTH), serotonin, alpha-fetoprotein (AFP), keratin, somatostatin, neuron specific enolase (NSE), calmodulin, and tubulin.

Adult bronchial cells, normal and abnormal, contain keratin, calmodulin, and tubulin, with calmodulin increasing at cell borders. Lung tumors frequently contain HCG and keratin (respectively, 80% and 75% of non-small cell tumors); somatostatin, found in keratinizing tissues and a few adenocarcinomas, rarely occur in lung tumors; likewise for ACTH, somatostatin, calcitonin, HCG, and AFP.

Digital imaging fluorescence microscopy (DIFM) is now being adapted for use in characterizing these cells. Spectrofluorometric Fura-2 (62+/15), previously used to measure cytosolic calcium in cultured human bronchial epithelial cells, is used in a series of tests including BCECF dye for pH, rhodamine 123 for mitochondrial membrane, and propidium iodide for viability to provide a valuable array of information on the cell injury indexes of these targets of airborne carcinogens.

Colon: All tissues were routinely examined for morphological and histochemical changes: apical vesicles by electron microscopy (EM) and by light microscopy (LM) with stains reacting with highly acid mucous in all segments; the HID-AB stain for sulphomucin in all regions of the organ; Carcinoembryonic antigen (CEA) in nonmalignant colon tissue was shown suppressed by fixation in 4% formaldehyde-1% gluteraldehyde (4F-1G).

Liver: Liver tissues and cultured cells are examined by EM and LM for histological and pathological evaluation. However, since liver has not been firmly established as a source of cell cultures, the thrust is on characterization of developmental aspects of the culture system. Therefore, the ultrastructural appearance of liver at autopsy is followed as a parameter for the viability of the cultured liver cell. The correlations have been very good for all tissues obtained from IA cases by the Hsu method. In this period, neutral red (NR) as an assay for viability has been compared to the release of lactic acid dehydrogenase (LDH), protein content, and morphology. There is also an effort to determine the differential sensitivity of NR and LDH release as an indicator of cytotoxicity from dimethylnitrosamine(s) and aflatoxin B₁. Samples are stored at -70°C. Specimens quick-frozen in liquid nitrogen are used in metabolic studies at the NCI.

Pancreas: Pancreatic tissues (24 from IA's; 4 from routine autopsy) were examined morphologically and histochemically, while maintained by organ explant and cell culture techniques. Two cultures were discarded for bacterial contamination; 2 IA and 3 intermediate cultures were frozen and thawed for further culturing; only one was successfully recultured. Five intermediate cultures were successfully grown in serum-free LHC-8 medium when the calcium was raised to 300 mM.

4. Epidemiological Profile Construction and Storage

Epidemiologic data (see below) are provided to facilitate the study of relationships between tumor type, selected risk factors and the amount of carcinogen exposure, i.e., benzo[a]pyrene (BP), alkylating agents such as nitrosoamines, and aflatoxin B₁ (AFB), respectively (e.g., in lung cancer and hepatoma), bound to DNA by the patient's noncancerous epithelium, potentially influencing tissue responses to in vitro experimentation and or examination (genetic analysis).

Medical records, donor histories, and computer storage of these combined as the environmental history of the collected tissues are essential requirements of this resource. In this period, 364 medical records were abstracted for surgery and autopsy patients (305 for thoracic and 59 for colonic); donor profiles were obtained (in interviews to complete an OMB approved questionnaire provided by the LHC) for 123 patients (80 bronchus and 43 colon); in data processing, a total of 339 (293 colonic and 46 thoracic) records were coded for computer storage and analysis.

The total number of cases with these data collected from the beginning of the contract (from the seven participating hospitals) are listed below:

	<u>Univ. Hosp.</u>	<u>LRVA</u>	<u>Un. Mem.</u>	<u>BCG</u>		
Bronchus	368(18)	179(7)	57(3)	1(0)		
Colon	405(32)	141(20)	33(3)	2(0)		
	<u>St. Ag.</u>	<u>South Baltimore General Hospital</u>	<u>Washington Veteran's Administration Hospital</u>	<u>Sinai</u>	<u>Medical</u>	<u>Total</u>
Bronchus	85(2)	10(0)	7(0)	10(1)	566(125)	1283(156)
Colon	0	0	51(0)	24(11)	0	656(66)

To date, the number of completions in the efforts 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>Refused Interview</u>			<u>Med. Ex.</u>
				<u>Patient/Doctor/UMH</u>			
Bronchus	1167(305)	541(80)	1155(293)	38(4)	6(0)	55(17)	546(125)
Colon	565(59)	455(43)	543(46)	28(2)	6(0)	33(3)	0
Total	1732(364)	996(123)	1698(339)	66(6)	12(0)	88(20)	546(125)

() = number accomplished in this report period.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05435-05, Z01CP05480-04 LHC and Z01CP00NEW-BGT

UNIVERSITY OF MARYLAND (NO1-CP-71012)

Title: Resource for Human Esophageal Tissues and Cells from Donors with Epidemiological Profiles

Current Annual Level: \$94,846

Man Years: 1.1

Objectives: To obtain human esophageal tissue specimens and cells, and, when possible, a medical and occupational profile from patients at the time of surgery and at autopsy; to provide fresh, well-characterized, and viable esophageal tissue for primary organ cultures at the NIH; to establish, characterize, and store monolayer cultures from esophageal tissues for delivery on request to the NIH.

Major Contributions:

1. Tissue Collections

Specimens were collected and characterized from 81 cases, including 71 intermediate autopsies, 6 immediate autopsies, and 4 surgical resections for esophageal carcinoma. Complete epidemiological profiles of donors have been provided when possible. The difficulties inherent in the collection of this tissue render autopsy the most reliable, lucrative source, many of which come from the medical examiner (ME). In instances of problematic deaths, a frequent occurrence with ME collections, the contractor is denied access to the next-of-kin for legal and/or emotional reasons.

Routinely excluded from the collection procedures were AIDS patients, known drug overdose victims, intravenous drug users, and HTLV-III-profiled individuals.

2. Viability and Characterization

Morphological, cytochemical, and immunocytochemical characteristics were determined for each collected tissue and are delivered to the NCI upon request. Each case was routinely sampled for histological evidence of cell injury, viability, and general condition. Assays are ongoing for specific biochemical markers (HCG_β, AFP, CEA, etc.) occurring in normal, premalignant, and malignant human esophageal epithelium. Tissues from each case are preserved in major fixatives (bouins, ethanol, and/or aldehydes) for additional characterization when required.

3. Cell Cultures

Organ cultures are routinely used to develop monolayer cultures. Explants from "normal" uninvolved immediate autopsy tissues and malignant human esophageal mucosa were cultured in this period. Plating efficiencies remain approximately 100% for cells, from surgical and IA tissues, and from tissues

obtained within 4 hours of death. All primary cultures are routinely assessed for viability. In this period, LHC has submitted SV40 T antigen (SVTA)-immortalized esophageal cells for characterization/comparison studies with primary esophageal cultures. Two cell lines of SVTA esophageal cells were assayed for epithelial properties by staining for keratin and differentiation (vimentin content):

- 1) The cultures grew in uniform populations of clusters or small sheets of cells;
- 2) Vimentin staining was heterogeneously distributed between small (intense) and large cells (little or no stain) typical of a pattern of differentiation;
- 3) Keratin staining was strong in all cell types, typical of epithelial cells, and was magnitudes greater than vimentin;
- 4) EM revealed cells connected by desmosome junctions and with well-developed filaments.

4. Cell Bank

A tissue culture cell bank of normal and malignant esophageal cells and tissue explants is maintained in a Queue -135° freezer in the contractor's facility (centralized tissue culture lab, MSTF 7-60). The cultures of cells from normal and malignant esophageal mucosa are frozen and thawed as viable cell stocks. Currently, there are approximately 549 (-12) vials of frozen stock, including 403 vials from 54 intermediate autopsy cases; 120 vials from 12 immediate autopsy cases; 727 vials from 8 (+4) surgical/tumor cases (one with 31 (+25) passages and 404 vials; one with 27 passages and 290 vials); 22 (+3) vials from 3 xenotransplanted esophageal tumors in nude mice.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05192-09 LHC, Z01CP05324-07 LHC, Z01CP05341-07 LHC, Z01CP05435-05 LHC, Z01CP05480-04 LHC, Z01CP00NEW-BGT and Z01CP00NEW-JFL

GEORGETOWN UNIVERSITY (N01-CP-85606)

Title: Collection and Evaluation of Human Tissues and Cells from Donors with an Epidemiological Profile

Current Annual Level: \$76,443

Man Years: 0.96

Objectives: To provide the NCI with (1) a source of human lung and bronchial tissues taken at surgery; (2) human bronchial alveolar cells and supernatants from bronchial lavage of normal, smoking and nonsmoking volunteers; and (3) completed epidemiological questionnaires for medical and environmental histories.

Major Contributions: In this second annual period of the new contract, the contractor has provided a total of 133 specimens: 26 specimens (10 benign, 16 malignant) of surgical bronchi with lung; 37 specimens of colon (26 normal, 11 tumor); and 2 specimens of esophagus (1 normal, 1 malignant) from 63 surgery patients (26 cardiothoracic, 36 colonic, 1 esophageal), 22 of which had malignancies (mostly adenocarcinomas). In addition, we received 21 sets of matching peripheral blood mononuclear (PBM) cells (95% lymphocytes), broncho-alveolar lavage cells (85% macrophages), and sera from normal volunteers (4 smokers and 17 nonsmokers) given chest x-rays and pulmonary function tests to qualify as donors to the project. From 11 different donors of bronchial lavage cells, we received the clarified supernatants. Epidemiological (medical and environmental history) profiles were completed for all participating patients and normal volunteers. These records were filed in the contractor's facility for future use by the NCI.

Tissues collected by this contractor were used in ongoing studies in the In Vitro Carcinogenesis and Biochemical Epidemiology Sections of the LHC: human bronchial and peripheral lung tissues in studies of chemical carcinogenesis in human lung cancer; lung and colon tissues for studying carcinogen DNA adducts, DNA repair, and genetic restriction fragment length (RFL) polymorphisms (RFLP).

This contract is unique among LHC resources. In addition to providing surgically-derived tissues, it is the sole provider of alveolar macrophages (i.e., bronchial lavage) and PBMs from normal, non-hospitalized, smoking and nonsmoking adult volunteers. Immunocompetent blood cells from healthy, adult populations serve as a source of macromolecules with which to establish a data base of normal levels for frequency and distribution of humans positive for certain carcinogen-induced damage to genes and gene products, chemical markers thought of as potential indices for individuals at risk for chemical carcinogenesis. Currently, studies are ongoing to detect carcinogen-DNA and carcinogen-hemoglobin adducts, and to associate these phenomena with enzymatic DNA repair in normal and endemically exposed populations.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05324-07 LHC, Z01CP05341-07 LHC, Z01CP05435-05 LHC, Z01CP05480-04 LHC, Z01CP00NEW-BGT and Z01CP00NEW-JFL

VETERANS ADMINISTRATION HOSPITAL (Y01-CP-70501)

Title: Resource for Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: \$64,874

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 cardiothoracic and colonic surgeries, the contractor delivered a total of 80 specimens: 24 lung (12 normal, 12 malignant); 30 colon (16 normal, 14 malignant); 6 bronchus; 20 pleura; and 1 malignant mediastinum. Of the 40 patients who participated, 25 (62.5%) had a malignancy: 6 (24%) had squamous cell carcinomas and 5 (20%) had adenocarcinomas of the lung; 9 (36%) had adenocarcinoma of the colon; 1 (4%) had bronchoalveolar carcinoma; and one each had mycetoma, neurilemoma, granuloma, fibrosis, and nonmalignant adenoma.

From a variety of surgical procedures, including local excision and biopsy, performed in this period, the contractor has provided numbers and types of tissues that contribute to the LHC program. Of the 25 malignancies diagnosed, 13 (52%) were in lung; 9 (36%) in colon and 1 (4%) in mediastinum. Epidemiological profiles are completed for each cooperating donor and filed in the contractor's facility.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05324-07 LHC, Z01CP05341-07 LHC, Z01CP05435-05 LHC, Z01CP05480-04 LHC, Z01CP00NEW-BGT and Z01CP00NEW-JFL

WALTER REED ARMY MEDICAL CENTER (Y01-CP-70500)

Title: Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: \$24,340

Man Years: 1.0

Objectives: This interagency agreement provides to the NIH for the study of carcinogen metabolism in human tissues (1) pathologically characterized specimens of malignant and noninvolved bronchus, lung, pleural effusions, and colonic epithelium (obtained at time of surgery for cancer or for benign lesions); and (2) epidemiological profiles (medical and environmental histories) for each donor.

Major Contributions: In this period the contractor has provided to the NIH a total of 95 specimens from 48 surgeries (31 cardiothorasic; 17 colonic), including 63 specimens of lung and bronchial epithelium (29 normal; 34 malignant) and 30 specimens of colon mucosa (17 normal; 13 malignant). Pleural fluids previously required for the collection of mesothelial cells are being phased out as unnecessary for the research program in the near future. Therefore, the attempt to collect them from this source has been discontinued in this period. However, the acquisition of histological and pathological reports has been successfully established during this period. All reports of epidemiological profiles for participating patients are received upon request and are of commendable quality. The scheduling and delivery of required reports are now among the best of our suppliers.

Much of the credit for the accomplishments in the administrative area of this procurement is due to the recent assignment of a new project director who has successfully addressed all previous areas of concern. Walter Reed has now materialized into the efficient supplier and collaborator for LHC that was envisioned 3 years ago.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05324-07 LHC, Z01CP05341-07 LHC and Z01CP05409-06 LHC

HAZELTON LABORATORIES, INC. (N01-CP-95670; REPLACES N01-CP-54001)

Title: Resource for Xenotransplantation Studies of Carcinogenesis of Human Tissues in Athymic Nude Mice

Current Annual Level: \$353,172

Man Years: 3.40

Objectives: To provide an immunodeficient animal model, the athymic nude mouse, for (1) long-term survival of human tissue xenografts; (2) long-term xenotransplantation, proliferation, and tumorigenicity studies of normal, premalignant, and malignant human tissues; and (3) study of the in vivo development of preneoplastic and neoplastic transformation in human tissues experimentally induced in vitro and in vivo by selected chemical agents, cellular manipulations, and genetic transfections.

Major Contributions: Human bronchus, pancreatic duct, colon, breast, prostate, and esophagus are maintained for 16 months and beyond as xenografts, as evidenced by viable-appearing epithelium with normal histology, detection of radiolabeled precursors into epithelial cells of the grafts, and positive test for human isozymes.

In the breeding stock, 824 (+67) Swiss litters contained 5,641 (+273) pups (6.8/litter), including 2,667 (47%) nu/nu pups. Forty-six percent (46%) of the nu/nu newborns survived, giving a total of 1,227 nudes or 1.5 nudes/litter. During the year, the contractor maintained a monthly average colony population of 1,249 mice: 178(+8) breeders, 293 (+75) newborn weanlings for new experiments, and 865 (+77 above previous period) mice in experimental protocols. An average of 13 (+4) experimental animals died and 98 (+63) were killed monthly.

As indicated for the breeder and experimental animal numbers above, important changes in colony procedures were maintained in this period which affected an increasing rate of experimental starts and the population levels for the ongoing experimental effort in the colony. Experiments used approximately 1,630 (+451) irradiated nude mice in this period, with 972 ongoing at the end. They included:

1) Experiments (totaling approximately 731 mice with about 517 ongoing) to study growth rates, morphology and tumorigenicity of xenotransplanted bronchial epithelial (BE) cell lines transformed by oncogene transfection and viral infection (e.g., BEAS-2B) in irradiated nudes; e.g., a) BEAS-2B zip-v-ha-ras control BE cells; b) BEAS-2B cells of different passage lines; c) BES-1A-1 cells; BEAS-2B cells; d) B39-TL tumor cells from BEAS-2B cells grown in nude mice; BZR-T33 cells for tumor suppression pilot experiments; 12 different BEAS-2B/ras-transfected constructs in nudes of increasing age--to list a few.

2) Experiments (totaling approximately 283 mice with some 223 ongoing) with mesothelioma cells and mesothelial cell lines to study growth, morphology, and transplantability by different routes with and without irradiation, and exposure to Amosite asbestos.

3) Experiments (totaling approximately 492 mice with about 118 ongoing) with the human lung carcinoma cell line, HuT-292, used as the host cell for the transfection of putative suppressor cells, subsequently implanted into nude mice to determine the resulting affects on the tumorigenicity

4) Experiments (totaling approximately 125 mice, 114 ongoing) performed for various miscellaneous objects of a pilot and/or study initiation or finalization design. These include studies with pancreatic duct, kidney, esophagus, and tissue culture cells containing varying genetically engineered gene constructs.

The contractor maintained a monthly average of 45 ongoing experiments requiring 788 mice, 681 of which survived the period. In brief, the increased level of colony performance relates directly to our accumulated experience in the use of experimental designs optimized to accomplish in vivo characterization of effects induced in vitro. All experiments are designed with a pre-determined shelf time (6 weeks, mostly, to 52 weeks as the maximum) and are routinely terminated by the contractor with proper notification to the individual investigators. In keeping with these standardizing formats, tumor transfers have been essentially discontinued as unrequired for the studies in the current program design; thus, the turnover is more rapid and there is more space available for new experiments. Consequently, the production rate of mice for experimental use is continuing at a maximum within the space constraints of the facility.

As reported in the past, tumorigenicity from human tissue chemical exposure in vitro still eludes observation in xenografts. However, the successful xenotransplantation of H-ras-transfected human bronchial epithelial (HBE) cells and zip-ras adeno 12 SV40 (hybrid virus)-transfected cell lines continue to produce tumors in nude mice. Inducers such as SV40 T antigen, and raf, PDGF or c-raf-modified PLJ retroviral vector are being studied. Cell lines treated with these substances are in long-term studies to define their tumorigenic properties.

All animals found dead or sacrificed at the request of the investigators are processed by the contractor for histological and pathological diagnosis by high resolution and/or electron microscopy, provided by an assigned veterinarian pathologist from the contractor's staff.

In this period we have promoted the hiring by the contractor of a computer consultant and programmer who has initiated and has achieved ~75% development of a very intricate and highly capable computerized (Dbase III) program for the collection, storage and analysis of the data generated in the colony. The program is scheduled to be completed near the end of this report period.

ANNUAL REPORT OF

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

October 1, 1988 to September 30, 1989

The Laboratory of Molecular Carcinogenesis (LMC) plans, develops, and conducts a research program designed to (1) clarify the molecular biology of chemical and physical carcinogenesis; (2) elucidate the fundamental nature of the interaction of carcinogenic agents, especially chemical, with biological systems in the induction of cancer; (3) identify those genetic, environmental, and endogenous factors which relate to and modify the carcinogenic process; and (4) clarify the genetic and environmental roles in the metabolic activation of chemical carcinogens and the detoxification and activation of xenobiotics such as drugs and environmental chemicals.

The goal of the Laboratory of Molecular Carcinogenesis is to understand the molecular basis of carcinogenesis with the view toward identifying susceptible populations and preventing human cancer. The research program is designed to understand the molecular basis by which carcinogenic agents cause malignant transformation and to identify and characterize those exogenous and endogenous factors involved in carcinogenesis. The Laboratory seeks to clarify the metabolic interaction of exogenous and endogenous agents in the living organism at the molecular, cellular, and organism levels, and seeks to understand the consequences of these interactions in terms of cell regulation and carcinogenesis. Emphasis is made on the role of carcinogen and drug activation and the genetics of the human population related to sensitivity to carcinogens, drugs, and environmental chemicals.

The Laboratory research program utilizes advanced techniques of molecular biology and immunology, as well as classical enzymology and protein chemistry. The staff are highly experienced in DNA recombinant and related molecular biology techniques, protein chemistry, and hybridoma technology. The power and precision of these technologies have promoted considerable progress in many of the projects of the Laboratory.

Office of the Chief - Studies (1) the nature of human genetic predisposition to cancer; and (2) the interaction of chemical and physical carcinogens with DNA, the repair of resulting damage and its relationship to human cancer formation.

Molecular, cellular, and clinical abnormalities in patients with xeroderma pigmentosum (XP), with the dysplastic nevus syndrome (DNS) of familial cutaneous melanoma, and with Bloom's syndrome (BS) are being studied. We have developed new assays utilizing plasmids as tools to measure DNA repair, ligation and mutagenesis at the molecular level in cultured human cells. Utilizing a shuttle vector plasmid, pZ189, we found that there is a restricted spectrum of mutations induced in UV-treated plasmid replicating in XP cells of complementation groups A and D. DNS cells introduced more mutations into UV-treated pZ189 than normal cells but had fewer tandem mutations. The major UV

photo-product, the T-T cyclobutane dimer, was found to be only weakly mutagenic in XP, DNS and normal lines. We determined that photoproduct frequency was not the major determinant of UV mutation frequency in plasmids replicated in human cells. Utilizing a linearized replicating plasmid we demonstrated reduced ability of BS cells to ligate plasmids in vivo and that this ligation process was error prone. The BS cells also introduced more deletion mutations than normal cells into the recircularized plasmids. Oxidative DNA damage to pZ189 produced by treatment with Cu(II) plus hydrogen peroxide produced site-specific damage at polyguanosines. A Registry of XP patients has been established. A 3-year clinical trial of cancer chemoprevention demonstrated that a high dose (2 mg/kg/da) of 13-cis retinoic acid (Accutane), administered orally, is effective in preventing formation of new skin cancers in patients with XP.

Metabolic Control Section - Studies (1) the metabolic activation and detoxification of the polycyclic hydrocarbons (PCH) and other carcinogens and drugs and the relationship of this metabolism to individual sensitivity and susceptibility to carcinogenesis; (2) the immunochemistry of cytochrome P-450 with the use of monoclonal antibodies for the detection, purification, and identification of forms of cytochrome P-450 responsible for different carcinogen and drug metabolism; (3) the protein structure and membrane topology of cytochrome P-450 with the goal of understanding structure-function relationships; and (4) use of expression vectors to produce pure P-450 for determination of functional specificity.

Mixed function oxidase (MFO) systems consist of three components: cytochrome P-450 (P-450), NADPH-P-450 reductase and phospholipid. The P-450s are the key components of MFO systems which metabolize many drugs, chemical carcinogens, fatty acids, prostaglandins and steroids. In some systems an additional electron carrier, cytochrome b₅, facilitates catalytic activity. We used monoclonal antibody (MAb)-directed procedures to make MAbs to b₅, reductase, P-450 3 (testosterone 7 α -hydroxylase) and triacetyl oleandomycin (TAO)-inducible P-450 (P-450 TA). Radioimmunoassay was used for screening of hybridomas which produced MAbs binding to the respective immunogens. Twenty-nine MAbs to b₅ were either IgG1(k), IgG2b(k), or IgG3(k). The inhibitory effects of the MAbs to b₅ with a NADH-reductase assay ranged from 0 to 77%. The MAbs had no effect on other catalytic activities. Fourteen MAbs to NADPH-P-450-reductase were either IgG(k) or IgM(k) but they had no effect on NADPH-reductase and AHH activity. The MAbs to P-450 3 and P-450 TAO belonged to IgM(k). These MAbs will be very useful in studying the role of the MAB in MFO systems and the identification of each component in animal and human tissues.

Identification and quantitation of P-450 isozyme species in tissues and organs may be a key to understanding individual differences in sensitivities to drugs and chemical carcinogens. Our approach is the use of epitope-specific monoclonal antibodies (MAbs) to P-450 isozymes. Eight libraries of MAbs to different forms of P-450 (LM2, LM4, MC-B, PB-B, PCN-E, ETOH, SCUP, and RLM5) were established and phenotyping of animal and human tissues were carried out by radioimmunoassay (RIA), reaction-phenotyping and immunohistochemistry. 3-methylcholanthrene (MC)-inducible P-450s were found in the lung, kidney, liver, and intestine of C57/BL mice and rats which were treated with MC. The MC forms of P-450 were also found in human placenta and lymphocytes of smoking women. However, the level of growth hormone- and male sex-dependent P-450 RLM5 was not greatly affected by MC induction. Ethanol-inducible P-450j was

found in human adult livers and pregnenolone 16 α -carbonitrile-inducible P-450 PCN-E was found in both adult and fetal human livers. Application of crude coal tar to skin of neonatal rats predominantly induced P-450 MC-B in both epidermis and liver but the P-450 PB-B form was also induced in liver. Treatment of adult male rats with chlortrimazole and diphenylhydantoin induced respectively, phenobarbital PCN and ethanol inducible-forms respectively in liver. These results indicate that MABs are useful probes for identification of P-450s which are induced by the administration of various drugs and chemical carcinogens.

Polyclonal (PAb) and/or monoclonal antibodies (MAB) have been raised against peptide sequences that are unique for a given P-450 enzyme, while the MABs are generated against purified, native P-450 enzymes. The production of anti-peptide PAB presents the opportunity to generate antibodies in a more rational and timely manner. In addition, the creation of P-450-specific anti-peptide PAB results in a reagent that is not only useful in numerous immunoassays but can also be used in the purification of the native molecule, which in turn can be used for production of MAB. The current research efforts center around the generation of specific anti-peptide PAB against sequences unique to either P-450d or P-450c. These two rat P-450 enzymes, induced following exposure to 3-methylcholanthrene, are key enzymes in the metabolism of carcinogens of the aromatic amine and polyaromatic hydrocarbon classes, respectively. While several unique regions have been identified in both enzymes, the initial emphasis will be on the creation of antisera against peptide sequences unique to P-450d, as there is no available antibody which specifically recognizes P-450d. The anti-peptide PABs are examined for use as reagents in ELISA and western blot analysis, and for inhibition of enzyme activity.

The focus of this project is the characterization of structure-function relationships the cytochrome P-450s. The quaternary structure of P-450s in intact rat liver microsomal membranes was examined by a cross-linking study which revealed specific association among P-450c and P-450 2a, forms which metabolize polycyclic hydrocarbons and testosterone, respectively. The cross-linking results also demonstrated a complex of P-450c with reductase. Such specific P-450 interactions may influence the disposition of P-450 substrates in secondary metabolism. The active site structure of purified and microsomal P-450c was examined in binding studies using the substrate benzopyrene (BP). BP fluorescence was enhanced upon binding to the microsomal membrane but quenched upon binding to P-450c. Quenching measurements on pure P-450c indicated that binding of MAB to this P-450 resulted in reduced mobility of the substrate binding site. Flash photolysis experiments of CO recombination to the P-450 heme yielded parallel kinetic data on the effect of BP on active site dynamics. We examined the iron spin state of testosterone-binding rat liver microsomal P-450s by absorption and electron spin resonance (ESR) spectroscopy. The data suggest at least two classes of testosterone binding P-450s: one P-450 class shifts microsomal P-450 from the low spin to the high-spin state, while the second class represents a perturbation from one low-spin state to another.

Factors governing the regulation of cytochrome P-450 expression were examined in several different systems. The tissue specificity of P-450c induction by 3-methylcholanthrene (MC) was probed both immunochemically with antibodies to P-450c, and enzymatically by aryl hydrocarbon hydroxylase (AHH) activity. The level of P-450c in rat tissue homogenates decreased in the following order:

liver, nasopharynx, lung, pancreas, kidney. There was no direct relation between the P-450c level and AHH activity, indicating that significant amounts of extrahepatic activity derive from P-450 forms other than P-450c, and/or the specific activity of P-450c varies among different tissues. The induction of rat liver microsomal and nuclear envelope P-450s by 2-acetylaminofluorene (AAF) was immunochemically probed with antibodies to P-450c and P-450d. Both P-450s were induced to different degrees in microsomes and nuclear envelopes. In addition, the antioxidant butylated hydroxytoluene accentuated AAF induction of P-450c. The effect of aging on P-450 catalyzed testosterone metabolism was studied. Although 24-month-old rats generally exhibited several lower hydroxylase activities, they had a higher level of 7 α -hydroxylase as well as the P-450a form responsible for this activity. The results demonstrate that although P-450-catalyzed activities generally decrease with senescence, expression of individual P-450s are selectively repressed or activated. A monoclonal antibody to rat P-450 2a detected a related P-450 in chicken liver microsomes obtained at various developmental stages. The developmental profile and induction characteristics of this P-450 and of several P-450 catalyzed activities were determined.

Nucleic Acids Section - Studies (1) the structure and evolutionary relationships of human and rodent cytochrome P-450 genes; (2) the mechanisms by which P-450s are induced by endogenous and xenobiotic substances; (3) the mechanisms by which P-450 genes are developmentally activated; (4) the enzymatic specificity of P-450s through the use of yeast and higher eukaryote cDNA expression vectors; (5) the molecular basis of enzymatic P-450 polymorphisms in man and rodents; (6) evolutionary, structural, and regulatory analysis of cellular peroxidases and the role of these enzymes in carcinogen metabolism and tumorigenesis.

P-450s are the principal enzymes involved in drug metabolism and carcinogen activation. A large number of P-450s have been purified from rodent tissues and their substrate specificities examined by in vitro reconstitution assays. Few P-450s have been purified from man, however, owing to the paucity of readily available human tissues. Purification of these enzymes from human tissue is further complicated by the large degree of genetic variability among individuals and the difficulties in obtaining homogeneous preparations of specific P-450 forms. We have applied a cDNA cloning and expression approach to the study of human P-450s. cDNA probes and antibodies to rodent P-450s are being used to screen λ gt11 expression libraries constructed from different human liver and lung RNA preparations. The cDNAs are being sequenced and then used to produce active enzymes in cell culture. The enzymes are examined for their abilities to carry out the oxidation of common therapeutically used drugs and to activate carcinogens and mutagens. We have isolated cDNAs and expressed several human P-450s including I1A1, I1A2, I1A3, I1B1, I1B2, I1C8, I1C9, I1D1, I1E1, I1F1, I1IA4, I1IA5, I1IA6 and I1VB1. The chromosomal locations of the genes in each P-450 subfamily have been determined. We have also cloned, sequenced, and expressed human microsomal xenobiotic epoxide hydrolase and NADPH-P-450 oxidoreductase. cDNA probes to human P-450s and antibodies against rodent P-450s were used to probe individual human liver specimens for P-450 gene expression to locate livers that do not express a particular P-450. These samples are further examined for the existence of mutant human genes.

Individual subjects vary considerably in their abilities to metabolize drugs. In addition, genetic influences are thought to govern individual susceptibility to chemically-induced cancers. The mixed function monooxygenase system is composed of multiple forms of P-450s that are the principal enzymes associated with drug and carcinogen metabolism. These enzymes can serve to detoxify and hasten the elimination of foreign agents or they can activate inert chemicals to harmful electrophilic metabolites that damage DNA and initiate the carcinogenic process. We have begun to study polymorphic drug oxidation in humans by establishing the mechanism of the debrisoquine 4-hydroxylase polymorphism. This polymorphism affects from 5% to 10% of the North American and European Caucasian population. These individuals, referred to as poor metabolizers, or PMs, cannot metabolize debrisoquine and many other related drugs, while the rest of the population, called extensive metabolizers, or EMs, rapidly hydroxylate this drug, resulting in the inactivation of its therapeutically active form. The DA rat strain has been proposed as a model for the human debrisoquine hydroxylase deficiency. To determine the mechanism by which the DA rat has lost debrisoquine 4-hydroxylase activity, we cloned and sequenced five cDNAs in the rat *CYP2D* gene subfamily. These sequences were used to develop probes specific for each mRNA. The DA rat is lacking mRNA for IID1 but contains mRNA for the remaining four IID genes. In contrast, SD rats, prototypes for human EMs, express all five IID mRNAs. The IID1 gene is, therefore, transcriptionally inactive in DA rat.

The majority of P-450 genes are regulated at the transcriptional level. During distinct stages of development, P-450 genes became transcriptionally activated. We are studying the genes coding for several P-450s that are activated during development. For example, the IIA1 gene is activated within 1 week after birth in males and females and becomes specifically suppressed in males at puberty. IIA2 becomes activated in males when they reach puberty and is never expressed in females. The rat IIA1 and IIA2 genes were isolated and completely sequenced. To study the *cis*- and *trans*-acting elements responsible for these gene activations, we are using a cell-free *in vitro* transcription system derived from rat liver. The IIA2 gene has been extensively studied. A unique nuclear extract has been developed that faithfully transcribes the IIA2 promoter. Only extracts derived from male rat nuclei are capable of accurately transcribing this promoter. Deletion analysis has been used to identify the boundaries of the DNA responsible for the sex dependent transcription.

To determine the mechanism by which P-450 genes are transcriptionally activated by inducing agents, genomic clones for two rat clofibrate-inducible genes designated IVA1 and IVA2 were isolated from a lambda EMBL3 library. These genes are both transcriptionally activated by hypolipidemic agents. A highly conserved 19 base pair region was identified just upstream of the start sites for these genes. This region may be involved in regulation of the IVA1 and IVA2 genes. Interestingly, the latter gene is constitutively expressed in kidney.

Peroxidase is a hemoprotein which is ubiquitously found from microorganisms to animals, and catalyzes biosynthesis and degradation of a variety of substances using hydrogen peroxide. During the peroxidative reaction, free radicals are produced that can irreversibly bind to DNA. This may suggest the involvement of peroxidase in tumorigenesis in various tissues. We have been studying

human thyroid peroxidase using molecular biological approaches. This peroxidase plays an important role in the biosynthesis of thyroid hormones and has been identified as one of autoantigens against which patients with autoimmune thyroid diseases such as Graves' disease and Hashimoto's thyroiditis, develop antibodies. When the human thyroid peroxidase cDNA nucleotide and deduced amino acid sequences, and the exon-intron junction structures of the gene were compared with those of human myeloperoxidase, a granulocyte protein with microbicidal function, it became clear that both enzymes that previously had been thought to be completely different in terms of protein structure, physicochemical properties, and physiological functions in their target tissues were in fact members of the same gene family and evolved from the common ancestral gene. We further expressed human thyroid peroxidase cDNA and obtained an enzymatically active protein using the vaccinia virus expression system. After immunoenrichment, the expressed protein exhibited a hemoprotein characteristic absorption spectrum when complexed with cyanide, and a more than 300-fold higher specific activity.

Protein Section - Studies (1) the relationship between chromatin structure and gene expression and (2) mechanisms by which chromosomal proteins affect the structure and function of chromatin.

The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes is being investigated. Present efforts are concentrated on determining the cellular function of two non-histone chromosomal proteins, HMG-14 and HMG-17. These two proteins are the only known nucleoproteins whose main binding site in the nucleus is on the nucleosome. Our experiments suggest that they may be involved in modulating the structure of transcriptionally active chromatin. We have isolated and sequenced the complete genes coding for both human and chicken chromosomal proteins HMG-14 and HMG-17. Presumably, these genes evolved from a common ancestor. The ancestral gene contained 6 exons and had features characteristic to both housekeeping genes and to cell cycle-regulated genes. The distribution of nucleotides in the gene is very unusual. The 5' half is highly enriched in G+C residues, while the 3' is enriched in A+T residues. The chicken HMG-14 gene has lost one of the introns present in the ancestral gene. Information derived from this gene family supports the theory that introns are lost rather than inserted during evolution. We mapped the human HMG-17 gene to chromosome 1 at position p 36.1 and the human HMG-14 gene to chromosome 21, near the SOD gene in the region associated with the development of Down's syndrome. The expression of the HMG-14 gene in cells taken from Down's syndrome patients is presently being studied. The human cDNAs coding for HMG-14 and HMG-17 have been introduced into yeast expression vector P176 under the control of the Gal 1 promoter. Northern and western analysis indicates that the human proteins are expressed in yeast. Induction of the proteins failed to produce detectable phenotypic changes in the growth pattern in liquid media; however, analysis by two-dimensional gels suggests that the presence of the human proteins changed the electrophoretic mobility of certain yeast proteins. These studies will advance the understanding of gene structure and function in normal and neoplastic cells and provide insights into the evolution of the nucleosome structure, a hallmark of all eukaryotic organisms.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP04496-12 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Chromosomal Proteins and Chromatin Function

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

PI:	Michael Bustin	Section Chief	LMC	NCI
Others:	David Landsman	Visiting Associate	LMC	NCI
	Thyagarajan Srikantha	Visiting Associate	LMC	NCI
	James Pash	IRTA Fellow	LMC	NCI
	Massimo Crippa	Visiting Fellow	LMC	NCI
	Donald Lehn	Guest Researcher	LMC	NCI
	Nirmolini Soares	Biologist	LMC	NCI
	O. Wesley McBride	Section Chief	LB DCBD	NCI

COOPERATING UNITS (if any)

Laboratory of Biochemistry, Georgetown University (Dr. M. Smulson)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

4.8

PROFESSIONAL

3.8

OTHER

1.0

CHECK APPROPRIATE BOX(ES)

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

~~THE ROLE OF CHROMOSOMAL PROTEINS IN MAINTAINING~~ the structure and regulating the function of chromatin and chromosomes is being investigated. Present efforts are concentrated on determining the cellular function of two non-histone chromosomal proteins, HMG-14 and HMG-17. These two proteins are the only known nucleoproteins whose main binding site in the nucleus is on the nucleosome. Our experiments suggest that they may be involved in modulating the structure of transcriptionally active chromatin. We have isolated and sequenced the complete genes coding for both human and chicken chromosomal proteins, HMG-14 and HMG-17. Presumably, these genes evolved from a common ancestor. The ancestral gene contained 6 exons and had features characteristic to both housekeeping genes and to cell cycle-regulated genes. The distribution of nucleotides in the gene is very unusual. The 5' half is highly enriched in G+C residues, while the 3' is enriched in A+T residues. The chicken HMG-14 gene has lost one of the introns present in the ancestral gene. Information derived from this gene family supports the theory that introns are lost rather than inserted during evolution. We mapped the human HMG-17 gene to chromosome 1 at position p 36.1 and the human HMG-14 gene to chromosome 21, near the SOD gene in the region associated with the development of Down's syndrome. The expression of the HMG-14 gene in cells taken from Down's syndrome patients is presently being studied. The human cDNAs coding for HMG-14 and HMG-17 have been introduced into yeast expression vector P176 under the control of Gal 1 promoter. Northern and western analysis indicate that the human proteins are expressed in yeast. Induction of the proteins failed to produce detectable phenotypic changes in the growth pattern in liquid media however, analysis by two-dimensional gels suggests that the presence of the human proteins changed the electrophoretic mobility of certain yeast proteins. These studies will advance the understanding of gene structure and function in normal and neoplastic cells and provide insights into the evolution of the nucleosome structure, a hallmark of all eukaryotic organisms.

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

Michael Bustin	Section Chief	LMC	NCI
David Landsman	Visiting Associate	LMC	NCI
Thyagarajan Srikantha	Visiting Associate	LMC	NCI
James Pash	IRTA Fellow	LMC	NCI
Massimo Crippa	Visiting Fellow	LMC	NCI
Donald Lehn	Guest Researcher	LMC	NCI
Nirmolini Soares	Biologist	LMC	NCI
O.W. McBride	Section Chief	LB DCBD	NCI

Objectives:

To understand the mechanism of gene regulation and its relation to neoplasia by studying the role of defined chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes in normal and transformed cells. Studies are designed to give insight into the chemical nature of chromosomal proteins, their immunological specificities, their cellular function, their mode of action, the manner in which they interact with DNA, and the regulation of the expression of genes coding for these proteins.

Methods Employed:

The regulated expression of the genetic information encoded in DNA is dependent on specific protein-nucleic acid interactions. We have elicited antibodies against specific proteins and used them to study the structure and function of specific chromosomal proteins and their interactions with DNA. Proteins are purified from isolated nuclei by differential precipitation, size exclusion chromatography, and ion exchange chromatography. Synthetic peptides are prepared by solid phase synthesis. Polyclonal and monoclonal antibodies are elicited in rabbits and mice, respectively. Chromatin is isolated from purified nuclei. The antigenic activity of the purified chromatin and isolated proteins is measured by enzyme-linked solid phase assay (ELISA), immunoblotting and radioimmunoassays. Nucleosomes are prepared by nuclease digestion. Immunoaffinity columns are prepared by the cyanogen bromide procedure using purified immunoglobulin (Ig). cDNA clones are isolated from expression libraries prepared from the mRNA isolated from transformed human cells. The clones are characterized, propagated in plasmids, and the DNA sequence determined. The cDNA clones are used to isolate the human genes coding for the proteins and to study gene expression in various tissues by analyzing the RNA. Genomic clones are isolated from human and chicken libraries using the cDNA fragment as a probe. Vectors used to transfect the cDNA into COS and yeast cells are constructed by inserting the cDNA fragment at selected restriction sites in the vector. COS cells are transformed by the diethyl amino ethyl (DEAE) cellulose method and yeast cells by the lithium acetate method. Transcription is determined by slot blot and northern analysis. Translation of the HMG protein is determined by extraction of whole

cells with 5% perchloric acid (PCA) and polyacrylamide gel electrophoresis. The cellular location of the protein is determined by immunofluorescence.

Major Findings:

The functional human HMG-17 and HMG-14 genes have been isolated using sets of oligonucleotides which could distinguish between the sequence of the functional gene and those of the respective retropseudogenes. The entire sequence of the genes coding for both human HMG-14 and HMG-17 has been determined. In addition the entire sequence of the chicken HMG-14 and HMG-17 genes also has been determined. The HMG-17 gene has features characteristic of both cell cycle-regulated and housekeeping genes. Comparison of the structure of these genes allows extrapolation as to the structure of the putative ancestral gene. The ancestral gene for the HMG-14/-17 gene family contained 6 exons. The 5' half of the genes is rich in G+C residues, while the 3' half is rich in A+T residues. Part of the regulatory sequences in the 5' region have been identified. The two HMG-14 genes lack a recognizable TATA box, while the HMG-17 genes have two TATA boxes. Conceivably, the transcriptional regulation differs between the two genes. The HMG gene family provides a compelling example of intron loss during evolution. The chicken HMG-14 gene has five exons, while the other three genes have six. Examination of the gene structure clearly reveals that the second intron of the ancestral gene has been spliced out of the chicken gene. However, three bases of the flanking sequences are still present in the gene. These three bases code for Valine. Indeed, among eight different known members of the HMG-14/-17 protein family, the chicken HMG-14 is the only protein containing a Valine in that particular position. The Valine interrupts the highly conserved DNA binding domain of this protein family. The chicken has an additional HMG-14 protein, and it is conceivable that this protein compensates for the original HMG-14 protein whose binding to DNA is impaired due to the Valine insertion.

The chromosome location of both human HMG-14 and HMG-17 gene was determined by linkage analysis and *in situ* hybridization. The functional gene for chromosomal protein HMG-17 is located on the short arm of chromosome 1 in region 1p36. The gene for chromosomal protein HMG-14 is located on the long arm of chromosome 21 in region q22.1. The Down's syndrome phenotype is associated with trisomy in region q22.1 of chromosome 21. The expression of chromosomal protein HMG-14 in patients suffering from this syndrome will be investigated.

Study of the amino acid sequence of all the known HMG-14 and HMG-17 proteins revealed a remarkable conservation in the DNA binding domain of these proteins. The evolutionary conservation suggests strict structural requirements for interaction with nucleosomes. Since the nucleosome is also a highly conserved structure, understanding the mechanism of binding of HMG to nucleosomes may provide insight into evolutionary differences between prokaryote and eukaryotes. Antibodies specific to various regions in the proteins have been elicited and are being used to study the organization of the proteins in chromatin.

The human proteins have been expressed in yeast. Expression of the cloned HMG-14 and HMG-17 does not affect the growth characteristics of the yeast;

however, two-dimensional polyacrylamide gel analysis reveals that the mobility of certain proteins has been altered.

Plasmids capable of expressing LEXA-HMG fusion proteins have been constructed. Expression of these proteins in yeast cells containing a reporter plasmid in which the LEXA receptor is adjacent to the promoter of β -Galactosidase will allow studies on the potential of the proteins to act as transcriptional activators.

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

PROJECT NUMBER

Z01CP04517-13 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

DNA Repair in Human Cancer-Prone Genetic Diseases

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

P.I.: K.H. Kraemer Research Scientist LMC NCI

Others:

S. Seetharam	Visiting Assoc.	LMC NCI	H. Waters	Tech (Biol)	LMC NCI
T. Runger	Guest Researcher	LMC NCI	J. DiGiovanna	Expert	DB NCI
M. Seidman	Spec Volunteer	LMC NCI	G. Peck	Sr. Invest.	DB NCI
R. Tarone	Biometrician	BB DCE NCI	K. Sanford	Sr. Invest.	LCMB NCI
D. Brash	Staff Fellow	LHC NCI	Y. Pommier	Sr. Invest	LMP NCI

COOPERATING UNITS (if any)

Dept of Pathology, NJ School of Medicine (W.C. Lambert); NY Blood Center (J. German); Dept of Dermatol Hosp Univ of Pa (W. H. Clark); Wistar Institute, Phila, PA (M. Herlyn); Bureau of Devices and Radiological Health, FDA (J. Sagripanti)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

4.0

3.0

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Molecular, cellular, and clinical abnormalities in patients with xeroderma pigmentosum (XP), with the dysplastic nevus syndrome (DNS) of familial cutaneous melanoma, and with Bloom's syndrome (BS) are being studied. We have developed new assays utilizing plasmids as tools to measure DNA repair, ligation and mutagenesis at the molecular level in cultured human cells. Utilizing a shuttle vector plasmid, pZ189, we found that there is a restricted spectrum of mutations induced in UV-treated plasmid replicating in XP cells of complementation groups A and D. DNS cells introduced more mutations into UV-treated pZ189 than normal cells but had fewer tandem mutations. The major UV photo-product, the T-T cyclobutane dimer, was found to be only weakly mutagenic in XP, DNS and normal lines. We determined that photoproduct frequency was not the major determinant of UV mutation frequency in plasmids replicated in human cells. Utilizing a linearized replicating plasmid, we demonstrated reduced ability of BS cells to ligate plasmids *in vivo* and that this ligation process was error prone. The BS cells also introduced more deletion mutations than normal cells into the recircularized plasmids. Oxidative DNA damage to pZ189 produced by treatment with Cu(II) plus hydrogen peroxide produced site-specific damage at polyguanosines. A Registry of XP patients has been established. A 3-year clinical trial of cancer chemoprevention demonstrated that a high dose (2 mg/kg/da) of 13-cis retinoic acid (Accutane), administered orally, is effective in preventing formation of new skin cancers in patients with XP.

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

K.H. Kraemer	Research Scientist	LMC NCI
S. Seetharam	Visiting Associate	LMC NCI
H. Waters	Biologist	LMC NCI
T. Runger	Guest Researcher	LMC NCI
M. Seidman	Special Volunteer	LMC NCI
G. Peck	Senior Investigator	DB NCI
R. Tarone	Biometrician	BB NCI
K. Sanford	Section Chief	LCMB NCI
D. Brash	Staff Fellow	LHC NCI
Y. Pommier	Senior Investigator	LMP NCI
J. DiGiovanna	Expert	DB NCI

Objectives:

Human cancer prone genetic diseases are being studied with a view toward identifying groups of people with an increased susceptibility to environmental carcinogenesis. We are attempting to 1) understand the molecular basis of their cellular hypersensitivity, 2) correlate cellular hypersensitivity with clinical abnormalities, 3) determine if there is genetic heterogeneity within such groups, 4) explore methods of cancer prevention in these patients, and 5) educate the medical community to the importance of early recognition and diagnosis of these disorders.

Methods Employed:

Plasmids for measurement of DNA repair or of mutagenesis are treated *in vitro* with ultraviolet radiation, with copper plus hydrogen peroxide, with damage-modifying enzymes or with restriction endonucleases to create linear plasmids. The extent and sites of damage are assessed by endonuclease-sensitive site assay, by polymerase chain termination assay with DNA sequencing, or with end labeling (Maxam-Gilbert) DNA sequencing. DNA-mediated gene transfer (transfection) is used to introduce the plasmids into cultured human cells. DNA repair is measured by transient expression of the encoded bacterial gene chloramphenicol acetyl transferase (CAT), or by autoradiographic measurement of unscheduled DNA synthesis (UDS). Plasmid mutation frequencies are measured by isolation of replicated plasmids from the human cells and transformation of indicator strains of bacteria. Mutant plasmids are isolated from bacterial colonies and purified. The DNA sequence of mutated plasmids is determined using a primer-directed dideoxy sequencing technique with double-stranded plasmids.

Patients with xeroderma pigmentosum (XP) or with the dysplastic nevus syndrome (DNS) are examined with particular emphasis on cutaneous abnormalities. Cultures of skin fibroblasts or peripheral blood lymphocytes are established for laboratory analysis. Physicians treating patients with XP are contacted and are encouraged to fill out a Xeroderma Pigmentosum Registry questionnaire

about their patients. New clinical forms of XP are investigated in depth. XP patients with multiple cutaneous neoplasms are being treated with oral 13-cis retinoic acid in a long-term study to attempt to reduce their rate of new skin tumor formation.

Major Findings:

We have developed host cell reactivation assays utilizing expression vector plasmids as tools to measure DNA repair and mutagenesis in cells from patients with cancer-prone genetic diseases. Transient expression of damaged plasmids depends on the competence of cellular repair enzymes. We found 100-fold differences in plasmid expression between normal and XP groups A and D cells.

In XP-A and XP-D cells, one pyrimidine dimer inactivated expression of the transfected gene. Selective enzymatic removal of pyrimidine dimers by pre-treatment with photoreactivating enzyme revealed that XP cells also cannot repair non-dimer photoproducts.

The shuttle vector plasmid, pZ189, was used to measure replication and mutagenesis after UV treatment and transfection in normal, XP A and D, and DNS cells. Plasmid survival was reduced in the XP cells reflecting their repair deficiency and cellular hypersensitivity to the cytotoxic effects of UV. Plasmid survival was normal in the DNS line, reflecting the normal UV survival of the DNS cell line. The frequency of mutations introduced by the cells into the UV-damaged pZ189 was greater than normal with the XP- A, XP-D and DNS lines, reflecting the cellular UV-hypermutable of these disorders. We demonstrated, for the first time, that there was a markedly reduced spectrum of mutations found with the XP-A, XP-D and DNS lines in comparison to the spectrum with normal cells. With the XP-A and XP-D lines there was a significant reduction in the frequency of plasmids recovered with multiple base substitutions and in transversion mutations. The XP-D line, arising from a patient who had several cutaneous melanomas, also had a reduced frequency of plasmids with tandem mutations. The DNS line, derived from a patient with multiple melanomas, was similar to the XP-D line in having a reduced frequency of plasmids with tandem mutations. Different mutagenic hot spots were present with each cell line. With all lines, more than 85% of the mutations were G:C to A:T transitions.

We found that the major UV photoproduct, the T-T cyclobutane dimer, was only minimally mutagenic in UV treated pZ189 introduced into all cell lines. When we eliminated 99% of the cyclobutane dimers by treatment with a photoreactivating enzyme prior to transfection, we found increased plasmid survival, decreased mutation frequency, and an altered spectrum of types of mutations. We determined that both dimer and non-dimer photoproducts were mutagenic in plasmids replicated in human cells. Cytosine-containing photoproducts produced 90-95% of the mutations. We measured photoproduct production at more than 50 sites of adjacent pyrimidines in the marker gene. There were large variations in the frequency of cyclobutane dimers and in formation of 6-4 pyrimidine-pyrimidone photoproducts at individual sites. Surprisingly, there was no correlation between photoproduct frequency and mutation frequency at the same sites. Thus, photoproduct frequency was not the major determinant of UV mutation hot spots or cold spots in plasmid DNA

replicated in human cells. Presumably, photoproduct-induced secondary structural alterations (that may be sequence-specific and cell polymerase-specific) are responsible for the mutational hot spots but these are not adequately measured by photoproduct frequency.

Cells from patients with Bloom's syndrome (BS) were reported by Lindahl to have reduced in vitro DNA ligase I activity, with levels 30 - 50% of normal. This enzyme is believed to be important in DNA replication and to have the unique ability to ligate double-stranded DNA pieces with blunt ends (in addition to being able to ligate pieces with overlapping ends). We utilized plasmid vectors to determine if this defect was also present in vivo. The non-replicating plasmid, pRSVcat, and the replicating plasmid, pZ189, was cut with restriction endonucleases to produce linear plasmids with either blunt or overlapping ends. These were transfected into BS and normal fibroblasts and lymphoblastoid cell lines, and recovery of CAT activity and ability of pZ189 to transfer ampicillin resistance was measured. No difference was found between the ability of BS and normal cells to express CAT activity with the linearized, non-replicating plasmid. However, there was a significantly reduced ability of the BS cells to repair pZ189 which must pass through a circular stage in order to replicate. In addition there was an approximately ten-fold higher frequency of mutations recovered in pZ189 replicated in BS in comparison to replication in normal cells. These results are consistent with the thesis that the ligase defect is present in vivo in BS.

A survey of the literature, our own unpublished data and unpublished information from others using pZ189 for mutagenesis studies, was performed to identify the sensitivity of the supF gene for detection of mutations. We determined that nearly 90% of the 85 base pairs in the tRNA region of the supF gene, when mutated, will reduce suppressor activity and thus be detectable.

A unique point mutation in the supF gene of pZ189 (A:T to T:A at position 136) was identified that results in the plasmid being 5- to 80-fold more sensitive to inhibition of transforming ability by UV-B radiation (295 nm) than the wild-type plasmid. A non-dimer photoproduct appears to be responsible since the hypersensitivity is still present after removal of cyclobutane dimers by enzymatic photoreactivation. This plasmid is not hypersensitive to UV-C radiation (254 nm). The mechanism of this hypersensitivity is under investigation.

Oxidative damage to DNA was examined by treating pZ189 with low levels (10^{-3} to 10^{-6} M) of copper and hydrogen peroxide. Cu(II) plus H_2O_2 was found to create DNA single- and double-strand breaks and to inactivate plasmid transforming ability at concentrations of each agent alone that did not alter the plasmid. Sequence analysis revealed site-specific DNA damage at sites of two or more adjacent guanosine residues.

Nevus cells and melanocytes were cultured from a patient with xeroderma pigmentosum who had defective DNA repair. We found a similar reduction in DNA repair of the cultured pigment cells as was present in the fibroblasts of this patient. This indicates that the DNA repair defect may be implicated in the high rate of melanomas in XP patients.

In a collaborative study designed to detect carriers of the XP gene, blood samples were obtained from patients with XP and from their parents (obligate heterozygotes). Coded samples were cultured and then exposed to X-rays. The frequency of chromosome breaks and gaps introduced by X-irradiation during the G2 phase of the cell cycle was determined. XP heterozygotes were found to have a level of chromosome breaks and gaps intermediate between the XP patients and the normal controls. This assay may thus form the basis for a test of XP heterozygote detection.

The XP Registry (a collaborative effort with Drs. Kraemer, Lambert, German, and Andrews) has collected clinical information on about 100 patients. We sent literature on XP to more than 500 physicians who requested information.

Clinical consultation, by telephone, concerning diagnosis or treatment of XP patients is provided to physicians who contact NIH at the rate of about 2-3 per month. We wrote and published the first information booklet for laymen describing XP.

A collaborative 3-year clinical trial of skin cancer prevention utilizing oral 13-cis retinoic acid (Accutane) in patients with XP was completed. XP patients were selected who had a documented high rate of skin tumor formation. All pre-existing tumors were surgically removed and a high dose (2 mg/kg/da) of 13-cis retinoic acid, administered orally, was given for 2 years. The drug was then stopped to allow an additional 1 year observation period. In the five XP patients who completed the study there was a greater than 60% reduction in frequency of skin cancer formation during the time of drug treatment and an increase to pre-treatment levels when the drug was withdrawn. All patients experienced the side effect of mucocutaneous toxicity; in addition some patients developed elevated liver function tests, elevated triglycerides and calcification of the tendons and ligaments. Patients who have completed the high dose protocol are presently being treated with a lower dose to determine if the effectiveness will remain with less toxicity.

Publications:

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

PROJECT NUMBER
 Z010P05086-11 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Phenotyping of Cytochrome P-450s in Animal and Human Tissues

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

P.I.:	Sang Shin Park	Expert	LMC	NCI
Others:	Harry V. Gelboin	Chief	LMC	NCI
	Choong S. Park	Guest Researcher	LMC	NCI
	David Ray	Research Chemist	LMC	NCI

COOPERATING UNITS (if any)

Univ. of Oulu, Finland (O. Pelkonen); IARC, Lyon, France (E. Hietanen, H. Bartsch); Univ. Hosp., Sweden (A. Rane); VA Med. Center, Cleveland, Ohio (W. A. Khan, H. Mukhtar); Seoul Natl. Univ., Seoul, Korea (N. D. Kim)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)

Cytochrome P-450s (P-450) are key components of mixed function oxidases which metabolize many drugs, chemical carcinogens, fatty acids, prostaglandins and some steroids. Identification and quantitation of P-450 isozyme species in tissues and organs may be a key to understanding the individual differences in sensitivities to drugs and chemical carcinogens. Our approach to this goal is the use of epitope-specific monoclonal antibodies (MAbs) to P-450 isozymes. Eight libraries of MAbs to different forms of P-450 (LM2, LM4, MC-B, PB-B, PCN-E, ETOH, SCUP, and RLM5) were established and the phenotypings in animal and human tissues were carried out by radioimmunoassay (RIA), reaction-phenotyping and immunohistochemistry. 3-Methylcholanthrene (MC)-inducible P-450s were found in the lung, kidney, liver, and intestine of C57/BL mice and rats which were treated with MC. The MC forms of P-450 were also found in human placenta and lymphocytes of smoking women. However, the level of growth hormone- and male sex-dependent P-450 RLM5 was not greatly affected by MC induction. Ethanol-inducible P-450j was found in human adult livers, and pregnenolone 16 α -carbonitrile-inducible P-450 PCN-E was found in both adult and fetal human livers. Application of crude coal tar to skin of neonatal rats predominantly induced P-450 MC-B in both epidermis and liver but P-450 PB-B form was also induced in liver. Treatment of adult male rats with chlortrimazole and diphenylhydantoin induced P-450 forms that are also induced by phenobarbital (PB), PCN and ethanol. These results indicate that MAbs are useful probes for identification of P-450s which were induced by the administration of many drugs and chemical carcinogens.

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

Sang Shin Park	Expert	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Choong S. Park	Guest Researcher	LMC	NCI
David Ray	Chemist	LMC	NCI

Objectives:

Cytochrome P-450s are key components of mixed function oxidase systems which metabolize many drugs and chemical carcinogens. The type and quantity of P-450 reflects individual differences in sensitivity to drugs and chemical carcinogens. The objective of this work is to utilize monoclonal antibodies (MABs) to determine the patterns of P-450 in tissues and organs after drug treatment.

Methods Employed:

Eight panels of MABs to different forms of P-450 (LM2, LM4, MC-B, PB-B, PCN-E, ETOH, SCUP, and RLM5) were established and phenotyping in animal and human tissues were carried out by radioimmunoassay (RIA), reaction-phenotyping (inhibitory of catalytic activity) and immunohistochemistry.

Major Findings:

3-Methylcholanthrene (MC)-inducible P-450s were found in the lung, kidney, liver, and intestine of C57/BL mice and rats which were treated with MC. The MC forms of P-450 were also found in human placenta and lymphocytes of smoking women. However, the level of growth hormone- and male sex-dependent P-450 RLM5 was not greatly affected by MC-induction. Ethanol-inducible P-450j was found in human adult liver and pregnenolone 16 α -carbonitrile-inducible P-450 PCN-E was found in both adult and fetal human liver. Application of crude coal tar to skin of neonatal rats predominantly induced P-450 MC-B in both epidermis and liver and the P-450 PB-B form was also induced in liver. Treatment of adult male rats with chlortrimazole and diphenylhydantoin induced P-450 forms that are also induced by phenobarbital (PB), PCN and ethanol. These results indicate that MABs are not only useful probes for identification of P-450s but also for obtaining P-450 isozyme patterns in animal and human tissues after drug and chemical carcinogen administration.

Publications:

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

PROJECT NUMBER

Z01CP05125-09 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Preparation and Characterization of Monoclonal Antibodies to P450

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

PI:	Sang Shin Park	Expert	LMC	NCI
Others:	Harry V. Gelboin	Chief	LMC	NCI
	David Ray	Chemist	LMC	NCI

COOPERATING UNITS (if any)

Dana-Farber Cancer Institute, Boston, MA (D.J. Waxman)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)

Mixed function oxidase (MFO) systems consist of three components: cytochrome P-450 (P-450), NADPH-P-450 reductase and phospholipid. The P-450s are key components of MFO systems which metabolize many drugs, chemical carcinogens, fatty acids, prostaglandins and some steroids. The substrate specificity depends on the P-450 species. In some systems an additional electron carrier, cytochrome b5, facilitates the catalytic activity. More than 20 P-450s were purified from different tissues and characterized by conventional chemical procedures. As a new approach, we adopted monoclonal antibody (MAb)-directed procedures. MABs to b5, reductase, P-450 3 (testosterone 7 α -hydroxylase) and triacetyl oleandomycin (TAO)-inducible P-450 (P-450 TAO) were prepared by fusion of primed spleen cells with myeloma cells. Radioimmuno-assay was adopted for screening of hybridomas which produced MABs binding to respective immunogens. Twenty-nine MABs to b5 belonged to either IgG1(k), IgG2b(k), or IgG3(k). The inhibitory effects of MABs to b5 on NADH-reductase ranged from 0 to 77%, but the MABs had no effect on other catalytic activities tested. Fourteen MABs to NADPH-P-450-reductase belonged to either IgG(k) or IgM(k) but they had no effect on NADPH-reductase and AHH activity. Each of the three MABs to P-450 3 and P-450 TAO belonged to IgM(k). These MABs would be very useful for studying their roles in MFO systems and identification of each component in animal and human tissues.

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

Sang Shin Park	Expert	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
David Ray	Chemist	LMC	NCI

Objectives:

The P-450s are key components of mixed function oxidase systems which metabolize many drugs, chemical carcinogens, fatty acid, prostaglandins and some steroids. In some MFO systems, cytochrome b5 enhances their functions. MAbs were prepared to cytochrome P-450 3, triacetyl oleandomycin (TAO)-inducible P-450 (P-450 TAO, NADPH-P-450 reductase and cytochrome b5 (b5). The MAbs will be used to identify and phenotype the components in animal and human tissues.

Methods Employed:

Balb/c female mice were immunized with purified b5, NADP-P-450-reductase, P-450 3, and P-450 TAO, separately. The primed spleen cells were fused with myeloma cells by polyethylene glycol; hybrid cells were grown in selective medium (HAT) and screened by radioimmunoassay (RIA) against each immunogen. The effect of MAbs on catalytic activity was measured in the microsomal systems.

Major Findings:

MAbs prepared against b5, NADPH-reductase, P-450 3 and P-450 TAO all had higher bindings in RIA than nonspecific MAbs to their respective immunogens. Twenty-nine MAbs to b5 belonged to either IgG1(k), IgG2b(k), or IgG3(k). The inhibitory effects of MAbs on NADH-reductase ranges from 0 to 77%, but the MAbs had no effect on acetanilide hydroxylase, aniline p-hydroxylase, 7-ethoxycoumarin deethylase, 7-ethoxy-resorufin deethylase, 7-penthoxyresorufin deethylase and benzphetamine demethylase activities. Fourteen MAbs to NADPH-P-450-reductase belonged to either IgG(k) or IgM(k) but they had no effect on NADPH-reductase and AHH activity. The three MAbs to P-450 3 and 3 Mabstp P-450 TAO belonged to IgM(k). These MAbs would be very useful for studying their roles in MFO systems and identification of each component in animal and human tissues.

PUBLICATIONS:

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Honkakoski P, Autio S, Juvonen R, Raunio H, Gelboin HV, Park SS, Pelkonen O, Lang M. Pyrazol is different from acetone and ethanol as an inducer of the polysubstrate monooxygenase system in mice: evidence that pyrazole-inducible P-450Coh is distinct from acetone-inducible P-450ac. Arch Biochem Biophys 1988;267:589-98.

Miller MS, Jones AB, Chauhan DP, Park SS, Anderson LM. Differential induction of fetal mouse liver and lung cytochromes P-450 by β -naphthoflavone and 3-methylcholanthrene. Cancer Res (In Press).

Park SS, Waxman DJ, Lapenson DP, Schenkman JB, Gelboin HV. Monoclonal antibodies to rat liver cytochrome P-450 2c/RLM5 that regiospecifically inhibit steroid metabolism. Biochem Pharmacol (In Press).

Song BJ, Veech RL, Park SS, Gelboin HV, Gonzalez FJ. Structure and regulation of the ethanol-inducible cytochrome P-450j. In: Stimmel B, ed. Advances in alcoholism and substance abuse. New York: Academic Press (In Press).

Song BJ, Veech RL, Park SS, Gelboin HV, Gonzalez FJ. Induction of rat hepatic N-nitrodimethylamine demethylase by acetone is due to protein stabilization. J Biol Chem 1989;264:3568-72.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05318-07 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Structural 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	Research Chemist	LMC	NCI
Others:	Yoshiaki Omata	Visiting Fellow	LMC	NCI
	Richard C. Robinson	Biologist	LMC	NCI
	Sang S. Park	Expert	LMC	NCI
	Harry V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)

Gerontology Research Center, NIA, Baltimore, MD (J. Rifkind)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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 unrounded type. Do not exceed the space provided.)

The focus of this project is the characterization of structure-function relationships of the cytochrome P-450s. The quaternary structure of P-450s in intact rat liver microsomal membranes was examined by a cross-linking study which revealed specific association among P-450c and P-450 2a, forms which metabolize polycyclic hydrocarbons and testosterone, respectively. The crosslinking results also demonstrated a complex of P-450c with reductase. Such specific P-450 interactions may influence the disposition of P-450 substrates in secondary metabolism. The active site structure of purified and microsomal P-450c was examined in binding studies using the substrate benzopyrene (BP). BP fluorescence was enhanced upon binding to the microsomal membrane but quenched upon binding to P-450c. Quenching measurements on pure P-450c indicated that binding of MAb to this P-450 resulted in reduced mobility of the substrate binding site. Flash photolysis experiments of CO recombination to the P-450 heme yielded parallel kinetic data on the effect of BP on active site dynamics. We examined the iron spin state of testosterone-binding rat liver microsomal P-450s by absorption and electron spin resonance (ESR) spectroscopy. The data suggest at least two classes of testosterone binding P-450s: one P-450 class shifts microsomal P-450 from the low spin to the high-spin state while the second class represents a perturbation from one low-spin state to another.

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

Fred K. Friedman	Research Chemist	LMC	NCI
Yoshiaki Omata	Visiting Fellow	LMC	NCI
Sang. S. Park	Sr. Staff fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI

Objectives:

To characterize the structure-function relationships of the multiple forms of cytochrome P-450 in both the purified and microsomal state.

Methods Employed:

Monoclonal antibodies (MAbs) were prepared to several rat liver P-450s. MAbs linked to Sepharose were used for immunopurification of P-450s from tissue microsomes. Cross-linking of microsomal membrane proteins was carried out with the crosslinker sulfosuccinimidyl(4-azidophenyl)dithio)propionate. Cross-linked P-450s were identified after disulfide cleavage. Analytical methods employed include gel electrophoresis, western blots, and spectral analyses. A laser flash photolysis apparatus was assembled from various components and used to monitor the kinetics of CO binding to P-450.

Major Findings:

Organization of P-450s in the microsomal membrane was studied by chemical and photochemical cross-linking of microsomal membrane proteins and subsequent immunopurification with MAbs to P-450. Results using the polycyclic hydrocarbon-inducible P-450c form demonstrated that it was not monomeric, but was associated with other proteins, including the constitutive testosterone metabolizing P-450 2a form. The data also demonstrated a complex between P-450c and reductase, a protein essential for expression of P-450 activity. Since P-450 substrates are often acted upon by multiple P-450s, the distribution of metabolites may be influenced by interactions among specific P-450s.

The active site structure of purified and microsomal P-450c was examined in binding studies using the substrate benzopyrene (BP). BP fluorescence was enhanced upon binding to the microsomal membrane but quenched upon binding to either purified or microsome-bound P-450c. Quenching measurements on pure P-450c indicated that binding of MAB to this P-450 resulted in reduced mobility of the substrate binding site. Flash photolysis experiments of CO recombination to the P-450 heme yielded parallel kinetic data on the effect of BP on active site dynamics.

Antibodies directed toward known P-450 amino acid sequences are a valuable tool for defining the structure-function role of various regions of the primary sequence. We are thus currently synthesizing peptides corresponding

to regions of P-450c which may be involved in binding to the BP substrate. These will be evaluated for their effect on P-450c activity and binding to BP.

We examined the iron spin state of testosterone-binding rat liver microsomal P-450s by absorption and ESR spectroscopy. Spectral perturbations by testosterone were used to identify testosterone-specific P-450 forms. Absorption difference spectra indicated that testosterone induced a greater conversion of P-450 to the high spin form in 3-month rats than in 24-month rats. ESR signals corresponding to total low spin P-450 were of higher intensity in the young rats and were increased by testosterone. Testosterone also interconverted one low spin P-450 species to another. The combined data suggest at least two classes of testosterone binding P-450s. One P-450 class, inferred from the absorbance titration data, shifts microsomal P-450 from the low spin to the high-spin state. The second class represents a perturbation from one low-spin state to another, as evidenced from the ESR data.

Publications:

Friedman FK, Robinson RC and Rifkind J. Age-related changes in the iron spin state of testosterone-binding rat liver microsomal cytochromes P-450. *Biochem Biophys Res Commun* 1989;158:480-4.

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

PROJECT NUMBER

Z01CP05436-05 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Mutagen Activation Analysis with Expressed P-450s and Monoclonal Antibodies

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

PIs:	Harry V. Gelboin	Chief	LMC NCI
	Frank Gonzalez	Acting Section Chief	LMC NCI
Others:	Toshifumi Aoyama	Visiting Associate	LMC NCI
	Sang Shin Park	Expert	LMC NCI
	Michael Myers	Senior Staff Fellow	LMC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section and Nucleic Acid Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda Maryland 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

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

Cytochrome P-450s metabolize xenobiotics such as drugs and carcinogens as well as endobiotics such as steroids and prostaglandins. Multiple forms of these enzymes are expressed constitutively or after administration of inducers. A single cytochrome P-450 may metabolize multiple substrates and a single substrate may be acted upon by either a single or several cytochrome P-450s. Cytochrome P-450s may engage normal detoxification or catalyze deleterious carcinogen or mutagen activation. This project has used previously cloned cDNAs of three P-450s to construct the three cDNAs into vaccinia virus vectors. These vectors express the incorporated cDNA into a single P-450 enzyme when infected into host cells.

cDNAs for rodent P₁-450, P₃-450, and P-450a were expressed in the modified vaccinia virus-T7 RNA polymerase system. Each P-450 exhibited its appropriate molecular weight and characteristic enzyme activity. Aryl hydrocarbon hydroxylase activity was catalyzed by P₁-450 and acetanilide hydroxylase by P₃-450. Each expressed P-450 was also analyzed for its ability to activate 19 carcinogens of diverse classes to their mutagenic forms. Most notable was the activation of several polycyclic aromatic hydrocarbons by P₁-450 and the activation of acetylaminofluorene, 4-aminobiphenyl, and several heterocyclic amine food pyrolysate products by P₃-450. P-450a, in contrast, showed slight mutagen activation only toward N-hydroxy-2-acetyl aminofluorene. The vaccinia virus-T7 RNA polymerase system described here can express cDNAs for diverse forms of P-450, each of which can then be characterized for substrate and product specificity and for mutagen activation.

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

Harry V. Gelboin		Chief	LMC
NCI			
Frank Gonzalez	Section Chief	LMC NCI	
Toshifumi Aoyama	Visiting Associate	LMC NCI	
Sang Shin Park	Expert	LMC NCI	
Michael Myers	Senior Staff Fellow	LMC NCI	

Objectives:

The goal of this project is to (a) express enzymatically active individual human and animal cytochrome P-450s from recombinant vaccinia virus vectors; (b) to analyze the expressed proteins for enzymatic activities; and (c) to test for mutagen activation and cell transformation activity of the individual P-450s. To use inhibitory monoclonal antibodies to different P-450s to determine the quantitative contribution of each P-450 to mutagen activation.

Methods Employed:

Construction of recombinant viruses requiring recombinant DNA methodology, DNA separation procedures, DNA transfections, cell culture techniques, virological procedures and genetic selection of cells and viruses. Detection and isolation of expression products requires subcellular fractionation, protein separations, electrophoresis and chromatography, immunological procedures employing monoclonal and polyclonal antibodies. Functional evaluation of the expressed proteins require a variety of enzymatic assays such as aryl hydrocarbon hydroxylase, ethoxyresorufin O-deethylase, 7-ethoxycoumarin O-deethylase, acetanilide hydroxylase and others. Monoclonal antibodies are produced by the hybridoma technology, and mutagen activation assays are carried out using the Ames test.

Major findings:

cDNAs for rodent P₁-450, P₃-450, and P-450a were expressed in the modified vaccinia virus-T7 RNA polymerase system. Each P-450 exhibited its appropriate molecular weight and characteristic enzyme activity. Aryl hydrocarbon hydroxylase activity was catalyzed by P₁-450 and acetanilide hydroxylase by P₃-450. Each expressed P-450 was also analyzed for its ability to activate 19 carcinogens of diverse classes to their mutagenic forms. Most notable was the activation of several polycyclic aromatic hydrocarbons by P₁ and the activation of acetylaminofluorene, 4-aminobiphenyl, and several heterocyclic amine food pyrolysate products by P₃-450. P-450a, in contrast, showed slight mutagen activation only toward N-hydroxy-2-acetyl aminofluorene. The vaccinia virus-T7 RNA polymerase system described here can express cDNAs for diverse forms of P-450, each of which can then be characterized for substrate and product specificity and for mutagen activation.

Aflatoxin B₁ (AFB₁), a potent hepatocarcinogen, is detoxified to aflatoxin M₁ (AFM₁) via a cytochrome P-450-mediated AFB₁-4-hydroxylase that is regulated by the aryl hydrocarbon (Ah) locus. Lysates from mammalian cells infected with recombinant vaccinia viruses containing cDNA expressed cytochrome P₁-450 or P₃-450 were tested for their ability to metabolize AFB₁ to AFM₁. The results identify cytochrome P₃-450 as an AFB₁-4-hydroxylase. This is the first assignment of a specific P-450 to an aflatoxin metabolic pathway.

Publications:

Aoyama T, Gonzalez FJ and Gelboin HV. Mutagen activation by cDNA-expressed P₁-450, P₃-450 and P-450a. *Molec Carcinogen* 1989;253-9.

Falleto JB, Koser PL, Battula N, Townsend GK, Maccubbin AE, Gelboin HV and Gurtoo HL. Cytochrome P₃-450 cDNA encodes aflatoxin B₁-4-hydroxylase. *J Biol Chem* 1988;263:12187-89.

Pacifici GM, Park SS, Gelboin HV and Rane A. 7-ethoxycoumarin and 7-ethoxyresorufin O-deethylase in human fetal and adult liver: studies with monoclonal antibodies. *Pharmacol Toxicol* 1988;63:26-9.

Honkakoski P, Autio S, Juvonen R, Raunio H, Gelboin HV, Park SS, Pelkonen O and Lang, MA. Pyrazole is different from acetone and ethanol as an inducer of the polysubstrate monooxygenase system in mice: evidence that pyrazole-inducible P-450Coh is distinct from acetone-inducible P-450ac. *Arch Biochem Biophys* 1988;267:580-98.

Gelboin HV. Chirality and cytochrome P-450: a perspective. *Biochem Pharmacol* 1988;36:103.

Pasanen M, Stenback F, Park SS, Gelboin HV and Pelkonen O. Immunohistochemical detection of human placental cytochrome P-450-associated monooxygenase system inducible by maternal cigarette smoking. *Placenta* 1988;9:1-9.

Gelboin HV, Park SS and Battula N. DNA recombinant and monoclonal antibody detected methods for determining cytochrome P-450 specificity. *Biochem Pharmacol* 1988;37:97-8.

Ladona MG, Park SS, Gelboin HV, Hammar L and Rane A. Monoclonal antibody directed detection of cytochrome P-450 (PCN) in human fetal liver. *Biochem Pharmacol* 1988;263:29-35.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05521-03 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Polymorphic Drug Oxidation: The Human and Rat Debrisoquine 4-hydroxylase Gene

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

PI:	Frank Gonzalez	Acting Section Chief	LMC	NCI
OTHERS:	Harry V. Gelboin	Chief	LMC	NCI
	Shioko Kimura	Visiting Scientist	LMC	NCI
	Nobumitsu Hanioka	Visiting Fellow	LMC	NCI
	Eiji Matsunaga	Visiting Fellow	LMC	NCI
	O. Wesley McBride	Section Chief	LB	DCBD NCI

COOPERATING UNITS (if any)

Argonne National Laboratory, Argonne, IL (James P. Hardwick); Biocenter, University of Basel, Switzerland (Urs A. Meyer)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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 un-reduced type. Do not exceed the space provided.)

Individual subjects vary considerably in their abilities to metabolize drugs. In addition, genetic influences are thought to govern individual susceptibility to chemically-induced cancers. The mixed function monooxygenase system is composed of multiple forms of P450s that are the principal enzymes associated with drug and carcinogen metabolism. These enzymes can serve to detoxify and hasten the elimination of foreign agents or they can activate inert chemicals to harmful electrophilic metabolites that damage DNA and initiate the carcinogenic process. We have begun to study polymorphic drug oxidation in humans by establishing the mechanism of the debrisoquine 4-hydroxylase polymorphism. This polymorphism effects from 5% to 10% of the North American and European Caucasian population. These individuals, referred to as poor metabolizers, or PMs, cannot metabolize debrisoquine and many other related drugs, while the rest of the population, called extensive metabolizers, or EMs, rapidly hydroxylate this drug, resulting in the inactivation of its therapeutically active form. The DA rat strain has been proposed as a model for the human debrisoquine hydroxylase deficiency. To determine the mechanism by which the DA rat has lost debrisoquine 4-hydroxylase activity, we cloned and sequenced five cDNAs in the rat *CYP2D* gene subfamily. These sequences were used to develop probes specific for each mRNA. The DA rat is lacking mRNA for IID1 but contains mRNA for the remaining four IID genes. In contrast, Sprague-Dawley (SD) rats, prototypes for human EMs, express all five IID mRNAs. The IID1 gene is, therefore, transcriptionally inactive in DA rat.

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

Frank Gonzalez	Acting Section Chief	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Shioko Kimura	Visiting Associate	LMC	NCI
Nobumitsu Hanioka	Visiting Fellow	LMC	NCI
Eiji Matsunaga	Visiting Fellow	LMC	NCI
O. Wesley McBride	Section Chief	LB DCBD	NCI

Objectives:

1. Isolate and characterize rat cDNAs in the IID gene family.
2. Express the rat cDNAs to determine the substrate specificities of each P450.
3. Determine the mechanism of the debrisoquine 4-hydroxylase polymorphism in DA rat.
4. Isolate and sequence all genes within the rat IID locus.

Methods Employed:

1. Construction of cDNA libraries in the λ gt11 vector was carried out by reverse transcription of mRNA and strand replacement.
2. Shotgun cloning into M13 phage and sequencing by the Sanger dideoxynucleotide method. Sequence data were compiled and analyzed by use of the Beckman Microgenie program.
3. Northern blotting was carried out by use of formaldehyde-agarose gels and the hybridization technique of Church and Gilbert.
4. Genomic cloning was performed using the λ EMBL3 vector and partial Sau3A-digested human DNA.
5. Southern blotting was carried out by alkaline transfer of DNA to nylon filters and hybridization using 50% formamide.

Major Findings:

The DA rat has been proposed as an animal model for the human debrisoquine 4-hydroxylase/bufuralol 1'-hydroxylase genetic deficiency. To determine the mechanism of this deficiency, we isolated and sequenced five cDNAs in the *CYP2D* gene subfamily including a new IID1 allele and two cDNAs of novel P-450s, designated IID3 and IID5. IID3 and IID5 cDNA-deduced amino acid sequences contained 500 and 504 residues with calculated molecular weights of 56,683 and 57,081 daltons, respectively. IID5 displayed 20 amino acid differences with the IID1, yet bore only 72% and 76% similarity to IID2 and

IID3. Despite an overall nucleotide similarity of 80% to 98% between the four cDNAs, a region of 134 nucleotides of sequence exists that contains only one base difference. This region is probably the result of gene conversion events between the P-450 IID genes. Although all IID cDNAs were expressed into immunodetectable proteins using the COS cell-SV40 based expression system, only IID1 could effectively catalyze the oxidation of the prototype substrate bufuralol. Expression of a cDNA isolated in an earlier study, previously called dbl and now designated IID1v, produced a protein with a drastically reduced activity as compared to cDNA-expressed IID1 despite only four amino acid differences between the two cDNA-deduced protein sequences. IID1 and IID1v appear to be allelic variants of the same gene. To determine the mechanism of the debrisoquine/bufuralol drug oxidation deficiency in DA rat, specific cDNA and oligonucleotide probes were used to quantitate levels of each mRNA in Sprague Dawley and DA rat livers. The former rat strain expressed IID1, IID2, IID3 and IID5 mRNAs, whereas the DA rat expressed only IID2, IID3 and IID5 mRNAs; the IID1 gene was not expressed in this rat strain. Moreover, immunoinhibition studies using a strongly inhibitory antibody suggested a major contribution of IID1 to bufuralol metabolism in SD but not in DA rats. These results establish that the DA rat drug oxidation polymorphism is due to the absence of expression of the IID1 gene.

Publications:

Matsunaga, E, Zanger, EM, Hardwick, JP, Gelboin, HV, Meyer, UA and Gonzalez, FJ. The *CYP2D* gene subfamily: analysis of the molecular basis of the deficiency debrisoquine 4-hydroxylase deficiency in DA rats. *Biochemistry* (In Press).

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

PROJECT NUMBER

Z01CP05522-03 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Structure and Characterization of Human Thyroid Peroxidase

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

PI: Shioko Kimura Visiting Scientist LMC NCI

OTHERS: Fumitaka Kikkawa Visiting Fellow LMC NCI

COOPERATING UNITS (if any)

Miyazaki Medical College Hospital, Miyazaki, Japan (Sachiya Ohtaki, Tomio Kotani)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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 un-reduced type. Do not exceed the space provided.)

Peroxidase is a hemoprotein which is ubiquitously found from microorganisms to animals, and catalyzes biosynthesis and degradation of a variety of substances using hydrogen peroxide. During the peroxidative reaction, free radicals are produced that can irreversibly bind to DNA. This may suggest the involvement of peroxidase in tumorigenesis in various tissues. We have been studying human thyroid peroxidase using molecular biological approaches. This peroxidase plays an important role in the biosynthesis of thyroid hormones and has been identified as one of autoantigens, against which patients with autoimmune thyroid diseases such as Graves' disease and Hashimoto's thyroiditis develop antibodies. When the human thyroid peroxidase cDNA nucleotide and deduced amino acid sequences, and the exon-intron junction structures of the gene were compared with those of human myeloperoxidase, a granulocyte protein with microbicidal function, it became clear that both enzymes that previously had been thought to be completely different in terms of protein structure, physicochemical properties, and physiological functions in their target tissues, were in fact members of the same gene family and evolved from the common ancestral gene. We further expressed human thyroid peroxidase cDNA and obtained an enzymatically active protein using the vaccinia virus expression system. After immunoenrichment, the expressed protein exhibited a hemoprotein characteristic absorption spectrum when complexed with cyanide, and a more than 300-fold higher specific activity.

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

Shioko Kimura	Visiting Scientist	LMC	NCI
Fumitaka Kikkawa	Visiting Fellow	LMC	NCI

Objectives:

1. Isolate and sequence genomic clones for human thyroid peroxidase.
2. Determine the mechanism of regulation of peroxidase levels in thyroid tissue.
3. Express human thyroid peroxidase in tissue culture cells and characterize the expressed peroxidase.
4. Examine individual variations in the expression of thyroid peroxidase in thyroid tissues from thyroid disease patients and normal individuals.

Methods Employed:

A genomic library in λ EBML3 and a cosmid library were constructed from human lymphocyte DNA by a partial Sau 3A digestion. These libraries were screened with human thyroid peroxidase cDNA and several clones were isolated. Eco RI-digested fragments from the isolated clones which hybridized to the cDNA were further subcloned and used to construct M13 shotgun libraries for sequencing. Sequencing was done by the dideoxy-nucleotide chain termination method. S1 mapping and primer extension were performed to determine the transcription start site of the gene. Genomic DNA characterization and mapping of genomic clones were carried out by Southern blot analysis.

Human thyroid peroxidase was expressed by means of the vaccinia virus expression system in human hepatoma Hep G2 cells. Expressed protein was analyzed by western blot and spectrophotometrical measurements were carried out to obtain enzyme activity and absorption spectrum. Monoclonal antibody-assisted immunoaffinity column chromatography was used to partially purify the expressed protein.

Major Findings:

All exons of the human thyroid peroxidase gene were cloned from phage and cosmid libraries and sequenced, including 2599 base pairs of upstream DNA. The gene contains 17 exons and covers at least 150 kilobase pairs of chromosome 2. The transcription start site was identified by both S1 mapping and primer extension; a typical TATA box was found 25 bases upstream of the putative start site. A comparison of the gene structures of thyroid peroxidase and a granulocyte protein, myeloperoxidase, revealed that the positions of the 3rd through 11th exon-intron junctions in thyroid coincide exactly with those of the 2nd through 11th exon-intron junctions in myeloperoxidase, except the 7th myeloperoxidase junction that does not have

any counterpart in thyroid peroxidase. The amino acid codon separation pattern in each junction is well conserved between both enzymes. We originally isolated and sequenced two human thyroid peroxidase cDNAs, designated phTPO-1 and phTPO-2. The phTPO-2 is almost identical to phTPO-1 except that it has lost 171 base pairs in the middle of the coding sequence. This 171 base pair sequence has intron splicing characteristic base pairs, GT and AG, at the beginning and the end of the sequence. This sequence was found to be exactly contained in exon 10. Four exons, unique to thyroid peroxidase, are located at the 3' end of the gene (exons 13-16), each of which encompasses a different protein module. Three of these modules, representing exons 13, 14 and 15, bear significant similarities to C4b- β 2 glycoprotein, the EGF-LDL receptor, and a typical transmembrane domain, respectively. The genes coding for these modules were probably fused to an ancestral peroxidase gene to generate the present thyroid peroxidase gene. The data suggest that intron loss, and/or insertion, and exon shuffling have played important roles in the evolution of the thyroid peroxidase gene.

hTPO-1 cDNA was expressed in human Hep G2 cells using a vaccinia virus cDNA-expression system. When examined by immunoblot analysis, hTPO-1 protein expression reached the maximum level approximately 24 hr after infection and remained at a similar level up to 72 hr post-infection. The expressed protein was enzymatically active as measured by guaiacol oxidation. Monoclonal antibody-assisted immunoaffinity column chromatography was used to partially purify vaccinia-expressed hTPO-1, resulting in more than a 300-fold higher specific activity and a measurable difference spectrum of the hTPO-1(Fe^{3+})-CN complex.

Publications:

Kimura, S, Hong, Y-S, Kotani, T, Ohtaki, S, Kikkawa, F. Structure of the human thyroid peroxidase gene: comparison and relationship to the human myeloperoxidase gene. *Biochemistry* (In Press).

Kimura, S, Kotani, T, Ohtaki, S, Aoyama, T. cDNA-directed expression of human thyroid peroxidase. *FEBS Lett* (In Press).

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

PROJECT NUMBER

Z01CP05561-02 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Transcriptional Regulation of Cytochrome P-450 Genes

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

PI:	Frank Gonzalez	Acting Section Chief	LMC	NCI
OTHERS:	Harry V. Gelboin	Chief	LMC	NCI
	Minoru Nomoto	Visiting Associate	LMC	NCI
	Takaki Ueno	Visiting Fellow	LMC	NCI
	Shioko Kimura	Visiting Scientist	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

3.0

PROFESSIONAL

3.0

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 majority of P-450 genes are regulated at the transcriptional level. During distinct stages of development, P-450 genes became transcriptionally activated. We are studying the genes coding for several P-450s that are activated during development. For example, the IIA1 gene is activated within 1 week after birth in males and females and becomes specifically suppressed in males at puberty. IIA2 becomes activated in males when they reach puberty and is never expressed in females. The rat IIA1 and IIA2 genes were isolated and completely sequenced. To study the cis- and trans-acting elements responsible for these gene activations, we are using a cell-free in vitro transcription system derived from rat liver. The IIA2 gene has been most extensively studied. A unique nuclear extract has been developed that faithfully transcribes the IIA2 promoter. Only extracts derived from male rat nuclei are capable of accurately transcribing this promoter. Deletion analysis has been used to identify the boundaries of the DNA responsible for the sex dependent transcription.

To determine the mechanism by which P-450 genes are transcriptionally activated by inducing agents, genomic clones for two rat clofibrate-inducible genes, designated IVA1 and IVA2, were isolated from a lambda EMBL3 library. These genes are both transcriptionally activated by hypolipidemic agents. A highly conserved 19 base pair region was identified just upstream of the start sites for these genes. This region may be involved in regulation of the IVA1 and IVA2 genes. Interestingly, the latter gene is constitutively expressed in kidney.

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

Frank Gonzalez	Acting Section Head	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Minoru Nomoto	Visiting Associate	LMC	NCI
Takaki Ueno	Visiting Fellow	LMC	NCI
Shioko Kimura	Visiting Scientist	LMC	NCI

Objectives:

1. Isolate and sequence genes coding for developmentally regulated P-450 genes and P-450 genes that are activated or induced by specific chemicals.
2. Northern blot analysis with specific oligonucleotide probes.
3. Develop an in vitro cell-free transcription system that faithfully transcribes P-450 gene promoters.
4. Determine the DNA sequence elements that are necessary for gene activation during development.
5. Characterize the protein factors that are needed to activate transcription during development.
6. Determine the sequences that bind to developmental, specific protein factors that activate transcription.

Methods Employed:

Genomic libraries were prepared from rat DNA using the λ EMBL3 vector. DNAs were sequenced by the dideoxynucleotide method and the sequence data were analyzed using the Beckman Microgenie program. Cell-free transcription extracts derived from rat liver were prepared by a modification of the method described by Gorski, Carneiro, and Schibler and the method of Dingham. Transcription was monitored by primer extension analysis. Human thymidine kinase and rat albumin promoters were included in the transcription extracts as internal positive controls. Upstream DNA was modified by deletion analysis using restriction enzymes and exonucleases. Factor-binding assays were performed using gel retardation assays of kinased, labeled fragments derived from upstream DNA sequence.

Major Findings:

P-450s IIA1 and IIA2 are steroid hydroxylases that are sex-specific and developmentally regulated in the rat. In order to explore the mechanism of their regulation, the IIA1 and IIA2 genes were isolated from a λ EMBL3 genomic library. The IIA1 gene was completely sequenced and spanned 12,831 bp. The IIA2 gene was sequenced except for 1 kbp and 12 kbp in its second and fifth

introns, respectively. This gene was about 10 kbp longer than IIA1. Both genes possess nine exons. DNA of 5449 and 5484 bp upstream from the IIA1 and IIA2 genes, respectively, was also sequenced and the transcription start sites were determined. Both genes had typical TATA boxes but no CCAAT boxes were found within several hundred bp upstream of the start sites. Search of the gene bank revealed the presence of a region of 255 bp at -3 kbp from the transcription start site of the IIA1 gene sharing significant nucleotide similarity with the polymerase gene of various retrovirus. The IIA2 gene contained two regions between -3.3 kbp to -4.4 kbp and -4.9 to -5.5 kbp that displayed 90% sequence similarity with the consensus long interspersed middle repetitive elements (LINE1). In addition, a 1.6 kbp-inserted sequence was detected between -200 bp to -1700 bp that appears to be a retroseudogene. Evidence was also uncovered that gene conversions that encompassed both exonic and intronic DNA have occurred between the IIA1 and IIA2 genes. A nuclear extract derived from adult hepatocytes was prepared and used to direct *in vitro* transcription of the IIA1 and IIA2 gene promoters. The IIA2 promoter was transcribed most efficiently in extracts from adult male rats, whereas the IIA1 promoter was transcribed from extracts made from both males and females. Deletion analysis was used to delineate the *cis*-acting DNA sequence elements required for sex-specific transcription.

To study P-450 gene regulation by inducing agents, the clofibrate-regulated P-450 IVA1 and IVA2 genes were isolated from a rat genomic library constructed in the vector λ EMBL3 and their complete sequences were determined. The IVA1 and IVA2 genes spanned 14,144 and 10,576 bp and contained 13 and 12 exons, respectively. The IVA1 gene contained an additional intron that splits exon 12 of the IVA2 gene, resulting in noncoding of the 13th exon in IVA1. The exon numbers of these genes were distinct among known P-450 genes, and yet several intron-exon junctions along the P-450 amino acid coding region were conserved with P-450 genes in the II, XI, and XXI gene families. Based on these data the number of exons in the putative ancestral P-450 gene was estimated. No consensus TATA sequence was found upstream of either gene's transcription start site. Comparison of the IVA1 and IVA2 promoters with other genes that lack TATA boxes did not reveal any strong consensus sequence in their immediate upstream regions. However, a conserved 19 bp sequence was located at the positions of 42 and 48 bp upstream of the IVA1 and IVA2 genes' start sites, respectively. This element might be involved in regulation of these two genes by clofibrate. The IVA2 gene also contained two 378 bp long direct repeats upstream of the start site. These repeats are derived from portions of the long interspersed middle repetitive element present in high copy numbers in the rat genome.

A novel P-450 cDNA, designated IVA3, was isolated from a λ gt11 clofibrate-treated rat liver library by screening with the lauric acid ω -hydroxylase, IVA1, cDNA probe. This cDNA encoded a protein with 507 amino acids and a calculated Mr of 58,239 daltons. The IVA3 cDNA shared 65% and 97% nucleotide and 72% and 96% DNA-deduced amino acid sequence similarities with the IVA1 and IVA2, respectively. The IVA gene family, designated the *Cyp4a* locus, was mapped to mouse chromosome 4 using a panel of mouse-hamster somatic cell hybrids. Levels of the IVA mRNAs were analyzed in rat tissues and cell cultures after treatment with the hypolipidemic drug clofibrate. The IVA1,

IVA2 and IVA3 mRNAs were present at very low levels in the livers of untreated rats and markedly induced in rats treated with clofibrate. Dose-response and time course studies revealed that all three genes were coordinately regulated in liver. In contrast to the coordinate induction in liver, only the IVA3 gene was induced in the rat hepatoma cell line McA-RH7777. In the kidney, IVA1 and IVA3 mRNAs were present at low levels and were induced by clofibrate in a manner similar to that in liver. In contrast, the IVA2 mRNA was expressed in the kidney of untreated rats at a level similar to that of the maximally-induced IVA2 mRNA in liver. These data indicate that the IVA1, IVA2 and IVA3 genes are coordinately regulated in liver, while only IVA2 is derepressed and constitutively activated in kidney.

Publications:

Kimura S, Hanioka N, Matsunaga E and Gonzalez FJ. The rat clofibrate-inducible *CYP4A* gene subfamily I. Complete intron and exon sequence of the IVA1 (*CYP4A1*) and IVA2 (*CYP4A2*) genes, unique exon organization, and identification of a conserved 19 base pair upstream element. DNA (In Press).

Kimura S, Hardwick JP, Kozak, CA and Gonzalez FJ. The rat clofibrate-inducible *CYP4A* gene subfamily II. cDNA sequence of IVA3 (*CYP4A3*), mapping of the *Cyp4a* locus to mouse chromosome 4, and coordinate and tissue-specific regulation of the *CYP4A* genes. DNA (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05562-02 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Identification and Characterization of New Human P-450

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

PI:	Frank Gonzalez	Acting Section Chief	LMC	NCI
OTHERS:	Toshifumi Aoyama	Visiting Associate	LMC	NCI
	Patson Nhamburo	Visiting Associate	LMC	NCI
	Shigeru Yamano	Special Volunteer	LMC	NCI
	Jun Tatsuno	Special Volunteer	LMC	NCI
	Shioko Kimura	Visiting Scientist	LMC	NCI
	Harry V. Gelboin	Chief	LMC	NCI
	O. Wesley McBride	Section Chief	LB DCBD	NCI

COOPERATING UNITS (if any)

Department of Pharmacology, Biocenter, University of Basel, Basel, Switzerland (U.A. Meyer); Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada (W. Kalow and T. Inaba)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

7.0

PROFESSIONAL

7.0

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

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

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

P-450s are the principal enzymes involved in drug metabolism and carcinogen activation. A large number of P-450s have been purified from rodent tissues and their substrate specificities examined by *in vitro* reconstitution assays. Few P-450s have been purified from man, however, owing to the paucity of readily available human tissues. Purification of these enzymes from human tissue is further complicated by the large degree of genetic variability among individuals and the difficulties in obtaining homogeneous preparations of specific P-450 forms. We have applied a cDNA cloning and expression approach to the study of human P-450s. cDNA probes and antibodies to rodent P-450s are being used to screen *tg*t11 expression libraries constructed from different human liver and lung RNA preparations. The cDNAs are being sequenced and then used to produce active enzymes in cell culture. The enzymes are examined for their abilities to carry out the oxidation of common therapeutically used drugs and to activate carcinogens and mutagens. We have isolated cDNAs and expressed several human P-450s including IA1, IA2, IIA3, IIB1, IIB2, IIC8, IIC9, IID1, IIE1, IIF1, IIIA4, IIIA5, IIIA6 and IVB1. The chromosomal locations of the genes in each P-450 subfamily have been determined. We have also cloned, sequenced, and expressed human microsomal xenobiotic epoxide hydrolase and NADPH-P-450 oxidoreductase. cDNA probes to human P-450s and antibodies against rodent P-450s were used to probe individual human liver specimens for P-450 gene expression to locate livers that do not express a particular P-450. These samples are further examined for the existence of mutant human genes.

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

Frank Gonzalez	Acting Section Chief	LMC	NCI
Toshifumi Aoyama	Visiting Associate	LMC	NCI
Patson T. Nhamburo	Visiting Associate	LMC	NCI
Shigeru Yamano	Guest Researcher	LMC	NCI
Jun Tatsuno	Guest Researcher	LMC	NCI
Shioko Kimura	Visiting Scientist	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
O. Wesley McBride	Section Chief	LB	DCBD NCI

Objectives:

1. Clone human P-450 cDNAs.
2. Determine the sequences of human liver and lung P-450s.
3. Produce human P-450s by vaccinia virus cDNA-expression system.
4. Determine the substrate specificities of individual expressed human P-450s.
5. Determine the specificities of individual forms of P-450 for activation of promutagens and procarcinogens.
6. Analyze the interindividual variability of P-450 protein and mRNA levels in human liver specimens.
7. Determine whether mutant P-450 genes exist in the population.

Methods Employed:

RNA was isolated from human liver and lung specimens that had been obtained from kidney donors. λ gt11 libraries were prepared using the strand replacement method. Libraries were screened with either antibody or cDNA probes derived from rat. Human cDNAs were sequenced using the dideoxynucleotide chain termination procedure. The cDNAs were inserted into vaccinia virus through use of the homologous recombination procedure and expressed in human hepatoma cell. Western immunoblots, spectral determinations and enzyme assays were performed using standard protocols. Polymerase chain reactions were carried out using oligonucleotides prepared on an Applied Biosystems 380B synthesizer and a Perkin Elmer Cetus DNA Thermal Cycler.

Major Findings:

By constructing cDNA libraries from RNA isolated from different human liver and lung specimens and screening these libraries with antibodies and cDNA probes derived from rodent P-450s, we have succeeded in isolating several novel human P-450 cDNAs. These cDNAs were expressed in human hepatoma cells using vaccinia virus and their respective P-450s were assayed for activities toward a variety of substrates.

A novel P-450 IIIA gene product was identified by screening a human liver bank with antibodies. Immunoblotting analysis of human liver microsome preparations revealed that human P-450 PCN1 (hPCN1, Mr 52,000) was expressed in each of 40 individual specimens examined. In about 10%-20% of the livers, an immunologically related protein having a lower electrophoretic mobility (Mr ~52,500) was also detected. A single liver was found that expressed only the lower mobility protein, designated hPCN3, and RNA isolated from this liver was used to construct a λ gt11 library. The library was screened with an hPCN1 cDNA probe resulting in the isolation of a unique, full-length cDNA that was sequenced and shown to encode hPCN3. The deduced amino acid sequence of this cDNA contained 502 residues, a calculated Mr of 57,115 daltons and displayed 84% similarity with hPCN1. The deduced amino terminal sequence of hPCN3 was identical to that of HFLa, a major P-450 expressed in human fetal liver that is immunologically cross-reactive with several family III P-450s. hPCN1 and hPCN3 cDNAs were expressed in Hep G2 cells using a vaccinia virus expression system and shown to encode active enzymes, both characterized by reduced (CO)-binding spectra with λ_{\max} at 450 nm. Enzymatic analysis revealed that both P-450s were similarly active in catalyzing oxidation of the calcium channel blocking drug nifedipine. Both enzymes also catalyzed 6 β -hydroxylation of the steroid hormones testosterone, progesterone and androstenedione, although hPCN1 exhibited several-fold higher expressed activity than hPCN3. Several minor oxidation products of these steroids (e.g., 15 β -hydroxytestosterone), comprising up to ~20% of the total metabolites, were formed by hPCN1 but not hPCN3, indicating that hPCN3 is a more highly regiospecific monooxygenase catalyst with steroid substrates. Clear differences were also detected in their catalytic activities toward the immunosuppressive drug cyclosporin, with two hydroxylated metabolites (M1 and M17) and one demethylated metabolite (M21) formed by hPCN1 but only one metabolite (M1) formed by hPCN3. These studies establish that hPCN3 is a newly described P-450 that is differentially expressed in the adult human population and that has overlapping substrate specificity compared to hPCN1 for metabolism of steroid and drug substrates.

Another P-450 in the IIB subfamily was identified through cDNA cloning. This cDNA, designated IIB1, was used as a probe to determine that the IIB1 gene is differentially expressed in man. The hIIB1 cDNA containing the entire coding sequence of a P-450 in the IIB gene family, was isolated from a human liver λ gt11 library by using the rat IIB1 cDNA as a probe. The hIIB1 protein, deduced from the cDNA sequence, contained 491 amino acids, had a calculated Mr of 56,286 daltons and displayed 76% amino acid similarity with the rat IIB1 protein. Expression of this cDNA, using the vaccinia virus system yielded a P-450 that had a reduced CO-binding spectrum with an absorption maximum of 452 nm. The human enzyme did not, however, metabolize substrates that are common for the rat IIB1 and IIB2 P-450s. Total RNA from 13 livers were probed for

levels of hIIB mRNA. Two livers had high levels, four contained moderate levels, and eight contained very low, or no detectable mRNA. These data suggest that either defective hIIB1 genes exist in humans or that the hIIB1 gene is regulated and variably induced in our liver specimens. To search for mutant mRNA transcripts, libraries were constructed from livers expressing low levels of hIIB1 mRNA. A cDNA, designated hIIB2, was isolated that was identical to the hIIB1 cDNA except for the presence of an unusual alteration of the DNA near its 5' end corresponding to the putative exon 4. This alteration was caused by a deletion of 29 bp and an insertion of 44 bp of nonhomologous DNA. This sequence replacement occurs at the junction of the third and fourth exon as predicted from the structure of the rat IIB1 gene, suggesting that a faulty splice might have given rise to the variant hIIB2 transcript. Due to the presence of an in frame termination codon in the inserted DNA, this variant transcript can only produce a prematurely terminated protein. A third cDNA, designated hIIB3, was identified in two separate libraries that displayed 95% nucleotide and 93% cDNA-deduced amino acid sequence similarities to hIIB1. This transcript was found to possess a C -> T change that resulted in a termination codon. The IIB genes (*CYP2B* locus) were localized to human chromosome 19 using the somatic cell hybrid mapping strategy. High frequency restriction fragment length polymorphisms were detected in both Bam HI and Bgl II digests.

In addition to liver we also constructed and screened cDNA libraries derived from lung. A cDNA coding for a P-450 expressed in human lung was isolated from a λ gt11 library constructed from lung mRNA using a cDNA probe to rat P-450 IVA1. The cDNA-deduced amino acid sequence of this P-450, designated IVB1, consisted of 511 amino acids and had a calculated Mr of 59,558 daltons. The IVB1 amino acid sequence bore 51%, 53%, and 52% similarities to rat IVA1, IVA2, and rabbit P-450_{p-2}, respectively. Comparison of the primary amino acid sequence of human IVB1 with rat IVA and rabbit p-2 P-450 sequences revealed a region of absolute sequence identity of 17 amino acids between residues 304 and 320. However, the functional significance of this conserved sequence is unknown. Human IVB1 also appears to be related to P-450 isozyme 5 that has been extensively characterized in rabbits. The IVB1 cDNA was inserted into a vaccinia virus expression vector and the enzyme expressed in human cell line. The expressed enzyme had an absorption spectrum with a λ_{max} at 450 nm when reduced and complexed with carbon monoxide, typical of other cytochrome P-450s. Unlike rabbit P-450 isozyme 5, however, human IVB1 was unable to activate the promutagen 2-aminofluorene. Human lung microsomal P-450s were also unable to metabolize this compound despite the presence of IVB1 mRNA in three out of four human lungs analyzed. In contrast to its expression in lung, IVB1 mRNA was undetectable in livers from 14 individuals, including those from which the lungs were derived. IVB1-related mRNA was also expressed in rat lung and was undetectable in untreated rat liver. In addition, this mRNA was abundant in both rat kidney and intestine. Human chromosome mapping, determined using the somatic cell hybrid strategy, allowed the assignment of the IVB subfamily to chromosome 1 (1p12-p34). Two restriction fragment length polymorphisms were identified by Hind III and Taq I that were linked to the IVB1 gene.

The cDNA containing the full coding sequence of human NADPH-P-450 oxidoreductase was also isolated and completely sequenced. This cDNA

contained 2398 base pairs, including 9 and 358 base pairs of 5' and 3' noncoding sequences, respectively. The human NADPH-P-450 oxidoreductase protein deduced from the cDNA has 677 amino acids with a calculated molecular weight of 76,656 daltons. The cDNA nucleotide and deduced amino acid sequences displayed 83% and 92% similarities, respectively, with those of the rat NADPH-P-450 oxidoreductase. By use of somatic cell hybrids, the NADPH-P-450 oxidoreductase gene was regionally localized to human chromosome 7 (7p15-q35). The levels of NADPH-P-450 oxidoreductase protein and mRNA were analyzed in 13 human liver specimens and less than threefold variation was found among the different livers. The NADPH-P-450 oxidoreductase cDNA was inserted into vaccinia virus and expressed in cell culture. The cDNA-expressed enzyme was active in reducing the electron acceptor cytochrome c. In addition, the NADPH-P-450 oxidoreductase stimulated the enzymatic activity of vaccinia virus expressed human P₄₅₀ when both recombinant viruses were used to coinfect human cells in culture. An approximate equal mol level of NADPH-P-450 oxidoreductase and P₄₅₀ was required to achieve maximal activity for both ethoxycoumarin O-deethylase and aryl hydrocarbon hydroxylase.

Publications:

Aoyama T, Yamano S, Waxman DJ, Lapenson DJ, Meyer UA, Fisher V, Tyndale R, Inaba T, Kalow W, Gelboin HV, Gonzalez FJ. Cytochrome P-450 hPCN3, a novel cytochrome IIIA gene product that is differentially expressed in adult human liver: cDNA and deduced amino acid sequence and distinct specificities of cDNA-expressed hPCN1 and hPCN3 for the metabolism of steroid hormones and cyclosporin. *J Biol Chem* (In Press).

Nhamburo PT, Gonzalez FJ, McBride OW, Gelboin HV, Kimura SK. Identification of a new P-450 expressed in human lung: complete cDNA sequence, cDNA-directed expression and chromosome mapping. *Biochemistry* (In Press).

Yamano S, Nhamburo PT, Aoyama T, Meyer UA, Inaba T, Kalow W, Gelboin HV, McBride OW, Gonzalez FJ. cDNA cloning and sequence, and cDNA-directed expression of human P-450 IIB1: identification of a normal and two variant cDNAs derived from the *CYP2B* locus on chromosome 19 and differential expression of the IIB mRNAs in human liver. *Biochemistry* (In Press).

Yamano S, Aoyama T, McBride OW, Hardwick JP, Gelboin HV, and Gonzalez FJ. Human NADPH-P-450 oxidoreductase: complementary DNA cloning, sequence and vaccinia virus-mediated expression, and localization of the *CYPOR* gene to chromosome 7. *Mol Pharmacol* (In Press).

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

PROJECT NUMBER

Z01CP05603-01 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Regulation of Cytochrome P-450

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

PI:	Fred K. Friedman	Research Chemist	LMC	NCI
Others:	Jewell Wilson	Biotech. Fellow	LMC	NCI
	Richard C. Robinson	Biologist	LMC	NCI
	Sang S. Park	Sr. Staff Fellow	LMC	NCI
	Harry V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)

Gerontology Research Center, NIA, Baltimore, MD (J. Rifkind)
 Cornell University, Ithaca, NY (N. Lorr)
 Oklahoma Medical Research Foundation, Oklahoma City, OK (R. Carubelli)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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 unrounded type. Do not exceed the space provided.)

Factors governing the regulation of cytochrome P-450 expression were examined in several different systems. The tissue specificity of P-450c induction by 3-methylcholanthrene (MC) was probed both immunochemically with antibodies to P-450c, and enzymatically by aryl hydrocarbon hydroxylase (AHH) activity. The level of P450c in rat tissue homogenates decreased in the following order: liver, nasopharynx, lung, pancreas, and kidney. There was no direct relation between the P-450c level and AHH activity, indicating that significant amounts of extrahepatic activity derive from P-450 forms other than P-450c, and/or the specific activity of P-450c varies among different tissues. The induction of rat liver microsomal and nuclear envelope P-450s by 2-acetylaminofluorene (AAF) was immunochemically probed with antibodies to P-450c and P-450d. Both P-450s were induced to different degrees in microsomes and nuclear envelopes. In addition, the antioxidant butylated hydroxytoluene accentuated AAF induction of P-450c. The effect of aging on P-450 catalyzed testosterone metabolism was studied. Although 24-month rats generally exhibited several lower hydroxylase activities, they had a higher level of 7 α -hydroxylase as well as the P-450a form responsible for this activity. The results demonstrate that although P-450-catalyzed activities generally decrease with senescence, expression of individual P-450s are selectively repressed or activated. A monoclonal antibody to rat P-450 2a immunodetected a related P-450 in chicken liver microsomes obtained at various developmental stages. The developmental profile and induction characteristics of this P-450 and of several P-450-catalyzed activities were determined.

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

Fred K. Friedman	Research Chemist	LMC	NCI
Jewell Wilson	Biotech. Fellow	LMC	NCI
Yoshiaki Omata	Visiting Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI

Objectives:

To examine the factors governing expression of P-450s, and to characterize the structure-function relationships and regulation of the multiple forms of cytochrome P-450 in animal and human tissues.

Methods Employed:

Monoclonal antibodies (MAbs) were prepared to several rat liver P-450s. Analytical methods employed include gel electrophoresis, western blots, enzyme assays for P-450 catalyzed activities, and spectral analyses.

Major Findings:

The effect of 3-methylcholanthrene (MC) treatment on the P-450 content of various rat tissues was probed both immunochemically using MAbs to P-450c, the major MC-inducible liver P-450, and enzymatically by aryl hydrocarbon hydroxylase (AHH) activity. Immunoblots revealed induction of P-450c in liver, lung, nasopharynx, kidney and pancreas, while P-450d induction was observed only in liver. The level of immunodetected P450c in the total tissue homogenates decreased in the following order: liver, nasopharynx, lung, pancreas, and kidney. The AHH activity of liver is significantly higher than that of any of the extrahepatic tissues assayed in this study, and decreased in the following order: liver, kidney, nasopharynx, lung, and pancreas. There was no direct relation between the levels of immunodetected P-450c and AHH activity, indicating that significant amounts of extrahepatic activity derive from P-450 forms other than P-450c, and/or the specific activity of P-450c varies among different tissues. This was most dramatically illustrated in kidney, where MC induced AHH more potently than P-450c.

The influence of dietary 2-acetylaminofluorene (AAF) on the P-450c and P-450d content of rat liver microsomes and nuclear envelope was immunochemically probed with monoclonal and polyclonal antibodies to P-450c and P-450d. An AAF diet elevated the levels of both P-450c and P-450d in nuclear envelope and microsomes. The degree of AAF induction of P-450c and P-450d differed: the ratio of P-450c to P-450d was lower in nuclear envelopes than in microsomes. We also examined the effect of the antioxidant butylated hydroxytoluene (BHT) on AAF-induction of P-450. BHT accentuated AAF induction of P-450c: nuclear envelopes and microsomes from rats fed both AAF and BHT had two and sevenfold higher levels of P-450c than fractions obtained from rats fed AAF alone. BHT alone had no effect on P-450c expression. Thus, the inductive effect of BHT on P-450c levels was only observed in concert with AAF feeding. The results

also demonstrate differential induction of P-450 in nuclear envelopes and microsomes.

We examined the age dependence of testosterone metabolism in liver microsomes from individual 3-month- and 24-month-old-male rats. Although the older rats exhibited lower 16 α , 6 β , and 2 α -hydroxylase activities than the young rats, they had higher levels of both 7 α -hydroxylase activity and P-450a, a known 7 α -hydroxylase. The mRNA for P-450a was measured with a cDNA probe and its level was fivefold higher in the old rats, whereas levels of mRNA coding for a 6 β -hydroxylase P-450 was markedly decreased. The increased expression of P-450a demonstrates that the generally observed decrease in P-450 catalyzed activities with senescence involves specific repression or activation of genes for constitutive P-450s.

We examined the developmental regulation of P-450s in chicken liver microsomes using MAbs to the rat liver P-450s induced by MC, phenobarbital, ethanol and pregnenolone-16- α -carbonitrile (PCN). Only the MAb to the PCN-P-450 immunodetected a related P-450 in chicken liver microsomes. It was most predominant in chickens at 1 day posthatching, while much lower levels were observed in the embryo and at 36 days posthatch. Chicken liver microsomal erythromycin demethylase, a characteristic activity of rat pregnenolone-16- α -carbonitrile-inducible P450, was similar in developmental profile and induction characteristics to that of the immunodetected P-450, while aldrin epoxidase, benzphetamine demethylase, ethylmorphine demethylase and aminopyrine demethylase were more similar to each other in development and induction and were less well correlated with the immunodetected P-450. This evidence suggests the presence in chicken liver of at least two types of P-450, one a form from the PCN-P-450 family. The evidence for this P-450 in chickens agrees with sequence information suggesting the early evolution of this form and demonstrates the suitability of the chicken for studies of P-450 evolution.

Publications:

Friedman FK, Miller H, Park SS, Graham SA, Gelboin HV and Carubelli, R. Induction of rat liver microsomal and nuclear cytochrome P-450 by dietary 2-acetylaminofluorene. *Biochem Pharmacol* (In Press).

Lorr NA, Bloom SE, Park SS, Gelboin HV, Miller H and Friedman FK. Evidence for a PCN-P450 enzyme in chickens and comparison of its development to that of other PB-inducible forms. *Mol Pharmacol* (In Press).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01CP05604-01 LMC

PERIOD COVERED

October 1, 1988 to September 30, 1989

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

Immunopharmacological Identification and Regulation of Cytochrome P-450

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

PI: Michael J. Myers Senior Staff Fellow LMC NCI

Others:	Haruko Miller	Bio. Lab. Tech.	LMC	NCI
	Gao Liu	Guest Researcher	LMC	NCI
	Fred Friedman	Research Chemist	LMC	NCI
	Richard Robinson	Biologist	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 20892

TOTAL MAN-YEARS

1.5

PROFESSIONAL

1.0

OTHER

0.5

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.)

Cytochrome P-450 enzymes metabolize a large array of both xenobiotic substances (such as carcinogens and drugs) as well as endobiotic substances such as steroids and prostaglandins. The focus of this research project is the regulation and function of the multiple forms of this enzyme, using either polyclonal (PAb) and/or monoclonal antibodies (MAb) in immunologically-based methodologies. The PABs are raised against peptide sequences that are unique for a given P-450 enzyme, while the MABs are generated against purified, native P-450 enzymes. The production of anti-peptide PAB presents the opportunity to generate antibodies in a more rational and timely manner. In addition, the creation of P-450-specific anti-peptide PAB results in a reagent that is not only useful in numerous immunoassays but can also be used in the purification of the native molecule, which in turn can be used for production of MABs. The current research efforts center around the generation of specific anti-peptide PABs against sequences unique to either P-450d or P-450c. These two rat P-450 enzymes, induced following exposure to 3-methylcholanthrene, are key enzymes in the metabolism of carcinogens of the aromatic amine and polyaromatic hydrocarbon classes, respectively. While several unique regions have been identified in both enzymes, the initial emphasis will be on the creation of antisera against peptide sequences unique to P-450d, as there is no available antibody which specifically recognizes P-450d. The anti-peptide PABs are examined for use as reagents in ELISA and western blot analysis, and inhibition of enzyme activity.

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

Michael J Myers	Senior Staff Fellow	LMC	NCI
Haruko Miller	Bio. Lab. Tech.	LMC	NCI
Gao Liu	Guest Researcher	LMC	NCI
Fred K. Friedman	Research Chemist	LMC	NCI
Richard C. Robinson	Biologist	LMC	NCI

Objectives:

To quantify the individual P-450 forms and their relative contribution towards the overall metabolism of a given substrate in different tissues and cells.

Methods Employed:

Synthetic peptides representing regions of the molecule which were unique to P-450d were prepared and coupled to a carrier protein. The resulting peptide-protein complex was used to generate rabbit anti-peptide antisera. Specificity of the resulting antisera was determined using western blot analysis and ELISA. Either native antisera or the IgG fraction of antisera was used in enzyme inhibition assays. P-450d activity is determined by measuring the metabolism of acetanalide to 4-hydroxyacetanalide spectrophotometrically.

Major Findings:

At present, antisera raised against two P-450d peptides (termed D1 and D2) have been raised. Preliminary data demonstrated that the anti-D1 antisera resulted in an increased capacity to metabolize acetanalide, while anti-D2 antisera inhibited this activity. Both antisera result in a positive ELISA signal, but give a weak signal in Western blot analysis. Current efforts are focused on further characterization of the modulatory effects of these two antisera on the metabolism of acetanalide. Also, efforts are underway to generate additional anti-P-450d peptide and anti-P-450c peptide antisera.

ANNUAL REPORT OF

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

October 1, 1988 through September 30, 1989

The Chemical and Physical Carcinogenesis Branch (CPCB) plans, coordinates and administers a national extramural program of basic and applied research consisting of grants and contracts, collectively concerned with the occurrence and the inhibition of cancer, caused or promoted by chemical or physical agents acting separately or together, or in combination with biological agents; plans, organizes and conducts meetings of scientists and otherwise maintains contacts with scientists-at-large, to identify and evaluate new and emergent research in, and related to, the fields of chemical and physical carcinogenesis; provides a broad spectrum of information, advice and consultation to scientists and to institutional science management officials, relative to the National Institutes of Health (NIH) and National Cancer Institute (NCI) funding and scientific review policies and procedures, preparation of grant applications and choice of funding instrument, based on individual need; plans, develops, maintains and allocates research resources necessary for the support of carcinogenesis research of high programmatic interest; and provides NCI management with recommendations concerning funding needs, priorities and strategies relative to the support of chemical and physical carcinogenesis research, consistent with the current state of development of individual research elements and the promise of potential, new initiatives.

Research and related activities supported under this program bear upon a broad range of subject matter areas, with principal emphasis on environmental carcinogenesis, mechanisms of action of chemical and physical carcinogens; the role of DNA damage and repair in carcinogenesis; properties of cells transformed by chemical and physical agents; inter- and intraspecies comparisons in the response to carcinogen exposure; the role of tumor promoters, hormones and other cofactors in cancer causation; experimental approaches to the inhibition of carcinogenesis; the role of diet and nutrition in carcinogenesis; the role of tobacco products and smoking in carcinogenesis; and in vitro carcinogenesis studies on human and other mammalian cells, tissues, and subcellular fractions. The program also supports the synthesis, acquisition, and distribution of a considerable spectrum of chemical standards, critically needed in the field of carcinogenesis research.

The Branch utilizes a variety of funding instruments to accomplish its objectives. These include: traditional individual research project grants (R01), program project grants (P01), first independent research support and transition (FIRST) awards (R29), conference grants (R13), cooperative agreements (U01), contracts (N01), small business innovative research (SBIR) grants (R43/44), SBIR contracts (N43/44), academic research enhancement awards (AREA) (R15), outstanding investigator grant (OIG) awards (R35) and the method to extend research in time (MERIT) awards (R37). Currently, the Branch administers 514 research grants with an annual budget of approximately 73.7 million dollars.

Grants and contracts administered by the staff of this Branch support six complementary categories of chemical and physical carcinogenesis research and associated

resources: Biological and Chemical Prevention, Carcinogenesis Mechanisms, Diet and Nutrition, Molecular Carcinogenesis, Smoking and Health and Research Resources.

The Biological and Chemical Prevention component is concerned with the experimental inhibition of carcinogenesis caused by chemical, physical and biological agents. Efforts are devoted to the identification, development and testing (both in vitro and in vivo) of agents intended to inhibit carcinogenesis. Areas of prime interest include mechanisms of action of candidate preventive agents, binding proteins and receptors, structure-function relationships, and the experimental use of combinations of preventive agents.

The Carcinogenesis Mechanisms category relates to the absorption and body distribution of carcinogens; metabolism, activation and inactivation of carcinogens; identification of proximate and ultimate carcinogenic forms; 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; molecular structure-carcinogenicity relationships; carcinogen-mutagen relationships; isolation, identification and synthesis of suspect carcinogens and their metabolites; factors which alter carcinogen activity; the characterization of carcinogen metabolizing enzymes; and the role of hormones in carcinogenesis.

The Diet and Nutrition category supports basic studies on the carcinogenic effects of diet and specific nutrients in animal systems and human cells in vitro.

The Molecular Carcinogenesis component focuses on changes in biological macromolecules and in cell functions as a result of carcinogen exposure; DNA damage and repair following exposure to carcinogens; the role of tumor promoters and the mechanism of tumor promotion in carcinogenesis; and studies on the genetics and mechanism of cell transformation and of the genetics and regulation of enzymes characteristically associated with the carcinogenesis process.

The Smoking and Health category supports studies on the toxicology and pharmacology of smoking and tobacco-related exposures. Both grant and contract mechanisms are used to support these activities.

The Research Resources component, consisting solely of contracts, is principally concerned with the synthesis and distribution of selected chemical carcinogens and certain of their metabolites through a repository. The inventory of over 700 compounds includes natural products, nitrosamines, dioxins, aromatic amines, and asbestos. Particular emphasis has been given to polynuclear aromatic hydrocarbon carcinogens, their metabolic intermediates, and analogous heterosubstituted compounds.

During the last year, the Branch awarded one new outstanding investigator grant of 7 years duration to Dr. Peter Jones (1 R35 CA49758-01), University of Southern California.

In addition, The Branch was successful in nominating five research grant applicants for the Method to Extend Research in Time (MERIT) award. These investigators were Dr. Errol Friedberg (2 R37 CA 12428-19), Stanford University; Dr. Jeffrey Rosen (2 R37 CA 16303-14), Baylor College of Medicine; Dr. Anthony

Pegg (2 R37 CA 18137-14), Pennsylvania State University, Hershey Medical Center; Dr. Charles King (2 R37 CA 23386-12), Michigan Cancer Foundation; and Dr. Martha Stampfer (2 R37 CA 24844-11), University of California, Berkeley.

During the first half of FY89 the Branch assumed program responsibility for three request for applications (RFAs) which were initiated by the staff of the Organ Systems Program in the Division of Cancer Prevention and Control (DCPC) prior to the transfer of the organ system related grant portfolios late in FY88. CPCB program staff assisted the Division of Extramural Activities review staff in identifying qualified review candidates for the three special study sections assembled to review the 46 bladder applications. In addition, they were responsible for interacting with applicants unfamiliar with the RFA process.

One RFA, suggested by the Breast Cancer Working Group, derived from the knowledge that assessment of the role of chemical carcinogens in human mammary cancer requires an understanding of the mechanism by which normal cells become malignant and the availability of a reproducible, quantitative system for transforming epithelial cells by exposure to carcinogens. In vitro treatment of mammary epithelial cells (MEC) with chemical carcinogens induces phenotypic changes, such as anchorage independent growth and multinucleation. Despite these advances, no one has, as yet, successfully developed a reproducible system for the complete transformation of MEC into malignant cells with chemical carcinogens. The main goal of the RFA was to achieve efficient, in vitro chemical or physical transformation of both rodent and human mammary cells paralleling in vivo mammary tumorigenesis insofar as possible. Of the 21 applicants who responded, four received a fundable priority score, for a period of five years. Of the four funded applications, three applicants have had considerable experience with culturing experimental animal mammary epithelial cells in vitro. This RFA will enable them to turn their attention to human mammary epithelial cell (HMEC) culture. The third applicant will conduct molecular and genetic studies on HMEC utilizing a new approach. The effect of the RFA has not been completely realized yet since a number of applicants who did not receive a fundable score revised and resubmitted their applications for review by regular Division of Research Grants (DRG) study sections.

The major goal of another RFA entitled "Identification of Genetic Alterations Involved in Bladder Carcinogenesis" is to increase understanding of the genetic alterations underlying multistage chemical carcinogenesis in the urinary bladder. Specific objectives of this RFA were: (1) determine which alterations (mutations, translocations, amplifications) in known cellular proto-oncogenes are important in multistage bladder carcinogenesis in experimental systems; (2) identify genes which might be involved in the pathogenesis of bladder cancers; (3) use cytogenetic studies to provide clues to the molecular alterations in bladder cells; (4) determine the mechanisms by which carcinogens activate proto-oncogenes in bladder tumorigenesis; and (5) determine the roles and timing of genetic changes during the multistage development of bladder neoplasia. There were seven applications in response to this RFA. Only one application received a priority score within the fundable range; however, it is hoped that revision and resubmission of the RFA may eventually result in new initiatives in this area.

A third RFA on "Mechanisms of Alcohol and Tobacco Related Carcinogenesis of the Oral Cavity" was announced, with a due date of May 16, 1988. There were 18 applications submitted and all were subsequently reviewed by a Special Review Committee constituted by the NCI Division of Extramural Activities.

Two applications were funded from this initiative. The overall response to the RFA was enthusiastic and the 18 applications covered the range of areas of announced interest. Eleven applications focused primarily on the role of tobacco and alcohol in the induction of oral cavity cancer. Of these, four applications addressed the effects of alcohol on the metabolic activations or DNA binding of carcinogens present in tobacco smoke or on subsequent DNA repair. Three applications involved the development of animal models to explore the mechanism of alcohol enhancement of tobacco-induced carcinomas. Three applications dealt with the establishment and use of in vitro models to study transforming and mutagenic effects of alcohol and tobacco. One application proposed studies on effects of alcohol on the permeability of the mucosal cell lining to tobacco components.

The remaining six applications addressed the role of snuff in the induction of oral cancer. Of these applications, three focused on the synergistic effects in snuff carcinogenesis of such factors as virus infection or alcohol use. Two proposed studies focused on the formation and repair of DNA adducts by snuff and tobacco-specific carcinogens. In one application, the major thrust was to develop a model for snuff-induced carcinogenesis.

The transfer to the Branch of all organ system grants related to Chemical and Physical Carcinogenesis was completed early this year. Together with grants that were already resident in CPCB, the current holdings are as follows: Bladder (12) \$1,660,644; Breast (54) \$6,991,506; Colon (8) \$3,043,586; Central Nervous System (1) \$142,116; Pancreas (8) \$756,630; Prostate (5) \$240,557; and Upper Aerodigestive (13) \$2,772,640.

Table I summarizes the number of grants and contracts in the Branch and the total cost for each type of funding instrument. In Table II the distribution of the grants and contracts by program component is summarized.

TABLE I
 CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
 (Extramural Activities - FY 1989 - Estimated)

	No. of Contracts/Grants	\$ (Millions)
Research Contracts	2	0.25
Research Grants	506	73.47
Traditional Research Grants (R01) (395 grants; \$46.01 Million)		
Conference Grants (R13) (11 grants; \$0.02 Million)		
FIRST Awards (R29) (23 grants; \$1.99 Million)		
New Investigator Research Grants (R23) (1 grants; \$0.00 Million)		
Program Project Grants (P01) (13 grants; \$11.55 Million)		
Cooperative Agreements (U01) (7 grants; \$3.10 Million)		
Small Business Grants (R43/R44) (4 grants; \$0.44 Million)		
Outstanding Investigator Grants (R35) (7 grants; \$3.89 Million)		
RFAs (R01) (25 grants; \$1.80 Million)		
MERIT Awards (R37) (17 grants; \$4.67 Million)		
AREA Grants (R15) (3 grants; \$0.00 Million)		
Research Resource Contracts	6	1.60
	<hr/> 514	<hr/> 75.32

TABLE II
 CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
 (Contracts and Grants Active During FY 1989)

FY 1989

	CONTRACTS		GRANTS	
	<u>No of Contracts</u>	<u>\$ (Millions)</u>	<u>No. of Grants</u>	<u>\$ (Millions)</u>
Biological & Chemical Prevention	1	0	80	11.26
Carcinogenesis Mechanisms	0	0	163	23.10
Diet and Nutrition	0	0	40	3.51
Molecular Carcinogenesis	0	0	210	33.52
Smoking and Health	1	0.25	13	2.08
Research Resources	6	1.60	0	0.00
TOTAL	8	1.85	506	73.47

SUMMARY REPORT

BIOLOGICAL AND CHEMICAL PREVENTION

The Biological and Chemical Prevention component of the Branch is responsible for research on agents that inhibit, arrest, reverse or delay the development of cancer in humans. Agents can derive from naturally occurring products such as foods consumed by man, from chemical synthesis, or from various biological sources. At the present time, there are 80 grants in this program area with FY89 funding of approximately \$11.26 million, one contract with zero FY89 funding, and seven cooperative agreements with FY89 funding of approximately \$3.10 million.

The currently active research grants consist predominately of Traditional Research Projects (60 R01s), as well as Research Program Projects (3 P01s), four First Independent Research Support and Transition Awards (4 R29s), four Academic Research Enhancement Awards (4 R15s), two Conference Grants (2 R13s), a MERIT Award (1 R37) and an SBIR grant (1 R43). The research grants can be categorized into retinoid; antioxidant; natural inhibitor; micronutrient including selenium, vitamin C and vitamin E, carotenoid, protease inhibitor, omega-3 polyunsaturated fatty acids, and miscellaneous areas. The miscellaneous area includes grants on protein kinase C inhibitors, vitamin D analogs, suicide substrates of cytochrome P-450, isothiocyanates, inhibitors of the arachidonic acid cascade, dehydroepiandrosterone, and nucleophilic compounds active against direct-acting carcinogens. They support diverse types of studies including the experimental inhibition of carcinogenesis, the inhibition or suppression of malignant transformation in culture, mechanisms of action and metabolism of preventive agents, synthesis of chemopreventive compounds, structure-function relationships, pharmacologic disposition, and toxicologic investigations. The most frequently used experimental approach is to study inhibition of carcinogenesis induced by chemical, physical, or biological agents against several stages of the tumorigenic process, and against the development of cancer at many organ sites. The modifying effects of anticarcinogens are investigated relative to a large number of biochemical and biological endpoints, which, in addition to tumorigenesis and transformation themselves, include the activity of the mixed-function oxidase system, free radical generation and quenching, cell proliferation, differentiation, activation/detoxification of carcinogens, DNA damage and repair, binding proteins or receptors for preventive agents, preneoplastic states, and selective attack and prevention of oncogene-specific neoplastic disease.

Grants Activity Summary

The CPCB has recently started several cooperative agreements under an RFA termed "National Collaborative Chemoprevention Projects (NCCPs)." These projects provide a mechanism to enhance and expand multidisciplinary/interdisciplinary investigations of chemoprevention through a funding mechanism that permits a combination of diverse research expertise from one or more institutions and the facilitating resources of the NCI. The stated objectives of the projects are to generate new approaches and strategies in the inhibition or suppression of the carcinogenic process and to bridge development from individual grant/contract-supported work up to the stage of preclinical/clinical testing of new agents for the protection or prevention of neoplasia.

In one NCCP, efforts are devoted to the development and understanding of the role of isothiocyanates in the chemoprevention of carcinogenesis. As is well known,

cruciferous vegetables contain a number of anticarcinogenic compounds which can be classified into several chemical classes. These compounds are non-nutritive, microconstituents of food. One such class of naturally-occurring inhibitors is the isothiocyanates which occur in these vegetables in precursor form as glucosinolates. Benzyliothiocyanate (BITC), for example, inhibits 7,12-dimethylbenzanthracene (DMBA)-induced rat mammary carcinogenesis whether given orally only shortly before or given in the diet only, after carcinogen. Such alteration of susceptibility to carcinogenic insult in the pre-initiation period, or modulation of the response to carcinogen in the post-initiation period, has also been shown by BITC against polycyclic aromatic hydrocarbon (PAH)-induced mouse pulmonary neoplasia. BITC has also been shown to be chemoprotective against mouse forestomach neoplasia when given orally shortly before either N-nitrosodiethylamine (DNA) or benzo(a)pyrene (BaP). Moreover, the naturally-occurring BITC glucosinolate, glucotropaeolin, has itself been shown to inhibit both DMBA-induced rat mammary carcinogenesis and BaP-induced mouse lung neoplasia. Other isothiocyanates have also shown anticarcinogenic efficacy. For example, phenethylisothiocyanate (PEITC) which also occurs in cruciferous vegetables as the glucosinolate, gluconasturiiin, inhibits DMBA-induced rat mammary carcinogenesis when given orally shortly before carcinogen, as well as DMBA-induced mouse forestomach tumorigenesis when given in the diet in the post-initiation period. In addition, the synthetic isothiocyanate, phenylisothiocyanate (PITC) has shown significant anti-initiating activity against DMBA-induced rat mammary carcinogenesis.

In this NCCP, the efficacy of these isothiocyanates has been investigated in a model system for lung tumorigenesis having particular interest. Both PAHs and nitrosamines are known tobacco carcinogens. Of the nitrosamines, the most potent is the tobacco-specific carcinogenic nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK induces lung, nasal cavity, liver and pancreatic tumors in F-344 rats, nasal cavity and lung tumors in hamsters, and lung tumors in mice. The organ-specific effect of NNK in lung tumor induction in all animal species tested, regardless of route of administration, suggests its possible role in the development of lung cancer among smokers. A recent study has tested PEITC, BITC and PITC for their capacities to inhibit NNK-induced lung tumorigenesis, O⁶-methylguanine (O⁶-mGua) formation and NNK metabolism in A/J mice. This model for pulmonary adenoma formation employs a single intraperitoneal injection of 10 micromoles of NNK resulting in 100% tumor incidence at 16 weeks at an average multiplicity of about 10 tumors per mouse. The inhibition experiments tested the efficacy of the isothiocyanates in an anti-initiation protocol by pretreatment before NNK administration. It was found that pretreatment by gavage with PEITC for four consecutive days at daily doses of 5 or 25 micromoles decreased tumor multiplicity from 10.7 to 2.6 or 0.3 tumors/mouse, respectively. Moreover, the higher 25 micromoles dose reduced the percentage of animals that developed tumors from 100% to 30%. On the other hand, neither PITC nor BITC (at 5 micromoles per dose x4 daily doses) inhibited NNK-induced pulmonary adenoma formation. In this particular system, PEITC appears not only to have greater efficacy than BITC and PITC, but also to be considerably less toxic.

Additional studies were performed in this NNK-induced mouse lung tumorigenesis system in order to investigate possible mechanisms of chemopreventive action of the isothiocyanates. In this regard, their effect on the NNK-induced formation of the promutagenic O⁶-mGua adduct in the target organ DNA, lung DNA, was studied under conditions of NNK dosing and isothiocyanate pretreatment used in the anti-tumorigenesis experiments. It was found that PEITC inhibited the formation of NNK-induced O⁶-mGua adducts and that PITC and BITC did not, results consistent

with the antitumorigenesis experiments. Adduct formation, just as pulmonary adenoma formation, was significantly reduced by both doses of PEITC, at both 2 and 6 hours post-carcinogen (at the higher PEITC dose, at six hours, O⁶-mGua adducts became undetectable). Moreover, studies on the effect of PEITC pretreatment on the metabolism of NNK in the target organ showed that this isothiocyanate quite effectively inhibited the microsomal metabolism of this tobacco-specific nitrosamine, including the formation of one metabolite known to result from alpha-hydroxylation of NNK. The total percentage of NNK metabolism decreased by approximately 90% upon pretreatment with either dose of PEITC used in the tumor inhibition experiments. Such an inhibition of NNK metabolism could readily account for the reduction of NNK-induced O⁶-mGua formation in vivo by PEITC, as well as the almost complete inhibitory activity of this isothiocyanate on NNK-induced pulmonary adenoma formation (30).

Studies in another NCCP emphasize the development of protease inhibitors as agents for antitransformation and anticarcinogenesis and their mechanisms of action. In vivo, protease inhibitors have prevented or suppressed carcinogenesis in a wide range of experimental systems. These include DMBA-initiated, phorbol ester-promoted two-stage mouse skin tumorigenesis (TPCK, TLCK, TAME, leupeptin, soybean diets rich in protease inhibitors), DMBA-induced cheek pouch carcinogenesis in hamsters (Bowman Birk inhibitor), 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in mice (crude and purified preparations of Bowman Birk inhibitor, epsilon aminocaproic acid), experimentally-induced colon and mammary carcinogenesis in rats (leupeptin) and spontaneous hepatocarcinogenesis in C3H/HeN mice (soybean based diet). Recent studies have shown that the soybean-derived Bowman Birk inhibitor (BBI) has the capacity to inhibit liver carcinogenesis in male CD-1 mice induced by low dose DMH administered over a long period of time. In these experiments, mice were prefed several types of BBI diets before commencing 20 weekly DMH treatments and continued on these diets to the termination of the study 60 weeks later. It was found that a semipurified preparation of BBI (containing 50% BBI) at 0.5% and 0.1% in the diet, as well as purified BBI at dietary levels of 0.1% and 0.01%, significantly suppressed the formation of angiosarcomas and nodular hyperplasia of the liver. Previous work has shown that chymotrypsin inhibition has been closely associated with the anticarcinogenic activity of many protease inhibitors. Similarly, in the case of BBI, which has both chymotrypsin and trypsin inhibiting domains, previous work has shown that only the chymotrypsin inhibitory portion of the molecule has the ability to suppress transformation. This was shown by enzymatically cleaving purified BBI into its active trypsin and chymotrypsin domains. It was also shown by inactivating the trypsin inhibitory activity of BBI by succinylation at its active site, while retaining antitransformation activity. However, in the present in vivo experiments, succinylated BBI as well as autoclaved BBI (loss of both enzyme inhibiting activities) failed to suppress angiosarcoma formation or nodular hyperplasia (35).

Another recent report from this group on the role of protease inhibitors in anti-transformation/anticarcinogenesis is of high interest. In these investigations, the ability of four protease inhibitors to suppress radiation-induced transformation of C3H/10T1/2 cells was investigated. The inhibitors tested included: (i) aprotinin (a serine protease inhibitor), (ii) N-acetyl-L-tyrosine ethyl ester (a chymotrypsin substrate and competitive inhibitor of protein degradation), (iii) carboxypeptidase inhibitor (a metallo-exopeptidase inhibitor) and (iv) potato protease inhibitor II (a chymotrypsin/trypsin inhibitor). While none of the inhibitors were toxic to the cells at the concentrations employed, only carboxypeptidase inhibitor and potato inhibitor II suppressed radiation-induced

transformation in a statistically significant manner. By use of fluorescent-labeled inhibitors, it was found that carboxypeptidase inhibitor and inhibitor II are internalized by these cells. Fluorescent-labeled inhibitors could be observed in the cells within 15 minutes of incubation and were present in distinct intracellular vacuoles within one hour. In general, at one and four hours, the labeled inhibitors were localized in the cytoplasm surrounding the nucleus. Previous work by this group has shown that the majority of cellular protein interacting with the potent anticarcinogenic protease inhibitor, Bowman Birk inhibitor, is present in the lysosomal per golgi subcellular fraction. These results are important since they indicate that a possible mechanism of anticarcinogenesis by protease inhibitors is internalization by target cells followed by inhibition of intracellular proteases (or other enzymes) involved in the conversion of a cell to the malignant state. An intriguing aspect of these studies was the unexpected observation that carboxypeptidase inhibitor (CPI) of potato origin is an inhibitor of radiation-induced transformation. CPI has been shown to inhibit metallo-exopeptidase activity, but has not been shown to inhibit any endopeptidase activities (C.A. Ryan, unpublished results cited in the open literature). Previously, the most effective suppressors of radiation transformation are those compounds which inhibit endopeptidase activities (BBI, chymostatin, chymotrypsin inhibitor, and TPCK). The results in this present study thus suggest that anticarcinogenesis/antitransformation may result from inhibition of either of two different types of protease activity involved in carcinogenesis: endo- and exopeptidase activities. These studies have shown, in fact, that CPI and potato inhibitor II, given together, inhibit radiation transformation at levels at which either one alone is ineffective (35).

In another NCCP, the efforts are devoted to the development of non-toxic, non-absorbable, nucleophilic inhibitors of gastrointestinal tract carcinogenesis. The objective is to find compounds that can trap direct-acting carcinogens within the lumen of the gastrointestinal tract and thus prevent these carcinogens from attacking tissues of the host. Since many direct-acting carcinogens are electrophiles, one possible strategy is to block their action by trapping them with nucleophiles. Emphasis in this project has been placed on trapping direct-acting carcinogens in two sites, the stomach and the large bowel. Electrophilic trapping in the stomach is complicated because of its acidity, since many nucleophiles are inactive in acid solution. The acidity of the stomach is known to vary with species and with food consumption. However, even in species with a very low fasting gastric acidity, as the human, the pH will rise rapidly to 4 or above shortly after the start of a meal. Although (ideally) nucleophiles effective in the low pH range would be desirable, those maintaining significant nucleophilicity at pH 4 and above could still be useful. One widely-used group of nucleophiles is the thiols. In general, aromatic thiols are the most potent of the thiol nucleophiles. Thiols lose most of their nucleophilicity when protonated so that for activity in the pH range of the gastric contents, it is important to employ thiols with relatively low pKa values. One such compound is 4-mercaptobenzene sulfonate (4-MBS). Recent efforts in this project have investigated the chemical and nucleophilic properties of this compound, its antimutagenicity and its anticarcinogenic activity against experimentally induced large bowel neoplasia in the rat and forestomach neoplasia in the mouse. Titration studies showed that the pKa of the thiol group of 4-MBS was 5.8. The interaction of 4-MBS with the direct-acting electrophilic carcinogen, beta-propiolactone (BPL), was then determined at pHs above and below the pKa of 4-MBS. It was shown, using radiolabeled BPL, that at pHs (7.4, 6.0) above the pKa (5.8) of 4-MBS almost all of the ¹⁴C-BPL reacts with 4-MBS to form an adduct. The reaction proceeds rapidly. At pHs below the

compound pKa, where protonation reduces thiol nucleophilicity, adduct formation diminished (pH 4.0, 6% BPL adduction; pH 2.0, 16% BPL adduction). These results are according to expectation. Antimutagenicity studies employed the Salmonella typhimurium strain TA-100 and tested the efficacy of 4-MBS to inhibit the mutagenic effects of BPL and another direct-acting carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). It was found that 4-MBS inhibited the mutagenicity of BPL by 64% and 97% at two different levels in the medium, and that of MNNG by about 35 to 65% depending on the concentration of 4-MBS employed. The anticarcinogenic efficacy of sodium 4-mercaptobenzene sulfonate (4-MBSNa) against BPL-induced neoplasia of the mouse forestomach was also determined in the female A/J mouse. In this experiment, 4-MBSNa was gavaged five minutes before BPL administration, also given by gavage. Forestomach papillomas per mouse were greatly reduced from 6.8 to 0.71. Moreover, the percent of mice with tumors decreased from 100% to 57%. It is noteworthy that sodium benzene sulfonate, also studied in this experiment, which differs from 4-MBSNa only in lacking the thiol group, did not inhibit tumor incidence at all, while showing only a slight but significant decrease in tumors/mouse from 6.8 to 4.2. Finally, the efficacy of 4-MBSNa in inhibition of BPL-induced neoplasia of the large bowel was determined. Both BPL and 4-MBSNa were administered to male F344 rats by intrarectal intubation, the latter again preceding carcinogen dosing by five minutes. The results showed strong chemopreventive efficacy against the induction of adenomatous polyps in this model: tumor incidence decreased from 100% to 21% and tumor multiplicity from 3.4 to 0.36 polyps per rat. These studies are somewhat unique in that an anticarcinogenic agent was designed de novo from first principles of physiology, chemistry, carcinogenesis and anticarcinogenesis (78).

In still another NCCP, efforts are directed at the conceptualization and creation of new vitamin D analogs which will function selectively and effectively to promote the differentiation of preleukemia and leukemia cells. The rationale for the efforts is that the disease of leukemia represents a form of cancer in which an inappropriate block of stem cell differentiation exists which is coupled to an enhancement of cell proliferation. Further, it is already known that the vitamin D metabolite (and hormonally active form of the vitamin) 1- α ,25(OH) $_2$ D $_3$, is a very potent mediator of stem cell differentiation and clonal proliferation. These effects are believed to result from the stereoselective interaction of 1- α ,25(OH) $_2$ D $_3$ with receptor present in these cells. However, a clinical trial of the natural hormone had to be terminated prior to detection of any beneficial effects due to the development of hypercalcemia. The objective of this project is therefore to develop synthetic analogs of 1- α ,25(OH) $_2$ D $_3$ which are capable of inducing differentiation of neoplastic cells without the calcitrophic effects of natural vitamin D (bone calcium mobilization and enhanced calcium absorption from the gut). Project areas include the chemical synthesis of vitamin D analogs, their assay in a variety of biological systems for toxicity and efficacy, and biochemical studies of mechanism. At this point, seven new analogs of 1,25(OH) $_2$ D $_3$ have been discovered to be either equivalent or more potent than 1,25(OH) $_2$ D $_3$ as assessed by (i) inhibition of clonal proliferation of HL-60, EM-2, U937 and patients' myeloid leukemic cells; and (ii) induction of differentiation of HL-60 promyelocytes. Furthermore, these analogs stimulated clonal growth of normal human myeloid stem cells. The most potent analog, 1,25-dihydroxy-16ene-23yne-vitamin D $_3$, was about 4-fold more potent than 1,25(OH) $_2$ D $_3$. This analog decreased clonal growth and expression of c-myc oncogene in HL-60 cells by 50% within 10 hours of exposure. Effects on calcium metabolism of these novel analogs in vivo were assessed by intestinal calcium absorption (ICA) and bone calcium mobilization (BCM). Each of the analogs mediated markedly less ICA and BCM as compared to

1,25(OH)₂D₃. To gain insight into the possible mechanism of action of these new analogs, receptor binding studies were done with 1,25(OH)₂-16ene-23yne-D₃ which showed that it competed only about 60% as effectively as 1,25(OH)₂D₃ for 1,25-(OH)₂D₃ receptors present in HL-60 cells and 98% as effectively as 1,25(OH)₂D₃ for receptors in chick intestinal cells. Thus, seven novel vitamin D analogs have been discovered which are more potent than physiologic 1,25(OH)₂D₃ as measured by a variety of hematopoietic assays. These compounds appear to have the potential to be markedly less toxic (induction of hypercalcemia). These novel vitamin D compounds may be superior to 1,25(OH)₂D₃ in a number of clinical situations including leukemia/preleukemia; and they will provide a tool to dissect the mechanism of action of vitamin D secosteroids in promoting cellular differentiation (40).

Another NCCP engages in efforts on the development of protein kinase C (PKC) inhibitors as potential chemopreventive agents. PKC is a critical enzyme in tumor promotion, growth regulation, differentiation and development. The underlying conceptualization is that PKC is regulated by positive and negative lipid second messengers derived from membrane glycerolipids and sphingolipids, respectively. Studies of structure-function relationships of these natural activators, *sn*-1,2-diacylglycerols (DAGs), and inhibitors, sphingosine (SPH), may lead to development of inhibitors of PKC. Such drugs would be expected to interfere with the promotion and progression steps of carcinogenesis. The project consists of four programs: 1) chemistry, where DAG and SPH analogues are prepared; 2) enzyme activity assessments/mechanisms, where the effects of these analogues on PKC activity and phorbol ester binding are determined as well as their effects on the enzymes of DAG and SPH metabolism; 3) cellular activity assessments, where the active analogues are tested on cellular PKC activity in human platelets, A431 human epidermoid carcinoma cells, human HL-60 cells and other transformed cultures; and 4) animal activity assessments, where pharmacokinetics, metabolism, toxicology and anti-promotion activity of selected agents are determined in mice. This new project has already designed several series of DAG and SPH analogs using computer-aided molecular modeling technology. The basic approach is to determine the structural features of DAG required for its tumor-promoting activity and, similarly, those of SPH required for its PKC inhibiting properties, and to develop structure-activity correlations. Analogs are then synthesized for activity determinations to determine the role that the structural features/modifications might play in their respective biological activities. With regard to SPH, for example, the data thus far indicate that the positive charge is an absolute but not a minimum requirement for PKC inhibition and that the chain length between the hydrophobic portion of the molecule and the positively-charged group appears to be optimum at 3. Furthermore, the stereochemistry of SPH does not appear to be critical since all four isomers are nearly equivalent in their ability to inhibit PKC. In addition, studies on the mechanism of SPH inhibition of PKC were performed. These studies investigated the effect of SPH on the autophosphorylation of PKC. SPH was shown to be a potent inhibitor of PKC autophosphorylation in the absence of protein substrate, and that addition of substrate had no effect in the SPH inhibition of autophosphorylation. These results support earlier work demonstrating that SPH inhibition occurs at the regulatory domain to inhibit activation of the catalytic domain of PKC.

Attempts to screen, fractionate, isolate and chemically identify anticarcinogenic compounds from natural sources require the selection of one or more bioassay systems in order to detect and follow the active chemopreventive agent in its purification. Since a large number of assays will typically be required, the selection

of the type of bioassay(s) represents a crucial decision in the discovery of naturally-occurring inhibitors of carcinogenesis. Recently, efforts have been devoted to the development and application of practical assays for activity-directed fractionation of plants that would be rapid, sensitive, convenient and capable of detecting alterations in carcinogen metabolism. These bioassays were used in conjunction with previously developed plant extraction and fractionation procedures to isolate a potentially-active anticarcinogenic agent from trifolium pratense L. Leguminosae (red clover). The fractionation scheme started with a 95% ethyl alcohol extract of fresh red clover leaves, flowers and stems followed by activity-directed fractionation by solvent partitioning, repeated silica gel chromatography and recrystallization from aqueous methanol. The assay system developed and applied in these studies measures effects of experimental extracts, fractions or compounds on the metabolism of [³H] benzo(a)pyrene (BaP) in early passage cultures of Syrian hamster embryo cells. This assay system provides a rapid measure of BaP metabolism in control and treated cultures and consists of several quantitative measures of such metabolism, including radioactivity assessment of unmetabolized BaP and its metabolites by extraction into organic and aqueous phases, analysis of the organic phase [³H]-BaP metabolites by high performance liquid chromatography (HPLC) employing authentic BaP metabolite standards, levels of total DNA binding of labeled BaP metabolites in control and treated cultures, and enzymatic digestion of such DNA to determine specific BaP: deoxyribonucleotide adducts. The assay system has the advantages that activity data are available in a few days and that a large number of different samples can be screened simultaneously. In addition, the assay measures the net result of modulating influences upon enzymes of BaP metabolism, that is, enzymes of carcinogen activation as well as detoxification. Thus, the assay is capable of detecting modifiers of carcinogen metabolism that act by a number of different mechanisms. Moreover, since a strong correlation exists between binding of aromatic hydrocarbons to DNA and their carcinogenic activity, the assay has additional potential in detecting inhibitors of carcinogenesis. The assay has thus far been used in screening over 70 species and varieties of plants and vegetables comprising 27 families. In the current example, extracts and fractions from the red clover have shown significant inhibition of BaP metabolism and binding to DNA. Activity-directed purification of these fractions has resulted in the isolation and purification of an isoflavone, biochanin A, which inhibits BaP metabolism by 54% and decreases BaP-DNA binding by 37 to 50%. Both major BaP-DNA adducts, syn- and (+) anti-BaP-7,8-diol-9,10-epoxide-deoxyguanosine, were reduced in biochanin A-treated cells. Further, additional experiments indicate that biochanin A is a potent inhibitor of BaP-induced mutation in a hamster embryo cell-mediated, V79 cell-HGPRT mutation assay (9).

BIOLOGICAL AND CHEMICAL PREVENTION

GRANTS ACTIVE DURING FY89

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ALWORTH, William L. Tulane University of Louisiana 5 R01 CA38192-02	Suicide Substrates of Cytochrome P-450 as Anticarcinogen
2. AWASTHI, Yogesh C. University of Texas Medical Branch 5 R01 CA27967-09	Mechanism of Anti-Carcinogenic Effect of Antioxidants
3. BAILEY, George S. Oregon State University 5 R01 CA34732-06	Mechanisms of Inhibition of Chemical Carcinogenesis
4. BANERJEE, Mihir R. University of Nebraska, Lincoln 5 R01 CA25304-08	Chemical Carcinogenesis Mammary Gland Organ Culture
5. BELL, Robert M. Duke University 5 U01 CA46738-02	Protein Kinase C Inhibitors as Chemopreventive Agents
6. BERTRAM, John S. University of Hawaii at Manoa 5 R01 CA39947-04	Inhibition of In Vitro Transformation by Retinoids
7. BIRT, Diane F. University of Nebraska Medical Center 1 R01 CA48028-01	Inhibition of Tumor Promotion by Sphingoid Bases
8. BYUS, Craig V. University of California, Riverside 1 R01 CA45707-01	Nutritional Modification of Cancer Cell Growth
9. CASSADY, John M. Ohio State University 2 R01 CA38151-04	Chemistry of Novel Natural Inhibitors of Carcinogenesis
10. CAVE, William T., Jr. University of Rochester 2 R01 CA30629-07A1	Promotion of Breast Cancer Lipid Hormone Interactions
11. CHUNG, Fung-Lung American Health Foundation 5 R01 CA41544-03	Glucosinolates and Environmental Nitrosamine Activation
12. COHEN, Leonard A. American Health Foundation 1 R01 CA47326-01	NMU Induced Mammary Cancer and Omega 3 Fatty Acids

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| 13. | COHEN, Leonard A.
American Health Foundation
1 R01 CA48741-01 | Voluntary Exercise as Means of
Mammary Cancer Prevention |
| 14. | CONNOR, Michael J.
University of California, Los Angeles
1 R01 CA47758-01 | Retinoid Metabolism and Skin
Carcinogenesis |
| 15. | COPE, Frederick O.
Southern Research Institute
5 R01 CA40894-03 | Retinoid Receptor Control in
Cytodifferentiation |
| 16. | CULLUM, Malford E.
Michigan State University
5 R29 CA45860-02 | Function of 13-Cis-Retinoic Acid
in HL-60 Cells |
| 17. | CURLEY, Robert W., Jr.
Ohio State University
5 R01 CA40967-03 | Affinity Probes for the Retinoic
Acid-Binding Proteins |
| 18. | DAWSON, Marcia I.
SRI International
5 R01 CA30512-07 | Novel Retinoids for Chemo-
Prevention of Cancer |
| 19. | DAWSON, Marcia I.
SRI International
5 R01 CA32428-07 | Retinoid Tumor Inhibitory
Activity-Toxicity Probe |
| 20. | DESCHNER, Eleanor E.
Sloan-Kettering Institute for Cancer Res
1 R01 CA46845-01 | Omega-3 Fatty Acids--Inhibitors
of Colon Cancer |
| 21. | DOERING, William V.
Harvard University
5 R01 CA41325-03 | Semi-Rigid Conjugated Polyenes
as Model Anticarcinogens |
| 22. | DURHAM, John P.
West Virginia University
5 R01 CA37060-03 | Calcium/lipid Protein Kinase
in Myeloid Differentiation |
| 23. | ERICKSON, Kent L.
University of California, Davis
1 R01 CA47050-01 | Dietary Fatty Acids, Eicosanoids
and Macrophage Function |
| 24. | FISCHER, Susan M.
University of Texas System Cancer Center
1 R01 CA46886-01 | Role of Omega-3 PUFA in
Cancer Prevention |
| 25. | GLAUERT, Howard P.
University of Kentucky
5 R01 CA43719-02 | Diet and Carcinogenesis by
Peroxisome Proliferators |
| 26. | GOULD, Michael N.
University of Wisconsin, Madison
5 R01 CA38128-04 | Anticarcinogenic Agents in
Orange Peel Oil |

27. GRUBBS, Clinton J.
University of Alabama at Birmingham
5 R01 CA44615-02
Mammary Chemoprevention-
Hormones/Vitamin A/Selenium
28. GUDAS, Lorraine J.
Dana-Farber Cancer Institute
5 R01 CA43796-02
Cellular Retinoic Acid
Binding Protein
29. HALL, Alan K.
University of Medicine & Dentistry of NJ
1 R01 CA49422-01
Molecular Actions of Retinoids
in Neoplastic Cell Growth
30. HECHT, Stephen S.
American Health Foundation
5 U01 CA46535-02
Isothiocyanates and Nitrosamine
Carcinogenesis
31. HILL, Donald L.
Southern Research Institute
2 P01 CA34968-04A1
Development of Chemopreventive
Retinoids
32. IP, Clement C. Y.
Roswell Park Memorial Institute
5 R01 CA27706-08
Selenium Chemoprevention of
Mammary Carcinogenesis
33. IP, Clement C. Y.
Roswell Park Memorial Institute
5 P01 CA45164-02
Mechanism of Selenium Chemo-
Prevention of Carcinogenesis
34. IP, Margot M.
Roswell Park Memorial Institute
5 R01 CA35641-05
Eicosanoids and Mammary Cancer
35. KENNEDY, Ann R.
University of Pennsylvania
7 U01 CA46496-02
Cancer Prevention by Protease
Inhibitors
36. KENSLER, Thomas W.
Johns Hopkins University
2 R01 CA39416-04A1
Mechanisms of Anticarcinogenesis
by Dithiolthiones
37. KEREN, David F.
University of Michigan at Ann Arbor
1 R01 CA47132-01A1
The Mucosal Immune Response to
Aflatoxin B1
38. KLINE, Kimberly
University of Texas, Austin
1 R29 CA45422-01A1
Micronutrients in Immuno-
modulations and Cancer
39. KOEFFLER, H. Phillip
University of California, Los Angeles
5 R01 CA33936-06
Action of Retinoids on Myeloid
Leukemia Cells
40. KOEFFLER, H. Phillip
University of California, Los Angeles
5 U01 CA43277-03
Vitamin D and Preleukemia/
Leukemia--Cancer Chemoprevention

41. KRINSKY, Norman I. Eighth International Symposium
Tufts University on Carotenoids
1 R13 CA45274-01
42. KRUMDIECK, Carlos L. Folate Deficiency--Preventable
University of Alabama at Birmingham Risk of Cancer
5 R01 CA40834-03
43. LAM, Luke K. Natural Chemopreventive Agents
LKT Associates
1 R43 CA47720-01
44. LANDOLPH, Joseph R. Inhibition of Chemical Trans-
University of Southern California formation by Aspirin
5 R01 CA41277-03
45. LIEBLER, Daniel C. Vitamin E Turnover and Chemical
University of Arizona Toxicity
1 R29 CA47943-01
46. LIEHR, Joachim G. Prevention of Estradiol-Induced
University of Texas Medical Branch Tumors by Vitamin C
5 R01 CA44069-03
47. LOTLIKAR, Prabhakar D. Mechanism of Anticarcinogenesis
Temple University by Antioxidants
5 R01 CA40885-03
48. LUTZ, Charles A. Role of Nitroxyl in the
Mills College Ascorbate-Nitrite Reaction
1 R15 CA47579-01
49. MACDONALD, Paul C. Dehydroisoandrosterone
University of Texas SW Med Ctr/Dallas Therapeutics--Biomolecular Basis
5 U01 CA43311-03
50. MC CORMICK, Anna M. Metabolism of Chemopreventive
University of Texas SW Med Ctr/Dallas Retinoids
5 R01 CA31676-07
51. MC CORMICK, David L. Arachidonic Acid Metabolism
IIT Research Institute and Cancer Chemoprevention
2 R01 CA40874-04A1
52. MEDINA, Daniel Biology of Mouse Mammary
Baylor College of Medicine Preneoplasias
2 R01 CA11944-16A1
53. MEHTA, Rajendra G. Hormone and Retinoid Interaction
IIT Research Institute in Mammary Tissue
2 R01 CA34664-04A2
54. MILNER, John A. Effects of Dietary Selenium on
University of Illinois, Urbana-Champaign the Initiation of DMBA
1 R01 CA44567-01A2

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| 69. | SHANKEL, Delbert M.
University of Kansas, Lawrence
1 R13 CA49398-01 | Intl Conf--Mechanisms of Anti-
mutagenesis/Anticarcinogenesis |
| 70. | SINHA, Dilip K.
Roswell Park Memorial Institute
5 R01 CA36139-05 | Protection Against Mammary
Carcinogenesis by Pregnancy |
| 71. | STONER, Gary D.
Medical College of Ohio at Toledo
2 R37 CA28950-07 | Carcinogenesis Studies in the
Esophagus |
| 72. | TALALAY, Paul
Johns Hopkins University
5 P01 CA44530-02 | Novel Strategies for Chemo-
protection Against Cancer |
| 73. | THOMPSON, Henry J.
AMC Cancer Research Center
1 R01 CA49212-01 | Antioxidants and Breast Cancer
Prevention |
| 74. | THOMPSON, John A.
University of Colorado at Boulder
5 R01 CA41248-03 | Bioactivation of Dietary Phenols
by Hemoproteins |
| 75. | VERMA, Ajit K.
University of Wisconsin, Madison
2 R01 CA42585-03A1 | Inhibition of Ornithine
Decarboxylase by Retinoic Acid |
| 76. | WALASZEK, Zbigniew
University of Texas System Cancer Center
1 R01 CA47342-01A1 | Mechanism of Glucarate
Inhibition of Mammary Cancer |
| 77. | WANG, Sho-Ya
State University of New York at Albany
5 R29 CA44955-03 | Differentiation of Terato-
carcinoma Cells: Regulation |
| 78. | WATTENBERG, Lee W.
University of Minnesota of Mnpls-St Paul
5 U01 CA43285-03 | Chemoprevention of Carcino-
genesis by Nucleophiles |
| 79. | WILKOFF, Lee J.
Southern Research Institute
5 R01 CA35593-03 | Mechanism of Retinoid Action
Against Prostate Lesions |
| 80. | YAVELow, Jonathan
Rider College
1 R01 CA47762-01 | Anticarcinogenic Mechanism of
Proteinase Inhibitors |

CONTRACTS ACTIVE DURING FY89

<u>Investigator/Institution/Contract Number</u>	<u>Title</u>
81. MCCORMICK, David L. IIT Research Institute N01-CP-41063	Toxicology and Pharmacology of Anticarcinogenic Agents

SUMMARY REPORT

CARCINOGENESIS MECHANISMS

The Carcinogenesis Mechanisms component of the Branch includes studies on the etiology of neoplasia in poikilothermic, aquatic animals; the metabolism, toxicity, physiological disposition, and mechanisms of action of carcinogens and their metabolites; syntheses of both known and suspect carcinogens or the development of derivatives for molecular structure-activity relationships; the development of carcinogenicity/mutagenicity testing procedures; the development of organ and cell culture systems and whole animal biological models for use in carcinogenesis studies; the development of procedures for the qualitative and quantitative analysis of body fluids and tissues and environmental specimens for the presence of chemical carcinogens and associated substances; genetics and regulation of enzymes characteristically associated with carcinogenesis; the identification of biochemical and molecular markers and properties of cells transformed by carcinogens; the hormone-related biochemistry of cancer and cancerous hosts. In FY89 there were four Research Program Project grants (P01), two Outstanding Investigator grants (R35), ten MERIT awards (R37), one Small Business Innovation Research award (R44), six FIRST awards (R29), one conference grant (R13) and 139 Traditional Research grants (R01) with a total funding level of \$23.1 million.

Grants Activity Summary

Fish RFA: A request for application (RFA) for "Studies on the Etiology of Neoplasia in Poikilothermic, Aquatic Animals: Finfish and Shellfish" was issued and a total of nine awards were made. The RFA was jointly sponsored by the National Cancer Institute (CPCB, DCE), the National Institute of Environmental Health Sciences (NIEHS) and the Department of the Army (Medical Research and Development Command). The Army funds for this initiative are being administered by the NCI through an Interagency Agreement. The following are the research accomplishments derived from the RFA grants.

One of the "Fish RFA" grants involves identification of a restriction fragment length polymorphism (RFLP) using a Drosophila DNA repair gene probe, and has used this in Southern blot analysis to map the gene in Xiphophorus backcross hybrids. Two different RFLPs for the src oncogene have been identified and a large number (>30) of individual backcross hybrids with a v-src probe have been analyzed. The construction of cDNA and genomic lambda libraries from Xiphophorus and related fish have been completed. The mapping assignments of oncogenes and DNA repair genes in this experimental system will provide valuable information concerning evolution of genes involved in carcinogenesis and DNA repair, and their roles in certain tumor types such as melanoma which are particularly amenable to investigation in Xiphophorus genetic hybrids (104).

Hemic neoplasia of bay mussels occurs in certain Puget Sound, Washington, populations at a prevalence of at least 45%. Experimental studies showed progressive disease in 20/40 mussels with 60% of these dying from the disease. Remission occurred in 20% of the experimental mussels and resulted from infiltration of granulocytes, secretion of an extracellular matrix which immobilized neoplastic cells and the formation of granuloma-like structures. Neoplastic cells showed reduced phagocytosis, in vitro, and diseased animals had reduced ability to clear injected bacteria compared to normal mussels. Further experiments demonstrated transplantation of hemic neoplasia to disease-free mussels with intact neoplastic

cells, transmission with cell-free homogenates and transmission by cohabitation of disease-free mussels with infected mussels. Cell cycling studies indicated that cell division of blood cell populations containing normal increments of DNA is first amplified as the disease process begins. Subsequently, discrete populations of cells with aneuploid DNA complements are formed from two anomalous cell cycles. DNA complements were as high as 14.7X haploid in the most advanced neoplastic population. Reverse transcriptase assays, ultrastructural examination of density- and velocity-gradient separated cell homogenates and whole tissues have so far failed to demonstrate the etiologic agent of the disease. The results support a continuous and synchronous cell transition model of the disease and indicate its infectious nature and the reduced immunocompetence of affected mussels (26).

Polycyclic aromatic hydrocarbons (PAH) are carcinogenic contaminants which can accumulate in sediments in aquatic ecosystems. As such, their availability to benthic organisms and potential transfer through aquatic food chains to man is a matter of environmental health concern. ^{14}C -benzo(a)pyrene (B[a]P) was used by another RFA awardee to investigate in vivo production and retention of PAH metabolites and bound residues and their potential trophic transfer in and between the polychaete (Nereis virens) and the winter flounder (Pseudopleuronectes americanus). Both the flounder and the polychaete metabolized single oral doses of B(a)P extensively to water soluble metabolites and bound residues which persisted for at least 4 to 20 days, respectively. In fish, bile, intestine and liver contained the highest percentage of the dose which in all cases was extensively metabolized, although the pattern of products accumulated differed between tissue type. Mixtures of metabolites produced by worms and fed to fish appeared to be accumulated and resulted in bound residue formation in fish livers. These experiments characterized the rapid in vivo metabolism of B(a)P in two species of marine organisms and demonstrated the potential for food chain transfer and further modification of metabolic products and bound residues (92).

The potential risks of eating contaminated shellfish were studied by measuring the extent of absorption and DNA binding of B(a)P in consumer target organs following exposure to pure B(a)P or meals of previously dosed spiny lobster (Panulirus argus). High performance liquid chromatography (HPLC) analysis of spiny lobster hepatopancreas (HP) indicated that 24 hours following an intrapericardial injection of [^{14}C]B(a)P (5 uCi/kg), 95% of the dose in HP had been metabolized to polar metabolites, including B(a)P-7,8 diol. In flounder fed HP from B(a)P-dosed lobster, $5.75 \pm 1.15\%$ of the dose was retained 24 hours after exposure. The liver contained $0.48 \pm 0.08\%$ of the dose. Exposure to HP spiked with pure [^{14}C]B(a)P resulted in the absorption of $12.74 \pm 0.64\%$ of the dose at 24 hours, with $0.92 \pm 0.18\%$ of the [^{14}C]B(a)P-equivalents retained in the liver. B(a)P DNA-adduct formation occurred in flounder liver following exposure to either HP containing B(a)P metabolites ($0.59 \pm 0.07 \text{ pmol } [^{14}\text{C}]BP\text{-equivalents/mg DNA}$) or B(a)P spiked HP ($1.43 \pm 0.15 \text{ pmol/mg DNA}$). These results indicate that the consumption of metabolized B(a)P present in contaminated shellfish can result in significant exposure and DNA damage to consumer organisms (60).

The intrahepatic biliary system was studied in the rainbow trout (Salmo gairdneri), a teleost known to form liver neoplasms after exposure to various carcinogens. Normal adults were examined using light microscopic, enzyme histochemical, and transmission and scanning electron microscopic methods. In light micrographs, longitudinal arrays of hepatocytes appeared as double rows incompletely divided by elongated darkly stained cells. Electron micrographs showed tubules of 5 to 9 pyramidally-shaped hepatocytes with their apices directed toward

a central biliary passageway and their bases directed toward sinusoids. Sequentially, beginning with hepatocytes, biliary passageways included canaliculi, preductules, ductules and ducts. Canaliculi were short and joined transitional passageways (preductules) formed by junctional complexes between plasma membranes of hepatocytes and small, electron-dense cells with a high nuclear to cytoplasmic ratio. Ductules, completely lined by biliary epithelial cells, occupied central regions of hepatic tubules. Relatively elongated, ductular cells were intimately associated with surrounding hepatocytes, separated from them by only a thin extracellular space devoid of a basal lamina. Epithelium of bile ducts included cuboidal through mucus-laden columnar cells, surrounded by basal lamina and, in larger ducts, by fibroblasts, smooth muscle cells and a capillary plexus. Bile ducts and hepatic arterioles, but not venules, were distributed together. The final objective of this study was to provide information that might be used in determining the role(s) of specific cell types in liver neoplasia. Although hepatocellular and cholangiocellular tumors are often found in the same liver following exposure of trout to various hepatocarcinogens, the contribution of the various segments of the intrahepatic biliary system to such lesions is not known. By electron microscopy correlated with immunohisto- and cytochemical methods, selective labeling of specific cell types may be achieved. Presence or absence of intermediate filaments, mucous granules, basal lamina, pericytes, connective tissue, and arterioles, sharing of junctional complexes with hepatocytes, and type and relative extent of plasma membrane specializations are some of the potential identifying characteristics emanating from the present study (54).

Cytochrome P-450 enzymes have been shown to be important in the metabolism of essential endogenous compounds and in the detoxification/bioactivation of many xenobiotics. Multiple forms or isozymes of P-450 identified in mammals have different functions and are subject to complex regulation. Only a limited set of P-450 isozymes have been identified in representative fish species. Among the forms seen in fish, scup cytochrome P-450E and trout P-450LM_{4b} are related enzymes. Both P-450E and P-450LM_{4b} are the primary P-450 forms involved in metabolizing PAH and they actively metabolize benzo(a)pyrene to benzo-ring dihydrodiols including 7,8-dihydrodiol, the direct precursor of the potent carcinogen 7,8-diol-9,10-epoxide. Furthermore, monoclonal and polyclonal antibodies directed against scup P-450E cross-react specifically with the beta-naphthoflavone (BNF)-induced trout P-450. The trout and scup enzymes also share regulatory properties. In scup, P-450E is the major isozyme induced by BNF, PAH or polychlorinated biphenyls (PCBs). Similarly, in trout, P-450LM_{4b} is the primary isozyme induced by BNF or by PCBs. The sensitivity of P-450E to induction by the various xenobiotics has led to the hypothesis that measuring levels of this P-450 isozyme in liver of feral fish could provide one means of detecting environmental pollution. A high incidence of tumors in fish occurs in several water systems of coastal North America. Association of such tumors with the presence of environmental chemicals such as PAH or polychlorinated hydrocarbons suggests a possible chemical etiology for their induction. Winter flounder (*Pseudopleuronectes americanus*) from areas of Boston Harbor, where sediments were heavily contaminated with PAH and other organic compounds, show a high incidence of liver lesions that include hepatocellular and cholangiocellular carcinomas, in contrast to a low incidence in livers of fish from adjacent less-polluted coastal areas. Livers of a natural population of winter flounder from a contaminated site in Boston Harbor were examined for the presence of oncogenes by transfection of DNA into NIH3T3 mouse fibroblasts.

Tissues analyzed contained histopathologic lesions including abnormal vacuolation, biliary proliferation, and, in many cases, hepatocellular and cholangiocellular carcinomas. Fibroblasts transfected with liver DNA samples from 7 of 13 animals were effective in the induction of subcutaneous sarcomas in nude mice. Further analysis revealed the presence of flounder c-K-ras oncogenes in all subcutaneous tumors examined. Direct DNA sequencing and allele-specific oligonucleotide hybridization following polymerase chain reaction DNA amplification of the tumor DNA showed mutations in the 12th codon in this gene. Analysis of DNA of all nude mouse tumors as well as 13 diseased flounder livers showed mutations at this codon. The mutations comprised G-C to A-T or G-C to T-A base changes resulting in amino acid substitutions of serine, valine, or cysteine for glycine. Liver DNA samples from five histologically normal livers of animals from a less polluted site were ineffective in the transfection assay and contained only wild type DNA sequences. The prevalence of mutations in this gene region correlated with the presence of liver lesions and could signify DNA damage resulting from environmental chemical exposure (138).

Polycyclic Aromatic Hydrocarbons: The microsomal monooxygenase system contains cytochrome P-450 as the terminal oxidase. The P-450s are a family of enzymes which have the ability to oxidize a wide variety of structurally unrelated compounds ranging from endogenous substrates such as steroids, bile acids and prostaglandins to exogenous substrates including drugs, insecticides, hydrocarbons and carcinogens. This diversity in substrate specificity is reflected in the large number of distinct P-450s as well as overlapping substrate specificities.

Work from many laboratories on hepatic microsomal cytochrome P-450 from rabbits and rats has established that many different forms of cytochrome P-450 exist, differing with respect to overall substrate specificity and even regio- or stereoselectivity for the metabolism of individual substrates. The relative proportions and total concentrations of various cytochrome P-450 isozymes in liver microsomes may be significantly altered following exposure of hepatocytes to foreign chemicals. The expression and activity of the phenobarbital (PB)-inducible P-450 isozymes, P-450b and P-450e, and the major 3-methylcholanthrene (MC)-inducible form, P-450c, were studied in primary cultures of adult rat hepatocytes in T1, Leibovitz L-15 (L-15) and a modification of Waymouth 752/1 (Way) media. P-450 isozymes in initially isolated hepatocytes, and control and PB-treated cultures were quantitated by Western blot analysis, and activity was determined with 7,12-dimethylbenz(a)anthracene (7,12-DMBA) as substrate. Data from the Western blot analysis correlated well with the metabolic activity toward DMBA. P-450b was consistently induced by PB in hepatocytes in T1 and to a lesser extent in Way media. P-450e protein was constitutive in initially isolated cells, expressed in control cultures at a reduced level, and increased or maintained by PB in all three media. DMBA metabolite formation associated with P-450b and P-450e activity was induced by PB in hepatocytes in T1 and Way and was inhibited by antibodies to P-450b. P-450c was only infrequently expressed in freshly prepared hepatocytes, but was detected in all control and PB-treated cultures although at a much higher level in T1. Thus, the amounts of P-450 isozymes, their inducibility by PB, and their activity toward DMBA were found to be dependent on the culture medium (61).

Cultures of adult mouse epidermal keratinocytes (MEKs) were utilized to determine whether the metabolism and metabolic activation of polycyclic aromatic hydrocarbons varied as a function of the stage of keratinocyte differentiation. Differentiation of MEKs in culture can be modulated by the Ca^{++} concentration in the medium. MEKs grown in low Ca^{++} -containing medium (0.05-0.10 mM) maintain basal

cell morphology and proliferate, while increasing the Ca^{++} concentration in the medium to 1.2-1.4 mM signals the cells to undergo terminal differentiation. Relative to cultures of undifferentiated MEKs (low Ca^{++}), cultures of differentiated MEKs that had been switched to high Ca^{++} medium 48 hours prior to treatment with benzo(a)pyrene (B[a]P) and DMBA exhibited more rapid overall metabolism of both hydrocarbons. The greatest differences in the metabolism of B(a)P and DMBA between the two types of cultures occurred after a 3-6 hour lag period. In addition, the levels of DNA-adducts formed from B(a)P and DMBA after a 24-hour exposure to the hydrocarbon were 4- and 3-fold higher respectively, in cultures of differentiated MEKs. Higher levels of mutagenesis and cytotoxicity were also observed in cocultures of Chinese hamster V-79 cells and differentiating or differentiated MEKs. In cocultures treated with hydrocarbons at the time of Ca^{++} shift, several hours elapsed before differences in mutagenesis were apparent between high and low Ca^{++} -containing cultures. This lag period was eliminated if the MEKs were switched to high Ca^{++} medium 24 hours prior to exposure to DMBA. Based on the present data, the expression and inducibility of certain monooxygenase activities involved in the metabolism of B(a)P and DMBA is dependent on the differentiation state of MEKs (21).

Dihydrodiol dehydrogenase has been implicated in the detoxification of trans-dihydrodiols of PAH. It has been suggested that this enzyme will oxidize these proximate carcinogens to innocuous catechols and thereby prevent the formation of the ultimate carcinogens, the anti-diol epoxides. Dihydrodiol dehydrogenase (DD) will oxidize non-K-region trans-dihydrodiols of polycyclic aromatic hydrocarbons; a reaction that can suppress the formation of anti-diol epoxides or ultimate carcinogens. Using benzenedihydrodiol ([\pm]-trans-1,2-dihydroxy-3,5-cyclohexadiene) as a substrate, 13 human liver biopsy and 8 human lung samples were examined for enzyme activity. In the human liver samples enzyme activity could be measured spectrophotometrically and specific activities ranged from 0.6-5.0 nmoles benzenedihydrodiol oxidized/min/mg. Highest activities were observed in liver specimens obtained from female donors. Western blot analysis of human liver cytosol using antisera raised against purified rat liver DD detected two bands $\text{Mr} = 34,000$ and $27,000$. The former mol. wt. is identical to that observed for the homogeneous rat liver enzyme. Gel-filtration experiments indicate that human liver DD activity elutes as a single peak and co-elutes with the purified rat liver enzyme, suggesting that the lower mol. wt. species is an artifact of degradation. Preparations of the human liver enzyme required NADP^+ for activity and were insensitive to inhibition by dicoumarol, indomethacin and 6-medroxyprogesterone acetate. These properties distinguish the enzyme from alcohol dehydrogenase, quinone reductase and rat liver DD. In human lung DD activity was barely detectable using a sensitive radiochemical assay in which catechol formation is linked to catechol-O-methyl transferase using [^3H]-S-adenosyl-methionine as methyl donor. Specific activities were approximately 1000th of that observed for human liver and ranged from 1-4 pmoles benzenedihydrodiol oxidized/min/mg. Western blot analysis of lung cytosol detected three bands $\text{Mr} = 34,000$, $31,000$ and $28,000$. The low level of DD in human lung, a known target of PAH-induced chemical carcinogenesis, suggests that DD may play a critical role in PAH detoxification (110).

PAHs are formed primarily as a by-product of incomplete combustion of organic matter and are present in relatively high concentrations as environmental contaminants. B(a)P and benzo(b)fluoranthene (B[b]F) are among the more prevalent PAHs detected. B(b)F is a non-alternant PAH which, like B(a)P, has incorporated in its structure a bay region. Although the mechanism(s) of activation for both alternant and nonalternant PAHs are not fully understood, much more is known about

the activation of alternant hydrocarbons such as B(a)P in comparison to nonalternant hydrocarbons such as B(b)F.

The types of B(b)F metabolites formed *in vitro* with rat liver homogenate and *in vivo* in mouse epidermis have been characterized. In addition, the mutagenic and tumorigenic activities of these metabolites have been evaluated. The results of these studies suggest that different mechanisms of activation are likely involved between alternant and nonalternant hydrocarbons. Unlike several alternant PAHs which are activated to carcinogenic bay region diol epoxides, B(b)F does not appear to be metabolically activated to a carcinogen by the formation of a simple bay region diol epoxide. This approach was used in this study to provide further insight into the metabolic activation of B(b)F. This approach is based on the assumption that fluorine can inhibit enzymatic oxidation at its position of attachment to the PAH system. Inhibition of metabolite formation at a position involved in metabolic activation would be expected to inhibit both DNA binding and tumorigenicity. The effects of fluorine substitution on B(b)F DNA adduct formation and tumorigenicity in mouse epidermis were investigated. Fluoro derivatives studied included 1-, 6-, 7-, 8-, 9-, and 11-fluoroB(b)F as well as 1,9- and 6,9-difluoroB(b)F. Each compound was applied topically to mice and hydrocarbon DNA adduct formation was assessed using the ³²P-postlabeling technique. All of the fluorinated compounds bound to DNA to a lesser extent than B(b)F. Among the fluorinated compounds, the greatest binding was observed for 8-fluoroB(b)F. The lowest levels of hydrocarbon DNA adduct formation from the fluoro derivatives were observed for 1-, 7-, 11-, and 6,9-difluoroB(b)F. The tumor-initiating activities on mouse skin of 7-, 9-, and 11-fluoroB(b)F were determined. All three compounds were significantly less tumorigenic than B(b)F. The results may be consistent with activation of B(b)F through multiple arene oxides. B(b)F-1,2-diol and B(b)F-11,12-diol are known metabolites of B(b)F which are formed by the hydration of the corresponding arene oxides. In addition, major known metabolites such as 4-, 5-, and 6-hydroxyB(b)F may also be formed in part from the 4,5-, 5,6-, and 6,7-oxides of B(b)F, respectively. If all of the above oxides, as well as small amounts of B(b)F-9,10-epoxide or B(b)F-9,10-diol-11,12-epoxide were involved in its tumorigenicity, the results would not be unexpected since one might anticipate that almost any monofluorinated derivative of B(b)F capable of inhibiting metabolism in rings A, B, and D would result in lower levels of DNA binding in comparison to B(b)F. Thus, the results observed in the tumorigenicity and DNA binding studies would be expected, since all the fluorinated compounds bound less to DNA than did B(b)F, but nevertheless retained some activity (51).

Enantiomeric compositions of epoxides formed in the metabolism of planar benz(a)-anthracene (BA), B(a)P, and chrysene (CR), and nonplanar benzo(c)phenanthrene (BcPh), 12-methylbenz(a)anthracene (12-MBA) and 7,12-DMBA by liver microsomes from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats are determined either by direct chiral stationary phase HPLC analysis or by the enantiomeric compositions of metabolically formed trans-dihydrodiols. Cytochrome P-450 isozymes contained in various liver microsomal preparations have varying degrees of stereoselectivity in catalyzing the epoxidation reactions at various formal double bonds of the polycyclic aromatic hydrocarbons studied. In general, cytochrome P-450c, the major cytochrome P-450 isozyme contained in liver microsomes from 3-methylcholanthrene-treated rats, has the highest degree of stereoselectivity. Regardless of absolute configuration, non-K-region epoxides are converted to trans-dihydrodiols by epoxide hydrolase-catalyzed water attack at the allylic carbon. The S-center of K-region S,R-epoxide enantiomers derived from planar BA, B(a)P and CR is the major site of epoxide hydrolase-catalyzed water attack. In

contrast, the R-center of K-region S,R-epoxide enantiomers derived from nonplanar BcPh, 12-MBA and 7,12-DMBA is the major site of epoxide hydrolase-catalyzed water attack. However, the K-region R,S-epoxide enantiomers of the six polycyclic aromatic hydrocarbons studied are hydrated by microsomal epoxide hydrolase with varying degrees of regioselectivity. Thus, the enantiomeric composition of a metabolically formed dihydrodiol is determined by (i) the stereoselective epoxidation at a formal double bond of a parent hydrocarbon by microsomal cytochrome P-450 isozymes, and (ii) the enantioselective and regioselective hydration of the metabolically formed epoxide by microsomal epoxide hydrolase (162).

Glutathione-S-transferases (GST's) have the capacity to detoxify electrophilic xenobiotics by catalyzing the formation of glutathione (GSH) conjugates. GST's are also thought to engage in the intracellular transport of a variety of hormones, endogenous metabolites and drugs, by virtue of their capacity to bind these substances. Multiple forms of the protein originate from dimeric combinations of different subunit types. Mammalian GST's have been subdivided into three different categories based on sequence homologies and other common properties. In the rat, Y_a and Y_c type subunits are classified as alpha forms, Y_b 's are in the mu family, and Y_p is in the pi category. There are subclasses within each group as the proteins are encoded by a multigene family. GST isoenzymes are expressed in a tissue-specific manner, and rat liver ordinarily has equivalent amounts of Y_a , Y_b , and Y_c subunits but lacks Y_p . A series of carcinogens, drugs, metabolites and related compounds were analyzed for binding to Y_{b2} -GST by the circular dichroic displacement assay. Broad specificity of binding is evident, but relative affinities are not determined by lipophilicity alone. This is exemplified by the striking differences between 2,3-benzanthracene, which was bound with high affinity and 1,2-benzanthracene which was classified as nonbinding. Distinct structural requirements emerged from these results. Steric factors appear to dictate binding; the poly-substituted anthracenes, such as the dibenzanthracenes, pyrene, chrysene, and other PAHs were not bound. Similarly, dibenz(a,j)acridine was not bound, whereas acridine and acridine orange were bound (80).

The adriamycin resistant subline Adr^RMCF-7, which was derived from the human breast adenocarcinoma cell line MCF-7, exhibits the multidrug resistant phenotype. To determine how these biochemical changes which include increased expression of the anionic isozyme of glutathione transferase affect the metabolism of the carcinogen B(a)P, MCF-7 and Adr^RMCF-7 cultures were exposed to [³H]B(a)P. The percentages of [³H]B(a)P converted to water-soluble metabolites were: MCF-7 cells 6 hours, 16.6%; 24 hours, 80.8%; 48 hours, 88.8%; Adr^RMCF-7 cells 6 hours, 1.4%; 24 hours, 2.2%; 48 hours, 4.5%. The organic-solvent extracts of the MCF-7 medium contained very polar B(a)P metabolites, whereas the Adr^RMCF-7 extract was composed almost entirely of unmetabolized B(a)P with very small amounts of BaP-7,8-diol and B(a)P-9,10-diol. Ion-pair HPLC of the water-soluble metabolites from the MCF-7 cultures revealed the presence of metabolite peaks that eluted in the regions of glutathione, glucuronide and sulfate conjugates. To examine the effect of elevated anionic glutathione transferase levels on an ultimate carcinogenic metabolite of B(a)P, cultures were exposed to [³H]anti-B(a)P-7,8-diol-9,10-epoxide (B(a)PDE). Preliminary results indicate that glutathione conjugates of B(a)PDE were formed in both MCF-7 and Adr^RMCF-7 cells and that the initial level of binding of anti-B(a)PDE to DNA and the DNA adducts formed were similar in both cell lines. Thus, Adr^RMCF-7 cells lack the ability of the parent MCF-7 cells to oxidize B(a)P to B(a)PDE, but are able to conjugate B(a)PDE to glutathione (5).

Alkylating Agents: An attractive hypothesis to explain the epidemiological relationship between gastric cancer and dietary intake of nitrate is based on the formation of N-nitroso compounds in the stomach. Nitrate is secreted in concentrated form in saliva and reduced to nitrite by oral bacteria. The rate at which nitrate is secreted is roughly proportional to its concentration in plasma, which in turn is influenced by the diet. The amount of nitrite formed in saliva from nitrate greatly exceeds typical dietary levels of nitrite. Upon swallowing, this nitrite gains access to the stomach, where the acidic conditions favor its reaction with amines and amides present in the diet, gastric juice or saliva to form nitrosamines or nitrosamides. Many such N-nitroso compounds are potent carcinogens, and may act in a variety of target organs in addition to the stomach. The gastric formation of nitrosamines from nitrite and amines is well documented in experimental animals.

To elucidate the factors governing the formation of N-nitrosamines in the stomach, the formation of N-nitrosoproline (NPro) was studied under controlled conditions, using a dog equipped with a Thomas cannula. Solutions containing nitrite, proline and in some cases ascorbic acid and/or SCN^- , were infused into the stomach and samples taken to determine gastric [nitrite], [NPro], [ASC], [SCN^-] and pH as functions of time. (Brackets indicate molar concentrations; ascorbic acid and ascorbate ion are denoted together by ASC.) Previous work from this group showed that the rapid decline of [nitrite] in the stomach was due primarily to absorption. Additional experiments in which ASC, proline, or NPro were infused together with a non-absorbable marker, in the absence of nitrite, demonstrated that there was negligible absorption or secretion of these substances in the stomach. Thus, changes in [ASC] and [NPro] with time could be interpreted quantitatively in terms of rates of chemical reaction and dilution of the stomach contents. It has been demonstrated that a detailed mathematical model, which combines appropriate mass balance equations with in vitro kinetic data, is capable of predicting rates of nitrosamine formation in the dog stomach under a wide variety of conditions. The model accounts for the inhibitory effects of various levels of ascorbic acid, the catalytic effects of thiocyanate and the effects of physical processes such as oxygen entry, absorption of nitrite and dilution of the stomach contents by secretions. The success of this model in predicting the present experimental findings supports the use of similar models to elucidate conditions which should either enhance or reduce the intragastric formation of N-nitroso compounds in humans. Indeed, a mathematical model has been developed by this group to estimate the rates of formation of nitrosamines and nitrosamides in the human stomach, under a variety of physiological and environmental conditions. The model combines a detailed description of kinetics of N-nitrosation with mass balance equations which account for gastric emptying, dilution and absorption. The simulations were based on a typical schedule of dietary inputs, and included variations in gastric pH and in the volume of the stomach contents over a 24-hour period. Consideration of these transient phenomena allowed a distinction to be made between amines or amides present in the diet and in gastric or salivary secretions. A comparison of the theoretical results with available data on the nitrosation of proline suggests that the model accurately predicts gastric rates of nitrosamine formation under controlled conditions, and correctly represents the strong catalytic effects of thiocyanate and the inhibitory effects of ascorbic acid or ascorbate ion. The results further suggest that NPro excretion is not an accurate index of gastric nitrosation under physiological (low-dose) conditions, even when corrections are made for dietary intake or NPro. The predicted rates of formation of N-nitrosodimethylamine (NDMA), even for a diet high in dimethylamine, were found to be a factor of 10^2 to 10^3 lower than published estimates of the dietary exposure to

performed NDMA. Thus, these findings do not support the hypothesis that gastric formation of NDMA from dietary dimethylamine poses a serious additional health risk (142).

Nitrosamines require metabolic activation for their cytotoxic and carcinogenic actions. The major activation step for N-nitrosodialkylamines is believed to be the oxygenation of the alpha-carbon catalyzed by a P-450-dependent enzyme system. The metabolism of NDMA, N-nitrosodiethylamine, N-nitrosobenzylmethylamine, and N-nitrosobutylmethylamine was investigated in incubations with human liver microsomes. All of the 16 microsomal samples studied were able to oxidize NDMA to both formaldehyde and nitrite at NDMA concentrations as low as 0.2 mM; the rates of product formation of the samples ranged from 0.18 to 2.99 nmol formaldehyde/min/mg microsomal protein (median, 0.53 nmol). At a concentration of 0.2 mM NDMA, the rates of denitrosation (nitrite formation) were 5 to 10% (median, 6.3%) those of demethylation (formaldehyde formation); the ratio of denitrosation to demethylation increased with increases in NDMA concentration, in a manner similar to rat liver microsomes. Immunoblot analysis with antibodies prepared against rat P-450_{ac} (an acetone-inducible form of cytochrome P-450) indicated that the P-450_{ac} orthologue in human liver microsomes had a slightly higher molecular weight than rat P-450_{ac} and the amounts of P-450_{ac} orthologue in human liver microsomes were highly correlated with NDMA demethylase activities ($r = 0.971$; $P < 0.001$). Analysis of four selected microsomal samples showed that human liver microsomes exhibited at least three apparent K_m and corresponding V_{max} values for NDMA demethylase. This result, suggesting the metabolism of NDMA by different P-450 enzymes, is similar to that obtained with rat liver microsomes, even though most of the human samples had lower activities than did the rat liver microsomes. The high affinity K_m values of the four human samples ranged from 27 to 48 μM (median, 35 μM), which were similar to or slightly lower than those observed in rat liver microsomes, indicating that human liver microsomes are as efficient as rat liver microsomes in the metabolism of NDMA (161).

Nitrosamines are a class of carcinogens which display a remarkable degree of organ and species specificity. Some factors thought to be important for this specificity include the absorption and distribution of the parent compound, cell-specific metabolic activation, and cell and species differences in DNA repair. The rates of uptake of the carcinogen N-nitrosobis(2-oxopropyl)amine (BOP) by hepatocytes isolated from Fischer rats and Syrian hamsters were determined in order to investigate species differences in cellular transport of the carcinogen. Initial rates of uptake of [^{14}C]BOP by hepatocytes were measured using a rapid centrifugation technique. At cell densities from 1.5 to 6 $\times 10^6$ cells/ml, initial rates of uptake were as much as 4-fold more rapid in hamster hepatocytes than in those of the rat. The cell/medium distribution ratio for hamster hepatocytes reached a value of 9.0 after a 20-minute incubation with an extracellular BOP concentration of 20 μM . Under the same conditions, the cell/medium distribution ratio for rat hepatocytes was only 2.4. These results indicated that BOP uptake proceeded against a concentration gradient and was more rapid in hamster hepatocytes. In both species, the rates of uptake were saturable with increasing concentration (2-685 μM) and displayed biphasic kinetics characteristic of high-affinity ($K_m < 20 \mu M$) and low-affinity ($K_m > 30 \mu M$) processes for the uptake of BOP. Evidence for the involvement of an ATP-dependent active carrier-mediated transport process was obtained from experiments in which hepatocytes were preincubated with metabolic inhibitors. Significant inhibition of uptake was observed in the presence of KCN, carbonyl cyanide-3-chlorophenylhydrazone, antimycin A, oligomycin, and other agents which interfere with electron transport or ATP generation.

Based on the reduction in uptake rates, rat hepatocytes were more sensitive to the effects of these inhibitors. These results suggest that the entry of BOP into hepatocytes is under cellular regulation and that the more rapid rate of uptake in liver cells of the hamster may be one factor responsible for the observation that BOP is a more potent hepatotoxin and carcinogen in this species (127).

The mutagenicity of BOP and of DMN were measured in the V79 assay. Homogenates of hepatocytes and pancreas acinar and duct tissue from Syrian golden hamsters and MRC-Wistar rats were used as the activating systems. Ouabain- and 6-thioguanine-resistance were the markers of mutagenicity. The order of effectiveness of the homogenates in generating mutagens from BOP was: hamster duct > hamster acinar > hamster hepatocyte > rat duct > rat acinar > rat hepatocyte. With NDMA, the order was: hamster acinar > hamster hepatocyte > hamster duct > rat duct > rat hepatocyte > rat acinar. These data show that there are differences in the activation of BOP by hamster acinar and duct tissue. The data presented show that there are significant differences between the ability of hamster pancreas acinar and duct tissue to activate BOP to mutagenic metabolites. It suggests that the abilities of acinar and duct tissue to metabolize BOP are comparable, but that the metabolism of BOP by acinar tissue largely leads to innocuous metabolites that alkylate DNA but not at sites that will produce mutations. The differences in metabolism would appear to be qualitative rather than quantitative. The data not only support the hypothesis that the target cells for BOP are part of the duct/ductular system of the pancreas but suggest that the greater susceptibility of the duct tissue to the carcinogenicity of BOP is due to a failure of this tissue to repair some potentially carcinogenic lesions on the DNA. The greater ability of the duct tissue to generate mutagens as compared to hepatocytes, questions an essential role for the liver in the production of carcinogenic and mutagenic metabolites from BOP, although some involvement of hepatic metabolism in the induction of pancreatic ductular adenocarcinoma in vivo cannot be ruled out (70).

Metabolism of the pancreatic carcinogen BOP in the hamster resulted in its reduction to N-nitroso(2-hydroxy-propyl)(2-oxopropyl)amine (HPOP) and N-nitrosobis(2-hydroxypropyl)amine (BHP) and their subsequent conjugation with sulfate and glucuronic acid. Assuming that HPOP and BHP glucuronides do not undergo further degradation, it can be estimated from the composition of the urine that at least 44% of the total BOP metabolism occurs via its reduction to HPOP. Levels of BOP and HPOP and its metabolites in blood showed that the half-life of BOP is relatively short (15 minutes) as compared to that of HPOP (1.5 hours). This difference resulted in the gradual increase of HPOP in the plasma from 8 to 84 nmoles, while the concentration of BOP declined from 60 to 5 nmoles between 1 and 2 hours, respectively. A parallel increase in HPOP during that time period was observed in the bile (from 2 to 10 nmoles) and pancreatic juice (from 0.4 to 13). Conjugates of HPOP, while present in the bile, were hardly detectable in the pancreatic juice. This, along with the absence of any other metabolites, suggests that BOP and HPOP present in the ducts are not metabolized to any significant extent by duct cells, and that HPOP present in pancreatic juice ducts may be transported there via the circulation (67).

The formation of methylphosphate during the metabolic activation of methylazoxymethanol (MAM) and NDMA by ethanol-induced rat liver microsomes, and during the spontaneous decomposition of methylnitrosourea (MNU) in phosphate buffer was investigated. In the case of MAM, the major metabolites found were labeled methanol and formic acid. In the case of NDMA, the major metabolites were labeled methylamine, methanol and formaldehyde. In both cases, however, an additional

minor product was detected when incubations were carried out in phosphate buffer, but not in Tris buffer. This same product, in addition to methanol, was obtained when ^{14}C -MNU was allowed to spontaneously decompose in phosphate buffer (but not Tris buffer), pH 8.0. The product of these three reactions in phosphate buffer was identified as ^{14}C -methylphosphate. These results indicate that the methyl-diazonium and/or the methylcarbonium ions, the only common denominators in the spontaneous decomposition of MNU and the metabolic activation of MAM and NDMA, react not only with water, to yield methanol, but also with inorganic phosphate to yield methylphosphate. This finding suggests the possibility of monitoring the formation of ^{32}P -alkylphosphates as a marker of the ability of various rodent and human tissues to metabolically activate unlabeled indirect alkylating carcinogens (30).

Markers and Properties of Transformed Cells: Research included in this subject area involves studies on the documentation of various growth and functional properties of initiated cells, preneoplastic cells and fully transformed cells, and the identification of biochemical and molecular markers for distinguishing these altered cell types from normal cells. The evidence obtained to date strengthens the supposition that the development of most cancers involves a multi-step process in which cells progress from normal to initiated, preneoplastic and premalignant stages to the end point of malignant neoplasia. In order to characterize cells at each stage, a detailed analysis and knowledge of the sequence of relevant biochemical and biological alterations associated with the development of chemically-induced carcinogenesis is needed. To achieve this purpose, a variety of model systems, both in vivo in animals and in cells in culture, are being used. Of the animal model systems, a predominant one currently in use is the rat chemically-induced hepatocarcinogenesis model. Although this model was established some time ago, the treatment regimens being employed have undergone a variety of changes depending on the purpose of the experiment and on the end point desired. Chronic or intermittent exposure regimens have been used, along with initiation-promotion type regimens in which various initiating carcinogens and promoting stimuli are used. The sequential appearance of foci of altered hepatocytes, nodules and hepatocellular carcinomas can be observed and analyzed. In addition, cell lines can be derived from either liver tumors or from normal tissues for further study. These cells, and cell lines which have been treated with a carcinogen in vitro, have been tested for the expression of various phenotypic markers which may be correlated with tumorigenicity.

Many cancer marker proteins have been described which are specific for one or more tumor types. While these tests are very useful, they would be complemented by a marker protein released into the circulation which can detect all cancers (tumors) regardless of the tissue of origin. A 60 kd oncofetal protein (OFP-60) has, in fact, been described which previous evaluations strongly suggested was a general cancer marker protein. This marker protein appears to concentrate in the plasma to levels higher than those present in the tumor itself. However, even here its concentration is very low. Although OFP-60 is present in the embryo and amniotic fluid in pregnancy, it does not cross the placental barrier to the maternal circulation. It is at nondetectable levels in the blood and tissues from normal individuals and those with non-neoplastic diseases or conditions. The specificity of OFP-60 for the presence of neoplastic cells has been confirmed in experimental animal systems. All of the above studies have been based on measurements using a biochemical assay which required preliminary separation of OFP-60 from a normal 35 kd protein (rats) or 25 kd protein (humans) present in all tissues and blood. These proteins, regardless of species, show a similar activity in the biochemical

assay. The development of simpler immunoassays would be greatly facilitated by the availability of monoclonal antibodies specific for OFP-60. In recent studies, monoclonal antibodies of good activity and specificity have now been prepared against the factor isolated from tumor-bearing rats. Inhibition ELISA and immuno-bioassays have been developed which give a linear response to the antigen. The purified monoclonal antibodies cross-react with the fetal protein, but not with a normal adult protein with similar biological activity. Western blots using the monoclonal antibodies indicate that the protein of approximately 60 kd is present in significant concentration in the cytosol fraction of both transplantable and primary tumors. It is shed to circulation, and in the case of mammary tumors also to the milk, but is not detectable in the plasma or tissue cytosol of normal animals (154).

It has been previously reported that epidermal growth factor (EGF) can induce or enhance the colony-forming ability in soft agar of chemically-treated or transfected rat liver epithelial cells. The emergence of EGF-induced anchorage-independence was shown to be the earliest and possibly the most reliable phenotype to indicate the imminent tumorigenic transformation of cultured rat liver epithelial cells. In contrast, transforming growth factor-beta (TGF-beta) has been shown to inhibit DNA synthesis and proliferation of normal cultured rat liver epithelial cells, but not chemically transformed cells. The differential sensitivity of normal versus malignant cells to the inhibitory effect of TGF-beta has been proposed to be one of the mechanisms by which TGF-beta exerts a role in tumorigenesis. Exposure of animals to phenobarbital (PB) results in pleiotropic effects on the liver which range from enzyme induction, hepatocyte hypertrophy and hyperplasia to the promotion of both hyperplastic lesions and hepatocellular carcinomas. Previous studies have suggested that PB promotes the formation of hepatic nodules by selectively inhibiting normal hepatocyte growth. The proliferation of hepatocytes in animals exposed to PB has been shown to be reduced in vivo in response to a partial hepatectomy and in vitro in response to EGF. A decrease in hepatocyte EGF receptor number and mRNA for the EGF receptor is also observed with the continual exposure of animals to PB. Though PB inhibits EGF-dependent mitogenesis of cultured normal hepatocytes at concentrations in excess of 1 mM, pre-neoplastic hepatocytes are relatively refractory to its inhibitory effects. These observations suggest that PB selectively alters the EGF-induced mitogenic signal transduction pathway in normal hepatocytes, and that the hepatocyte EGF receptor may be a probable site of PB action. Pre-exposure of normal hepatocytes to EGF causes approximately a 60% decrease in EGF receptor number in both the presence and absence of PB. Additionally, hepatocytes which are removed from animals exposed to a promotional regimen of PB (i.e., 0.1% in the drinking water for two months), though having significantly fewer initial receptors on the cell surface, also respond to EGF in culture by down-regulating the receptors by 60%. Thus, PB's inhibition of EGF-dependent hepatocyte proliferation in culture is not caused by inhibition of EGF receptor down-regulation. Not only is the magnitude of the down-regulation in control and PB-treated cells comparable, but also the kinetics of the internalization is similar. Thus, PB does not mediate its inhibitory effect on EGF-dependent proliferation of hepatocytes by either directly interfering with EGF interaction with its surface receptor, or modifying the process of EGF-induced down-regulation (63).

A transient reduction of EGF binding is another common response of cultured cells to tumor promoters. The response to many of these agents is a consequence of protein kinase C activation, although reduced EGF binding also occurs with the skin tumor promoter, palytoxin, in the absence of protein kinase C activation.

Thus, attenuation of EGF binding either through a protein kinase C-dependent or -independent mechanism may be an important component of the promotional phase of carcinogenesis. Consequently, whether PB also decreased EGF binding by adult rat hepatocytes in culture was investigated. The results of these studies clearly show that PB directly applied to cultured, adult rat hepatocytes at a concentration >1 mM decreases cellular surface binding of EGF. This effect of PB, resembles that of the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), in that both drugs decrease EGF receptor number but do not affect receptor affinity. The effects of the two promoters differ, however, in that only TPA reduces the high affinity EGF binding by A431 cells. They also differ in that TPA, but not PB, causes redistribution of protein kinase C from a soluble to a membranous hepatocyte subcellular fraction. These data indicate that decreased EGF binding is a common hepatocyte response to the tumor promoters TPA and PB, but that this response can be mediated by either a TPA-activated, protein kinase C-dependent pathway or a PB sensitive, protein kinase C-independent pathway (63).

Hepatocytes from normal rat liver have high affinity, saturable receptors for TGF-beta, and their mitogen-induced DNA synthesis is inhibited by TGF-beta in a dose-dependent manner. Hepatocytes from early regenerating rat liver lose high affinity binding for TGF-beta at early time points and concomitantly lose sensitivity to the inhibitory effects of TGF-beta. However, TGF-beta does not appear to inhibit DNA synthesis once it has started, since it does not inhibit the DNA synthesis *in vitro* of hepatocytes obtained 20 hours after partial hepatectomy or in normal hepatocytes at the time of DNA synthesis. TGF-beta mRNA levels are increased during hepatic regeneration, with the peak occurring at approximately 36 hours with a plateau until 96 hours before starting to decline. The liver cell which produces this strong TGF-beta mRNA signal has not yet been identified but may not be the hepatocyte. Whether this is reflected in TGF-beta protein production and at sufficient levels to cause inhibition of growth *in vivo*, has yet to be determined. Human and rat hepatomas have decreased sensitivity to the inhibitory actions of TGF-beta, yet retain normal overall levels of TGF-beta binding, although the receptor characteristics are quite different from normal. Primary rat hepatomas appeared to produce TGF-beta, although this was not seen in the rat hepatoma cell lines. This would appear to indicate that some cells other than the carcinogen-altered hepatocyte are producing the TGF-beta in primary rat hepatomas. In contrast, five human hepatoma cell lines overproduced TGF-beta mRNA and TGF-beta protein compared to normal human or rat liver (13).

Transforming growth factor alpha (TGF-alpha) is a mitogenic polypeptide which shares structural and functional homology with EGF. TGF-alpha, which binds to the EGF receptor and activates the receptor's intrinsic tyrosine kinase activity, was originally detected in the culture medium of retrovirally transformed fibroblasts, and very little is known about the regulation of its expression. TGF-alpha mRNA levels were examined in a set of cloned neoplastic cell lines derived by chemical transformation of a normal rat liver epithelial cell. The untransformed parental cell line, WB-344, did not express a detectable level of TGF-alpha mRNA, whereas GP6ac, a transformed line capable of autonomous growth in soft agar, expressed TGF-alpha. When GP6ac cells were treated with agents thought to regulate protein kinase C activity, e.g., the tumor promoter, TPA, TGF-alpha mRNA levels increased by 8- to 11-fold. The induction of TGF-alpha mRNA was detectable at 2 hours, was maximal at 8-12 hours, and declined by 24 hours. Angiotensin, bradykinin, epinephrine, and EGF also increased TGF-alpha mRNA by 2- to 5-fold. In contrast, parental WB cells neither expressed TGF-alpha mRNA, nor responded to TPA. TPA also increased EGF receptor mRNA in GP6ac cells but the effect was less prolonged;

maximal levels were seen at 4 hours after TPA exposure and returned to control levels by 12 hours. TPA increased TGF- α mRNA in GP6ac cells, in part, by increasing transcription of the TGF- α gene as measured by run-on transcription rates in isolated nuclei. In addition, the induction of TGF- α by TPA was blocked by concurrent incubation with agents that inhibit protein synthesis. However, if TPA was present for at least 2 hours, subsequent addition of cycloheximide enhanced the effect of TPA. This indicates that the induction of TGF- α in GP6ac cells is comprised of at least two phases demarcated by the requirement for protein synthesis. The time course of induction and the sensitivity to inhibition of protein synthesis distinguish the effect of TPA on TGF- α mRNA from that of other genes regulated by TPA; e.g., c-*myc* and c-*fos*. These data also suggest that chemical transformation of rat liver epithelial cells leads to expression of TGF- α mRNA, and that once expressed, TGF- α mRNA can be modulated in a protein kinase C-dependent manner (39).

Genetics and Regulation of Enzymes Associated with Carcinogenesis: Many of the different cytochrome P-450 forms are regulated differentially according to age, tissue, inheritance and their ability to be induced in response to different types of agents. Therefore, variations in the expression of different P-450s depending on factors such as age, the level of induction following exposure to certain agents, or the genetic events that contribute to heritable differences, can alter the balance between the formation of toxic and non-toxic products. The macrolide antibiotic, rifampicin, elicits a >10-fold elevation of liver microsomal concentrations of cytochrome P-450 3c (P450III_{A6}) in 7-day old rabbits 24 hours after treatment. A cDNA for P450III_{A6} was cloned and used to monitor the transcription of the corresponding Cyp3A6 gene and changes in the concentration of P450III_{A6} mRNAs. An 8-fold increase in the rate of Cyp3A6 transcription was evident at 12 hours following administration of rifampicin. Increases in the corresponding mRNAs and of microsomal P450III_{A6} concentrations were observed that persisted after rates of transcription diminished at 18 hours following treatment with rifampicin. These temporal changes in the rate of transcription, in mRNA concentration and of the accumulation of P450III_{A6} protein were similar to those elicited in 7-day old rabbits by the synthetic glucocorticoid dexamethasone. The 7-day old rabbits exhibit lower basal concentrations of P450III_{A6} than adults which exhibit 2- to 10-fold higher concentrations at four weeks of age and older. Rifampicin also elicits a >10-fold increase of liver microsomal progesterone 6- β -hydroxylase activity in 7-day old rabbits which is activated *in vitro* by the effector α -naphthoflavone. Transient transfection of COS-1 cells with the cDNA for P450III_{A6} leads to the expression of the enzyme in microsomes. The stimulation of progesterone 6- β -hydroxylase activity by α -naphthoflavone is evident in cultured cells in which P450III_{A6} is expressed (147).

Certain PAHs, such as B(a)P, and certain halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are important environmental pollutants. Many of these compounds are extremely toxic and carcinogenic. These same compounds also bind the soluble Ah receptor and are thereby capable of inducing cytochrome P-450IA1. Cytochrome P-450IA1-dependent aryl hydrocarbon hydroxylase (AHH) activity in turn plays a central role in metabolism of PAHs to their ultimate carcinogenic derivatives. Pathogenesis by HAHs also depends upon action of the Ah receptor. However, in the case of these compounds, the HAHs themselves, rather than metabolites, are the pathogenic agents, and P-450IA1 activity is not involved.

The 300 kd mouse Ah receptor appears to dissociate to a 100 kd ligand-binding polypeptide upon treatment with high salt which is the same size as the receptor detected under denaturing conditions. This suggests that the receptor is polymeric but does not shed light on whether it contains one, or more than one, type of subunit. Besides their intimate involvement in chemical carcinogenesis, cytochrome P-450IA1 and the Ah receptor are very interesting for at least two other reasons. (i) They provide a good model for studying gene regulation in mammalian cells, since P-450IA1 is highly inducible, and is expressed to widely different degrees in different tissues and at different times in development. (ii) The Ah receptor appears to be involved in the regulation of cell division and cell differentiation in certain tissues (49).

Occupational and environmental exposures to arylamine chemicals are a major factor in the etiology of human bladder cancer. The chemicals are not carcinogenic until they undergo metabolic activation by host enzymes to highly reactive intermediates. One hypothesis for the metabolic activation of arylamine chemicals is that oxidation and glucuronidation take place in the liver, followed by transport of N-hydroxy-N-glucuronide conjugates through the kidney and into the bladder lumen. In the acidic environment of the urinary bladder, the conjugate may undergo hydrolysis and protonation to a highly reactive arylnitrenium ion intermediate capable of forming arylamine-DNA adducts. This model suggests a relatively passive role for the bladder epithelium in the metabolism of arylamine carcinogens. Alternatively, the bladder epithelium may play a very active role in the metabolic activation and deactivation of arylamine carcinogens. Many studies have shown that bladder cells and/or tissue from several mammalian species exhibit the metabolic capacity to activate arylamine carcinogens to genotoxic products. Of particular interest is the description of acetyltransferase activity in bladder epithelium, because the genetic regulation of bladder acetyltransferase(s) may be important with respect to the incidence of arylamine-induced bladder cancer. Acetyltransferase enzymes expressed in hepatic and extrahepatic tissues are products of an acetyltransferase gene locus. Acetylation capacity is regulated by simple autosomal Mendelian inheritance of two codominant alleles at this locus. Human slow-acetylators are predisposed to bladder cancer from arylamine chemicals. The role of the bladder in arylamine metabolism and of bladder acetyltransferases in the etiology of bladder cancer is not fully understood, but the acetylator genotype-dependent expression of arylamine N-acetyltransferase and N-hydroxyarylamine O-acetyltransferase in bladder cytosol may contribute towards the genetic predisposition of human slow-acetylators to arylamine-induced bladder cancer (52).

Development of In Vitro Systems: Several investigators have found that cells transfected with oncogenes exhibit an in vitro phenotype characteristic of malignancy. Results reported by one investigator suggested that a single genetic change, imposed on rat mammary tumor cells by transfection, was able to convert growth-factor-dependent cells to growth factor autonomy. These cells when transfected with c-Ha-ras gene were able to grow in the absence of epidermal growth factor (EGF) and insulin. This independence from growth factors in serum-free culture correlated with a highly malignant phenotype in vivo (28). Similar results were obtained by another investigator with a spontaneously immortalized human mammary epithelial cell line which was phenotypically normal for almost three years, but when transfected with c-Ha-ras oncogene it exhibited phenotypic changes of malignancy (126). Elsewhere it was found that, unlike other rodent mammary carcinogenesis systems where the activation of H-ras oncogene is frequently observed, K-ras oncogene seems to play an important role in an in vitro

N-methyl-N-nitrosourea (MNU)-induced mouse mammary transformation system. Results suggested that the activated K-ras oncogene may be involved in preneoplastic hyperplastic nodulogenesis and can be maintained during progression to mammary carcinomas (106). A K-ras oncogene activation was also reported in the Wistar rat prostatic carcinoma induced by MNU (8).

A valuable tool to study normal mammary gland development and tumorigenesis in transgenic animals and to study the role of oncogene expression in mammary tumorigenesis has been developed. A MERIT awardee established lines of transgenic mice carrying an entire rat beta-casein genomic DNA fragment. Explant cultures derived from these mice were grown in serum-free medium to study how the level and timing of neu expression affects normal mammary development and carcinogenesis. The neu (c-erb-2) oncogene has been implicated in the development of human breast cancer (125).

Studies on development and progression of bladder cancer in vitro yielded significant results. The first in vitro conversion of uroepithelial cells to malignancy by aromatic amines was achieved. A multistep in vitro transformation system has been developed which provided an opportunity to study mechanisms of arylamine transformation of human uroepithelial cells (HUC) (122).

Hormones: Hormones are associated with several cancers in humans and are known to induce tumors in experimental animals. In search of methods to study estrogen carcinogenesis in vitro, it was discovered that estrogen induces proliferation of cultured hamster renal proximal tubular cells. Results demonstrated that there is a strong correlation between estrogen-induced proliferation of the epithelium in culture to in vivo estrogen carcinogenicity. Estrogens that exhibited high or moderate ability to affect renal tubular proliferation in culture showed appreciable carcinogenic activity at this organ site. In contrast, the estrogens that had minimal ability to cause tubular cell proliferation in culture, exhibited little or no carcinogenic activity in the hamster kidney (75).

A group of investigators have been studying which factor(s) is responsible for modulation of the susceptibility of epithelial cells to malignant transformation. Simultaneous treatment of ethinylestradiol (EE), with 17-beta estradiol (E₂), diethylstilbestrol (DES) or Moxestrol (MOX) completely prevented the induction of renal tumors in the hamster. The inhibition of estrogen carcinogenesis by EE was not reversed by continued treatment of these carcinogenic estrogens. The prevention of carcinogenesis by concomitant EE treatment did not appear to involve a hormonal mechanism (76). In another experimental system, hormones were of importance in prevention of cancer. Results showed that the incidence of mammary carcinomas induced in Sprague Dawley rats by 7,12-DMBA was modulated by variations in the number of terminal end buds (TEBs). Both estrogenic-progestagenic combination and chorionic gonadotropin hormone treatment exerted a powerful differentiating and protective effect from neoplastic transformation, whereas the prostatic agent medroxyprogesterone acetate tended to induce no protection or to increase mammary cancer risk (126). Another investigator, who studied the prevention of estrogen-induced tumors by chemical means, reported that 4-hydroxy-catechol-estrogens (4-OH-CE) may play a special role in estrogen-induced carcinogenesis in hamster kidney where their detoxification by O-methylation is inhibited by 2-HO-CEs and where they can, therefore, exert their effects as potent estrogens and serve as substrates for redox cycling (77).

Primary liver cancer occurs more frequently in men than in women. A liver cancer rat model which exhibited a much faster outgrowth of preneoplastic foci and an earlier development of hepatocellular carcinomas in males than in females has been developed. This sexual dimorphism seems to be mediated by the sex-differentiated secretion pattern of growth hormone (GH), which in turn is regulated by the pituitary. The study of the influence of GH on the expression of growth-regulated genes during early stages of carcinogenesis offers the possibility to pinpoint the mechanisms of regulatory alterations during carcinogenesis. The expression of both *c-myc* and *c-fos* oncogenes was increased in preneoplastic and neoplastic stages in males. The *c-fos* expression was short lived, but the *c-myc* expression remained elevated for several weeks. This observation strengthens the view that the *c-myc* gene is one of the important genetic targets in rat liver carcinogenesis (46).

Adenocarcinoma of the prostate is almost exclusively a disease of older men. The Noble rat was chosen to acquire a comprehensive understanding of the role sex hormones may play in human prostatic carcinogenesis. Results in this laboratory are the first to demonstrate an elevation in prostatic nuclear type II estrogen binding sites that are associated with the presence of a proliferative lesion in the prostate gland. Simultaneous implantation of these rats with testosterone and estradiol (E_2) caused atypical hyperplasia (dysplasia) and enlargement exclusively in the dorsolateral prostates of all animals. Treatment of rats with testosterone caused enlargement but not dysplasia, implicating estrogen as a key factor in the genesis of the proliferative lesion (71). The Wistar rat is another potential animal model of human prostatic carcinogenesis. This rat model has a number of important characteristics in common with human prostatic carcinomas: the rats develop slow-growing adenocarcinomas that metastasize, elevate plasma acid phosphatase, require testosterone (T) to develop, can cause urinary obstruction, and show great diversity in degree of histologic differentiation. Adenocarcinomas of the dorsolateral prostate have been induced at low incidence by a single injection of MNU, but only if MNU was administered when prostatic cell proliferation was stimulated. The incidence of these carcinomas was markedly increased by long-term post-initiation treatment with T at low, near physiological doses. Since only a single carcinogen injection is needed in this model to develop adenocarcinomas and not repeated administration, it provides excellent opportunities for studying the promotion stage of prostatic carcinogenesis and its modification by environmental factors (8).

Other Agents: A method known as the ^{32}P -postlabeling technique, which has been developed for the detection of the in vivo formation of carcinogen-DNA adducts where the use of radiolabeled test compounds is not required, has come into increasingly widespread use as the technique has become more refined and standardized. The method involves the reaction of DNA with chemicals in vitro or in vivo and the purification and enzymatic digestion of DNA to deoxyribonucleoside 3'-monophosphates. These are converted to ^{32}P -labeled deoxyribonucleoside 3',5'-bisphosphates after incubation with (γ - ^{32}P) adenosine triphosphate (ATP) and T4 polynucleotide kinase. The ^{32}P -labeled digests are then fingerprinted by using reversed-phase liquid chromatography and anion-exchange thin-layer chromatography on polyethyleneimine-cellulose followed by detection by autoradiography and quantitation by scintillation counting. One refinement to the procedure involves the chromatographic removal of normal DNA nucleotides prior to ^{32}P -labeling. A further minor modification of this procedure has been recently reported which entails the postincubation of DNA digests with *Penicillium citrinum* nuclease P1 before ^{32}P -labeling. Nuclease P1 was found to cleave deoxyribonucleoside

3'-monophosphates of normal nucleotides to deoxyribonucleosides which do not serve as substrates for polynucleotide kinase, while most adducted nucleotides were found to be totally or partially resistant to the 3'-dephosphorylating action of nuclease P1. These refinements and modifications have enhanced the technique's sensitivity of adduct detection to about one adduct in 10^{10} nucleotides for a 10 microgram DNA sample. The new procedure was found to be simple, highly reproducible, and applicable to the detection and measurement of aromatic or bulky non-aromatic DNA adducts formed with many structurally diverse carcinogens. This method has been applied to answer questions about the tissue specificity of carcinogen adduct formation, the degree of persistence of DNA adducts in cells, and to detect adducts in cells of humans exposed to carcinogenic chemicals.

I-compounds are non-polar covalent DNA modifications of as yet undetermined structure that tend to accumulate in an age-dependent manner in tissues of untreated animals. They are detectable by ^{32}P -postlabeling assay because of their adduct-like properties and chromatographically resemble DNA nucleotides containing bulky/hydrophobic moieties. To determine which factors may be involved in their formation, I-compounds were examined by ^{32}P -postlabeling in liver and kidney DNA of female and male Sprague-Dawley rats and Syrian hamsters of different ages (1,4 and 10 months and 1,2.5 and 9.5 months, respectively). The following results were obtained: (i) Every tissue DNA studied contained characteristic I-compounds. (ii) Patterns and amounts of I-compounds were reproducible among animals of the same kind. (iii) There were pronounced organ and species differences. (iv) I-compound patterns were sex-dependent. (v) I-compound levels increased with age in all tissues studied, except in male hamster kidney, a target organ of estrogen-induced carcinogenesis. The highest levels were observed in liver and kidney of 10-month-old female rats. (vi) The rise of I-compound levels was less steep during the latter part of the observation period for female but not male animals. (vii) Gonadectomy decreased I-compound levels in female hamster kidney DNA, while causing a slight increase in male animals later in life. These I-compounds were identical to previously reported DNA modifications that increased in male hamster kidneys after prolonged estrogen treatment. Points, iv, vi and vii strongly implicate sex hormones in I-compound formation. The qualitative effects of species, tissue differentiation, gender, and sex hormones on these DNA modifications support the hypothesis that I-compounds are formed by the binding of endogenous electrophiles to DNA. As persistent DNA alterations, they are likely to affect DNA replication and to play a role in spontaneous and chemically-induced carcinogenesis and in aging (119).

Exposure to certain arylamines has been associated with a high incidence of human bladder tumors. These agents produce tumors of the bladder as well as other organs in various experimental animals. The organ-specificity is thought to be related in part to the distribution of a variety of xenobiotic-metabolizing enzymes in certain organs but not in the others. These include enzymes that are capable of conjugation of sulfate with N-arylhydroxamic acids and arylhydroxylamines, N-deacetylation of N-arylhydroxamic acids and the generation of O-acetylhydroxylamines by either intramolecular N,O-acetyltransfer of N-arylhydroxamic acids or direct O-acetylation of hydroxylamines.

Unscheduled DNA synthesis (UDS)-inducing activity was used as a parameter to estimate the abilities of rat mammary epithelial cells and urothelial cells from various species to activate carcinogenic aromatic amine derivatives. The N-hydroxy, N-hydroxy-N-acetyl, N-hydroxy-N-glucuronosyl derivatives of 2-aminofluorene (2-AF) and 4-aminobiphenyl (4-ABP) induced UDS in primary cultures of rat mammary

epithelial cells, but 2-AF, the O-glucuronide of N-hydroxy-N-acetyl-2-AF(N-OH-AAF) and 4-ABP did not. Neither the activity of N-OH-AAF, N-hydroxy-N-formyl-2-AF, nor N-acetoxy-N-acetyl-2-AF was significantly altered by paraoxon, an inhibitor of microsomal N-deacetylase. Although N-hydroxy-3,2'-dimethyl-4-aminobiphenyl (N-OH-DMABP) also induced UDS, its N-acetyl derivative, which can not be activated by intramolecular N,O-acetyltransfer, did not. Similarly, rat urothelial cells were responsive to the UDS-inducing activity of this hydroxylamine, but not the hydroxamic acid. In contrast, dog urothelial cells were responsive to both compounds. The UDS-inducing activity of N-OH-AAF was inhibited by paraoxon in the dog, but not in rat urothelial cells. N-Hydroxy-N,N'-diacetylbenzidine induced UDS in the urothelial cells of dog, rat, and rabbit, and a human urothelial cell line, HCV-29, whereas benzidine, N-acetylbenzidine, and N,N'-diacetylbenzidine did not. Co-treatment with 12-O-tetradecanoylphorbol-13-acetate did not enable benzidine to induce UDS in dog urothelial cells. Rat mammary epithelial cells activated N-OH-DMABP by acetyl coenzyme A-dependent O-acetylation and N-OH-AAF by N,O-acetyltransfer. They could not N-deacetylate N-OH-AAF. These results suggest that rat mammary and bladder epithelial cells are capable of activating N-arylhydroxylamine metabolites of these carcinogens, probably by N,O-acetyltransfer and O-acetylation, whereas dog urothelial cells are more likely to activate these metabolites by N-deacetylation and a reaction that has yet to be identified (65).

1,3-Indandione (IDD) which can be ionized to an enolate or a carbanion may be a trapping agent of ultimate carcinogens and mutagens. This hypothesis was tested in the Ames system using the direct-acting mutagens, methylnitrosourea and 2-nitrofluorene, and the indirect-acting mutagens, aflatoxin B₁ and B(a)P, that require activation by rat liver S-9. IDD, 10 μmol/plate, inhibited the mutagenicity of 2-nitrofluorene and methylnitrosourea by 90%. However, it did not inhibit the mutagenicity of the latter when it was added one hour after the addition of the mutagen. At 20 μmol per plate, IDD completely inhibited the mutagenicities of aflatoxin B₁ and B(a)P. When tested in an in vitro system employing the binding of these carcinogens to tRNA, 0.5 mM IDD inhibited rat liver microsome-catalyzed binding of aflatoxin B₁ to tRNA by 50%, and cytosol-catalyzed binding of N-hydroxy-2-acetylaminofluorene to tRNA by 90%. However, its effect on the binding of B(a)P to tRNA catalyzed by microsomes was not as remarkable. Reaction of IDD with an activated 2-aminofluorene (2-AF) yielded a compound which, upon hydrolysis, produced IDD, and 1-hydroxy- and 3-hydroxy-2-aminofluorene. These data demonstrate that IDD can trap ultimate carcinogens, and suggest that IDD may possess anticarcinogenic activity (152).

N-Arylhydroxamic acids are mammary gland (MG) carcinogens in the rat upon systemic administration or direct application to the glands. These compounds are also locally carcinogenic at sites of injections. The tumorigenic responses and histology of tumors suggest that N-arylhydroxamic acids are metabolically activated within the epithelium of MG and tissues of mesenchymal origin (MG stroma, subcutaneous and muscle tissue, serosa of intraperitoneal cavity). These sites may contain intracellular peroxidases, e.g., prostaglandin H synthase (PHS) or peroxidases of leukocyte origin (myeloperoxidase, eosinophil peroxidase, PHS). The hydroxamic acids, N-hydroxy-N-2-fluorenylacetylacetamide (N-OH-2-FAA) and N-hydroxy-N-2-fluorenylbenzamide (N-OH-2-FBA) are metabolized by mammalian peroxidases/H₂O₂ via either one electron (1e⁻) oxidation to nitroxyl free radical which dismutates to the ester of the hydroxamic acid (N-AcO-2-FAA or N-Bzo-2-FBA, respectively) and 2-nitrosofluorene (2-NOF) or Br⁻-dependent oxidative cleavage to 2-NOF. Thus, the relative extents of the two pathways are determined from the ratios of 2-NOF: ester. Peroxidative activity extracted from rat uterus (UT) (chiefly of

eosinophil origin) oxidized N-OH-2-FAA via both pathways in 0.004% cetyltrimethylammonium Br⁻ (Cetab) which approximated physiologic levels of Br⁻ (-0.1 mM), whereas at 0.4% Cetab the Br⁻-dependent pathway was greatly enhanced and le⁻ oxidation was negligible. At both concentrations of Cetab, extracts of MG, which had a much lower specific activity, yielded 2-NOF and no N-AcO-2-FAA indicating Br⁻-dependent oxidative cleavage and lack of le⁻ oxidation. PHS, which may contribute to peroxidative activity in these and other tissues, metabolized N-OH-2-FAA via le⁻ oxidation in the presence of H₂O₂; in the presence of arachidonic acid, 2-nitrofluorene was the major metabolite possibly arising from oxidation of 2-NOF. Since the leukocytes may be a chief source of peroxidative activities at the site of i.p. injections, metabolism of N-OH-2-FAA and N-OH-2-FBA was also examined by eosinophil-rich cell suspensions from rat i.p. fluid. Like UT extracts, cells suspended in 0.4% Cetab metabolized the hydroxamic acids largely to 2-NOF, indicating Br⁻-dependent oxidative cleavage as a predominant peroxidative pathway. The results suggest that N-arylhydroxamic acids may be activated in vivo by peroxidative metabolism. In tissues of mesenchymal origin infiltrated by leukocytes, 2-NOF would most likely be a major product. The mechanism(s) by which 2-NOF and other nitrosoaromatics are mutagenic and carcinogenic has not yet been elucidated, although their reduction to the hydroxylamines yielding nitrenium ions is one possibility (87).

Under physiological conditions the concentration of plasma heme is very low, but large amounts of circulating heme are found in acute and chronic states of hemolysis or during heme therapy for porphyria. It is generally assumed that plasma heme is transported to hepatocytes, where it is catabolized to bilirubin. However, not all heme that enters the hepatocyte is immediately catabolized. Some heme is incorporated into microsomal cytochromes P-450 and other intracellular proteins. Exogenous heme also has several metabolic effects. Heme causes accumulation of cytosolic delta-aminolaevulinate synthase (ALA-S) and decreases the mRNA for ALA-S. Thus, heme taken up by hepatocytes can enter at least three cellular compartments, i.e., those affecting feedback inhibition on ALA-S, incorporation into cytochromes P-450 and heme degradation.

Several mechanisms of heme uptake by cells have been proposed, some of which appear to be carrier-protein-mediated, and others of which are not dependent on a specific circulating carrier protein. Experiments in vivo with rats and in vitro with freshly isolated rat liver cells indicated that hemopexin, a serum protein which binds heme at an equimolar ratio and which possess an exceptionally high affinity for heme, has a role in hepatic heme uptake. Results of experiments performed in vivo in other species and in patients with elevated plasma heme concentrations owing to hemolysis or heme therapy for porphyrias were considered to be consistent with this hypothesis.

Whether a major portion of heme is delivered to liver cells by a hemopexin-mediated process under physiological conditions, however, remains unclear. One grant has examined this question in primary cultures of chick-embryo and adult rat liver cells (28). The following are the results of four sets of experiments which indicate that hemopexin retarded heme uptake by hepatocytes in culture. 1) Heme bound to bovine serum albumin is known to repress the activity of 8-aminolaevulinate synthase in chick cultures as indicated by decreased porphyrin accumulation. When heme-albumin was added in the presence of excess purified or freshly secreted chicken hemopexin, no heme-mediated repression of porphyrin production was observed. The heme-mediated repression of porphyrin accumulation was partially prevented when human, but not chicken, albumin was added to cultures. This

finding reflected the higher affinity of human albumin for heme compared with that of chicken albumin. 2) Hemopexin inhibited the ability of heme to be incorporated into cytochrome P-450 induced in the chick cultures in the presence of the iron chelator desferrioxamine. 3) The rate of association of [^{55}Fe]heme with cultured rat hepatocytes when [^{55}Fe]heme-hemopexin was added was one-eighth of the rate observed when [^{55}Fe]heme-bovine serum albumin was used as the heme donor. 4) The presence of hemopexin also diminished the catabolism of heme by both rat and chick-embryo liver cell cultures. It is concluded that the uptake and subsequent metabolic effects of heme are inhibited in cultured hepatocytes by proteins such as hemopexin which have a high affinity for heme (132).

Aflatoxin B₁ (AFB₁) is one of the most potent hepatocarcinogens known, and environmental contamination by various aflatoxins is a serious problem in many parts of the world. The prototype AFB₁ is not particularly biologically active in its native form, and oxidation is necessary for interaction with DNA and biological damage. With regard to the specific forms of cytochrome P-450 involved in AFB₁ activation, there is ambiguity in assignment of the involved rat liver P-450s in the literature, and these studies do not point clearly to human orthologs. If the form of human P-450 involved in aflatoxin bioactivation could be identified and appropriate *in vivo* assays could be devised for populations in which aflatoxin exposure is high, then the general hypothesis concerning cancer risk might be tested.

In vitro studies with human liver indicate that the major catalyst involved in the bioactivation of the hepatocarcinogen aflatoxin B₁ (AFB₁) to its genotoxic 2,3-epoxide derivative is cytochrome P-450_{NF}, a protein that also catalyzes the oxidation of nifedipine and other dihydropyridines, quinidine, macrolide antibiotics, various steroids and other compounds. Evidence was obtained using activation of AFB₁ as monitored by umuC gene expression response in Salmonella typhimurium TA1535/pSK1002 and enzyme reconstitution, immunochemical inhibition, correlation of response with levels of P-450_{NF} and nifedipine oxidase activity in different liver samples, stimulation of activity by 7,8-benzoflavone and inhibition of activity by troleandomycin. Similar results were obtained when levels of 2,3-dihydro-2-(N'-guanyl)-3-hydroxyaflatoxin B₁ formed in DNA were measured. P-450_{NF} or a closely related protein also appears to be the major catalyst involved in the activation of aflatoxin G₁ and sterigmatocystin, the latter compound being more genotoxic than AFB₁ in these systems. Several drugs and conditions are known to influence the levels and activity of P-450_{NF} in human liver, and the activity of the enzyme can be estimated by noninvasive assays. These findings provide a test system for the hypothesis that a specific human disease state (liver cancer) is linked to the level of oxidative metabolism in populations in which aflatoxin ingestion is high (41).

CARCINOGENESIS MECHANISMS

GRANTS ACTIVE DURING FY89

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ABUL-HAJJ, Yusuf J. University of Minnesota of Mnpls-St Paul 5 R01 CA41269-02	Macromolecular Binding of Estrogens and Carcinogenesis
2. ALBERTINI, Richard J. University of Vermont & St Agric College 5 R01 CA30688-06	Direct Mutagenicity Testing in Man
3. ALLFREY, Vincent G. Rockefeller University 5 R01 CA14908-15	Nuclear Proteins in Carcino- genesis of the Colon
4. ARCHER, Michael C. Ontario Cancer Institute 5 R01 CA26651-09	Mechanism of Nitrosamine Alkylation of DNA and RNA
5. BAIRD, William M. Purdue University West Lafayette 5 R37 CA28825-09	Modifiers of Chemical Carcino- genesis in Cell Culture
6. BEATTIE, Craig W. University of Illinois at Chicago 5 R01 CA45355-02	Estrogen and NMU Synergism in Carcinogenesis
7. BARTSCH, Helmut Intl Agency for Res on Cancer 1 R01 CA47591-01A1	Bacteria, DNA Damage in Stomach and Bladder Cancers
8. BOSLAND, Maarten C. New York University 5 R01 CA43151-03	Chemical Induction of Prostatic Adenocarcinomas
9. BRASITUS, Thomas A. Michael Reese Hosp & Med Ctr (Chicago) 5 R37 CA36745-06	Colonic Epithelial Cell Plasma Membranes
10. BRESNICK, Edward University of Nebraska Medical Center 5 R01 CA36106-06	Polycyclic Hydrocarbon Metabolism and Carcinogenesis
11. BRESNICK, Edward University of Nebraska Medical Center 1 R13 CA47350-01	Biology and Chemistry--Nitroso Related Compounds
12. BUHLER, Donald R. Oregon State University 5 R01 CA22524-10	Pyrrolizidine Alkaloid Toxicity, Metabolism and Binding

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| 13. | CARR, Brian I.
City of Hope National Medical Center
1 R29 CA44602-01A1 | TGF-B Receptors in Hepato-
carcinogenesis |
| 14. | CAVALIERI, Ercole L.
University of Nebraska Medical Center
1 R01 CA44686-01A1 | Radical Cations of PAH in
Carcinogenesis and Metabolism |
| 15. | CAVALIERI, Ercole L.
University of Nebraska Medical Center
1 P01 CA49210-01 | Mechanisms of 7,12-dimethyl-
benzanthracene Carcinogenesis |
| 16. | CHANG, Chia-Cheng
Michigan State University
1 R01 CA50430-01 | Transformation of Human Breast
Epithelial Cells In Vitro |
| 17. | COLBY, Howard D.
Philadelphia College of Pharmacy-Science
7 R01 CA43604-03 | Adrenal Carcinogen Metabolism |
| 18. | CUCHENS, Marvin A.
University of Mississippi Medical Center
2 R01 CA33111-04A1 | Carcinogenesis of B-Lymphocytes
in Rat Peyer's Patches |
| 19. | CURPHEY, Thomas J.
Dartmouth College
5 R01 CA30650-06 | Pancreas and Liver Carcinogen
Metabolism in Two Species |
| 20. | DETRISAC, Carol J.
IIT Research Institute
5 R29 CA40501-04 | Transitional Cell Carcinoma:
In Vitro/In Vivo Correlates |
| 21. | DIGIOVANNI, John
University of Texas System Cancer Center
5 R01 CA36979-05 | Role of DNA-Binding in Skin
Tumor Initiation |
| 22. | DUFFEL, Michael W.
University of Iowa
5 R01 CA38683-05 | Aryl Sulfotransferase in Drug
and Xenobiotic Metabolism |
| 23. | EATON, David L.
University of Washington
1 R01 CA47561-01 | Species Differences in
Glutathione-S-Transferase |
| 24. | EL-BAYOUMY, Karam E.
American Health Foundation
5 R01 CA35519-05 | Nitroaromatics--Carcinogenicity
and Metabolism |
| 25. | ELMETS, Craig A.
Case Western Reserve University
1 R01 CA48763-01 | Skin Cancer: Immunotoxic
Mechanisms |

26. ELSTON, Ralph A.
Battelle Pacific Northwest Division
5 R01 CA44269-03
Biology of Hemic Neoplasia in
the Marine Mussell
27. ENSLEIN, Kurt
Health Designs, Inc.
5 R44 CA37494-03
SAR Estimation of Carcinogenesis
Bioassay Results
28. ETHIER, Stephen P.
University of Michigan at Ann Arbor
2 R01 CA40064-04A1
Growth Factor Independence in
Mammary Neoplasia
29. FAGAN, John B.
Maharishi International University
5 R01 CA38655-03
Cytochrome P-450 Gene Structure
and Regulation
30. FIALA, Emerich S.
American Health Foundation
2 R01 CA31012-07
Disposition of Hydrazines--
Species and Strain Effects
31. FLESHER, James W.
University of Kentucky
5 R01 CA45823-02
Bioalkylation in Chemical
Carcinogenesis
32. FLOSS, Heinz G.
Ohio State University
5 R01 CA37661-03
Biochemical Mechanisms of
Nitrosamine Carcinogenesis
33. GLUSKER, Jenny P.
Institute for Cancer Research
5 R01 CA10925-39
Application of Crystallographic
Techniques
34. GOLD, Avram
University of North Carolina, Chapel Hill
1 R01 CA47965-01
Pathways of Activation and DNA
Adducts of Cyclopenta PAH
35. GOLD, Barry I.
University of Nebraska Medical Center
5 R01 CA29088-05
Activation and Transportation
of Nitrosamines
36. GOLDMAN, Peter
Harvard University
5 R01 CA34957-06
Carcinogen Metabolism By Host
Intestinal Bacteria
37. GOULD, Michael N.
University of Wisconsin, Madison
5 R01 CA30295-08
Mammary Carcinogenesis--
Interspecies Comparisons
38. GREENBERGER, Joel S.
University of Massachusetts Medical Sch
2 R37 CA39851-05
Stem Cell Age and X-Ray
Chemotherapy Leukemogenesis

39. GRISHAM, Joe W.
University of North Carolina, Chapel Hill
5 R01 CA29323-08
Clonal Analysis of Carcinogenesis In Vitro
40. GROVER, Philip L.
University of London
5 R01 CA21959-12
Mechanisms of Activation of Polycyclic Hydrocarbons
41. GUENGERICH, F. Peter
Vanderbilt University
5 R35 CA44353-02
Enzymic Activation of Chemical Carcinogens
42. GUENGERICH, F. Peter
Vanderbilt University
5 R01 CA30907-07
Purified Human Enzymes and Carcinogen Metabolism
43. GUENTHNER, Thomas M.
University of Illinois at Chicago
5 R01 CA34455-06
Toxicologic Implications of Multiple Epoxide Hydrolases
44. GUENTHNER, Thomas M.
University of Illinois at Chicago
1 R01 CA46129-01
Xenobiotic-Metabolizing Enzymes in Human Lung
45. GURTOO, Hira L.
Roswell Park Memorial Institute
5 R01 CA25362-08
Genetics of Aflatoxin Metabolism Role in Carcinogenesis
46. GUSTAFSSON, Jan-Ake
Caroline Institute
5 R01 CA40037-03
Binding and Metabolism of Toxic Agents in the Prostate
47. GUSTAFSSON, Jan-Ake
Caroline Institute
5 R01 CA42054-02
Hormonal Regulation of Liver Carcinogenesis
48. HAM, Richard G.
University of Colorado at Boulder
2 R01 CA30028-07A1
Human Mammary Cell Growth and Function in Defined Media
49. HANKINSON, Oliver
University of California, Los Angeles
5 R01 CA28868-09
Carcinogen Activation and Screening in Variant Cells
50. HARVEY, Ronald G.
University of Chicago
5 R01 CA36097-05
Mechanism of Carcinogenesis of Polycyclic Hydrocarbons
51. HECHT, Stephen S.
American Health Foundation
5 R35 CA44377-02
Metabolic Activation of Carcinogens

52. HEIN, David W.
Morehouse School of Medicine
5 R01 CA34627-05
Pharmacogenetics of Drug and
Carcinogen Metabolism
53. HENDRICKS, Jerry D.
Oregon State University
5 R01 CA44317-03
Neoplasia--Tumor and Mechanism
Studies
54. HINTON, David E.
University of California, Davis
5 R01 CA45131-03
Cell and Matrix Biology in
Fish Liver Carcinogenesis
55. HIXSON, Douglas C.
Rhode Island Hospital (Providence, RI)
2 R01 CA42715-03
Cellular Origins of Liver
Cancer
56. HIXSON, Douglas C.
Rhode Island Hospital (Providence, RI)
5 R01 CA42716-04
Membrane Glycoproteins During
Hepatocarcinogenesis
57. HOLLENBERG, Paul F.
Wayne State University
2 R37 CA16954-13
Hemoprotein-Catalyzed
Oxygenations of Carcinogens
58. HUNT, John M., Jr.
University of Texas Hlth Sci Ctr, Houston
5 R01 CA37150-05
Alloantigens as Probes for
Hepatocarcinogenesis
59. IVERSEN, Patrick L.
University of Nebraska Medical Center
1 R01 CA49135-01
Gene Specific Inhibition of
Cytochrome P-450 Isoforms
60. JAMES, Margaret O.
University of Florida
5 R01 CA44297-03
Carcinogen Biotransformation by
Aquatic Invertebrates
61. JEFCOATE, Colin R.
University of Wisconsin, Madison
5 R01 CA16265-14
Metabolism of Polycyclic
Hydrocarbons and Carcinogenesis
62. JENSEN, David E.
Temple University
5 R01 CA31503-07
N-Nitroso Compound
Detoxification
63. JIRTLE, Randy L.
Duke University
5 R01 CA25951-08
Survival and Carcinogenesis in
Transplanted Hepatocytes
64. KAUFFMAN, Frederick C
University of Maryland Balt Prof School
2 R01 CA20807-09A2
Pharmacology of Carcinogen
Activation in Intact Cells

65. KING, Charles M. Michigan Cancer Foundation
5 R01 CA23386-11 Mechanistic Approaches to Carcinogenesis
66. KLEIN-SZANTO, Andres J. Fox Chase Cancer Center
2 R01 CA44981-02 Carcinogenesis of Xeno-Transplanted Human Epithelia
67. KOKKINAKIS, Demetri M. Northwestern University
1 R01 CA42983-01A1 DNA Damage Induced by Pancreatropic Nitrosamines
68. KOREEDA, Masato University of Michigan at Ann Arbor
2 R01 CA25185-10 Synthesis and Properties of Arene Oxides and Analogs
69. KRAUTER, Kenneth S. Yeshiva University
2 R01 CA39553-04 Induction of Gene Expression by Chemical Carcinogens
70. LAWSON, Terence A. University of Nebraska Medical Center
1 R01 CA43646-01A2 Alkylation and Mutagenesis by Pancreas Carcinogens
71. LEAV, Irwin Tufts University
5 R01 CA15776-11 Prostatic Differentiation and Sex Hormone Metabolism
72. LEE, Mei-Sie Michigan Cancer Foundation
2 R01 CA37885-04 Metabolic Activation of Unsubstituted Hydroxamic Acid
73. LEHR, Roland E. University of Oklahoma, Norman
5 R01 CA22985-12 Diol Epoxide and Other Derivatives of PAH and Aza-PAH
74. LEVINE, Walter G. Yeshiva University
5 R01 CA14231-15 Role of Metabolism in the Biliary Excretion of Drugs
75. LI, Jonathan J. University of Minnesota of Mnpls-St Paul
2 R01 CA22008-10A1 Estrogen Carcinogenicity and Hormone-Dependent Tumors
76. LI, Jonathan J. University of Minnesota of Mnpls-St Paul
5 R01 CA41387-03 Sex Hormones and Hepatocellular Carcinomas
77. LIEHR, Joachim G. University of Texas Medical Branch
5 R01 CA43232-03 Prevention of Estrogen-Induced Tumors by Chemical Means

78. LILLY, Frank
Yeshiva University
5 P01 CA31855-07
Mechanisms of Chemical
Lymphomagenesis
79. LINDAHL, Ronald G.
University of Alabama in Tuscaloosa
5 R01 CA21103-09
Gene-Enzyme Correlates of
Liver Aldehyde Dehydrogenases
80. LISTOWSKY, Irving
Yeshiva University
5 R01 CA42448-03
High Affinity, High Capacity
Steroid and Carcinogen
81. LOEPPKY, Richard N.
University of Missouri, Columbia
5 R37 CA26914-08
Carcinogenesis: Nitrosamine
Formation and Inhibition
82. LONGNECKER, Daniel S.
Dartmouth College
1 R01 CA47327-01
Transgenic Mouse Models of
Pancreatic Carcinogenesis
83. LOTLIKAR, Prabhakar D.
Temple University
5 R01 CA31641-06
Modulation of Mycotoxin
Carcinogenesis By Glutathione
84. LU, Lee-Jane W.
University of Texas Medical Branch
5 R01 CA44163-03
DNA Damage during Pregnancy
85. LUDLUM, David B
University of Massachusetts Medical Sch
1 R01 CA47103-01
The Role of DNA Adducts in
Ethyl Eneoxide Exposure
86. MAGEE, Peter N.
Temple University
5 R37 CA43342-02
Formation and Metabolism of
Nitrosamines
87. MALEJKA-GIGANTI, Danuta
University of Minnesota of Mnpls-St Paul
2 R37 CA28000-09
Mammary Carcinogenesis by
N-Substituted Aryl Compounds
88. MANGOLD, Bonnie L. K.
University of Connecticut, Storrs
1 R29 CA48972-01
Sulfotransferase Inactivation
by Carcinogens
89. MARCHOK, Ann C.
Oak Ridge National Laboratory
2 R01 CA42798-03
Preneoplastic Markers in
Specific Lesion Cell Populations
90. MARLETTA, Michael A.
University of Michigan at Ann Arbor
7 R01 CA37770-03
Properties of a Carcinogen
Binding Receptor-Like Protein

91. MC CORMICK, J. Justin
Michigan State University
5 R01 CA21289-11
In Vitro Transformation of
Human Cells
92. MC ELROY, Anne E.
University of Massachusetts, Amherst
5 R01 CA44289-03
Trophic Transfer of PAH and
Metabolites
93. MEDINA, Daniel
Baylor College of Medicine
1 R01 CA47112-01
Early Events in Chemical Car-
cinogen Induced Mammary Tumors
94. MEHTA, Rajendra G.
IIT Research Institute
1 R01 CA47329-01
Nutrients and Growth Modifier
Interaction--Mammary Cancer
95. MELIKIAN, Assieh
American Health Foundation
5 R01 CA43910-02
Mechanism of Catechol Co-
carcinogenesis With B(a)P
96. MICHALOPOULOS, George K.
Duke University
5 R01 CA30241-08
Cell Culture and Transplantation
of Human Hepatocytes
97. MICHALOPOULOS, George K.
Duke University
5 R01 CA43632-02
A1 Adrenoreceptor, Liver
Carcinogenesis and Regeneration
98. MILO, George E.
Ohio State University
2 R01 CA25907-07A1
Neoplastic Transformation of
Human Epithelial Cells
99. MIRVISH, Sidney S.
University of Nebraska Medical Center
5 R01 CA35628-05
Nitrosamine Metabolism in the
Esophagus
100. MORIZOT, Donald C.
University of Texas System Cancer Center
5 R01 CA39729-03
Multistep Chemical Initiation
in Genetic Melanoma Models
101. MORRIS, Rebecca J.
University of Texas System Cancer Center
5 R29 CA45293-02
Epidermal Stem Cells in Two-
Stage Carcinogenesis
102. MORRISON, Harry A.
Purdue University, West Lafayette
5 R01 CA18267-10
Cutaneous Photobiology
103. NAGEL, Donald L.
University of Nebraska Medical Center
5 R01 CA31016-06
An In Vitro Model for Alkylation
by Pancreas Carcinogens

104. NAIRN, Rodney S.
University of Texas System Cancer Center
5 R01 CA44303-03
Genetic Determinants of
Carcinogenesis
105. NANDI, Satyabrata
University of California, Berkeley
5 P01 CA05388-29
Biology of Normal and Tumor
Mammary and Genital Epithelia
106. NANDI, Satyabrata
University of California, Berkeley
1 R01 CA49374-01
Transformation of Human Breast
Epithelial Cells In Vitro
107. PAQUETTE, Leo A.
Ohio State University
5 R01 CA12115-18
Cytotoxic, Cocarcinogenic and
Antileukemic Agents
108. PARTHASARATHY, Rengachary
Roswell Park Memorial Institute
5 R01 CA23704-09
Stereochemistry of Thiol-
disulfide Interchanges
109. PAULI, Bendicht U.
Cornell University, Ithaca
1 R01 CA48642-01
Carcinogenesis Testing of
Sintered Porous COCrMp Implants
110. PENNING, Trevor M.
University of Pennsylvania
5 R01 CA39504-03
Dihydrodiol Dehydrogenase and
the Aspirin-Like Drugs
111. PEREIRA, Michael A.
Environmental Health Res. and Testing
2 U01 CA45110-02
Initiation-Promotion Bioassay
in Mouse Liver
112. PIETTE, Lawrence H.
Utah State University
5 R01 CA42013-04
ESR Studies of Biological Free
Radical Mechanisms
113. PIZER, Lewis I.
University of Colorado Hlth Sciences Ctr
5 R01 CA42444-03
Mutagenic Activity of Anti-
Herpes Drugs
114. POUR, Parviz M.
University of Nebraska Medical Center
2 R01 CA35042-04A2
Prevention of Nasal Cavity
Tumors by Castration
115. POUR, Parviz M.
University of Nebraska Medical Center
5 R01 CA42133-02
Transplacental Induction of
Pancreatic Cancer
116. PRETLOW, Theresa P.
Case Western Reserve University
1 R01 CA48032-01
Colonic Putative Preneoplastic
Foci

117. PURDY, Robert H.
Southwest Foundation for Biomedical Res
5 R01 CA41569-03
Role of Catechol Formation
in Estrogen-Mediated Cancer
118. RAJALAKSHMI, S. R.
University of Toronto
5 R01 CA45361-02
Glycosylation in Experimental
Liver Carcinogenesis
119. RANDERATH, Kurt
Baylor College of Medicine
5 R37 CA32157-07
³²P-Labeling Test for Nucleic
Acid Damage by Carcinogens
120. REINERS, John J., Jr.
University of Texas System Cancer Center
2 R01 CA40823-04
Epidermal Polycyclic Aromatic
Hydrocarbon Metabolism
121. REINISCH, Carol L.
Tufts University
1 R01 CA44307-01A1
Unique Antigens on Neoplastic
Cells of Mya Arenaria
122. REZNIKOFF, Catherine A.
University of Wisconsin, Madison
5 R01 CA29525-08
Transformation In Vitro of
Human Uroepithelial Cells
123. RICE, Robert H.
Harvard University
1 R01 CA46928-01
Carcinogen Suppression of
Keratinocyte Differentiation
124. ROGAN, Eleanor G.
University of Nebraska Medical Center
2 R01 CA25176-07
Binding of Aromatic Hydrocarbons
to Nucleic Acids
125. ROSEN, Jeffrey M.
Baylor College of Medicine
5 R01 CA16303-13
Hormonal Regulation of Breast
Cancer
126. RUSSO, Jose
Michigan Cancer Foundation
2 R01 CA38921-04A1
Human Breast Susceptibility
to Transformation
127. SCARPELLI, Dante G.
Northwestern University
5 R01 CA34051-06
Metabolism of Pancreatic Car-
cinogens: Species Differences
128. SCHATZ, Robert A.
Northeastern University
1 R01 CA47671-01
Solvent and Ethanol Interaction
on B(a)P Metabolism
129. SHARMA, Minoti
Roswell Park Memorial Institute
1 R01 CA46896-01A2
Fluorescence Postlabeling Assay
for DNA Damage

130. SHAY, Jerry W.
University of Texas SW Med Ctr/Dallas
1 R01 CA50195-01
In Vitro Transformation of Human
Mammary Epithelial Cell
131. SIEGAL, Gene P.
University of North Carolina, Chapel Hill
5 R29 CA45727-02
Chemical Progression and
Inhibition of Neoplasia
132. SINCLAIR, Peter R.
Dartmouth College
5 R01 CA25012-10
Liver Cell Cultures for Study
of Carcinogen Activation
133. SIRICA, Alphonse E.
Virginia Commonwealth University
2 R01 CA39225-04
Hepatic Oval Cells in Culture
and In Vivo
134. SOLT, Dennis B.
Northwestern University
2 R01 CA34160-05
Sequential Analysis of Oral
Carcinogenesis
135. SOROF, Sam
Institute for Cancer Research
2 R01 CA05945-24
Ligand-Protein Complexes,
Growth and Carcinogenesis
136. STAMBROOK, Peter J.
University of Cincinnati
5 R01 CA36897-05
Mammalian Cell Assay for
Mutagenesis and Carcinogenesis
137. STAMBROOK, Peter J.
University of Cincinnati
1 R01 CA48118-01
Genotoxicant-Induced Deletion
and Rearrangement
138. STEGEMAN, John J.
Woods Hole Oceanographic Institution
5 R01 CA44306-03
Environmental Tumorigenesis
139. STEVENS, James
W. Alton Jones Cell Science Center
1 R01 CA48197-01
Renal Proximal Tubule Cell
Transformation
140. STROBEL, Henry W.
University of Texas Hlth Sci Ctr, Houston
5 R01 CA42995-02
Human Large Bowel Cancer and
Cytochromes P-450
141. SUDILOVSKY, Oscar
Case Western Reserve University
2 R01 CA35362-03
DNA Content of Dysplastic
Lesions in Human and Rat Liver
142. TANNENBAUM, Steven R.
Massachusetts Institute of Technology
5 P01 CA26731-09
Endogenous Nitrite Carcino-
genesis in Man

143. TAYLOR, John-Stephen A.
Washington University
2 R01 CA40463-04
DNA Photolesion Structure-
Activity Relationships
144. TERZAGHI-HOWE, Margaret
Oak Ridge National Laboratory
5 R01 CA34695-06
Cell Interactions: Expression
of Preneoplastic Markers
145. TOLBERT, Laren M.
Georgia Institute of Technology
5 R01 CA43806-02
Bio-Oxidation of Arylalkyl
Hydrocarbons
146. TOTH, Bela
University of Nebraska Medical Center
5 R01 CA31611-05
Carcinogenesis and Chemistry
of Cultivated Mushrooms
147. TUKEY, Robert H.
University of California, San Diego
5 R01 CA37139-05
Cytochrome P-450 Genes and
Chemical Carcinogenesis
148. UNDERWOOD, Graham R.
New York University
1 R01 CA47599-01
Mechanistic Studies of Arylamide
Carcinogens
149. VESSELINOVITCH, Stan D.
University of Chicago
5 R01 CA25522-09
Role of Sex Hormones in
Hepatocarcinogenesis
150. VOLLHARDT, K. Peter C.
University of California, Berkeley
5 R01 CA20713-11
Activated Mutagenic and Aromatic
Hydrocarbons
151. WALKER, Bruce E.
Michigan State University
5 R01 CA41599-03
Mechanism of DES Transplacental
Carcinogenesis
152. WANG, Ching Y.
Michigan Cancer Foundation
2 R01 CA23800-10
Mechanisms of Bladder
Tumorigenesis
153. WANG, Ching Y.
Michigan Cancer Foundation
1 R01 CA49783-01
Genetic Alterations Involved in
Bladder Carcinogenesis
154. WEBB, Thomas E.
Ohio State University
5 R01 CA42886-02
60 Kd Oncofetal Protein
Associated With Carcinogenesis
155. WEBER, Wendell W.
University of Michigan at Ann Arbor
5 R01 CA39018-04
Acetylation Pharmacogenetics:
Arylamines and DNA Damage

156. WEISSMAN, Bernard E.
Children's Hospital of Los Angeles
5 R01 CA39602-03
Chemical Transformation of
Mouse Epidermal Keratinocytes
157. WEYAND, Eric H.
Rutgers the State Univ, New Brunswick
1 R29 CA49826-01
Initiation of Mammary Carcino-
genesis in the Rat
158. WHALEN, Dale L.
University of Maryland, Balt Co Campus
5 R01 CA17278-09
Kinetic Studies of Aryl Epoxide
Reactions
159. WILLIAMS, Gary
American Health Foundation
5 R01 CA39545-03
Biochemical Toxicity of Agents
Increasing Reactive O2
160. WHITLOCK, James P., Jr.
Stanford University
5 R37 CA32786-06
Carcinogen-Metabolizing Enzymes:
Action in Variant Cells
161. YANG, Chung S.
Rutgers the State Univ, New Brunswick
7 R37 CA37037-05
Metabolic Activation of
N-Nitrosamines
162. YANG, Shen K.
U.S. Uniformed Services Univ of Hlth Sci
5 R01 CA29133-08
Metabolic Activation of
Monomethylbenzanthracenes
163. ZENSER, Terry V.
St. Louis University
5 R01 CA28015-09
Bladder Cancer: Metabolism of
Carcinogens and Prevention

SUMMARY REPORT
DIET AND NUTRITION

The Diet and Nutrition component within the Chemical and Physical Carcinogenesis Branch contains 40 active grants with FY89 funding of \$3.51 million. The component supports laboratory investigations searching for cancer etiologic factors related to diet and nutrition. These investigations include mechanistic studies of cancer induction by a variety of dietary constituents. Research supported focuses on the role of mutagens/carcinogens in foods in human cancer causation (15 grants), the influence of fats of varying sources and saturation levels on tumor induction (9 grants), dietary constituents such as protein and orotate in cancer induction (2 grants), the role of nutrients and micronutrients in carcinogenesis (2 grants), the effects of calorie consumption and energy expenditure on cancer causation (3 grants), dietary lipotropes and cancer (3 grants), and the role of compounds associated with the gut and the influence of microflora in cancer induction (4 grants).

Grants Activity Summary

Mutagens in Human Foods: In December 1984, a Request for Applications (RFA) entitled "Mutagens in Human Foods" was published. The purpose of this RFA was to accelerate the development of additional understanding relative to the possible role, fate, and cancer relevance of known dietary mutagens commonly present in human foods. The RFA resulted in the award of nine grants, two of which have been successfully recompleted and four others which are still active. Three of these projects focus on the quantification and/or metabolism of aminoimidazoazaarene type compounds present in foods such as fried beef, two others deal with the genotoxicity of 1,2-dicarbonyl compounds and the biological effects of their nucleic acid adducts, and one deals with the gastric formation of N-nitroso compounds in a miniature pig model. Progress resulting from a number of these projects are summarized below.

It has been suggested that certain types of cancer, such as cancer of the stomach, large intestine or other organs, may be attributed to traditional food and lifestyles in certain regions of the world. Studies have shown that the cooking of foods, including the grilling, frying and broiling of meat, can produce genotoxic substances at common household cooking temperatures. Such methods of cooking meat produce several structurally related compounds of the aminoimidazoazaarene family. These include 2-amino-3-methylimidazo(4,5-f)quinoline (IQ), 2-amino-3,4-dimethylimidazo(4,5,-f)quinoline (MeIQ), 2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo(4,5-f)quinoxaline (DiMeIQx) and 6-phenyl-2-amino-1-methylimidazo(4,5-f)pyridine (PhIP). Although these compounds are only found in minute quantities in cooked foods, they have been shown to be very potent mutagens. Studies on this group of compounds include those to isolate and identify them from human foods and studies to elucidate their activation, metabolism, and mutagenic and carcinogenic activity.

Although aminoimidazoazaarene mutagens have been identified using conventional analytical chemistry methods, these methods are very labor intensive for the quantification of the mutagens in large numbers of samples. In order to provide a solution to this problem, investigators have developed six monoclonal antibodies to mutagens of the aminoimidazoazaarene (AIA) type and have demonstrated their

usefulness in immunoassays for the mutagens in cooked food. Antibodies were produced that will recognize four of the five identified AIA mutagens (IQ, MeIQ, MeIQx, and DiMeIQx). Cooked beef extracts were assayed that differed 200-fold in mutagenic activity. The high-mutagen extract contained significantly more cross-reactive material than did the low-mutagen extract. The antibodies were sufficiently sensitive to allow the detection of AIAs at levels present in cooked meat (ng/g). In addition, the antibodies did not react with high levels of structurally similar compounds that might be present in beef extract, such as creatinine, nucleic acids, and amino acids. Each antibody was found to possess its own unique binding selectivity pattern; some are class-specific, whereas others are compound-specific. Although none of the antibodies thus far produced recognize PhIP, work is continuing to produce antibodies specific for this mutagen. These antibodies may prove to be useful not only in the quantification of known AIAs in cooked meats but also may aid in the isolation and identification of unknown AIA mutagens (12).

Other investigators have been examining the occurrence of heterocyclic amines in cooked food and their absorption and metabolism in humans. As part of these studies, these researchers have reported the development of a gas chromatographic-mass spectrometric assay for the measurement of MeIQx and DiMeIQx in fried beef. This assay utilizes capillary column gas chromatography, electron capture negative ion chemical ionization mass spectrometry with a stable isotope labeled analogue of MeIQx as an internal standard. Lean beef patties were cooked by heating on a hot frying pan, with no added fat or oil, at a temperature near 200 degrees Centigrade until done but without charring of the exterior. The sample was extracted with a simple three-step extraction procedure, derivatized with 3,5-bistrifluoromethylbenzyl bromide, and analyzed by gas chromatography - mass spectrometry. Both MeIQx and DiMeIQx were found to be present in readily measurable (nanogram) quantities. On the other hand, these compounds were not found to be present in raw uncooked beef, confirming that the MeIQx and DiMeIQx detected in the fried beef were formed during the cooking process. While it is suggested that the amounts of these compounds ingested in a normal meat-containing diet could be on the order of hundreds of nanograms per day, the significance to human cancer remains to be determined (10).

IQ has been shown to be not only a potent mutagen in the Ames Salmonella assay, but also a carcinogen in Fischer-344 (F-344) rats and CDF1 mice. Since the formation of DNA adducts is thought to be required for the initiation of the carcinogenic process, researchers have studied the in vivo formation of such adducts in the major target organs (liver, small intestine, and large intestine) in the F-344 rat. The ³²P-postlabeling method was used to isolate and quantitate IQ-DNA adducts 24 hours following i.p. doses of IQ (5-50 mg/kg). Five adducts were identified in each of the target organs and the patterns of these adducts were qualitatively similar in each organ. The liver and large intestine were found to contain an average of 18.1 and 2.4 times as many adducts, respectively, as that found in the small intestine. Vehicle treated animals showed no evidence of adduct formation. These studies represent the first demonstration that IQ forms specific DNA adducts in target tissues of the F-344 rat. Investigations are continuing towards the identification of the major IQ-DNA adduct (28).

Caffeine (1,3,7 trimethylxanthine) is widely consumed in coffee, colas, teas, and chocolates, and is used as an additive in many prescription and non-prescription medications. However, the literature about the relationship of caffeine consumption to cancer risk has been inconsistent and inconclusive. In order to

help resolve this controversy, the modifying effects of caffeine consumption on 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary gland tumorigenesis in female Sprague-Dawley rats and a number of mouse strains has been explored. The effects of caffeine consumption via the drinking water on both the initiation and promotion phases were also examined. In initiation studies, both moderate (100-400 mg) and high doses (860 mg) of caffeine administered to rats fed a commercial chow diet resulted in significant reduction of mammary carcinoma multiplicity (number of tumors per rat). The consumption of high to moderate doses of caffeinated coffee also significantly reduced mammary carcinoma multiplicity, while consumption of high to moderate doses of decaffeinated coffee did not. Addition of caffeine to the decaffeinated coffee resulted in a sharp reduction in mammary carcinoma multiplicity. The percentage of rats with mammary carcinomas or the mean latency period of tumor appearance were not effected by caffeine or coffee consumption. In studies of the promotion phase, an apparent stimulatory effect of caffeine on mammary carcinoma multiplicity that was temperate and transitory was observed. However, prolonged consumption of moderate doses of caffeine or moderate to high dose levels of caffeinated coffee or decaffeinated coffee had no significant effect on mammary carcinoma multiplicity. Similar results were obtained with chemically defined diets containing standard or high levels of unsaturated fat. The latter studies provided further evidence that the promotion phase may be modulated by high (20%) corn oil diets fed during the promotion phase compared to rats fed standard levels (5%), but that when fed during the initiation phase, high levels of dietary fat do not modulate this carcinogenic process.

In similar experiments, the effect of caffeine consumption on the development of DMBA-induced mammary carcinomas in BD2F₁ female mice and spontaneous mammary carcinomas in nulliparous C3H mice was examined. In both mouse strains, caffeine administered via the drinking water resulted in an increase in mammary carcinoma multiplicity but did not effect the percentage of mice bearing mammary carcinomas or the mean latency period of mammary tumor appearance. Although these results are not in total accord with the results obtained in rats in which prolonged caffeine consumption during promotion did not significantly increase the yield of mammary carcinomas, they coincide more closely to the temperate and transient stimulatory effect of caffeine observed during the early promotional stages of rat mammary gland carcinogenesis. These results indicate that caffeine or caffeinated coffee consumption can significantly influence chemical carcinogenesis in the mammary gland of female rats and mice and that this effect is dependent upon the dose level, duration, and time-span of caffeine administration as well as the animal model investigated.

In another series of experiments, the effect of caffeine consumption on mammary gland development in female BALB/c mice was assessed *in vivo* and *in vitro*. In mice consuming caffeine via the drinking water, mammary gland development was significantly increased compared to control mice. This increase in mammary gland development was more dramatic in mice treated with mammotropic hormones. In addition, the mammary glands derived from caffeine consuming mice were more responsive *in vitro* to a mammotropic hormonal developmental growth stimulus than were mammae derived from control mice. These results indicate that caffeine administration *in vivo* significantly increases the development of the BALB/c mouse mammary gland and that the caffeine-induced increase in mammae development is more conspicuous after administering a mammotropic hormonal developmental growth stimulus. It is suggested that one mechanism by which caffeine may modulate rodent mammary tumorigenesis is by altering, systemically, the secretion of a hormonal factor or factors affecting the developmental growth of the normal and

neoplastic mammary epithelium. The mechanisms by which caffeine modulates rodent mammary gland tumorigenesis will be further explored (38).

Although it is well documented that caloric restriction can inhibit tumorigenesis in rodent models, the underlying mechanism for this effect remains elusive. It has been pointed out that in previous studies, food restriction has amounted to an approximately 50% reduction of the average daily ration, possibly resulting in drastic hormonal imbalances that could have not only disrupted the normal physiology of the animals, but may have masked other effects of dietary restriction as well. Investigators have now demonstrated that dietary restriction in the form of under-feeding can inhibit DMBA-induced mammary carcinogenesis, even when the restriction is at much lower levels (20%) than those previously reported. It was found that the 20% reduction in food intake resulted in no significant changes in estrogen levels as measured by radioimmunoassay. In addition, the rats in the 20% restricted group appeared to be cycling normally and showed no observable differences in breeding capacity. Thus, it was concluded that no significant hormonal imbalances resulted from the dietary restriction. These investigators are now exploring the possibility that changes in the immune system, in addition to observed reductions in the levels of cell proliferation, act synergistically to inhibit the induction of mammary tumorigenesis by chemical carcinogens (29).

Several epidemiological studies have demonstrated that a high intake of dietary fat is generally correlated with an increased colonic cancer risk in humans. The results of animal studies suggest that the effect of dietary fats on tumor promotion not only depends on the amount of dietary fat consumed, but also the types of saturated and polyunsaturated fats consumed. Sources of dietary fats containing high levels of unsaturated fatty acids such as corn oil or safflower oil have been shown to increase chemically-induced colon tumors, whereas sources of dietary fats containing high levels of saturated fatty acids such as olive and coconut oil had no tumor enhancing effect. While the mechanism for the enhancement of tumorigenesis by dietary fat is a subject of intense investigation, the precise role of dietary fat remains to be elucidated.

In order to clarify the mechanism by which dietary fat enhances tumorigenesis, investigators are exploring the possibility that unsaturated fatty acids enhance tumorigenesis via the formation of biologically active autoxidation products. Since the induction of ornithine decarboxylase (ODC) and stimulation of DNA synthesis are associated with tumor promotion, these investigators have determined the structural features of oxidized fatty acids that are required for such activity. The mitogenic activity of hydroperoxy and hydroxy fatty acids derived from oleate and stearate were investigated as well as ricinoleic acid and the alpha, beta-unsaturated ketone derived from oleic acid. Compounds were instilled intrarectally and either three hours later (ODC activity) or 12 hours later (tritiated thymidine incorporation), the animals were sacrificed and the colonic mucosa harvested for measurement of mitogenic activity. It was found that a carbon-carbon double bond adjacent to an oxidized group is the minimal requirement for mitogenic activity. In addition, all of the active acids tested contained a trans double bond within the hydrocarbon portion of the molecule as well as an oxidized functionality. However, the presence of a trans double bond was necessary but not sufficient to confer mitogenic activity. The oxidized group adjacent to the double bond could be a hydroperoxide, alcohol, or carbonyl group with no significant difference in activity, raising the possibility that a common intermediate is involved. Activity of the fatty acid was lost by saturation of the hydrocarbon backbone, separation of the oxidized group from the double bond by

a methylene group or the absence of an oxidized group. These results indicate that the autoxidation products of unsaturated fatty acids may play a role in the enhancement of tumorigenesis by high levels of dietary fat and suggest a possible mechanism of action for the active compounds, such as the involvement of an alpha, beta unsaturated ketone which could conceivably bind to cellular constituents to produce the biological responses observed. Further studies of the metabolic fate of the active compounds will be pursued (7).

Although epidemiological studies have shown that Eskimos in Alaska and Greenland have a lower cancer incidence than North Americans who consume food containing high dietary fat, food consumed by Eskimos contains large amounts of fish oils. These oils contain high levels of omega-3 fatty acids such as eicosapentaenoic acid (c20:5, n-3) and docosahexaenoic acid (c22:6, n-3); thus, their role in health and disease is of recent interest. In order to determine the optimum dietary levels of omega-3 and omega-6 fatty acids that elicit maximum inhibition of colon tumors, the modulating effect of these fatty acids during the promotional phase of colon carcinogenesis was investigated. Four days after receiving s.c. injections of azoxymethane, groups of male F344 rats were fed one of the following diets until termination of the experiment at week 38 after carcinogen treatment: 4% Menhaden oil + 1% corn oil (Diet 1); 23.5% corn oil (Diet 2); 17.6% corn oil + 5.9% Menhaden oil (Diet 3); 11.8% corn oil + 11.8% Menhaden oil (Diet 4); or 5.9% corn oil + 17.6% Menhaden oil (Diet 5). It was found that feeding of the high fat diets with increasing levels of Menhaden oil (Diet 3, Diet 4, or Diet 5) significantly inhibited the incidence (percentage of rats with tumors) of colon adenocarcinomas compared to that of the 23.5% corn oil diet (Diet 2). The multiplicity (number of tumors/rat) of adenocarcinomas was only inhibited in the groups fed the 5.9% corn oil + 17.6% Menhaden oil diet (Diet 5) compared to those fed the 23.5% corn oil diet. Both the incidence and the multiplicity were greater in rats fed the 23.5% corn oil diet compared to those fed the 5% corn oil diet. While it might be argued that the inhibition of colon tumor incidence might be due to the decreased corn oil in the high fat diets, it was found that the inhibition of colon tumor incidence by decreasing the level of corn oil or increasing the ratio of omega-3 to omega-6 fatty acids in the diets was not dose-dependent. Thus, it was concluded that high fat intake is necessary but not sufficient for colon tumor promotion and that it may be the relative proportions of omega-3 and omega-6 fatty acids in the diet that are determinants of the high fat effect (26).

Fecapentaene-12 (FP-12) and fecapentaene-14 (FP-14), potent mutagens which have been isolated from human feces, have been implicated in the etiology of human colon cancer. These compounds are synthesized from thus far unidentified precursors by resident bacteria of the genus *Bacteroides* in the human gastrointestinal tract. Investigators have recently described the direct mutagenic activities of this pair of naturally-occurring fecapentaenes and several synthetic fecapentaenes in the Ames/Salmonella assay. It was found that the most sensitive procedure for FP-12 was strain TA100 with preincubation. Tests using FP-12, FP-14 and the synthetic isomer, cis-FP-12, gave similar mutagenic activities in the range 1000-2000 TA100 revertants per microgram of compound, suggesting that the size of the terminal carbon chain (at least up to 4) attached to the pentaene conjugated system and the configuration at the double bond adjacent to the ether group may not be important determinants of mutagenic activity. Synthetic derivatives in which the glycerol functionality was replaced with a methoxy group, MFP-12 and MFP-14, were found to exhibit greater activity than their parent compounds. Although these results do not agree with an earlier report by other investigators that the methoxy analog of FP-12 is less active than its parent

compound, MFP-12 and MFP-14 were found to display greater mutagenic activity than their respective parents in assays using L5178Y mouse lymphoma cells. The addition of post-mitochondrial supernatant solutions of homogenates of livers (S9) from Aroclor 1254-induced male Sprague-Dawley rats reduced the activities of all the fecapentaenes tested in a dose-dependent manner (23).

In another laboratory, the stereochemistry of fecapentaene-12 was investigated. The absolute stereochemistry of fecapentaene-12 was determined to be an S configuration during its initial structural elucidation. However, the stereochemistry of the unsaturated system has remained unclear, although the all-E isomer was shown to be a significant component of the natural product mixture. Investigators have now reported the results of a comparison of natural fecapentaene-12 with two different synthetic samples. From the analysis of proton nuclear magnetic resonance and high performance liquid chromatography (HPLC) data, it is suggested that one probable explanation for the results obtained is that the natural fecapentaene-12 consists of a mixture of the 1-Z, 3-Z and 5-Z isomers, together with the all-E isomer; however, it is recognized that these results are not unambiguous and that many E/Z isomers are possible.

In a collaborative case-controlled study with Dr. Mark H. Schiffman of the Environmental Epidemiology Branch, NCI, these same investigators have screened over 1000 fecal samples for excretion of fecapentaenes and their precursors. Groups studied included both cancer patients and several control groups. It was shown that cancer patients excrete significantly less fecapentaenes than normal controls, and that the mutagenicity of moderately polar extracts such as those obtained using ether or acetone, is due almost exclusively to the fecapentaenes. Quantitation of fecapentaenes by HPLC correlated greater than 95% with the mutagenicity results. As part of these same studies, it was shown that fecapentaenes and their precursors are uniformly distributed throughout the colon with no significant decreases or increases of either as the colonic contents progress from proximal to distal regions. While this observation is consistent with the presence of precursor-producing bacteria that are continually producing precursors in vivo at the same time that the Bacteroides species are converting the precursors to fecapentaenes, efforts to isolate such organisms have been unsuccessful. The possibility that the precursors are a product of host metabolism is also consistent with these observations; thus, additional approaches to screen host tissues for precursors will be developed (40).

A number of laboratories have demonstrated that diets devoid of choline and low in methionine induce hepatocellular carcinoma in rats. The temporal sequence of biochemical and cellular later events in the livers of rats exposed to such diets have been shown to include triglyceride accumulation followed by nuclear lipid peroxidation, DNA alteration, mitochondrial lipid peroxidation, cell death and proliferation, initiation of carcinogenesis, and finally induction of cancer. Investigators are exploring the following possible bases for the involvement of free radicals in the lipid peroxidation produced by a choline devoid diet: 1) the excessive generation of free radicals such as superoxide or hydroxyl radicals in the liver of animals fed the choline devoid diet; 2) the diminution in free radical scavengers; and 3) increased susceptibility of nuclear membranes to interactions with normally-generated free radicals. As part of these studies, the levels and activities of glutathione S-transferase, glutathione peroxidase, superoxide dismutase, catalase and DT-diaphorase were investigated since these enzymes are related to the metabolism of free radicals. However, these enzymes showed neither increased nor decreased activity as assayed between 12 hours and 8 days on

the diet. Glutathione levels in the liver of Fischer F-344 male rats fed a choline-devoid diet were found to be elevated at 3, 6 and 8 days, and showed significant elevation in hepatic concentration at times when lipid peroxidation was maximal. Thus, it appears that the diminution in free radical scavengers as a basis for the nuclear and mitochondrial lipid peroxidation resulting from choline-devoid diets has no objective evidence so far, at least where water-soluble scavengers are concerned. As noted by these researchers, the major lipid-soluble scavenger in membranes, vitamin E, has yet be assessed in the nuclear and mitochondrial membranes of the liver in the animals fed a choline-devoid diet (14).

DIET AND NUTRITION
GRANTS ACTIVE DURING FY89

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ABRAHAM, Samuel Children's Hospital Med Ctr N CA-Oakland 5 R01 CA29767-05	Effect of Dietary Fat on Mammary Neoplasia
2. AUSMAN, Lynne M. Center For Molecular Medicine & Immunolo 5 R01 CA42352-03	Nutritional Influences on Colon Cancer in the Tamarin
3. BARCH, David H. University of Illinois at Chicago 2 R01 CA40487-04	Role of Zinc and Ethanol in Esophageal Carcinogenesis
4. BIRT, Diane F. University of Nebraska Medical Center 5 R01 CA42986-03	Dietary Fat, Calories and Two- Stage Carcinogenesis
5. BOKKENHEUSER, Victor D. St. Luke's-Roosevelt Inst for Hlth Sci 5 R01 CA25763-12	Bacterial Metabolism of Flavonoid Glycosides/Aglycones
6. BRYAN, George T. University of Wisconsin, Madison 5 R01 CA31127-06	Intestinal Carcinogenicity of Quercetin and Flavonols
7. BULL, Arthur W. Oakland University 5 R01 CA47912-03	Hydroperoxide Metabolism by Colonic Mucosa
8. CAMPBELL, T. Colin Cornell University Ithaca 5 R01 CA34205-05	Dietary Protein and Chemical Carcinogenesis
9. CHATTORAJ, Sati C. Boston University 5 R01 CA39381-03	Catechol Estrogens, Diet and Breast Cancer
10. DAVIES, Donald S. University of London 5 R01 CA40895-03	The Metabolic Fate of Mutagenic Amines in Animals and Man
11. ESSIGMANN, John M. Massachusetts Institute of Technology 5 R01 CA40817-04	Biological Effects of Cyclic Nucleic Acid Adducts Formed
12. FELTON, James S. Univ of Calif-Lawrence Lvrnr Nat Lab 2 R01 CA40811-03A1	Quantification of Cooked-Food Mutagens by Immunoassay

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| 13. | FLEMING, Sharon E.
University of California Berkeley
5 R01 CA40845-03 | Fiber Volatile Fatty Acids and
Colonic Cell Biology |
| 14. | GHOSHAL, Amiya K.
University of Toronto
5 R01 CA41537-03 | Diet and Cancer--Choline and
Methionine in Liver Cancer |
| 15. | HOTCHKISS, Joseph H.
Cornell University Ithaca
5 R01 CA40833-04 | Gastric Formation of N-Nitroso
Compounds |
| 16. | IP, Margot M.
Roswell Park Memorial Institute
5 R01 CA33240-06 | Lipid Modulation of Mammary
Neoplastic Growth |
| 17. | ISSENBERG, Phillip
University of Nebraska Medical Center
5 R01 CA43589-02 | Cruciferous Vegetables/
Endogenous Nitrosamine Forms |
| 18. | KINGSTON, David G.
Virginia Polytechnic Inst and St Univ
2 R01 CA40821-04 | Anaerobic Metabolism of Mutagens
in Human Foods |
| 19. | KRITCHEVSKY, David
Wistar Institute of Anatomy and Biology
1 R01 CA43856-01A3 | Caloric Restriction in Tumor
Promotion: Mechanisms |
| 20. | LEA, Michael A.
University of Medicine & Dentistry of NJ
1 R01 CA46442-01 | Nucleotide Metabolism and
Promotion of Carcinogenesis |
| 21. | NEWBERNE, Paul M.
Boston City Hospital
5 R01 CA40080-03 | Zinc, Nitrosamine and
Esophageal Cancer |
| 22. | NEWBERNE, Paul M.
Boston City Hospital
1 R01 CA46288-01A1 | Lipotrope Deficiency and Liver
Cancer in Rats |
| 23. | PETERS, John H.
SRI International
5 R01 CA40918-03 | Fecapentaenes Mechanistic
Studies |
| 24. | POUR, Parviz M.
University of Nebraska Medical Center
1 R01 CA43550-01A1 | High Fat Diet in Experimental
Prostatic Cancer |
| 25. | RAPOPORT, Henry
University of California, Berkeley
5 R01 CA40984-04 | Metabolism and Bioavailability
of MeIOx from Fried Beef |
| 26. | REDDY, Bandaru S.
American Health Foundation
2 R01 CA37663-04A1 | Calories, Energy Expenditure and
Fish Oil in Colon Cancer |

27. SARKAR, Nurul H.
Medical College of Georgia
5 R01 CA45127-03
Effect of Diet on Murine Mammary Tumorigenesis
28. SCHUT, Herman A.
Medical College of Ohio at Toledo
1 R01 CA47484-01
Carcinogenesis of Heterocyclic Amines
29. SINHA, Dilip K.
Roswell Park Memorial Institute
5 R01 CA42853-03
Prevention of Mammary Cancer by Caloric Restriction
30. TAYLOR, Robert T.
Univ of Calif-Lawrence Lvrnr Nat Lab
5 R01 CA40816-04
Genotoxicity of Food Related 1,2-dicarbonyl Compounds
31. TOTH, Bela
University of Nebraska Medical Center
5 R01 CA40989-03
Capsaicin: Chemistry, Carcinogenesis, and Mode of Action
32. TOTH, Bela
University of Nebraska Medical Center
5 R01 CA44075-02
False Morel Hydrazines: Carcinogenesis and Chemistry
33. VISEK, Willard J.
University of Illinois Urbana-Champaign
5 R01 CA41707-03
Calorie Consumption and Experimental Colon Cancer
34. WAINFAN, Elsie
New York Blood Center
1 R01 CA47600-01
Diet Cancer and Oncogene Expression in Inbred Mice
35. WALKER, Bruce E.
Michigan State University
1 R01 CA39456-01A2
Transplacental Carcinogenesis and Diet
36. WEISBURGER, John H.
American Health Foundation
5 R01 CA42381-03
Metabolism of the Carcinogen Aminomethylimidazoquinoline
37. WEISBURGER, John H.
American Health Foundation
5 R01 CA45720-02
Identification of Carcinogens
38. WELSCH, Clifford W.
Michigan State University
2 R01 CA37613-04
Caffeine and Experimental Mammary Gland Tumorigenesis
39. WELSCH, Clifford W.
Michigan State University
5 R01 CA42876-03
Can Dietary Fat--Mammary Gland Growth Processes
40. WILKINS, Tracy D.
Virginia Polytechnic Inst and St Univ
5 R01 CA23857-11
Relationships of Fecal Mutagens to Colon Cancer

SUMMARY REPORT

MOLECULAR CARCINOGENESIS

The Molecular Carcinogenesis component of the Branch includes 210 grants with FY89 funding of approximately \$33.52 million. There are no contracts in this area. The currently active grants consist of 170 R01 (Research Project) grants, 1 R23 (Young Investigator) grant, 13 R29 "FIRST" (First Independent Research Support & Transition) awards, 1 R15 "AREA" (Academic Research Enhancement Award) grant, 1 R43 and 1 R44 (Small Business) grants, 5 P01 (Program Project) grants, and 5 R35 Outstanding Investigator grants. In addition, 6 grants have been approved as R37 "MERIT" (Method to Extend Research In Time) Awards (2 additional from R01s in FY89). Also included are 7 R13 conference grant awards. Research supported by this component focuses on the role of exocyclic DNA adducts in carcinogenesis (7 grants), the characterization of carcinogen-macromolecular interactions (21 grants), changes in biological macromolecules and cell functions as a result of carcinogen or cocarcinogen exposure (19 grants), the mechanisms of carcinogen-induced mutagenesis and genetic damage (24 grants), the role of DNA repair in carcinogenesis (27 grants), the genetics and mechanisms of cell transformation (49 grants), the role of oxygen radicals in carcinogenesis (16 grants), and the identification and properties of tumor promoters and mechanisms of tumor promotion (47 grants). Expanded descriptions of individual subject areas, along with examples of research accomplishments, are provided below.

Grants Activity Summary

Exocyclic Adducts in Carcinogenesis: In January 1986 a Program Announcement entitled "Biological Role of Exocyclic Nucleic Acid Derivatives in Carcinogenesis" was issued. The purpose of this Announcement was to stimulate basic research on a class of important compounds which have the capability of forming exocyclic nucleic acid adducts. It was expected that studies relevant to many of the above-listed subject areas would be stimulated by this program initiative. Two applications directly relevant to the focus of the Program Announcement were submitted prior to its publication. These were both funded. One grant supported a project to develop monoclonal antibodies to various exocyclic nucleic acid adducts. This grant is now pending renewal. The second grant supported studies on the reaction of crotonaldehyde and 4-hydroxynonenal with guanine nucleotides and with DNA, both in vitro and in vivo. This grant will be renewed in FY89 for an additional 5 years and will focus on testing the hypothesis that the epoxidation of enals could constitute an important activation pathway in enal mutagenesis and tumorigenesis. Between January 1986 and January 1987, five applications (including one Small Business grant application) were submitted in response to the Program Announcement. One grant, which is focused on a study of acrolein as a possible causative agent in human bladder cancer, was funded and the Small Business grant, whose goal is to develop monoclonal antibodies specific for the major nucleic acid adducts of bifunctional carbonyl compounds (i.e., malonaldehyde, acrolein, methyl glyoxal) in human DNA, was also funded. In FY89 the phase II application of this SBIR grant was funded to allow the continued development of the above mentioned monoclonal antibodies. In early 1987 the proceedings of the meeting which led to the generation of the Program Announcement were published by the International Agency for Research on Cancer (IARC) Scientific Publications. The availability of this publication and the relatively low number of responses received up to that time led to the decision, in April 1987, to reissue the Program Announcement. In FY88 five additional responses

resulted from this reissuance. Three of these grant applications were funded. One grant supports a study to test the hypothesis that N-nitroso-2-pyrrolidone is the proximate carcinogen derived from two successive metabolic oxidative transformations of N-nitrosopyrrolidine and that N-nitrosopyrrolidine genotoxicity is derived from the formation of transient DNA adducts. The second grant supports studies on the in vitro mutagenic potential of N²,3-ethenoguanine, the evaluation of the effects of site-specific 1,N⁶-ethenoadenine and 3,N⁴-ethenocytosine lesions on duplex DNA structure and replication fidelity, as well as the investigation of whether adducts other than the known lesions are formed by metabolites of vinyl chloride. Previous support for the above studies had been included in one large grant which has now been divided into two more focused grants, one of which represents this response to the Program Announcement. The third grant supports studies on the mechanisms of mutagenesis of ethenocytidine and ethenoadenine adducts induced in DNA by metabolites of the carcinogen, vinyl chloride. Eight grant applications relevant to this area were received in FY89. Two renewal applications and the phase II SBIR application were mentioned previously. One new P01 and three new R01 applications were received, but none will be funded. In addition, one grant that focuses on continuing investigations of the biological role of cyclic nucleic acid adducts will be renewed for another 5 years. This grant had originally been submitted in response to an RFA on "Mutagens in Foods."

Malondialdehyde is a product of polyunsaturated fatty acid oxidation that is ubiquitously distributed in the plant and animal kingdom. It can arise as a side product of the arachidonic acid cascade and is the major carbonyl-containing end product of lipid peroxidation, the nonspecific oxidative degradation of unsaturated fatty acid containing lipids. Malondialdehyde was shown to be mutagenic to bacteria and mammalian cells and carcinogenic to mice. Chemically, malondialdehyde is a prototype beta-dicarbonyl compound but actually exists as a tautomer, beta-hydroxyacrolein in polar solvents. It was expected that malondialdehyde would be biologically similar to the structurally analogous molecules methyl glyoxal and acrolein. However, malondialdehyde was shown to induce frameshifts and base-pair substitutions, whereas methyl glyoxal and acrolein induce only base-pair substitutions. In order to explain the unusual pattern of mutagenicity displayed by malondialdehyde, malondialdehyde and a series of acrolein derivatives substituted in the beta-position with good leaving groups were reacted with guanine and guanine nucleosides to form two different types of adducts. One adduct had spectroscopic properties consistent with the structure, 3-beta-D-erythro-pentofuranosyl-pyrimido[1,2-a]purin-10(3H)-one (PyP-ribose). The second guanosine adduct was shown to be an equal mixture of diastereomers that had spectroscopic and chemical properties to suggest an oxadiazabicyclo[3.3.1]nonene (Oxa) structure. This structure was apparently formed by the reaction of a second malondialdehyde molecule to the PyP adduct. The formation of multimeric adducts such as Oxa is considered to be unique to malondialdehyde among all known chemical mutagens and carcinogens. Comparison of the deoxyguanosine adducts of malondialdehyde to those of the structurally related carbonyl compounds, methyl glyoxal and acrolein, provided a structural basis to explain the unique ability of malondialdehyde to induce frameshift mutations in bacterial mutagenesis systems. The PyP adduct is fluorescent, aromatic and hydrophobic and bears a striking similarity to acridine or proflavine dyes that are well established frameshift mutagens. All other adducts, in contrast, are nonfluorescent, puckered and contain polar hydroxyl or carbonyl functionalities in the base-pairing region. These would not be expected to generate frameshift mutations (122).

Vinyl chloride is a human carcinogen that was shown to be metabolized to the unstable epoxide, chloroethylene oxide, which rapidly rearranges to a stable metabolite, chloroacetaldehyde. This can initially react with the exocyclic amino group of adenine, guanine or cytosine in monomers and polymers to form an unstable product. The initial adduct can then cyclize rapidly to the adjacent endo nitrogen which then dehydrates to form the well-known etheno derivatives, 1,N⁶-etheno-adenine, 1,N²-ethenoguanine, N²,3-ethenoguanine and 3,N⁴-ethenocytosine. In addition, cross-linking to a base on the opposite strand is possible. In one study chloroacetaldehyde was shown to form interstrand cross-links in vitro in salmon sperm DNA and in the alternating copolymer, poly(dA-dT). Formation of the cross-link was shown to be a function of both time of reaction and concentration of chloroacetaldehyde. The extent of cross-linking was quantitated using the relative fluorescence of ethidium bromide after denaturation and reannealing at 40°C. Three percent cross-linking in poly(dA-dT) was detected after 10 min reaction with 20 mM chloroacetaldehyde at 24°C. Three times as much cross-linking was shown to occur in DNA as compared to poly(dA-dT) under identical reaction conditions. The postulated structure for an interstrand cross-link in poly(dA-dT) is a hydroxy-ethyl bridge across the strands between the N⁶- amino groups of alternate adenine residues. In DNA, other amino groups in the proper configuration can be involved. Further investigation of the range of cross-links produced by bifunctional aldehydes is under investigation (173).

Carcinogen-Macromolecular Interactions: The projects in this subject area focus on studies on the identification, quantitation and characterization of carcinogen-nucleic acid adducts. Interest in the identification and characterization of DNA adducts stems from the role that alterations in DNA play in the initiation of carcinogenesis. Most of the carcinogens used in these studies are ones which are metabolized by cellular xenobiotic metabolizing enzymes to a variety of metabolites of which one or a few are reactive and bind to nucleic acids and/or proteins. The identification and quantitation of the binding species are generally determined by chromatographic and radioisotopic techniques. A technique using fluorescence line-narrowing spectrometry, which has a detection level of about five adducts per 10⁶ bases, is being developed for the purpose of analyzing complex mixtures of DNA adducts. In experiments to evaluate this technique, intact DNA-polycyclic aromatic hydrocarbon (PAH) and globin-PAH adducts, as well as polar PAH metabolites were examined. A detection limit of about three modified bases in 10⁶ for a DNA adduct formed with a diol epoxide of benzo(a)pyrene has been reported. The methodology can be used to analyze exposures to complex mixtures of PAHs and has the advantage that the structures of the adducts need not be known and the DNA need not be digested prior to analysis (196). In addition, the development of monoclonal antibodies to various carcinogen-nucleic acid adducts has led to the increasing use of radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) as a sensitive means of adduct detection. For derivatives such as exocyclic adducts, highly sensitive immunoassays need to be developed in order to provide the necessary sensitivity for the detection of those adducts in the DNA of exposed cells. Other methods which are being developed for detecting and analyzing low levels of adducts such as the exocyclic nucleic acid derivatives involve the coupling of systems such as liquid chromatography-mass spectrometry.

An interlaboratory comparison of immunoassays using antisera elicited against benzo(a)pyrene diol epoxide (BPDE-I) modified DNA was carried out resulting in standardization of antisera, competitors and assay conditions. The need for such a comparison was due to the suggestion from several studies that there may be some

variability in different laboratories in the quantitation of BPDE-I adduct levels in human samples. The assays used included competitive enzyme-linked immunosorbent assays (ELISA) with color and fluorescent endpoint detection (laboratories of R. Santella, Columbia University and M. Poirier, National Cancer Institute) and an ultrasensitive enzyme radioimmunoassay (USERIA) with a radioactive endpoint (laboratory of C. Harris, National Cancer Institute). Three different antisera were compared, two of which were obtained from different rabbits immunized with the same BPDE-I-DNA and a third from an animal immunized with another BPDE-I-DNA sample. Samples of standardized BPDE-I-DNA with high and low modification levels were prepared and used in each laboratory. Since the antisera were all elicited against highly modified DNAs, they detected adducts in a slightly modified DNA sample with lower efficiency, the discrepancy varying between 1.4- and 11.2-fold depending on the antiserum and assay. To ascertain the quantitative capability of the immunoassays, the modification level of mouse keratinocytes treated with ^3H -benzo(a)pyrene was determined by radioactivity and immunoassay. These results indicated that when a biological sample is assayed against a BPDE-I-DNA standard modified in the same range as the biological samples, quantitative recovery of adducts is achieved by immunoassay. From these studies it was realized that interlaboratory differences in immunoassay procedures can have significant consequences for data comparison. It was recommended that, where possible, it is preferable for laboratories to use the same antisera and modified DNA standards (196).

In a different study the use of antibodies to BPDE-I-DNA adducts to quantitate adduct levels and determine their distribution by electron microscopy was reported. For this purpose the immunoglobulin fraction of rabbit antiserum specific for BPDE-I-DNA adducts was digested with papain and the Fab fragments were purified by affinity chromatography on protein A-sepharose and subsequently cross-linked to ferritin. For the experiments, DNA from cells treated with BPDE-I in culture was reacted with ferritin-labeled Fab fragments, separated from unreacted Fab using a Sepharose CL-4B column and examined by electron microscopy. An aliquot of the same DNA was used to determine the level of BPDE-I adduction using ELISA. Close agreement was found between the levels of adduction determined by ELISA and electron microscopy. A good correlation was also found between the level of adduction measured by electron microscopy and scintillation spectrometry when DNA was modified by ^3H -BPDE-I in vitro. The use of Fab fragments avoids the possibility of cross-linking of two separate adducts by the same IgG molecule. The procedure was also determined to allow the analysis of very limited quantities of DNA samples, as little as 1.5 micrograms of DNA. The strong motivation for applying electron microscopy technology to the study of carcinogen binding derives from its unique capability of providing information, not only on average levels of adduction but also on the distribution of DNA adducts (95).

A method known as the ^{32}P -postlabeling technique, which has been developed for the detection of the in vivo formation of carcinogen-DNA adducts where the use of radiolabeled test compounds is not required, has come into increasingly widespread use as the technique has become more refined and standardized. The method involves the reaction of DNA with chemicals in vitro or in vivo and the purification and enzymatic digestion of DNA to deoxyribonucleoside 3'-monophosphates. These are converted to ^{32}P -labeled deoxyribonucleoside 3',5'-bisphosphates after incubation with (gamma- ^{32}P) adenosine triphosphate (ATP) and T4 polynucleotide kinase. The ^{32}P -labeled digests are then fingerprinted by using reverse-phase liquid chromatography and anion-exchange thin-layer chromatography on polyethyleneimine-cellulose followed by detection by autoradiography and quantitation

by scintillation counting. Refinements to the procedure involve the enrichment of adducts by removing normal DNA nucleotides prior to ^{32}P -labeling by butanol extraction, reverse phase chromatography or *Penicillium citrinum* nuclease P1 treatment. Nuclease P1 was found to cleave deoxyribonucleoside 3'-monophosphates of normal nucleotides to deoxyribonucleosides which do not serve as substrates for polynucleotide kinase, while most adducted nucleotides were found to be totally or partially resistant to the 3'-dephosphorylating action of nuclease P1. These refinements and modifications have enhanced the technique's sensitivity of adduct detection to about 1 adduct in 10^9 or 10^{10} nucleotides for a 10 microgram DNA sample. The new procedures have been found to be simple, highly reproducible, and applicable to the detection and measurement of aromatic or bulky non-aromatic DNA adducts formed with many structurally diverse carcinogens. This method has been applied to answer questions about the tissue specificity of carcinogen adduct formation, the degree of persistence of DNA adducts in cells, and to detect adducts in cells of humans exposed to carcinogenic chemicals.

To further explore the question of adduct recoveries, the above mentioned enhancement procedures as well as a newly developed polynucleotide kinase enrichment procedure were compared in parallel for a large number of different carcinogen-DNA adducts (>70) of known and unknown chemical nature. When compared with the butanol extraction procedure, arylamines bound to the C8 position of guanine were shown to be almost completely lost (0.2-4% recovery) in the nuclease P1 assay, while the presence of a polar group in the aromatic amine moiety (such as 2-acetylaminofluorene) rendered a recovery similar to the butanol procedure. In contrast, aromatic amines and PAHs bound to the exocyclic positions of guanine or adenine showed extensive or as complete recovery in the nuclease P1 procedure as in the extraction procedure. The recovery pattern of most adducts examined in the polynucleotide kinase enrichment assay was found to be essentially the same as found in the nuclease P1 assay, except that overall lower values were obtained. The data obtained suggest that a given DNA sample, particularly DNA specimens of humans exposed to low levels of unknown carcinogens, should be analyzed by different versions of the ^{32}P -adduct assay. The observation that the chemical structure of an adduct may be detrimental in its recovery in the enzyme and extraction mediated enrichment procedures may serve as a probe in the structural characterization of adducts of unknown carcinogens (74).

The levels and persistence of specific DNA adducts are often related to the organ specificity of the carcinogen and may indicate which of the adducts are biologically relevant. For many carcinogens, such as the polycyclic aromatic hydrocarbons, alkyl nitrosamines, N-2-acetylaminofluorene, and aflatoxin B₁, the reactive metabolites and the identity of the various nucleoside adducts are known. The chemical nature and physical conformation of the adducts is thought to determine the biological effect of the adduct. For this reason, several investigators are focusing on the chemical and biophysical characterization of carcinogen-DNA adducts and on the resultant conformational changes the adducts may introduce into the DNA molecule. In many of the studies, defined polydeoxynucleotide sequences containing a modified base are synthesized for analysis. Several different techniques have been utilized for the characterization of carcinogen-nucleic acid adducts. These include high pressure liquid chromatography, absorption and fluorescence spectroscopy, nuclear magnetic resonance, optically detected magnetic resonance, linear and circular dichroism spectroscopy and x-ray crystallography. Another determinant of the biological effect of carcinogen DNA adducts is their potential site or sequence-specific interaction on the DNA molecule. The examination of this possibility for aromatic amine, polycyclic aromatic hydrocarbon and

metal carcinogens is the focus of several studies. The development and use of sophisticated molecular biology techniques to analyze site-specific interactions of carcinogens has made this a growing area of interest. In addition, computer analysis of possible carcinogen-DNA adduct conformations in a defined DNA sequence has allowed the building of molecular models for the most likely conformations. The results of these studies give information as to the possible mechanisms by which a carcinogen may cause a mutation or other alteration in the DNA structure.

The reaction of chemical carcinogens with DNA is well documented, but whether chemical carcinogens react specifically or nonspecifically with particular sites in chromatin is not known. The observations available indicate that carcinogen binding to chromosomal DNA is of a nonrandom nature. As an example, the linker regions of nucleosomes have been shown to be better targets for chemical carcinogens than core regions. Experiments with benzo(a)pyrene and aflatoxin B₁ have suggested that transcriptionally active chromatin is a more reactive target than inactive chromatin. In order to further understand the interaction between chemical carcinogens and nuclear DNA, the *in vivo* reaction of a known carcinogen, chloroacetaldehyde, with the active and inactive major immediate early gene of human cytomegalovirus was examined in one laboratory. It was found that during active transcription of this gene, chloroacetaldehyde reacted with a unique DNA site in the 5' flanking sequence of the major immediate early gene. No reaction was detected in infected nonpermissive cells in which the gene was inactive. The chloroacetaldehyde-reactive site was found to be located at -836 ± 10 bp from the mRNA cap site in the part of the regulatory region that can both negatively and positively affect promoter activity. The results obtained suggest, at least in the case of chloroacetaldehyde, the possibility that the molecular mechanism of chemical carcinogenesis involves a chemical reaction at specific sites in chromatin within the sequences responsible for regulation of gene expression. Previous studies with bromoacetaldehyde suggested that regulatory regions of various genes, when transcriptionally active, contain a non-B DNA structure with impaired DNA bases at specific loci that are particularly susceptible to chemical reaction. These results suggest that an important step for chemical carcinogenesis may be a specific interaction of a carcinogen with chromatin that results in the significant impairment of the regulation of gene expression (102).

The interactions of BPDE-I with chromosomal components have been shown to be highly selective with the three-dimensional structure of chromatin, as well as the DNA sequence, playing an important role in determining the accessibility and removal of BPDE-I DNA adducts. BPDE-I, like other bulky carcinogens, has been shown to preferentially modify chromosomal linker DNA and to preferentially target active genomic sequences in eukaryotic cells. DNA damage in active DNA sequences is also known to be preferentially repaired. In another laboratory a photochemical method was used to describe clusters of highly preferred BPDE-I binding sites in a 300 bp region 5' to the beta-globin gene transcription start site. These preferential BPDE-I binding sites were shown to be associated with repeating poly(dG) DNA sequences. In a recent study the analysis of BPDE-I binding sites was extended to the Chinese hamster ovary aprt gene, in particular the 5' flanking region of the gene. For these studies, a plasmid (pGAL) containing the entire hamster aprt gene including the 3' and 5' flanking regions was inserted into the Bam HI site of the multiple cloning sites of pGEM so that the T7 promoter was 5' to the aprt gene. In vitro transcription of BPDE-I-modified pGAL, using the T7 RNA polymerase, revealed two prominent transcriptional stop sites, one in the first exon of the aprt gene. The second transcriptional stop site was determined to be located about 150 bp upstream from the translational start site. This

latter region was shown to contain two perfect GC-box consensus sequences that are potential Spl binding sites. Using a specific laser cutting technique to map BPDE-I DNA binding sites in the 5' flanking region of the aprt gene, the DNA region containing the GC-box consensus sequences was shown to be a hot spot for BPDE-I modification (117).

Changes in Cellular Macromolecules and in Cell Functions: The types of research activities in this subject area include studies on alterations in the composition and amounts of various proteins and small molecules, and changes in the pattern of DNA methylation in cells induced by carcinogens to the preneoplastic or neoplastic state. Biochemical and immunochemical methods have been used to isolate, identify and characterize nonhistone chromosomal proteins, phosphoproteins and cytosolic proteins which are either altered or specifically appear in chemically induced hepatocarcinogenesis models. Neoplastic cells have been shown to manifest a variety of morphological and biochemical phenotypes, different from their normal cell counterparts. This generation of heterogeneity and phenotypic instability in cancer is presumed to be due, in part, to changes in the control of gene expression during the transformation of normal cells to neoplastic cells. One possible manner in which the derepression and repression of genes could occur is by alterations in nuclear DNA-nuclear protein complexes. There is also much evidence showing that the state of DNA methylation regulates gene expression and is also involved in the control of cell differentiation. Thus, a greater understanding of the effects of chemical carcinogens and other oncogenic agents on production of aberrant DNA methylation patterns during carcinogenesis is warranted. Several studies are being supported which seek to define the role of altered chromosomal protein-DNA complexes in carcinogenesis and to understand the role of DNA methylation in the control of gene expression and carcinogenesis. Some of the latter studies are focused on elucidating the properties and regulation of DNA methyltransferase, the enzyme responsible for the postreplication methylation of cytosine residues in DNA. Other studies are focused on the state of methylation of specific DNA sequences or genes as a result of carcinogen exposure. The biological effects of DNA hypomethylation, i.e., altered cell differentiation or induction of cell transformation, are being studied by using compounds such as 5-azacytidine, which are known to affect the transfer of methyl groups to DNA.

Approximately 3 to 6% of cytosine residues in the DNA of all vertebrates is modified to 5-methylcytosine, which is predominately found in the dinucleoside sequence 5-CpG. Methylation patterns appear to be tissue-specific and the hypomethylation of many genes is correlated with their active expression. For example, the inhibition of genomic methylation by 5-azacytidine can result in the reactivation of genes on the transcriptionally inactive X chromosome, the induction of tissue-specific gene expression and the expression of differentiated phenotypes in cultured cells. Hypermethylation of gene sequences has been shown to preclude gene expression. Despite such correlations, the relationship between methylation patterns and expression of some genes remains unclear and it is not known how changes in the methylation patterns of certain genes alter expression, while the activities of other genes remain unaffected. Recent studies have demonstrated that cytosine methylation may directly influence the binding of regulatory or transcriptional factors to promoter regions of some, but not all genes. Alternatively, the inhibitory effect of DNA methylation may be mediated directly by protein-DNA interactions which render chromatin inactive. Since DNA methylation is only one part of a multifactorial mechanism for the regulation of eukaryotic genes, and a large number of 5-methylcytosine residues are presumably not involved in the direct control of gene expression, the function of the excess methylation

may quite possibly be unrelated to gene control. In order to assess the influence of methylation on gene expression and other functional roles of 5-methylcytosine residues, three clonal lines with extremely low DNA methylation levels were derived by multiple consecutive treatments of C3H 10T½ Cl8 cells with 5-aza-2'-deoxycytidine (5-aza-CdR). The methylation status of genes in the three methyl-deficient clones was examined to assess the specificity of the induced hypomethylation. Complete demethylation of virtually all 5'-CCGG-3' sites was observed in four genes examined, but some sites common to all three clones were found to be persistently methylated even after further exhaustive 5-aza-CdR treatment. Thus, cells have a subset of methylation sites which can never be stably demethylated. The extensive demethylation of cell DNA was not always associated with changes in the level of RNA expression in the genes examined, but was found to be strongly correlated with an altered chromatin structure of the unexpressed α_1 -globin gene and the muscle determination gene, MyoD1. The latter was determined by nuclease assay. The data obtained provide circumstantial evidence that DNA methylation inhibits gene expression by inducing condensation of chromatin structure. It remains unclear whether DNA methylation is a cause or an effect of chromatin compaction. However, the results strongly suggest that alterations in DNA methylation are associated with changes in the interactions between DNA and nuclear proteins and that these structural alterations may indirectly contribute to the regulation of gene expression (91).

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.

O^2 -Alkylpyrimidines are known to be formed in DNA treated in vivo with carcinogenic N-nitroso alkylating agents. The biological significance of deoxythymidine alkylation is not yet clear, although in vitro experiments indicate that it may act similar to the coding found for O^4 -alkyl-T or -U. O^2 -Methyl-T and O^4 -methyl-T in poly d(A-T) have been shown on replication with Pol I to lead to A:T to G:C transitions. Mutagenesis studies have not yet been performed with larger analogues. The amount of O^2 -ethyl-T formed in alkylated DNA has been shown to be 50 to 100 times that of O^2 -methyl-T and to be similar to the amount of O^6 -ethyl-G or O^6 -methyl-G, a well-known promutagen. The O^2 -alkyl-T lesion has been shown to be relatively persistent and may have biological significance since ethylation of T in DNA leads to A:T to G:C transitions. No differentiation has yet been made between the mutagenic contribution of O^2 - compared to O^4 -ethyl-T. The effect of alkyl group size on the ability to act as dTTP was studied for the carcinogen products O^2 -methyl-, O^2 -ethyl- and O^2 -isopropyl-dTTP by using three types of nucleic acids as templates and DNA polymerase I (Pol I) or Klenow fragment as the polymerizing enzyme. Previous kinetic studies of the same series of O^2 -alkyl-dTTPs indicated that they could all be incorporated by polymerases in the replication of DNA. Apparent Km and Vmax values were determined on primer extension of M13 DNA at a single defined site, in poly d(A-T) and in nicked DNA. These data were used for the calculation of the relative rate of insertion opposite A, relative to dTTP. The insertion rate for any O^2 -alkyl-dTTP was shown to be much

higher than for a mismatch between unmodified dNTPs. Unexpectedly, it was found that O²-isopropyl-dTTP was more efficiently utilized than O²-methyl-dTTP or O²-ethyl-dTTP on each of the templates. O²-isopropyl-dTTP substituted for dTTP over extended times of DNA synthesis at a rate only slightly lower than that of dTTP. Parallel experiments with O⁴-methyl-dTTP under the same conditions showed that it was incorporated opposite A more frequently than was O²-methyl-dTTP. Therefore, both the ring position and size of the alkyl group was shown to influence polymerase recognition. Once formed, all O²-alkyl-T:A termini were shown to permit elongation, as did O⁴-methyl-T:A. In contrast to the relative difficulty of incorporating the O-alkyl-dTTPs, formation of the following normal C:G base pair occurred rapidly when dGTP was present. This indicated that a single O-alkyl-T:A pair does not confer significant structural distortion recognized by Pol I (172).

Genomic heterogeneity is a hallmark of HIV-1. Studies on sequential HIV-1 isolates from persistently infected individuals suggest that HIV-1 evolves at a rate approximately a million times as great as that of eukaryotic genomes. This hypermutability could be central to the pathogenesis of HIV-1 and could thwart efforts to develop effective vaccines. The high rate of HIV-1 mutagenesis is known to be shared by other retroviruses and presumably originates in mechanisms unique to the retroviral life cycle. Replication of retroviral genomes has been shown to proceed by a series of enzymatic reactions involving virus-coded reverse transcriptase and integrase as well as host cell-coded DNA polymerases and RNA polymerase II. The high error rate demonstrated by avian myeloblastosis virus reverse transcriptase in vitro, with both deoxyribonucleotide and ribonucleotide templates, implicates reverse transcriptases as major contributors to retroviral mutagenesis. In one study, three different methods were used to evaluate the fidelity of DNA synthesis by HIV-1 reverse transcriptase. From these studies, it was shown that HIV-1 reverse transcriptase introduced base-substitution errors in DNA from the bacteriophage phiX174 amber 3 at estimated frequencies of 1/2000 to 1/4000. Analyses of misincorporation rates opposite a single template A residue showed that HIV-1 reverse transcriptase catalyzed nucleotide mismatches with a specificity of A:C >> A:G > A:A. The high error rate of HIV-1 reverse transcriptase in vitro translates to about 5 to 10 errors per HIV-1 genome per round of replication in vivo. The high error rate suggests that misincorporation by HIV-1 reverse transcriptase is, at least in part, responsible for the hypermutability of the AIDS virus. A detailed analysis of misincorporation by HIV-1 reverse transcriptase and host cell DNA polymerases should yield insights that could guide the design of antiviral nucleoside analogs that are preferentially incorporated by HIV-1 reverse transcriptase (115).

The principal enzyme involved in the replicative process in animal cells is thought to be DNA polymerase-alpha. A four-subunit DNA polymerase complex has been purified by immunoaffinity chromatography from calf thymus and other cells. The DNA polymerase-alpha activity was shown to be associated with a polypeptide whose molecular size ranges between 140 and 180 kDa. DNA primase activity was associated with subunits of 50 and 60 kDa. The purified DNA polymerase-alpha was shown to exhibit many properties during catalysis in vitro that would be expected of an enzyme involved in DNA replication. The fidelity of the DNA polymerase-alpha-primase complex from calf thymus has been analyzed by measuring mutagenesis in vitro and by site-specific nucleotide misinsertion and misrepair extension. Using the phiX174 am 3 DNA reversion assay, errors were detected at the amber 3 site only when dATP and dCTP were significantly biased during in vitro copying reactions. Measurements of misinsertion rates opposite template A showed that the

rates of dAMP or dCMP misinsertion were similar and occur 40 - 50 times more rapidly than dGMP misinsertion. Nucleotide misinsertions to generate all 12 possible mispairs have been measured kinetically on phiX174 DNA templates that contained either A, C, G or T at position 587. The measured misinsertion frequencies were shown to range from 1/4000 to 1/10⁶ depending on the mispairs generated. Extension from all 12 different mispairs was examined by starting with oligonucleotide primers that contained different 3'-terminal mispairs. Rates of extension from mispairs was shown to be 10³ to 10⁶ times slower than from correctly paired bases. This lack of extension of misincorporated bases suggests the involvement of exonucleolytic proofreading to enable continued DNA synthesis and to guarantee the high fidelity of eukaryotic DNA replication (115).

Mechanisms of Mutagenesis and Genetic Damage: The projects that are being supported in this subject area seek to understand how mutations and DNA or chromosome damage are generated by carcinogenic chemicals. Specifically synthesized oligonucleotides of defined base sequences are being used to examine the molecular mechanism of base-pair substitution and frameshift mutagenesis. The base sequence specificities of the interactions of mutagens with oligonucleotides are being studied and correlated to their mutagenic activity in *E. coli*. Newer studies in this area have focused on the use of specific genes which will either be cloned into plasmids or are present in cellular DNA as targets for the mutagenic action of various chemical carcinogens. The c-Ha-ras oncogene was cloned into a plasmid, the lac gene was introduced into M13 phage DNA, and the dihydrofolate reductase gene in Chinese hamster ovary (CHO) cells are being used as target genes to assess the mutagenic action of chemicals such as benzo(a)pyrene diol epoxide (BPDE), N-acetoxy-2-acetylaminofluorene (N-AcO-AAF), and other aromatic amines. DNA sequence techniques and effects of lesions on DNA synthesis will be used to determine the mechanisms of mutagenesis.

In bacteria, benzo(a)pyrene diol epoxide (BPDE) has been shown to cause mainly G:C to T:A transversions and to a lesser extent A:T to T:A transversions. Single base insertions and deletions are also known to be induced, tending to occur at clusters of consecutive T:A or G:C base pairs. The analysis of mutational mechanisms in mammalian cells has been considered to be difficult due to the large genome size and the relative paucity of selectable markers. Despite the difficulties, it is desirable to obtain comparative mutagenesis data using endogenous mammalian genes located at natural chromosomal locations. In one laboratory the endogenous CHO gene encoding dihydrofolate reductase (*dhfr*) has been used as a model to study spontaneous, chemical and radiation-induced mutations in mammalian cells. Recessive *DHFR*⁻ mutants are readily selectable in a cell line that is hemizygous at this locus and probes for the region containing the 25-kb gene are available. In this system the types of mutations documented at this locus include point mutations, deletions, inversions and other gene disruptions. In one study CHO cells were mutagenized with BPDE and mutants at the *dhfr* locus were isolated. Of 15 mutants analyzed by Southern blotting, one contained a large deletion that spanned all six exons of the 25-kb *dhfr* gene, while the rest exhibited no detectable changes. Three of the putative point mutations were localized by the loss of a restriction site--a *SacI* site in exon III, an *MspI* site in exon III, and a *KpnI* site in exon VI. The affected regions in two of these mutants were cloned and sequenced. The *SacI*⁻ mutant was shown to be caused by a G:C to T:A transversion that resulted in an amber termination codon. A single C:G was shown to be deleted in the *MspI*⁻ mutant which resulted in a frameshift and a downstream ochre termination codon. On the basis of overlapping restriction site sequences, the *KpnI*⁻ mutant was deduced to be a splicing mutant involving the most 3' G in intron V.

RNA heteroduplex mapping was used to locate these and the other remaining 11 putative point mutations. Mismatched bases between riboprobes complementary to wild-type *dhfr* mRNA and mutant mRNA molecules were detected in 10 of the 14 mutants analyzed. These mutations were mapped to four of the six exons or exon splice sites. An unexpected result that was obtained was that many of the point mutants exhibited greatly reduced (approximately 10-fold) steady-state levels of *dhfr* mRNA (73).

In other laboratories, methods for the analysis of mutations induced in human or other mammalian cells at the DNA sequence level are being developed. The approaches used depend on the development of recombinant DNA shuttle vectors composed of the simian virus 40 (SV40) early region, the Epstein-Barr virus (EBV) *oriP* element or some other sequence that allows the plasmid to be replicated in human or mammalian cells, sequences derived from the bacterial plasmid, pBR322, which permits the plasmid to also replicate in *E. coli*, and some selectable target genes for mutant selection. The studies seek to determine the types of DNA sequence changes induced by chemical carcinogens or mutagens and to characterize host processes that determine the frequency or types of mutations induced specifically in mammalian cells.

In one laboratory an SV40-based shuttle vector pZ189, containing the bacterial suppressor tRNA target gene (*supF*), was used to investigate at the DNA sequence level the kinds of mutations induced when DNA containing covalently bound carcinogen residues replicates in human cells. The advantage of the *supF* gene as the target for mutation studies at the sequence level is stated to be its small size and the fact that it has been shown to be highly responsive to base changes. The human cell line 293 used as the eukaryotic host for replicating the plasmid was shown to offer the advantage of a background mutant frequency of 1.4×10^{-4} , which was considered to be low enough to allow one to observe an increase in mutant frequency induced by carcinogen treatment of the plasmids. Using this system, the number of covalently bound residues per plasmid was determined in an experiment in which a plasmid was treated with radiolabeled PAHs. The progeny plasmids were rescued and assayed for the frequency of *supF* mutants by being used to transform indicator bacteria carrying an amber mutation in the beta-galactosidase gene. The agents tested in this study were BPDE, 1-nitrosopyrene (1-NOP), N-AcO-AAF and its trifluoro derivative (N-AcO-F₃-AAF) which yields deacetylated adducts. With each agent a linear increase in the frequency of *supF* mutants as a function of the number of DNA adducts formed was demonstrated, reaching frequencies as high as 20×10^{-4} to 40×10^{-4} . When compared on the basis of adducts formed per plasmid, BPDE, which forms its principal DNA adduct at the N² position of guanine, was shown to be about 4 times more mutagenic than 1-NOP, NAcO-AAF and N-AcO-F₃-AAF, which bind principally or exclusively to the C8 position of guanine. This was thought to be due to the approximate 3-fold greater rate of removal of 1-NOP versus BPDE adducts by the excision repair system. Agarose gel electrophoresis and DNA sequencing analysis of 35 mutants derived from untreated plasmids showed that the majority (70%) involved deletions, insertions, or gross rearrangements. In contrast, the majority of those derived from carcinogen-treated plasmids were shown to be base substitutions. DNA sequencing of 86 independent mutants derived from BPDE-treated plasmids and 60 from 1-NOP-treated plasmids showed that 60% and 80%, respectively, contained a single base substitution, 5 - 10% had two base substitutions, and 4 - 10% had small insertions or deletions of one or two base pairs. The majority (84 - 86%) of the base substitutions in mutants from BPDE- or 1-NOP-treated plasmids were shown to be transversions, with 73% of these being G:C to T:A. BPDE and 1-NOP were shown to produce their own spectrum of mutations in

that of the 7 hot spots for base substitution mutations produced with BPDE; two were the same as seen with 1-NOP-treated plasmids. However, 4 of the other hot spots were cold spots for 1-NOP-treated plasmids. Conversely, the 3 other hot spots seen with 1-NOP-treated plasmids were shown to be cold spots for BPDE-treated plasmids. The mutational spectrum produced by AF and AAF adducts is still being determined. Preliminary indications are that the location of the mutational hot spots induced by AF adducts corresponds to at least one of those found with 1-NOP, but also includes a unique position (119).

In another laboratory the system used is a retroviral shuttle vector into which the target gene for mutagenesis, the *E. coli* *gpt* gene which codes for the enzyme xanthine-guanine phosphoribosyltransferase (GPT), is incorporated. This retroviral vector, pZipNeoSV(X)1, was introduced into murine A9 cells, which lack activity of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT), a mammalian enzyme that is similar in function to GPT. In HGPRT-deficient cells, transformants that produce functional GPT can be selected in HAT medium and those that have lost the ability to produce functional GPT can be selected with 6-thioguanine. In one study, mutant cell lines containing *gpt* genes with single base changes were used to study the mechanisms of mutagen-induced reversion after exposure of the mutant lines to ethyl methanesulfonate (EMS). EMS did not increase the frequency of revertants for cell lines with mutant *gpt* genes carrying G:C to A:T transitions or A:T to T:A transversions, whereas it increased the frequency 50-fold to greater than 800-fold for cell lines with mutant *gpt* genes carrying A:T to G:C transitions and for one cell line with a G:C to C:G transversion. The *gpt* genes of 15 independent revertants derived from the EMS-revertable cell lines were recovered and sequenced. All revertants derived from cell lines with A:T to G:C transitions was shown to have mutated back to the wild-type *gpt* sequence via G:C to A:T transitions at their original sites of mutation. Five of six revertants derived from the cell line carrying a *gpt* gene with a G:C to C:G transversion had mutated via G:C to A:T transition at the site of the original mutation or at the adjacent base in the same triplet; these changes generated non-wild-type DNA sequences that code for non-wild-type amino acids that are apparently compatible with GPT activity. The sixth revertant was shown to have mutated via C:G to G:C transversion back to the wild-type sequence. The results of this study define certain amino acid substitutions in the GPT polypeptide that are compatible with enzyme activity. The results obtained also establish mutagen-induced reversion analysis as a sensitive and specific assay for mutagenesis in mammalian cells (36).

Role of DNA Repair in Carcinogenesis: The types of projects in this subject area include studies on the characterization of DNA damage produced by bulky chemical carcinogens, alkylating agents, ultraviolet light and ionizing radiation; the isolation and characterization of proteins responsible for DNA nucleotide excision repair and base excision repair; the cloning and characterization of the DNA nucleotide and base excision repair genes; and the determination of the role of chromosome structure, location and site of DNA lesions and poly(ADP-ribosylation) in the repair of DNA damage. A variety of rodent, frog, yeast, bacterial and normal and repair-deficient human cells are being used in these studies. Since DNA modification by chemical carcinogens has been shown to lead to mutations or other alterations in gene expression, DNA repair may have evolved as a defense mechanism to eliminate such damage and thus restore the correct genetic information and DNA structure. The significance of this process to human well being was emphasized by the demonstration that, in contrast to normal cells, cells from individuals with xeroderma pigmentosum, a hereditary genetic disease, did not

remove UV radiation-induced pyrimidine dimers from their DNA. These individuals develop multiple skin cancers at an early age only on exposed surfaces, thus establishing a link between genetic damage, defective repair of the damage, and cancer in the exposed tissue.

Defects in DNA repair deficient mutants isolated from established rodent cell lines have been shown to be genetically complemented by human genes using DNA-mediated gene transfer or cell fusion. The first human repair gene (ERCC-1) was cloned by a Dutch group using DNA-mediated gene transfer to the UV-sensitive Chinese hamster ovary (CHO) cell line 45-3B and shown to abolish most of the DNA repair deficiency in this and other mutants belonging to complementation group 1. Using this system, the pattern of preferential DNA repair of UV-induced pyrimidine dimers was studied in the laboratory of an OIG awardee. Repair efficiency was measured in the active dihydrofolate reductase (dhfr) gene and in its flanking, non-transcribed sequences in three cell lines, wild type CHO cells, a UV-sensitive excision deficient CHO mutant, and the transfected line of the mutant carrying the expressed ERCC-1 gene. In all normal mammalian cells studied so far, evidence was found for preferential repair of active genes. It was deemed important to examine whether this function had been restored in mutants transfected with repair genes. The results showed that the CHO cells transformed with the ERCC-1 gene repaired the active dhfr gene much more efficiently than the non-transcribed sequences, a pattern similar to that seen in wild-type CHO cells. This pattern differs from that previously reported in CHO cells transfected with the denV gene of bacteriophage T4, in which both active and non-transcribed DNA sequences were efficiently repaired. The ERCC-1 gene is said to code for an enzyme roughly twice the size of the denV product, T4 endo V, which has an estimated MW of 32 kDa. Given that the preferential DNA repair of the dhfr gene in CHO cells has been shown to be largely due to the selective repair of the transcribed strand, the function of the ERCC-1 gene product is thought to be to locate the active genes in the genome for the repair complex based on a specific recognition rather than just chromatin accessibility (76).

Previous studies have shown that cell specificity exists for the alkylation of DNA from lung cells following treatment of rats with the tobacco-specific carcinogen 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The concentration of the promutagenic adduct O⁶-methylguanine (O⁶MG) was found to be greatest in Clara cells followed by macrophages, type II cells and alveolar small cells. One major pathway for the removal of alkylation damage is the removal of O⁶-alkylguanine adducts by a protein called O⁶-methylguanine-DNA methyltransferase (O⁶MGMT), which transfers the alkyl group to a cysteine acceptor site present on the enzyme and restores the DNA structure in a single step. The cysteine site is not regenerated and thus new protein must be generated prior to removal of another O⁶-alkyl adduct. The rate of resynthesis of this protein may thus be a critical factor in the ability of a tissue to repair promutagenic damage prior to cell replication. O⁶MGMT activity has been shown to vary quite dramatically among tissues and cell specificity has been shown to exist in the liver where enzyme activity is much greater in hepatocytes than in non-parenchymal cells. There is, at present, little information concerning the distribution of pathways involved in either activation of chemical carcinogens or repair of DNA adducts in rat lung, an organ which contains greater than 40 different cell types and is a major target tissue for exposure to environmental chemicals. In previous studies with lungs from rabbits, the distribution of cytochrome P-450 isozymes and monooxygenase activity was shown to be greatest in Clara cells but O⁶MGMT activity was undetectable in those cells while present in type II cells and alveolar macrophages. The purpose

of one study was to measure the activity of the repair protein O⁶MGMT and to determine whether differences exist for the removal of O⁶MG among pulmonary cell types. Constitutive activity of O⁶MGMT was shown to be 2-fold greater in macrophages and type II cells than in alveolar small cells and Clara cells. NNK treatment had no effect on O⁶MGMT activity in macrophages, but was shown to decrease activity in alveolar small cells and type II cells by 57 and 84%, respectively. O⁶MGMT activity was reduced to below limits of detection of Clara cells. The effect of NNK on O⁶MGMT activity was consistent with the rates of removal of O⁶MG in macrophages and Clara cells. The results obtained indicate that the activity of O⁶MGMT and the rate of resynthesis of this repair enzyme differ considerably among pulmonary cells following methylation of DNA. The high concentration of O⁶MG in Clara cells and the low rate of repair of this promutagenic lesion may be critical factors in the potent pulmonary carcinogenicity induced by the tobacco-specific carcinogen NNK (142).

Bloom's syndrome is an autosomal recessive human genetic disease that clinically presents low body weight at birth, stunted growth, cutaneous rash and immunological deficiency. Individuals with Bloom's syndrome are predisposed to infection and are cancer-prone. Currently, individuals with this disease cannot be readily identified prior to the appearance of clinical symptoms. Bloom's syndrome cells are characterized by their high rates of chromosome aberration, spontaneous hypermutability, hypersensitivity to environmental agents and a unique series of temporal alterations in the proliferation-dependent regulation of DNA repair. An early finding raised the possibility that the uracil DNA glycosylase, an enzyme which excises uracil from DNA during base-excision repair, in Bloom's syndrome could be potentially characterized by a unique reaction to a monoclonal antibody raised against the uracil DNA glycosylase of normal human placenta. Three such monoclonal antibodies were raised and tested in one laboratory to determine whether one could be used as a negative marker for Bloom's syndrome. As defined by enzyme-linked immunosorbent assay (ELISA), monoclonal antibody 40.10.09, which reacted with normal human glycosylase, was shown to neither recognize nor inhibit native uracil DNA glycosylase from any of five separate Bloom's syndrome cell strains. Immunoblot analysis demonstrated that the denatured glycosylase protein was immunoreactive with the 40.10.09 antibody. Each native enzyme was shown to be immunoreactive with two other anti-human placental uracil DNA glycosylase monoclonal antibodies. In contrast, ELISA reactivity was observed with all three monoclonal antibodies in reactions of glycosylases from five normal human cell types and 13 abnormal human cell strains. These results verified the specificity of the aberrant reactivity of the Bloom's syndrome uracil DNA glycosylase. The results obtained raise the possibility that determination of the lack of immunoreactivity with antibody 40.10.09 may have value in the early diagnosis of Bloom's syndrome (174).

Individuals homozygous for xeroderma pigmentosum (XP) are hypersensitive to UV radiation and have a high incidence of skin cancers. XP cells are known to be defective in the repair of damaged DNA containing UV-induced pyrimidine dimers. So far, the molecular basis for the XP defect has remained undefined. Fusion of cells from different patients have defined nine genetic complementation groups (A through I), which implies that DNA repair in humans involves multiple gene products. In one laboratory, the gel electrophoresis binding assay was extended to the identification of proteins that bind to damaged DNA rather than to specific DNA sequences. At least one nuclear factor was identified that bound to DNA damaged by UV radiation or the antitumor drug cisplatin. It was shown to be absent in cells from complementation group E. From the data obtained, it appeared

that the factor may participate in a versatile DNA repair pathway at the stage of binding and recognition (27).

Genetics and Mechanisms of Cell Transformation: In the subject area of genetics and mechanisms of cell transformation are studies designed to test the somatic cell mutation hypothesis of cell transformation and to attempt to identify those specific genes which are responsible or have an influence on cell transformation. There is a large body of data demonstrating a high correlation between the mutagenicity and carcinogenicity of various chemicals. This evidence supports the hypothesis that somatic mutations are involved in the process leading to neoplasia. Also, efforts are being made to develop animal models for hereditary cancer which would allow the examination of genetic mechanisms of carcinogenesis. A rat strain which develops hereditary renal carcinoma is being studied in one laboratory. This may yield a model analogous to human retinoblastoma and Wilms' tumor. In another laboratory, a mouse model for susceptibility to hepatocarcinogenesis is being developed. It has been shown that C3H/HeJ male mice are 20- to 50-fold more susceptible to the induction of liver tumors than are male C57BL/6J mice and that about 85% of this difference in susceptibility to liver tumor induction results from an allelic difference at a single locus, designated the hepatocarcinogen sensitivity locus (Hcs). Further studies have been planned to characterize the model and the susceptibility gene. In one subsequent study, the development of putatively preneoplastic hepatic lesions that are deficient in glucose-6-phosphatase (G6Pase) in mice treated at 12 days of age with N-ethyl-N-nitrosourea (ENU) was followed in order to determine whether the Hcs locus affected initiation or promotion of hepatocarcinogenesis. In ENU-treated male mice of both C3H/HeJ and C57BL/6J strains, the number and size of G6Pase-deficient hepatic foci was shown to increase over time between 12 and 24 weeks of age. However, the rate of growth was 1.7 times faster for the lesions in C3H/HeJ male mice. No significant difference between the growth rates of the foci in female C3H/HeJ and C57BL/6J mice were observed. The phenotypic effect of the Hcs locus thus appeared to be dependent on promotion of liver tumor induction by the male hormonal environment. The data obtained from ³H-thymidine labeling studies suggest that the Hcs locus may affect the promotion phase of hepatocarcinogenesis in male mice by increasing the proliferative rate of both normal and preneoplastic hepatocytes (145).

Breast cancer is a major health problem affecting approximately one in ten American women. While there are a number of suspected contributing factors, such as diet and hormone levels, genetic predisposition is known to play an important role in many cases. For this reason, it was deemed important to determine the mechanisms by which genes control susceptibility to mammary cancer. The use of inbred rat strains facilitates the study of genetic control of susceptibility to carcinogenesis. Previous studies in one laboratory indicated that inbred Wistar/Furth (WF) and Fischer 344 (F344) rats were comparable to outbred Sprague-Dawley and Long-Evans rats, respectively, in their susceptibilities to 7,12-dimethylbenz(a)-anthracene (DMBA)-induced mammary cancers. Genetic analyses indicated that the WF rat possessed multiple independently segregating dominant "enhancer" genes, each of which confers susceptibility to chemically-induced tumorigenesis. These genes were shown to be absent in the F344 rat. A second susceptibility model studied was the Copenhagen (Cop) rat, which was shown to be totally resistant to DMBA-induced and spontaneous mammary carcinogenesis. Cop rats have been shown to possess a single dominant autosomal "suppressor" gene, which confers complete resistance to mammary tumorigenesis. F344 rats apparently did not contain either of the gene types. The mechanisms of action and potential relationship of the mammary carcinogenesis enhancer and suppressor genes are currently unknown. Using a

mammary cell transplant system, both the WF enhancer and the Cop suppressor genes were found to be active in the mammary epithelial cells themselves. Since DMBA metabolism is a necessary first step in the activation of this carcinogen, this process has been studied in mammary epithelial cells in vitro. In order to determine whether the susceptibility-modifying genes present in the WF and Cop rats affect the early stages in DMBA-induced carcinogenesis, DMBA metabolism and DNA binding was examined in mammary epithelial cells isolated from WF, F344 and Cop rats. Quantitative analyses of DMBA metabolites and DMBA-DNA adducts showed that they were essentially identical. These data suggest that the genes controlling susceptibility and resistance to mammary carcinogenesis in these rat strains are likely to be active at later stages of the carcinogenic process. Studies are in progress to identify and characterize the mammary-specific enhancer and suppressor genes and their products. This information may suggest a specific role for these genes in controlling susceptibility to mammary carcinogenesis (68).

Several studies on the role of specific genes and gene products in chemically induced cell transformation have been initiated and are ongoing. Recombinant DNA, gene cloning, and DNA sequencing techniques have been employed in this research which has resulted in a veritable explosion of publications demonstrating the isolation and characterization of genes responsible for the transformation of cells to malignancy. To date, several different transforming genes have been isolated from different human tumor cells and their homology to various viral oncogenes has been established. Studies involving the identification of specific transforming genes or the activation of known, previously identified oncogenes are currently ongoing or have just been initiated in several laboratories. Various chemically induced animal or cell model systems are being utilized, which include rat hepatocellular, mouse thymic lymphoma, rat nasal carcinoma, skin carcinoma, mouse bladder carcinoma, human pancreas and in vitro hamster and human fibroblast and epithelial cell transformation systems. In other studies the mouse two-stage skin carcinogenesis model is being used to determine whether the altered expression of specific genes (several oncogenes, murine leukemia virus proviral sequences, and long terminal repeat sequences) coincides with particular stages of carcinogenesis and/or tumor development. The interaction of chemicals and viruses such as Epstein-Barr virus, murine mammary tumor virus and adenovirus type 5 are being examined by several laboratories to determine their role in carcinogenesis.

The majority of human tumor cells, and particularly those of mesenchymal origin, have the ability to grow in semisolid medium. This anchorage-independent growth phenotype is rarely observed in nonneoplastic human cells. Treatment of diploid human fibroblasts with an alkylating mutagen has been shown to induce stable, anchorage-independent cell populations at frequencies consistent with an activating mutation. Mutagen-induced acquisition of anchorage-independent growth in many aneuploid immortal rodent cell lines has been shown to correlate well with acquisition of tumorigenic growth, whereas in diploid primary human fibroblasts, mutagen treatment has been shown to induce anchorage-independent cell populations that do not yield progressively growing tumors. When neoplastic transformation is viewed in the context of a multiple-step transition from normal to neoplastic cells, mutagen-induced anchorage independence is likely to represent one of these discrete steps, where this acquired phenotype is related mechanistically to the final tumorigenic state. It was of interest in one laboratory to determine whether mutagen-induced soft agar growth could be assigned to an activating mutation at a specific human gene. For these studies, human foreskin fibroblasts were treated with benzo(a)pyrene diol epoxide (BPDE) and selected in soft agar. Seventeen different anchorage-independent clones were isolated and expanded, and their

cellular DNA was used to cotransfect NIH 3T3 cells along with pSV2neo. DNA from 11 of the 17 clones were shown to induce multiple NIH 3T3 cell tumors in recipient nude mice. Intact human Ha-ras sequences were observed in 2 of the 11 tumor groups, whereas no hybridization was detected when human Ki-ras or N-ras probes were used. The results from the direct sequence analysis of a part of the Ha-ras gene demonstrated that exposure of normal human cells to a common environmental mutagen yields Ha-ras G:C to T:A codon 12 transversions that have been commonly observed in human tumors. In addition, in the majority of the anchorage-independent human clones, an as yet to be identified gene or genes was shown to be responsible for the observed human cell anchorage-independence and associated NIH 3T3 cell transformation (48).

It was previously reported that rat nasal squamous cell carcinomas induced by inhalation of the direct-acting alkylating agents methylmethane sulfonate (MMS) and dimethylcarbonyl chloride (DMCC) appeared to contain significant differences in the transforming activity of their DNAs, suggesting carcinogen specificity in the activation of oncogenes. In a recent study, two novel oncogenes activated in rat nasal squamous cell carcinomas induced by MMS and beta-propiolactone (beta-PL) in a carcinogen-specific manner were detected. The DNAs containing activated oncogenes were shown to have no homology to any member of the ras family. The novel NIH 3T3 transforming oncogenes from tumors induced by beta-PL and MMS were shown to be distinct from each other based on restriction analysis. The gene isolated from beta-PL-induced tumors was shown to be between 6 and 9 kb in size. None of the tumors induced by DMCC contained positive DNA in the NIH 3T3 focus assay or in the nude mouse cotransfection assay. From these studies, it was concluded that the rat nasal tumor model is well suited for analysis of the roles of carcinogen and tissue specificity in oncogene activation, especially related to novel (non-ras) transforming oncogenes (63).

Mutations in cellular ras genes have been strongly implicated in various stages of mammalian tumorigenesis. In human tumors, the point mutations which have been identified have largely been localized to codons 12, 13 or 61 of either c-Ha-ras-1, c-Ki-ras-2 or N-ras genes. Although DNA transfection experiments utilizing NIH 3T3 mouse cells as recipients in a focus-forming assay have detected mutated ras oncogenes in 10 to 30% of "spontaneous" human tumors, recent analyses by more sensitive detection methods have demonstrated that as many as 40 to 50% of colon carcinomas, myeloid leukemias, and 80% of pancreatic carcinomas contain activating mutations at a single codon. In human colorectal carcinomas, mutations in codons 12, 13, and, more rarely, 61 of the c-Ki-ras-2 gene have been found in fresh tumors using the techniques of DNA amplification and oligonucleotide hybridization, as well as by RNase A mismatch cleavage analysis. Subsequent analyses have suggested that mutational activation of ras may be an early event in the temporal development of these lesions and may contribute directly to tumorigenic progression in colon carcinomas. The heterogenous mixture of cells present in human tumors often makes the quantitation of the frequency of ras mutations in these tumors and the assignment of the change in sequence to a particular nucleotide position difficult. In order to overcome these difficulties, the laboratory of an OIG recipient utilized a combination of techniques, including histological enrichment, purifying subpopulations of tumor cells by flow cytometric sorting of nuclei based upon the presence of an aneuploid DNA content, followed by direct sequencing of the desired gene fragment after amplification via the polymerase chain reaction. Using these procedures, point mutations have been identified in the c-Ki-ras gene in DNA extracted from as few as 1000 sorted nuclei from aneuploid and diploid subpopulations of a carcinoma. Mutations in codon 12 were shown to be

present in both aneuploid and diploid subpopulations of sorted carcinomas, suggesting that these mutations precede ploidy alterations in the progression of these neoplasms. The method has been further adapted for use with paraffin-embedded material after fixation in a variety of fixatives, including methyl carnoys, formalin and Hollande's solution. This procedure should allow one to retrospectively reconstruct the temporal relationship between the occurrence of mutations and sequential morphological changes during tumorigenic progression (115).

Another possible mechanism of cell transformation by chemicals could involve the induction of DNA sequence rearrangements, chromosome alterations, or the involvement of mitochondrial genes and metabolism or specific proteases. These changes could result in the altered cell growth and other properties characteristic of transformed cells. In one study the occurrence of DNA sequence rearrangements during hepatocarcinogenesis in rats and whether such rearrangements involve transforming genes is being examined using gene cloning, restriction endonuclease analysis and DNA transfection technologies. The success in developing a reliable method for obtaining chromosome preparations from fresh solid tumors has allowed a study to determine whether chromosomal alterations are mechanistically related to the origin and/or progression of chemically induced mouse skin tumors. In an initial study, it has been shown that aneuploidy is an early event in mouse skin tumor development. In another project the role of DNA recombinational events, free radical intermediates, cell growth modification, patterns of cell differentiation, and the induction of specific proteases is being examined in the mouse embryo C3H 10T1/2 cell line and in a human diploid cell line transformed by chemicals and radiation.

Amphibole asbestos is a major cause of malignant mesothelioma in humans. In spite of widespread interest and considerable research, its mechanism of carcinogenesis remains obscure. The clastogenic effects of asbestos on cultured cells have been investigated by several investigators. Exposure of cells in vitro to asbestos was found to increase the number of transformation foci and the frequency of binucleate cells. Tetraploid and aneuploid cells were also commonly observed in cultures exposed to asbestos. In one laboratory a comprehensive study of mesotheliomas induced in rats by crocidolite or chrysotile asbestos was undertaken in order to study the significance of the observed chromosomal anomalies and their relationship to asbestos tumorigenesis. Cytogenetic analysis was conducted on cells of 15 asbestos-induced rat mesotheliomas. The tumor cells were shown to be diploid, triploid or tetraploid. All tumor lines exhibited aneuploidy and marker chromosomes. The loss of at least one copy of the X chromosome was observed in each of the tumors analyzed, and loss of copies of chromosomes 8, 16, 20 or 18 was shown to characterize at least six of the tumors. Translocations were observed in 12 tumors, with six chromosome rearrangements present in at least two different tumors, but with nonidentical breakpoints. However, translocations involving chromosomes 5, 10 and 13 were shown to exhibit repeated breakage at the same loci. Such specific and repetitive translocations may be involved in the process of asbestos-induced tumor development (35).

Another focus of projects in this subject area are studies designed to test the cell cycle specificity of the induction of cytotoxicity, mutagenesis and neoplastic transformation by chemical carcinogens. Also, the quantitative relationship between the level, persistence and species of carcinogen-nucleotide adducts and the concomitant cell transformation frequency are being determined. There is a substantial amount of information supporting the hypothesis of cell cycle

specificity of carcinogenesis. It has been shown that in mouse embryo C3H10T1/2 cells, G₁ and S phase cells are susceptible to cytotoxicity and mutation, while only S phase cells (in synchronized cultures) are susceptible to neoplastic transformation by exposure to alkylating agents. In adult rat liver, the hepatocytes are generally resistant to carcinogenesis by a single exposure to agents capable of inducing cancer in other tissues. Hepatocyte susceptibility to carcinogenesis is increased by certain treatments which stimulate the proliferation of carcinogen-damaged cells. Additional work is in progress to determine more specifically in rat liver the phase of the cell cycle which is most susceptible to the initiating effect of carcinogenic chemicals.

In one study the temporal order of replication of several genes was determined in C3H 10T1/2 cells synchronized by release from confluence-induced arrest of proliferation followed by treatment with aphidicolin. Replicating DNA was labeled with bromodeoxyuridine for subsequent separation from bulk DNA. Using ³²P-labeled probes Ha-ras was shown to be among the first genes replicated at the onset of the S phase. The myc proto-oncogene was shown to be replicated later but within the first hour of the S phase. The replication of Ki-ras, raf, and mos was detected between hour 1 and 2 of the S phase. The dihydrofolate reductase gene was shown to replicate early (0 - 2 hr) and the myb proto-oncogene replicated in mid-S phase (2 - 4 hr). An immunoglobulin V_H sequence and the beta-globin gene were shown to replicate late in C3H 10T1/2 cells, 4 - 6 hr after removal of aphidicolin. Replicating DNA has been shown to be preferentially adducted by chemical carcinogens, and replication of damaged proto-oncogenes before they are repaired may activate their transforming potential. The observed replication of proto-oncogenes during the early S phase may thus underlie the enhanced sensitivity of C3H 10T1/2 cells to chemically induced transformation at this point in the cell cycle (95).

Many lines of evidence suggest that the oncogene c-myc regulates cellular proliferation and plays an important role in the malignant transformation of cells. C-myc is an inducible gene that has been shown to be transiently induced when quiescent fibroblasts are stimulated with mitogens that initiate DNA synthesis. Several different observations have supported the concept that the rise in c-myc expression which occurs before the onset of DNA synthesis is causally related to the progression of cells through the cell cycle. It is believed by many investigators that c-myc acts as a competence factor in stimulating cells to proliferate. However, in one laboratory, it was observed that c-myc RNA levels do not rise in stimulated cycling C3H 10T1/2 and 3T3 cells that have been grown to confluence in the presence of the protease inhibitor antipain. Cells were shown to continue to progress from the G₀/G₁ phase to S phase of the cell cycle in the absence of an increase in c-myc RNA. The kinetics of ³H-thymidine incorporation after serum stimulation were comparable to those observed in cells in which c-myc RNA levels rise after serum stimulation. The observations made were considered to be significant because they suggest a dissociation between the transient rise of c-myc RNA which occurs before DNA synthesis and the events that initiate DNA synthesis in quiescent fibroblasts. The results suggest that c-myc may not play as central a role in stimulating noncycling, quiescent cells to progress through the cell cycle as has been generally assumed (97).

Role of Oxygen Radicals in Carcinogenesis: This new subcategory area of research was recently created in order to better highlight progress in this subject area. This is due to increased attention and interest by investigators on studies on the role of oxygen radicals and other oxidation reaction products on tumor initiation, cell transformation and tumor promotion. The types of research included in this

subject area include studies on the identification of oxidative damage in DNA and the mechanisms by which oxygen radicals generate mutations in DNA and activate transforming genes in cells. The hypothesis that the phorbol ester, TPA, and carcinogenic metals, such as Cd, may exert their effects through the generation of oxygen radicals will be tested in one project. In terms of cancer initiation, studies are ongoing that focus on the examination of the role of oxygen radicals in the induction of renal tumorigenesis by estrogens and in the induction of liver carcinogenesis by peroxisome proliferating agents and choline devoid diets. Studies are also being supported which focus on the hypothesis that the promoting activity of tumor promoters involves the formation of active oxygen species and subsequently, oxidized bases in DNA. The role of the arachidonic acid pathway, the participation of inflammatory cells and the formation of polyunsaturated fatty acid peroxides in tumor promotion in mouse skin and in cells in culture are also being investigated.

Reactive oxygen species such as the superoxide radical, hydrogen peroxide and the hydroxyl radical are formed in vivo during aerobic metabolism as well as during radiation exposure. Although cells have developed various enzymatic and nonenzymatic systems for controlling excited oxygen species, a certain fraction escapes the cellular defense and may cause permanent or transient damage to proteins, lipids and nucleic acids. Oxidative damage has been suggested to contribute to aging and a host of diseases including cancer, chronic inflammation, ischemia and autoimmune diseases. The critical targets that may be affected by excited oxygen during aging and in diseases have not yet been identified with certainty. Much attention, however, has been given to oxidative DNA damage, especially in relation to aging and cancer. A high rate of oxidative damage to mammalian DNA has been previously demonstrated in the laboratory of an OIG recipient by measuring oxidized DNA bases excreted in urine after DNA repair. The rate of oxidative DNA damage was shown to be directly related to the metabolic rate and inversely related to life span of the organisms. This work did not distinguish between damage to nuclear and mitochondrial DNA (mtDNA). For one study the oxidized base, 8-hydroxydeoxyguanosine (8-OHdG), one of about 20 known radiation damage products, was assayed in the DNA of rat liver. 8-OHdG was shown to be present at a level of 1 per 130,000 bases in nuclear DNA and 1 per 8,000 bases in mtDNA. Mitochondria treated with various prooxidants were shown to have an increased level of 8-OHdG. The high level of 8-OHdG in mtDNA was thought to be caused by the immense oxygen metabolism, relatively inefficient DNA repair and the absence of histones in mitochondria. This may also be responsible for the observed high mutation rate of mtDNA (3).

Peroxisome proliferators are chemicals which induce hepatomegaly and peroxisome proliferation in liver cells. These include structurally diverse compounds such as the lipid lowering drugs clofibrate, aprofibrate and gemfibrozil and an industrial phthalate ester plasticizer. Long-term administration of these chemicals to rats and mice results in the development of hepatocellular carcinomas. The mechanism by which nongenotoxic peroxisome proliferators induce hepatocellular carcinomas in rats and mice remains intriguing. The available experimental evidence suggests that the proliferation of peroxisomes and induction of peroxisome-associated enzymes results in oxidative stress which then leads to tumorigenesis. However, so far no direct evidence for oxidative DNA damage in livers of peroxisome proliferator-treated animals has been established. In one study to obtain such evidence, the DNA obtained from the livers of rats treated with ciprofibrate was examined for variable periods of time for 8-OHdG. Administration of ciprofibrate in the diet for 16, 28, 36 or 40 weeks was shown to result in progressively

significant increases in the levels of 8-OHdG. This increase in 8-OHdG was attributed to persistent peroxisome proliferation resulting from chronic ciprofibrate treatment as no increase in 8-OHdG was observed in liver DNA of rats that received a single dose of ciprofibrate. The results of this study clearly demonstrated, for the first time, that the persistent proliferation of peroxisomes leads to specific oxidative DNA damage (148).

Benzene is strongly suspected of being an animal and human carcinogen, but the mechanisms by which benzene induces tumors of lymphoid and hematopoietic organs are unknown. Binding studies *in vivo* have shown a very low level of covalent binding to the DNA of bone marrow elements. Since several metabolites of benzene have the potential to autooxidize and thereby generate reactive oxygen intermediates, the hypothesis that benzene metabolites can induce DNA damage through the generation of oxygen radicals was tested in one laboratory. Hydroquinone (HQ), benzoquinone (BQ), catechol and 1,2,4-benzenetriol (BT) were first tested for their ability to generate superoxide at a physiological pH. BT, and, to a lesser extent HQ, were autooxidized and shown to produce significant quantities of superoxide. No detectable superoxide was produced by catechol or BQ. Similarly, BT was shown to be very efficient at degrading DNA, and this degradation was inhibited by scavengers of superoxide, H_2O_2 , and hydroxyl radical. HQ was shown to induce single- and double-strand breaks but to not degrade DNA. In contrast to the action of BT, the breakage of DNA by HQ was not inhibited by scavengers of reactive oxygen intermediates. The metabolites which did not produce superoxide (catechol and BQ) did not induce significant breakage of DNA. The data obtained support the hypothesis that certain benzene metabolites can induce DNA damage through the production of oxygen radicals. The data also suggest that other metabolites may act via another mechanism to damage DNA (110).

A substantial amount of evidence has been accumulating in the last few years suggesting that the generation of free radicals, such as superoxide anion and hydroxyl radical, may be involved in the tumor promotion stage of multistage skin carcinogenesis. The most direct evidence for free radical involvement comes from studies in which free radical generating compounds, such as benzoyl peroxide, were shown to be complete tumor promoters. Indirect evidence comes from studies in which various antioxidants were shown to be inhibitors of TPA-induced tumor promotion. A chemiluminescence assay was recently developed for measuring TPA-induced and phospholipase C-induced oxidant generation in mouse epidermal cells. The relationship of this response to tumor promotion is suggested by the findings that most classes of known inhibitors of TPA promotion also inhibit this response. In addition, a comparison between SSIN (inbred SENCAR) and C57BL/6J mice showed that the extent of the oxidant response was found to correlate with the degree of sensitivity to TPA as a tumor promoter. Since C57BL/6J mice had been previously shown to be essentially refractory to TPA as a promoter, although sensitive to benzoyl peroxide promotion, it was hypothesized that while the C57BL/6J mice are able to respond to free radical generating agents, the diminished oxidant production in these mice may be the basis for the refractoriness to TPA promotion. Also, C57BL/6J mice were shown not to respond to topical application of TPA with the hyperplasia and inflammation observed in promotion-sensitive mice such as the SSIN.

Inflammatory cells are an abundant source of reactive oxygen intermediates in the body and release large quantities when exposed to phorbol esters. Both protein kinase C, the phorbol ester receptor, and the NADPH oxidase which generates reactive oxygen intermediates are known to be calcium and magnesium-dependent. The

requirements for calcium and magnesium of macrophages from mouse strains that are sensitive (SENCAR) and resistant (C57BL/6) to the promotion of tumors by phorbol esters were investigated in one laboratory. Macrophages from SENCAR mice were shown to require much lower levels of calcium or magnesium to mount a full respiratory burst, as measured by the release of H_2O_2 in response to phorbol esters, than macrophages from C57BL/6 mice. Conversely, when the particulate stimulus zymosan was used, little difference between the macrophages from the two strains was observed. The results obtained demonstrate that differences in sensitivity to divalent cations by macrophages from these two strains was selective for phorbol ester stimulation and that lower requirements for calcium and magnesium for reactive oxygen intermediate release correlated with sensitivity to the promotion of tumors by phorbol esters. The molecular basis for differences in macrophages from SENCAR mice for the release of reactive oxygen intermediates during exposure to phorbol esters remains to be established. An attractive hypothesis being considered is that molecular differences in protein kinase C are responsible for differences in the respiratory burst between these strains of mice (110).

Properties and Mechanisms of Tumor Promotion: Research in this subject area involves projects designed to analyze the various cellular, biochemical and molecular activities and pleiotropic effects induced in cells upon exposure to tumor promoters. The phorbol ester tumor promoters are, by far, the most widely used compounds in these studies. They have been shown to exert their effects by binding to specific receptors on cell surface membranes. A number of grants support studies on the characterization of the phorbol ester receptor protein. The results of phorbol ester binding include alterations in membrane phospholipid metabolism, membrane structure and function, alterations in the transport of small molecules, the activation of macromolecular synthesis, the induction or inhibition of terminal cell differentiation by normal or neoplastic cells, the mimicry of the transformed phenotype by normal cells and the enhancement of transformation by chemicals and oncogenic viruses. Studies in the laboratory of a MERIT awardee are focused on the perturbation of ion fluxes by the tumor promoter, 12-O-tetradecanoylphorbol 13-acetate (TPA). Since the action of TPA may be mediated by the phosphorylation of proteins and lipids, several studies are focused on the purification and characterization of protein kinase C (PKC), a calcium and phospholipid-dependent protein kinase whose activity is stimulated by TPA. A characterization of the proteins phosphorylated by this enzyme is included in some of these studies. In addition, several laboratories are attempting to clone the genes for what has been shown to be a family of these enzymes. The activation of expression of certain genes is thought to occur during neoplastic progression of cells. The possible activation of oncogene sequences and other viral and cellular gene sequences by TPA and other promoters is the focus of several studies. Another property of tumor promoters is their apparent ability to disrupt cell-cell communication. The mechanism of this phenomenon is the focus of at least three studies, and systems exhibiting this phenomenon are being developed as indicators of potential tumor promoting agents.

Increasing evidence implicates membrane transport systems as important targets of tumor-promoting phorbol esters. There is evidence supporting the idea that binding of phorbol esters to the enzyme PKC is required for modulation of transport activity to occur. What is still not clear for any transport system are the precise biochemical mechanisms responsible for phorbol ester-induced modification of transport function. Recent studies from the laboratory of a MERIT awardee have suggested that phorbol esters may trigger changes in gene expression and cell proliferation in BALB/c 3T3 cells via modulation of a specific membrane ion

transport system, the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ -cotransporter. The experimental approach taken was to isolate, by positive selection from mutagenized cell populations, cloned cell lines that were defective in $\text{Na}^+/\text{K}^+/\text{Cl}^-$ -cotransport activity, and to compare the responses of parental cells and cotransport-defective cells to the phorbol ester TPA. This experimental system showed promise as a useful model in which to examine the relationship between early ionic signals elicited by phorbol esters and longer-term responses, such as gene expression and DNA synthesis. Even though the phorbol ester TPA is known to bind to and activate PKC, it is still not certain that all cellular responses to phorbol esters are necessarily mediated by PKC. In BALB/c 3T3 preadipose cells, TPA has previously been shown to rapidly inhibit $\text{Na}^+/\text{K}^+/\text{Cl}^-$ -cotransport activity, stimulate 2-deoxyglucose uptake and induce ornithine decarboxylase activity. The cell-permeable diacylglycerol sn-1,2-dioctanoylglycerol (DiC_8) was used in order to distinguish between PKC-dependent and -independent responses of BALB/c 3T3 cells. DiC_8 was shown to modulate $^{86}\text{Rb}^+$ fluxes in BALB/c 3T3 cells in the same manner as TPA, while in cotransport-defective cells no effect was observed. In contrast, stimulation of 2-deoxyglucose uptake by DiC_8 did not occur in either parental or cotransport-defective cell lines, even though TPA was a very effective inducer of this transport system in both cell types. The PKC-dependent phosphorylation of an acidic 80-kDa protein was shown to be stimulated by both TPA and DiC_8 in parental and cotransport-defective cell lines, suggesting that a gross defect in the primary effector system used by both TPA and diacylglycerols cannot explain the results seen. The results obtained in these experiments suggested that PKC was essential for some phorbol ester membrane transport responses (such as inhibition of $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransport), but do not allow a conclusion that other responses (such as stimulation of 2-deoxyglucose uptake) necessarily require PKC activation (135).

Several laboratories have recently isolated cDNA clones encoding distinct forms of PKC, thus demonstrating that PKC is encoded by a multigene family. The multiple forms of PKC have been shown to exhibit considerable tissue specificity, which suggests that there may be subtle differences in the functions of each of the distinct forms. In order to further elucidate the role of specific forms of PKC in growth control and tumor promotion, it was thought desirable to generate cell lines that stably produce elevated levels of one form of this enzyme. For this purpose a series of rat fibroblast cell lines that stably overexpress a full-length cDNA encoding the beta-1 form of PKC was generated in one laboratory. These cell lines were shown to contain a 20- to 53-fold increase in PKC activity and to exhibit dramatically enhanced morphologic changes following exposure to the tumor promoter TPA. They were shown to grow to a high saturation density in monolayer cultures and, when maintained at postconfluence, to develop small, dense foci. In contrast to control cells, which display complete anchorage dependence, PKC-overproducing cells were shown to form small colonies in soft agar in the absence of TPA and large colonies in the presence of TPA. The mere overproduction of a single form of PKC was thus sufficient to confer multiple growth abnormalities in rat fibroblasts. The results obtained provide direct evidence that PKC plays a critical role in growth control and that it mediates several of the cellular effects of the phorbol ester tumor promoters. The studies also suggest that the activation of PKC may be of central importance in the process of multi-stage carcinogenesis. Further studies to define the role of PKC are in progress (197).

Although there is evidence supporting the fact that during the process of carcinogenesis tumor promoters may act by mediating clonal expansion of previously initiated cells, it is also possible that genetic effects involving alterations in the

regulation of gene expression may play a role in tumor promotion. Phorbol ester tumor promoters have been shown to modulate the transcription of a number of cellular and viral genes *in vitro*. In contrast to extensive cell culture studies, very few animal experiments have been reported in which the effects of tumor promoters on the expression of specific genes have been studied. In one such animal model system the multistage model of mouse skin tumor formation which consistently produces benign papillomas and invasive squamous cell carcinomas was used. During the progression of benign mouse skin papillomas to malignant squamous cell carcinomas induced by a two-stage protocol, it was previously shown that transin RNA, a 1.9-kb RNA coding for a novel, secreted proteinase, was overexpressed. A high degree of similarity has been demonstrated between rabbit stromelysin, a secreted metalloproteinase that degrades proteoglycans found in the basement membrane and the amino acid sequence predicted in rat transin cDNA. The expression of proteolytic enzymes by tumors and malignantly transformed cells suggests that matrix-degrading proteinases are required for invasion and metastasis. The inappropriate expression of transin may, therefore, be causally involved in one or more steps in tumor progression from a benign papilloma to a malignant, invasive, and eventually metastatic squamous cell carcinoma. For this reason, the expression of transin in the very late steps of progression from a nonmetastatic to a metastatic squamous cell carcinoma was investigated in a further study. DNA sequencing of a mouse cDNA isolated from a squamous cell carcinoma (DMBA initiation, TPA promotion) showed greater than 85% nucleotide similarity and 90% amino acid similarity to the rat transin-1 cDNA nucleotide and predicted amino acid sequences. Enhanced levels of transin mRNA transcripts were found in squamous cell carcinomas induced by a protocol giving rise to metastatic tumors (repeated N-methyl-N-nitroso-N'-nitroguanidine (MNNG) treatments) compared with the level found in squamous cell carcinomas induced by a protocol that had a lower probability of giving rise to metastatic tumors (MNNG initiation, TPA promotion). Southern blot analyses of DNA isolated from epidermis, papillomas and squamous cell carcinomas indicated that neither transin gene amplification nor rearrangement accounted for increased levels of the transin mRNA transcripts. The data obtained suggest a role for enhanced levels of transin production in the invasion and metastasis of chemically induced squamous cell carcinomas. Further studies to define this role is considered necessary (12).

VL30 is a proliferation-associated gene which was initially identified as a multi-gene family of approximately 200 homologous but not identical members in the mouse. Expression of the 30S VL30 RNA in mouse AKR-2B cells has been shown to be induced by either epidermal growth factor (EGF) or activators of PKC. Structurally, VL30 was shown to possess many characteristics of a retroviral gene, including the presence of long terminal repeats and sequences homologous to known retroviral enhancers. The cell line RVL-3, which contains a single mouse VL30 element with full responsiveness to both EGF and PKC activation, was used in one laboratory to study the ability of EGF and PKC activators to regulate the expression of a single VL30 element at the levels of RNA transcription and RNA accumulation. Both EGF and the PKC activators TPA and DiC₈ were shown to induce the transcription of the single VL30 element within 5 min of stimulation. Following TPA-induced depletion of PKC activity, EGF stimulation of VL30 transcription and accumulation was shown to be unaffected, while TPA effects were inhibited. These results imply that EGF and TPA act by separable pathways (118).

Gap-junction-mediated intercellular communication has been proposed to have an important role in the process of tumor promotion. Many known tumor promoters have been shown to inhibit metabolic cooperation, a specific type of intercellular

communication in which small molecular weight, possible growth regulatory molecules are passed between adjacent cells in physical contact via membrane structures called gap-junctions. Correlations have been reported linking the tumor promoting efficacy of tumor promoters with their ability to inhibit metabolic cooperation. In one laboratory, transformed foci of BALB/c 3T3 cells produced in vitro by chemicals or by oncogene transfection did not communicate with their normal counterparts. An absence of direct cytoplasmic exchange of molecules, including growth regulating factor, between tumorigenic BALB/c 3T3 cells and their non-transformed counterparts was thought to prevent the control of growth that is exerted by normal cells. Since greater than 90% of human tumors are of epithelial origin, it was then of interest to see whether selective communication occurred between non-transformed and transformed cells in an epithelial model. For this purpose, the homologous and heterologous communication capacities of four rat liver epithelial cell lines were compared with their expression of transformed phenotypes. All four cell lines demonstrated reasonably good homologous junctional communication capacity, as measured by the dye-transfer assay. In heterologous co-cultures, the non-transformed cell line IAR 20 was shown to not communicate with the transformed cell lines IAR 6-1 or IAR 27 F. These two cell lines showed a high degree of transformed phenotypes such as cell morphology, growth in soft agar and expression of gamma-glutamyltranspeptidase activity. The IAR 27 E cell line, which showed the least degree of transformation, was shown to communicate with IAR 20 cells. From these results, it appears that there is an inverse correlation between the extent of expression of transformed phenotypes by rat liver epithelial cells and their ability to communicate with non-transformed counterparts. Further studies, especially those in vivo, are considered necessary to show whether selective intercellular communication plays an important role in carcinogenesis (207).

Since humans are not normally exposed to phorbol ester tumor promoters, it was deemed necessary, in 1981, to stimulate more research on agents more relevant to human exposure which might function as tumor promoters. To accomplish this a Request for Applications (RFA) was issued inviting grant applications from interested investigators for both basic and applied studies that would seek to provide insight and approaches to an understanding of the role of tumor promoters, hormones and other cofactors in human cancer causation. The studies were to be focused on one or more of five different categories: (1) the development of non-phorbol tumor promotion or cocarcinogenesis models in experimental animals using the breast, colon, lung, prostate, stomach, urinary bladder and/or uterus organ systems; (2) the development of nonphorbol tumor promotion or cocarcinogenesis models in human and/or nonhuman cell and/or organ culture systems; (3) the study of the possible tumor promotion role of hormones and substances such as bile acids, saturated/unsaturated dietary fat, alcohol, salt or oxygen-free radicals; (4) the identification and elucidation of the mechanisms of action of nonphorbol tumor promoters and/or cocarcinogens; and (5) interdisciplinary studies involving epidemiologists and experimentalists to test hypotheses concerning tumor promotion generated by either.

In FY82, 12 grants were funded from applications submitted in response to this RFA; ten were approved for 3 years of funding and two for 4 years. The role of dietary fat on DMBA-induced mammary carcinogenesis in rats or mice was the focus of two of the studies. The cocarcinogenic action of ethanol with nitrosamines in the oral cavity, esophagus and larynx of rats, mice and hamsters was the focus of one study. The rates of metabolic activation of nitrosamines in the target organ and cell cultures was to be measured. In a mouse lung tumor model, the mode of

action of butylated hydroxytoluene (BHT) as a tumor promoter was to be examined. The metabolism of BHT, the activation of cyclic GMP- and calcium-dependent protein kinase, the effect of glucocorticoids on urethane tumorigenesis and tumor promotion and the effect of BHT on glucocorticoid receptor localization was to be studied. Using a heterotopically transplanted rat bladder system, one laboratory was to investigate the promoting effect of urine components on bladder carcinogenesis induced by N-methyl-N-nitrosourea and N-butyl-N-(3-carboxypropyl)-nitrosamine. The hypothesis that asbestos and selected nonasbestos minerals act as tumor promoters in carcinogenesis of the respiratory tract was to be studied using a hamster trachea model. The determination of whether EBV-related oncogenic mechanisms in in vitro virus-cell interaction models involves promotion was to be made in one laboratory. The hypothesis given is that a viral-mediated increase in an intracellular protein that blocks the viral lytic cycle and interferes with cell differentiation leads to uncontrolled cell proliferation and the ultimate selection of neoplastic cells. A study of the tumor-promoting activity of a number of anthracene derivatives, such as chrysarobin and its synthetic analogs and homologs, which are related to anthralin, was to be conducted using the 7,12-dimethylbenz(a)anthracene skin tumor model system. Two in vitro model systems for testing for tumor promoters were to be developed. One model system used hepatocytes or liver cells from carcinogen-treated rats which were then promoted in culture using selected compounds. The other model system used various rodent and human cells to test the hypothesis that the induction of mutations at the hypoxanthine guanine phosphoribosyl transferase (HGPRT) locus by promoters in hypermutable cells is a common property of cancer cells. In one study on the mechanism of action of promoters, the ability of promoters to stimulate gene amplification to methotrexate resistance was to be studied. Finally, one of the studies involved a biochemical epidemiology project in which sex hormone levels in breast and prostatic cancer were studied.

In FY85, two of the projects were successfully renewed. In FY86 support was continued for three additional projects, mainly due to successful revised renewal applications. Two other projects were renewed and funded in FY87. Three of the projects will not be renewed as no renewal applications have been submitted and none are anticipated. One renewed project was for continuation of studies on the mechanism of action of a nonphorbol ester tumor promoter, chrysarobin. This was renewed for an additional five years in FY88. The second project, funded in FY85, supported studies on the potential role of oxygen-free radicals in asbestos-induced bronchogenic carcinoma. This project was not renewed in FY88.

Of the projects receiving continued support in FY86, one involved the further investigation of mechanisms of urinary bladder carcinogenesis utilizing the heterotopically transplanted bladder system and natural bladder models in rats. In the second project, the goal was changed to investigate the possible role played by the endogenous mouse mammary tumor virus gene and cellular oncogene components in murine mammary tumorigenesis, alone and in combination with chemical carcinogens. This project was successfully renewed for an additional five years in FY89. The third project involved studies on the role of Epstein-Barr virus in neoplastic transformation. Of the two projects renewed in FY87, one involved further studies on the mechanisms of butylated hydroxytoluene stimulation of mouse lung tumor multiplicity. The second project involved the development of mammary cell culture models for further studies on the role of dietary fat in mammary carcinogenesis. It is also evident that this RFA has stimulated more studies on nonphorbol tumor promoters of relevance to humans. Some recently funded projects

seek to study the activity of compounds such as orotic acid, cyclosporine, endogenous growth factors, hormones and dietary L-tryptophan as tumor promoters. In addition, epithelial cell and organ culture systems from human endometrium are being developed to study the process of tumor promotion by a variety of agents such as hormones and TPA.

In one study of the mechanism of action of the non-phorbol ester tumor promoter, chrysarobin, a single topical application of chrysarobin to SENCAR mouse skin was shown to produce alterations in epidermal polyamine levels that were distinctly different from that following a single topical treatment with TPA. Putrescine and spermidine levels were shown to be elevated prior to the induction of epidermal ornithine decarboxylase (ODC), considered to be the rate limiting enzyme in the biosynthesis of polyamines. The association between increased cellular levels of polyamines and cell proliferation has been noted in many other experimental systems. Also, a good correlation exists between tumor-promoting activity by TPA and increased ODC activity followed by elevated polyamine levels, and it has been proposed that the induction of ODC and elevated polyamines by TPA are essential for tumor promotion, especially later stages of tumor promotion. Information on the changes in epidermal polyamine levels brought about by non-phorbol ester tumor promoters in mouse skin is generally lacking. Chrysarobin is an anthrone tumor promoter which was shown to induce ODC in mouse epidermis by an initial mechanism which appears to be different than TPA. In addition to differences in polyamine levels, the time courses for changes in epidermal DNA synthesis in mouse skin following single treatments with TPA or chrysarobin also showed considerable differences. Treatment with chrysarobin was associated with an initial, dramatic inhibition in epidermal DNA synthesis which was much more extensive than that elicited by TPA. Inhibition of epidermal DNA synthesis following treatment with chrysarobin was observed within a few hours after treatment and remained depressed until about 36 hr after treatment. Following treatment with both chrysarobin and TPA, higher levels of epidermal DNA synthesis was shown to correlate closely with higher molar levels of spermidine/spermine, indicating a strong relationship between epidermal spermidine levels and epidermal cell proliferation induced by both promoters. Since anthrone derivatives did not interact with the phorbol ester receptor and did not activate PKC *in vitro*, the data obtained suggest that TPA and chrysarobin bring about initial changes in epidermal polyamines by distinct mechanisms. Both compounds, however, ultimately lead to a dramatic stimulation of epidermal DNA synthesis. The present data support the working hypothesis that anthrones promote skin tumors by an initial mechanism different from that of the phorbol esters. Anthrone derivative may also be useful compounds for studying alternate pathways of polyamine metabolism involved in cell proliferation (41).

Butylated hydroxytoluene (BHT) is an important preservative added to foods and other consumer products because of its antioxidant properties. Although shown not to be carcinogenic, BHT has exhibited a variety of tumor modulatory actions in rats and mice. When administered before a carcinogen such as urethane or concurrently with it, the number of tumors induced in various organs is often less than in animals treated with the carcinogen only. This same BHT treatment, however, can also enhance tumor development in certain cases. BHT has been shown to be extensively metabolized and several lines of evidence have indicated that promotion is associated with its biotransformation, and especially with the generation of free radical species. BHT has been shown to undergo three routes of oxidative attack: hydroxylation of the 4-methyl group to form BHT-MeOH, hydroxylation of a tert-butyl group to form BHT-BuOH, and oxidation of the pi electron system leading

to non-aromatic products. Many of the primary metabolites can be further oxidized. In order to determine which metabolite is involved in promotion, the oxidative metabolism of BHT by liver microsomes and lung tumor promotion by BHT were compared in three inbred mouse strains which differed in their susceptibility to BHT tumor promotion. This differing susceptibility was shown to correlate with their ability to produce a particular metabolite, BHT-BuOH. Chronic BHT administration, according to the treatment regimen for promotion, was shown to selectively induce the BHT oxidization pathway leading to BHT-BuOH. The hypothesis that BHT-BuOH formation leads to the tumor promoting effects of BHT was tested directly by the chronic administration of BHT, BHT-BuOH, and two other metabolites, 2,6-di-tert-butyl-4-hydroxymethyl phenol and 2,6-di-tert-butyl-1,4-benzoquinone to MA/MyJ mice following a single injection of urethane. The only metabolite that enhanced lung tumor formation was shown to be BHT-BuOH, which was effective at one-fourth the effective dose of BHT. Thus, both indirect and direct evidence implicated BHT-BuOH formation as an important step in the chain of events leading to promotion of lung tumors. Further studies show that BHT-BuOH is oxidized to at least four products by liver microsomes, but none of these are currently available in quantities sufficient for additional animal studies (121).

MOLECULAR CARCINOGENESIS
GRANTS ACTIVE DURING FY89

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ADAIR, Gerald M. University of Texas System Cancer Center 2 R01 CA28711-07	Expression of Genetic Variation in Cultured Cells
2. ALDAZ, Claudio M. University of Texas System Cancer Center 1 R29 CA48922-01	Chromosomal Abnormalities in Rat Mammary Carcinogenesis
3. AMES, Bruce N. University of California, Berkeley 5 R35 CA39910-04	Mutagenesis and Carcinogenesis
4. ANANTHASWAMY, Honnavara N. University of Texas System Cancer Center 1 R01 CA46523-01A1	Mechanisms of Induction of Skin Cancers by UV Light
5. ASHENDEL, Curtis L. Purdue University, West Lafayette 5 R01 CA36262-05	Interactions of Tumor Promoters with Receptors
6. AUST, Steven D. Gordon Research Conferences 1 R13 CA49187-01	1989 GRC Oxygen Radicals in Biology
7. AVADHANI, Navayan G. University of Pennsylvania 5 R01 CA22762-11	Mitochondrial DNA Damage during Chemical Carcinogenesis
8. BAIRD, William M. Purdue University, West Lafayette 5 R01 CA40228-04	Molecular Mechanisms of Hydrocarbon DNA interactions
9. BAXTER, C. Stuart University of Cincinnati 5 R01 CA34446-05	In Vivo Immunotoxicology of Tumor-Promoting Agents
10. BIGGART, Neal W. San Diego State University 1 R29 CA46818-01	Reactive Oxygen-Mediated Mutagenesis by CdCl2 and TPA
11. BILLINGS, Paul C. University of Pennsylvania 5 R01 CA45734-02	Target Proteases of Anticarcino- genic Protease Inhibitors
12. BOWDEN, George T. University of Arizona 2 R01 CA40584-04	Oncogene Activation During Skin Tumor Progression

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| 13. | BOX, Harold C.
Roswell Park Memorial Institute
1 R01 CA44808-01A1 | DNA Damage, Promotion and the
Prooxidant State |
| 14. | BOYNTON, Alton L.
University of Hawaii at Manoa
2 R01 CA39745-04 | Assays for and Mechanisms of
Action of Tumor Promoters |
| 15. | BOYNTON, Alton L.
University of Hawaii at Manoa
5 R01 CA42942-03 | Tumor Promoters, Second
Messengers, and Carcinogenesis |
| 16. | BRESNICK, Edward
University of Nebraska Medical Center
5 R01 CA36679-06 | DNA Repair after Polycyclic
Hydrocarbon Administration |
| 17. | BRIGGS, Robert C.
Vanderbilt University
5 R01 CA26412-09 | Experimental Hepatocarcino-
genesis |
| 18. | BROIDO, Michelle S.
Hunter College
5 R29 CA46713-02 | NMR Studies of Phosphate-
Alkylated DNA Oligomers |
| 19. | BROYDE, Suse B.
New York University
5 R01 CA28038-08 | Carcinogen-DNA Adducts--Linkage
Site and Conformation |
| 20. | BUTEL, Janet S.
Baylor College of Medicine
5 R01 CA33369-06 | Mammary Cancer Stage: Viral
and Chemical Interactions |
| 21. | BUTLER, Andrew P.
University of Texas System Cancer Center
1 R01 CA46629-01 | Regulation of Ornithine
Decarboxylase by Phorbol Esters |
| 22. | CALOS, Michele P.
Stanford University
5 R01 CA33056-07 | Mutation in Human Cells at
The DNA Sequence Level |
| 23. | CARTER, Timothy H.
St. John's University
5 R01 CA37761-05 | Regulation of Transcription by
a Tumor Promoter |
| 24. | CHEN, Fu-Ming
Tennessee State University
5 R01 CA42682-03 | Conformational Lability of
Poly(dG-m5dC): Poly(dG-m5-dC) |
| 25. | CHRISTMAN, Judith K.
Michigan Cancer Foundation
7 R01 CA25985-09 | Response of Phagocytic
Leukocytes to Tumor Promoters |
| 26. | CHRISTMAN, Judith K.
Michigan Cancer Foundation
1 R01 CA45028-01A1 | Mechanism of 5-AzaCR-Mediated
Alteration--Gene Activity |

27. CHU, Gilbert
Stanford University
5 R01 CA44949-02
Finding the Molecular Defect
in Xeroderma Pigmentosum
28. CHUNG, Fung-Lung
American Health Foundation
5 R01 CA43159-03
Enals in Tumorigenesis
29. CLAWSON, Gary A.
George Washington University
2 R01 CA21141-13
Pathology of Chemical Carcino-
genesis
30. COHEN, Samuel M.
University of Nebraska Medical Center
5 R01 CA44886-02
Acrolein and Urinary Bladder
Carcinogenesis
31. COHEN, Samuel M.
University of Nebraska Medical Center
5 R01 CA32513-07
Studies on Experimental Bladder
Tumors
32. CONNER, Mary K.
University of Pittsburgh at Pittsburgh
5 R01 CA39401-03
Murine Lymphocyte SCE Model
for Predicting Genotoxic Risk
33. CONTI, Claudio J.
University of Texas System
5 R01 CA42157-03
Chromosome Alteration during
Chemical Carcinogenesis
34. COSTA, Max
New York University
5 R01 CA43070-04
Mechanism of Metal Carcino-
genesis
35. CRAIGHEAD, John E.
University of Vermont & St Agric College
2 R01 CA36993-04
Experimental Asbestos-Induced
Mesothelioma
36. DAVIDSON, Richard L.
University of Illinois at Chicago
2 R01 CA31781-08
Mechanisms of Chemical Muta-
genesis in Mammalian Cells
37. DAY, Rufus S. III
Cross Cancer Institute
1 R01 CA49936-01
Cellular DNA Repair Response
to Methylating Agents
38. DERUBERTIS, Frederick R.
University of Pittsburgh at Pittsburgh
2 R01 CA31680-07
Phospholipid Derived Signals
in Colon Epithelial Growth
39. DIAMOND, Leila
Wistar institute of Anatomy and Biology
5 R01 CA23413-11
Tumor Promoters and Cell
Differentiation
40. DIAMOND, Leila
Wistar institute of Anatomy and Biology
5 R01 CA37168-04
Chemical Transformation Neo-
plastic Progression and Oncogene

41. DIGIOVANNI, John
University of Texas System Cancer Center
2 R01 CA37111-06
Mechanism of Skin Tumor
Promotion by Chrysarobin
42. DIGIOVANNI, John
University of Texas System Cancer Center
2 R01 CA38871-04
Genetics of Susceptibility to
Skin Tumor Promotion
43. DRESLER, Steven L.
Washington University
5 R01 CA37261-04
Molecular Analysis of
Carcinogen-Induced DNA Repair
44. DUKER, Nahum J.
Temple University
5 R01 CA24103-08
Molecular Pathology of
Carcinogenic DNA Damage
45. ECHOLS, G. Harrison, Jr.
University of California, Berkeley
5 R01 CA41655-04
Mutagenesis and Its Control
in *E. coli*
46. ELLIOTT, Mark S.
Old Dominion University
5 R29 CA45213-02
Modulation of Quenue Levels
with Tumor Promoters
47. ESSIGMANN, John M.
Massachusetts Institute of Technology
5 R01 CA43066-02
Extrachromosomal Probes for
Mutagenesis
48. FAHL, William E.
University of Wisconsin, Madison
5 R37 CA42024-04
Carcinogen-Transformed Human
Cells: Genetic Traits
49. FAUSTO, Nelson
Brown University
2 R01 CA35249-04A1
Protooncogenes and Cell Lineages
in Liver Carcinogenesis
50. FEINBERG, Andrew P.
University of Michigan at Ann Arbor
1 R01 CA48932-01
Identification of the Earliest
Steps in Transformation
51. FISCHER, Susan M.
University of Texas System Cancer Center
5 R01 CA34443-05
Role of Arachidonate Metabolites
in Tumor Promotion
52. FISCHER, Susan M.
University of Texas System Cancer Center
5 R01 CA42211-03
Tumor Promoter-Induced Oxidants
from Epidermal Cells
53. FISHER, Paul B.
Columbia University, New York
5 R01 CA35675-05
Analysis of Progression of the
Transformed Phenotype
54. FISHER, Paul B.
Columbia University, New York
5 R01 CA43208-02
Multifactor Interactions in
Carcinogenesis

55. FLOYD, Robert A.
Oklahoma Medical Research Foundation
5 R01 CA42854-02
Oxygen Free Radicals in
Carcinogenesis
56. FOILES, Peter
American Health Foundation
5 R01 CA42235-03
Monoclonal Antibodies Specific
for Cyclic DNA Adducts
57. FOSTER, Patricia L.
Boston University
2 R01 CA37880-04A1
Mechanisms of Mutagenesis by
Chemical Carcinogens
58. FOX, C. Fred
University of California, Los Angeles
1 R13 CA47041-01
Mechanism and Consequence of
DNA Damage Process
59. FRENKEL, Krystyna
New York University
2 R01 CA37858-04
Tumor Promoters Effecting Base
Modification in DNA
60. FRIEDBERG, Errol C.
Stanford University
5 R01 CA12428-18
DNA Repair and Its Relationship
to Carcinogenesis
61. FRIEDBERG, Errol C.
Stanford University
5 R01 CA44247-02
DNA Repair and Cancer-Prone
Hereditary Human Disease
62. GALLAGHER, Patricia E.
West Virginia University
5 R29 CA47457-02
Enzymatic Repair of Carcinogenic
Damage to Human DNA
63. GARTE, Seymour J.
New York University
5 R01 CA36342-05
Transforming Genes in Inhalation
Carcinogenesis
64. GEACINTOV, Nicholas E.
New York University
5 R01 CA20851-11
Characterization of Carcinogen
Nucleic Acid Complexes
65. GERSON, Stanton L.
Case Western Reserve University
5 R01 CA45609-02
Prevention of Leukemogenesis
in Hematopoietic Precursors
66. GOLD, Barry I.
University of Nebraska Medical Center
2 R01 CA38976-04
Metabolism and Genotoxicity of
Nitrosamines
67. GORDON, Nancy R.
American University
1 R15 CA43552-01
Cr-VI Induced DNA-Protein
Cross-Links
68. GOULD, Michael N.
University of Wisconsin, Madison
2 R01 CA28954-07A1
Factors Controlling Susceptibil-
ity to Mammary Cancer

69. GOULD, Michael N.
University of Wisconsin, Madison
5 R01 CA44387-02
Characterizing Early Events in
Mammary Carcinogenesis
70. GRIFFITH, O. Hayes
University of Oregon
2 R01 CA11695-19
Photoelectron Imaging of
Eucaryotic Cells
71. GRISHAM, Joe W.
University of North Carolina, Chapel Hill
2 R01 CA24144-10
Mechanism of DNA Dependent
Cytotoxicity by Chemicals
72. GROLLMAN, Arthur P.
State University New York, Stony Brook
5 R01 CA17395-14
Molecular Pharmacology of
Tumor and Virus Inhibitors
73. GRUNBERGER, Dezider
Columbia University, New York
2 R01 CA39547-04
Mechanism of Mutation Induced
in Mammalian Genes
74. GUPTA, Ramesh C.
Baylor College of Medicine
5 R01 CA30606-08
Reaction of Carcinogenic
Aromatic Amines with DNA
75. HAMILTON, Joshua W.
Dartmouth College
1 R29 CA49002-01
Effect of Carcinogens on Gene
Expression In Vivo
76. HANAWALT, Philip C.
Stanford University
5 R35 CA44349-02
Cellular Processing of Damaged
DNA: Role in Oncogenesis
77. HENDERSON, Earl E.
Temple University
5 R01 CA35471-03
Carcinogen Enhancement of EBV
Induced Transformation
78. HERSCHMAN, Harvey R.
University of California, Los Angeles
5 R01 CA42887-03
Molecular Mechanisms--Single-
and 2-stage Transformation
79. HITTELMAN, Walter N.
University of Texas System Cancer Center
5 R01 CA27931-08
Chromosome Aberrations with
Therapeutic Agents
80. HITTELMAN, Walter N.
University of Texas System Cancer Center
5 R01 CA39534-03
Visualization of Chromosome
Events in DNA Repair
81. HOGAN, Michael E.
Baylor College of Medicine
2 R01 CA39527-04
Mapping Carcinogen Binding Sites
on Genes

82. HUMAYUN, M. Zafri
University of Medicine & Dentistry of NJ
5 R01 CA27735-08 Mutagenesis by Carcinogenes:
A Molecular Approach
83. HUMAYUN, M. Zafri
University of Medicine & Dentistry of NJ
1 R01 CA47234-01 Mechanisms of Mutagenesis by
Cyclic DNA Adducts
84. HUTCHINSON, Franklin
Yale University
5 R01 CA40195-03 DNA Sequence Changes in Mutated
Mammalian Cells
85. HUTCHINSON, Franklin
Gordon Research Conferences
1 R13 CA48592-01 Gordon Research Conference on
Mutagenesis, 1988
86. ISAACS, John T.
Johns Hopkins University
5 R01 CA42954-02 Genetic Factors and the
Suppression of Mammary Cancer
87. IVARIE, Robert D.
University of Georgia
5 R01 CA34066-05 Inactivation of Gene Expression
by DNA Agents
88. JACOBSON, Myron K.
Texas College of Osteopathic Medicine
5 R01 CA43894-03 Alteration of NAD Metabolism
by Chemical Carcinogens
89. JAKEN, Susan
W. Alton Jones Cell Science Center
1 R01 CA48137-01 Decreased Cellular Sensitivity
to Phorbol Esters
90. JENSEN, David E.
Temple University
5 R01 CA38077-04 The Biochemistry of DNA Alkyl
Phosphotriesters
91. JONES, Peter A.
University of Southern California
5 R01 CA39913-05 5-Azacytidine Induced
Differentiation
92. JONES, Peter A.
University of Southern California
1 R35 CA49758-01 DNA Methylation in Development
and Cancer
93. KALLENBACH, Neville R.
New York University
5 R01 CA24101-10 Ligand Interactions of DNA
Junctions
94. KAUFMAN, David G.
University of North Carolina, Chapel Hill
5 R01 CA31733-07 Promotion of Chemical Carcino-
genesis in Uterine Tissue
95. KAUFMAN, David G.
University of North Carolina, Chapel Hill
1 P01 CA42765-01A1 Cycle-Dependent Mechanisms of
Chemical Carcinogenesis

110. LEWIS, James G.
Duke University
5 R29 CA44734-02
Xenobiotics, Inflammation and
Carcinogenesis
111. LIEBERMAN, Michael W.
Fox Chase Cancer Center
2 R37 CA39392-05
Carcinogen Activation of
Unexpressed Mammalian Genes
112. LIEBERMAN, Michael W.
Fox Chase Cancer Center
5 R01 CA40263-04
Gene Expression in Carcinogen-
Induced Liver Cancer
113. LIEBERMAN, Michael W.
Baylor College of Medicine
1 R01 CA50684-01
Metallothionein Activation:
A Model of Carcinogen Action
114. LIEHR, Joachim G.
University of Texas Medical Branch
5 R37 CA43233-04
Mechanism of Estrogen-Induced
Renal Carcinogenesis
115. LOEB, Lawrence A.
University of Washington
5 R35 CA39903-04
Fidelity of DNA Replication
116. LOMBARDI, Benito
University of Pittsburgh at Pittsburgh
5 R01 CA23449-10
Choline Deficiency and Hepato-
carcinogenesis
117. MACLEOD, Michael C.
University of Texas System Cancer Center
5 R01 CA35581-06
Specificity of Diol Epoxide:
Chromatin Interactions
118. MAGUN, Bruce E.
University of Arizona
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Mechanisms of Tumor Promotion
In Vitro
119. MAHER, Veronica M.
Michigan State University
2 R01 CA21253-12
Interaction of Carcinogens
with DNA: Spectra of Mutation
120. MAHER, Veronica M.
Michigan State University
1 R01 CA48066-01A1
Mechanisms of Homologous
Recombination in Human Cells
121. MALKINSON, Alvin M.
University of Colorado at Boulder
2 R01 CA33497-05A1
Promotion of Lung Tumors by BHT
122. MARNETT, Lawrence J.
Wayne State University
1 R35 CA47479-01
Polyunsaturated Fatty Acid
Metabolism and Carcinogenesis
123. MATRISIAN, Lynn M.
Vanderbilt University
1 R01 CA46843-01
Role of Transin in Tumor
Promotion and Progression

138. OYASU, Ryoichi
Northwestern University
5 R01 CA33511-06
Experimental Urinary Bladder
Carcinogenesis
139. OYASU, Ryoichi
Northwestern University
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Role of Stroma in Urinary
Bladder Carcinogenesis
140. PATEL, Dinshaw J.
Columbia University, New York
1 R01 CA46533-01
DNA Damage Sites--Mutagenic and
Carcinogenic Lesions
141. PATEL, Dinshaw J.
American Association for Cancer Research
1 R13 CA49267-01
Conference On Molecular Events
in Mutation/Cancer
142. PEGG, Anthony E.
Pennsylvania State Univ Hershey Med Ctr
5 R01 CA18137-13
Persistence of Alkylated DNA
in Carcinogenesis
143. PEGG, Anthony E.
Gordon Research Conferences
1 R13 CA48663-01
Gordon Research Conference on
Mammalian DNA Repair
144. PELLING, Jill C.
University of Nebraska Medical Center
2 R01 CA40847-04
Two-stage Skin Carcinogenesis
and Altered Gene Expression
145. PITOT, Henry C.
University of Wisconsin, Madison
2 P01 CA22484-11
Biochemical Studies in Chemical
Carcinogenesis
146. PITOT, Henry C.
University of Wisconsin, Madison
5 R01 CA45700-02
Instability of Tumor Promotion
in Hepatocarcinogenesis
147. PRESTON, Bradley D.
Rutgers The State Univ, New Brunswick
1 R29 CA48174-01
Mutagenesis by Chemical Carcino-
gens
148. RAO, M. Sambasiva
Northwestern University
2 R01 CA36130-04
Gamma-Glutamyltranspeptidase
Negative Hepatocarcinogenesis
149. REDDY, Arram L.
University of Washington
5 R01 CA32716-05
Skin Tumorigenesis Studied with
Cell Markers
150. REINERS, John J., Jr.
University of Texas System Cancer Center
1 R01 CA49935-01
Immunomodulation and Chemically
Induced Carcinogenesis
151. RICH, Alexander
Massachusetts Institute of Technology
5 R01 CA29753-08
Chemical Carcinogenesis and DNA
Structure

152. RICHIE, Ellen R.
University of Texas System Cancer Center
2 R01 CA37912-04A2
Mechanisms of MNU Induced
Lymphoma in AKR Mice
153. RIGBY, James H.
Wayne State University
2 R01 CA36543-03A2
Synthesis of Cocarcinogenic
Diterpenes
154. ROKITA, Steven E.
State University New York, Stony Brook
5 R01 CA43593-02
Oligonucleotide Photochemistry
155. ROMANO, Louis J.
Wayne State University
5 R01 CA35451-05
In Vitro Function of DNA
Containing Carcinogen Adducts
156. ROMANO, Louis J.
Wayne State University
5 R01 CA40605-03
Biological Consequences of Site-
Specific Damage to DNA
157. ROSENSTEIN, Barry S.
Brown University
5 R01 CA45078-02
Repair of 290-320 NM Induced
Non-Dimer DNA Damage
158. ROSNER, Marsha R.
University of Chicago
5 R01 CA35541-06
Modulation of Cellular Phos-
phorylation by Tumor Promoter
159. ROSSMAN, Toby G.
New York University
2 R01 CA29258-07A1
Mutagenesis by Metals of
Environmental Significance
160. SAFFRAN, Wilma A.
Queens College
5 R01 CA42377-03
Psoralen-Induced Mutation and
Recombination
161. SARMA, D. S.
University of Toronto
5 R01 CA37077-05
Promotion of Liver Carcino-
genesis by Orotic Acid
162. SARMA, D. S.
University of Toronto
1 R01 CA46261-01
Cell Proliferation and Liver
Carcinogenesis
163. SEVILLA, Cynthia L.
Proteins International
2 R44 CA45878-02
Immunoassays for Mutagenic and
Carcinogenic Adducts
164. SHANK, Peter R.
Brown University
1 R01 CA47363-01
Non-ras Oncogenes in Chemically
Induced Liver Tumors
165. SHARMA, Surendra
Roger Williams General Hospital
5 R01 CA46959-02
Regulation of DNA Repair in
Human B and T Lymphocytes

166. SHAW, Barbara R.
Duke University
5 R01 CA44709-02
Ionized Base Pairs and Cross-Strand Mutagenesis
167. SHAY, Jerry W.
University of Texas SW Med Ctr/Dallas
5 R01 CA40065-03
A Cytoplasmic Role in Carcinogen Induced Tumorigenicity
168. SHINOZUKA, Hisashi
University of Pittsburgh at Pittsburgh
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Dietary Modification and Promotion of Liver Carcinogenesis
169. SHINOZUKA, Hisashi
University of Pittsburgh at Pittsburgh
5 R01 CA36175-05
Cyclosporine A: Promoter of Lymphoma Induction
170. SHINOZUKA, Hisashi
University of Pittsburgh at Pittsburgh
5 R01 CA40062-03
Liver Cancers by Hypolipidemic Peroxisome Proliferators
171. SIDRANSKY, Herschel
George Washington University
5 R01 CA41832-03
Tryptophan and Hepatic Carcinogenesis
172. SINGER, Bea A.
Univ of Calif-Lawrence Berkeley Lab
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Alkylation of Polynucleotides In Vitro and In Vivo
173. SINGER, Bea A.
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Biochemical Mechanisms of Vinyl Chloride Carcinogenesis
174. SIROVER, Michael A.
Temple University
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Regulation of DNA Repair in Human Carcinogenesis
175. SLAGA, Thomas J.
University of Texas System Cancer Center
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Critical Changes and Factors in Skin Tumor Progression
176. SMULSON, Mark E.
Gerogetown University
5 R01 CA25344-10
Carcinogens and Chromatin Structure and Function
177. SNOW, Elizabeth T.
New York University
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Mechanisms of Metal Mutagenesis: Cr, Ni, and Be
178. STAMPFER, Martha R.
Univ of Calif-Lawrence Berkeley Lab
5 R01 CA24844-10
Characterization of Human Mammary Cells
179. STATES, J. Christopher
Children's Hospital Med Ctr (Cincinnati)
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Genetics of Human DNA Repair

180. STRAUSS, Bernard S.
University of Chicago
5 R01 CA32436-07
Error Prone DNA Synthesis and
Oncogene Mutagenesis
181. STRAUSS, Bernard S.
University of Chicago
5 P01 CA40046-04
Etiology of Treatment-Induced
Secondary Leukemia
182. SUDILOVSKY, Oscar
Case Western Reserve University
5 R01 CA45716-02
Pathobiology of High Dose
Sucrose
183. SUKUMAR, Saraswati
Salk Institute For Biological Studies
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Role of *ras* Oncogenes in
Chemical Carcinogenesis
184. SUMMERHAYES, Ian C.
New England Deaconess Hospital
5 R23 CA42944-03
Oncogenesis of Bladder
Epithelial Cells
185. SUMMERS, William C.
Yale University
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DNA Repair, Recombination and
Mutagenesis
186. TANG, Eric M.
University of Texas System Cancer Center
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Repair of Antitumor Antibiotic
Induced DNA Damage
187. TEEBOR, George W.
New York University
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Damage to DNA
188. TEEBOR, George W.
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Phylogeny of DNA Repair
189. TELANG, Nitin T.
Memorial Hospital for Cancer/Allied Dis
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In Vitro Induction and Modula-
tion of Mammary Preneoplasia
190. TOPAL, Michael D.
University of North Carolina, Chapel Hill
5 R01 CA46527-10
Molecular Basis of Environ-
mentally Induced Mutations
191. TROSKO, James E.
Michigan State University
5 R01 CA21104-11
Mutation and Derepression of
Genes in Carcinogenesis
192. VERMA, Ajit K.
University of Wisconsin, Madison
5 R01 CA35368-05
Ca²⁺-Dependent Processes
Involved in Phorbol Ester Tumor
193. VOLSKY, David J.
St. Luke's-Roosevelt Inst for Hlth Sci
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Epstein-Barr Virus and Tumor
Promotion

208. YAMASAKI, Hiroshi
World Hlth Org, Intl Agcy Res on Cancer
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Perinatal and Multigenerational
Carcinogenesis
209. YU, Fu-Li
College of Medicine at Rockford
2 R01 CA30093-07
Aflatoxin B1 and Nucleolar RNA
Synthesis
210. ZURLO, Joanne
Dartmouth College
5 R01 CA42419-02
Mechanisms of Mutagenesis by
Environmental Agents

SUMMARY REPORT

SMOKING AND HEALTH

The Smoking and Health component within the Chemical and Physical Carcinogenesis Branch includes 13 grants with FY89 funding of \$2.08 million and one Interagency Agreement with FY89 funding of \$0.25 million. Support is continuing for research directed toward understanding and mitigating the deleterious effects of tobacco products on health. Significant past efforts have included identifying smoking-related diseases and the chemical analyses of major whole smoke components and their subsequent metabolic products. These efforts have been expanded to include smokeless tobacco products, since these items have shown increased usage in recent years, especially among younger people. Current activities are focused on the toxicological and pharmacological aspects of the problem, with emphasis on nicotine and nicotine metabolites, and their effect on tobacco dosimetry in humans.

The use of tobacco products is the single most important risk factor for cancer development in the United States. The 1982 Surgeon General's report entitled "The Health Consequences of Smoking" estimates that 30% of all cancer mortality is related to smoking. An International Agency for Research on Cancer (IARC) working group calculated that the occurrence of malignant tumors of the respiratory tract is causally related to the smoking of various forms of tobacco, and that cigarette smoking also causes malignant tumors of the bladder, renal pelvis and pancreas. It has been estimated that 50-85% of the lung, oral, laryngeal and esophageal cancer deaths in the United States are associated with smoking. Although the prevalence of cigarette smoking has declined in recent years there are still approximately 50 million smokers in the United States and hundreds of millions of smokers worldwide.

In addition to smoking, exposure to tobacco can occur through the use of smokeless tobacco products. In 1985 over 12 million people used smokeless tobacco products, half of those at least once a week. The use of these products has become particularly popular among teenage and young adult males. Studies of women in the southern United States who use smokeless tobacco indicate that the relative risk for development of oropharyngeal cancer can be as high as 60 and is related to the length of time the product has been used. An IARC working group and the U.S. Surgeon General have concluded that oral use of snuff causes oral cancer.

Dependence on nicotine is the primary reason for continued tobacco use. Nicotine is the most abundant alkaloid present in tobacco products, comprising 1 to 2% of the tobacco. As a tertiary amine, nicotine can react with nitrite to form nitrosamines. The major nitrosamines formed from nicotine are N'-nitrosornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). These nitrosamines are present in mainstream and sidestream smoke at levels of 30-770 ng per cigarette and in smokeless tobacco products at levels of 100-89,000 ppb.

Studies under a program project on experimental tobacco carcinogenesis have revealed that tobacco contains seven N-nitrosamino acids and a nicotine-derived N-nitrosamino acid, 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC). The recently identified iso-NNAC and the most abundant tobacco N-nitrosamino acid, 3-(methylnitrosamino)propionic acid (NMPA; 0.6-13.1 ug/g tobacco) had never been bioassayed for carcinogenicity. NMPA proved to be highly carcinogenic, inducing

lung adenoma and lung adenocarcinoma in strain A mice. However, iso-NNAC was also inactive in this *in vivo* bioassay. This result is of major significance since *in vitro* studies under physiological conditions have shown that iso-NNAC can be formed from the major nicotine metabolite, cotinine and from the metabolite 4-(methylamino)-4-(3-pyridyl)butyric acid. This opens the possibility that iso-NNAC is endogenously formed from nicotine and/or from some of its metabolites. If this can be confirmed, we may have a marker for the endogenous formation of tobacco-specific N-nitrosamines, which are powerful animal carcinogens. This important aspect is under further study (6).

Investigations by another grantee seek to determine if NNK, a nicotine-derived N-nitrosamine and potent respiratory tract carcinogen in adult rodents, is metabolized by fetal respiratory tract tissue of Syrian golden hamsters, nonhuman primates (*Cynomolgus*), and humans, and if such metabolism leads to cancer in the offspring of pregnant females injected with the compound. Specific DNA-lesions such as 7-methylguanine induced by NNK are also being assayed. Carcinogenicity bioassays are complementing such biochemical studies.

Fetal trachea and lung explants were cultured *in vitro* with [5-³H] NNK and metabolites released in the culture media were analyzed by HPLC. With lung explants, levels of pyridine N-oxidation of NNK and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and alpha-carbon hydroxylation were increasing from day 12 to day 15 of gestation. Oncogenic development of the two pathways were non-parallel. With lung explants of 12 day-old fetuses, metabolism of NNK by pyridine N-oxidation and alpha-carbon hydroxylation was low but still higher than with heat-treated (control) explants.

Culture of maternal and fetal lung explants with [CH₂-³H] NNK resulted in methylation at O-6 and N-7 guanine sites of explant DNA. O⁶-MeGua was persistent in the explants, while 7-MeGua was removed. These results indicated that fetal trachea and lung tissues activate NNK by alpha-carbon hydroxylation and that some electrophilic intermediates were methylating explant DNA.

Pregnant hamsters on day 14 of gestation were injected with [5-³H] NNK (200mg/kg). Extraction and HPLC analysis of fetal lung, liver and amniotic fluid indicated that NNK could cross the placental barrier. NNK is rapidly reduced to the N-nitrosamine NNAL in hamster and this metabolite was the most abundant in placenta, amniotic fluid, fetal lung and liver. These results also indicated that NNK could cross the placental barrier. Hamsters on day 14 of gestation were injected *i.p.* with NNK (25, 50, 100 and 200mg/kg) and sacrificed 18 hours later. Fetal lung and trachea were cultured *in vitro* for 1 to 2 weeks. A significant number of primary epithelial cell cultures were established from explant out-growths. Chromosome aberrations were scored from well-spread metaphases. The number of chromosomal aberrations per mitosis was increasing from 3.23 (25 mg/kg) to 12.82 (200mg/kg) in lung epithelial cells. A significant number of chromosomal aberrations was observed in epithelial cells derived from tracheal explants at 50, 100 and 200 mg/kg doses. Maternal exposure to NNK was apparently leading to formation of genotoxic metabolites in fetal respiratory tract tissues.

Micronucleus (MN) induction in fetal liver and maternal bone marrow polychromatic erythrocytes were assayed after *i.p.* injection of NNK (0-200 mg/kg). The frequency of MN induction reached a maximum 18 hours after treatment. The relationship of dose of NNK to MN frequency was significant ($P < 0.005$). No significant MN induction was observed in adult bone marrow polychromatic erythrocyte nuclei. These results suggest that NNK could be activated to clastogenic intermediates in fetal

liver. These intermediates were not generated in adult bone marrow either because NNK could not reach bone marrow or no activating enzymes were present in this tissue. V79 cells were incubated with NNK and liver or lung homogenates from adult or fetal hamsters. Genotoxic damage was quantified by scoring micronuclei in the interphase, chromosomal aberrations in the metaphase and abnormal divisions in the anaphase-telophase. Fetal liver and lung homogenates from fetuses on the 15th day of gestation were activating NNK to intermediates and inducing micronuclei, but to a lower degree than adult tissue homogenates. These results further support the investigator's conclusions that NNK can be activated by fetal tissues.

If carcinogenic metabolites are formed by the fetal tissues, morphological changes suggestive of neoplastic transformation should be detectable after in vitro incubation with NNK. Explants of fetal tracheas and lung were therefore incubated for 24 hours with NNK (1 micromolar) in CMRL-1066 medium. They were then maintained in vitro in the same medium without NNK for 2 weeks and analyzed by light and electron microscopy. Control explants of tracheas and lungs showed normal morphology, while tracheal explants incubated with NNK demonstrated a variety of changes suggestive of neoplastic transformation: Explants of lungs incubated with NNK showed evidence of disturbed surfactant production but no neoplastic changes. This data correlates well with the metabolism data of NNK in this system which indicate that the fetal tracheas metabolize NNK to a greater extent than the fetal lungs. These data support the theory that NNK is a potential transplacental carcinogen. In a short-term acute toxicity experiment, the offspring were sacrificed at 4 weeks of age and all organs processed for histopathology evaluation. Although there were no detectable abnormalities in any of the offspring surviving to this age, there was a pronounced effect of NNK on the number of babies delivered per litter. When NNK was administered as a single injection to pregnant females on day 15 of gestation, there was an almost linear decrease in the number of male babies with increasing dose levels. The opposite was observed for the number of female babies which were lowest in the low dose group (10mg/kg) and highest in the high dose group (200 mg/kg). The overall number of babies born female at a single dose of NNK on day 15 of gestation was substantially lower (between 46 and 72%) than in the controls. But no clear cut dose-response relationship for each sex separately was evident.

In the corresponding multiple dosing study, pregnant females were injected with either 10 mg/kg or 100 mg/kg NNK on each of the three last days of gestation. All offspring were sacrificed and analyzed by histopathology at the age of 4 weeks. Again no abnormalities were found in any organs by histopathology. However, there was a linear decrease in the number of female babies with high dose levels of NNK, while the number of male babies was lowest in the low dose (10mg/kg) group. Although the mechanism of this transplacental effect of NNK require further study, it is of interest that such pronounced sexual dimorphism occurs. In any event, it is very likely that lower numbers of babies per litter are a reflection of higher fetal mortality.

In a chronic bioassay experiment, groups of pregnant hamsters were injected with dose levels of NNK identical to those used in the toxicity test on either day 15 of gestation or on the 3 last days of gestation. Animals were allowed to survive until weight loss or other clinical symptoms of disease and/or discomfort occurred. So far (12 months after the transplacental injections) almost 50% of the hamsters which received a single dose of 100mg/kg NNK on day 15 of gestation are dead. Many of them had macroscopic lesions in their respiratory tract (nasal

cavity, larynx, trachea, lung) which may be tumors. Between 10 and 30% of the animals in the other dose groups are dead and exhibit basically the same organ distribution of macroscopically visible lesions (12).

Preliminary animal studies at another institution have shown that the repeated instillation of commercial snuff, which is produced for oral consumption, into a surgically created lip canal can lead to neoplastic changes in the lip canal as well as in the oral cavity of rats. However, such bioassays with snuff have not led to a significant rate of malignant tumors in the oral cavity of rats. Current studies by these grantees have modified the surgically created lip canal in such a manner that the narrow opening is towards the outside and the wider opening is facing the oral cavity.

For a long-term bioassay on snuff, 280 male F344 rats were purchased at the age of 6-7 weeks; 260 of these were selected by weight and 250 were subjected to lip surgery, while 10 remained as sentinels. Thus, 3 groups were set up: 125 male rats received 2 snuff treatments daily (group 1), 125 male rats with lip canal remain untreated (group 2), and 10 male rats without lip canal remain untreated (group 3).

The rats in group 1 remained untreated for 3 weeks. Then they were gradually introduced to snuff instillations over a period of 2 weeks. Thereafter, they received 10 instillations of snuff per week.

After 6 months of treatment, 3 of the rats of group 1 (treated) and 2 rats of group 2 (controls) were sacrificed. Histological examination of the tissue of the lip canal and surrounding oral tissues revealed inflammatory reactions and acanthosis especially around the opening of the lip canal in the treated animals. These symptoms were not observed in the untreated controls.

After 7 months of snuff treatment, 122 male rats in group 1 survive. Their average weight is 381 g. In group 2 there are 123 rats with an average weight of 417 g. All 10 rats in group 3 are alive with an average body weight of 422 g. Snuff treatment of the rats will continue as scheduled for a full 24 months or until there are fewer than 20% survivors in the snuff group.

Prior to examining whether and to what extent DNA adduct formation by tobacco-specific nitrosamines in the oral cavity had occurred, it was necessary to improve the purity and yield of DNA. A new method of DNA isolation from small tissues was designed. DNA was isolated from the tissue of individual animals by HPLC on a DEAE ion exchange column. Prior to HPLC separation, homogenized samples were incubated with protease in 0.1% SDS and extracted once with chloroform: isoamyl alcohol (24:1). The aqueous layer was injected onto a Protein Pak 5W HPLC column. Peaks eluted in three major regions, designated I, II, III. The early eluting peaks (2-8 min) contained a mixture of compounds, such as tRNA molecules, proteins and other contaminants. The majority of the material eluting between 20-26 min (Fraction II) appears to be RNA. When the sample was treated with ribonuclease A prior to HPLC analysis, essentially all the peaks in this region disappeared. Also, fractions collected in this region had A_{260}/A_{280} absorbance ratios between 1.97 and 2.06. A ratio of 2.00 is characteristic of RNA. The third peak (III) coeluted with calf thymus DNA and rat liver DNA.

The UV absorbance measured in this fraction as obtained from oral tissue samples showed a ratio of A_{260}/A_{280} between 1.77 and 1.87 and averaged 1.83 ± 0.04

(n=10). A ratio of 1.8 is typical for double-stranded DNA. The extent of RNA contamination of DNA purified in this manner was measured and found to be less than 1% contamination by weight. This DNA was also shown to be essentially free of protein (less than 1 microgram/100 microgram DNA).

To confirm the usefulness of this method of DNA isolation, it was applied to the study of DNA methylation by the tobacco-specific carcinogen NNK (0.4nmol/kg) in the nasal mucosa. The levels of alkylation measured in individual samples were 2105 ± 900 pmol 7mG/micromole guanine and 205 ± 40 pmol O^6 mG/micromole guanine. These values agree well with those determined previously in DNA purified by a modified Marmur method from pooled tissues.

The methylation and pyridyloxobutylation of oral DNA by NNK were studied. To measure methylation, a dose of 0.40 nmol/kg NNK was administered by gavage. Neither N⁷methylguanine (<150 micromol/mol guanine) nor O^6 methylguanine (<50 umol/mol guanine) was detected.

Treatment of rats with NNK also results in the 4-(3-pyridyl)-4-oxo-butylation of DNA in the liver and in the lung. Therefore, the possible formation of this adduct in oral DNA was studied. [$5-^3$ H]-NNK was administered by gavage and DNA (300 ug/rat) was isolated. Three fractions were collected and the radioactivity in each was determined. The majority of the radioactivity, >95%, eluted in the first fraction. This would contain NNK and NNK metabolites. Only 100 dpm of radioactivity above background eluted in the DNA region (304 ug DNA). If this radioactivity represents a DNA adduct formed from NNK metabolites, the amount would be 0.2 pmol per micromol guanine.

The metabolism of NNN and NNK was determined in whole tissue from the oral cavity. Tissue removed from inside the cheeks, lips and from the roof of the mouth, was placed on filters in a culture dish containing Williams Medium E. NNK or NNN, respectively (100 nmol/ml, 10 uCi/ml), was incubated with the tissue for 3 hours at 37° C. The medium was removed and the metabolites present were analyzed by HPLC with a radioflow detector. The extent of metabolism of NNN (943 pmol/ml medium) by oral tissue is eightfold greater than that of NNK (121 pmol/ml medium), excluding the formation of NNAL. Whether or not this difference in metabolism observed in vitro results in differences in the extent of DNA adduct formation by these two nitrosamines is currently being investigated (7).

In the previous year, investigators on another grant made the observation that pretreatment of Salmonella with a subthreshold dose of N-nitroso-N-methylurea (MNU) resulted in greatly enhanced numbers of revertants of TA100 induced by smokeless tobacco, and elimination of the threshold in the dose response curves in mutagenesis induced by various fractions of tobacco extracts. This observation proved important because it provided a method of mutagenesis leading to a more meaningful interpretation of fractionation studies (see below). This threshold probably results from the repair of the premutagenic DNA adduct, O^6 -methylguanine by a constitutive repair activity present in Salmonella. This activity is irreversibly deactivated upon removal of the methyl group from O^6 -methylguanine, and once exhausted, mutagenesis rapidly increases. A similar enhancing effect of pretreatment by MNU on Salmonella resulted in greatly enhanced mutagenesis induced by N-nitroso-N-methyl compounds, but not by higher homologue nitroso compounds or other classes of compounds.

Fractionation of smokeless tobacco resulted in a relatively low activity in most fractions because mutagens were distributed into many fractions. As a result of this low activity the threshold portion of the dose-response curve became extremely prominent and complicated comparisons of mutagenic activities in the various fractions. Although in principle it should have been possible to overcome the threshold with higher doses of the fractions, this was often difficult because of toxicity and inhibitory effects at high doses. With the use of MNU-pretreated TA100, near-linear dose response curves were obtained for most of the fractions.

Two tobacco samples were fractionated into a number of subfractions and the mutagenic activity in each fraction was determined using MNU-pretreated Salmonella. The crude aqueous extract was first extracted with methylene chloride, then after adjusting to pH 12 the aqueous layer was again extracted with methylene chloride. After acidification, it was again extracted with methylene chloride. This procedure yielded neutral, alkaline and acidic organic extracts and the corresponding residual aqueous layers. Only about 10-20% of the mutagenic activity extracted into each organic layer with the bulk of the activity remaining in the aqueous layer. The organic acidic layer contained more activity than the other organic layers. Mutagenic activity in the organic layers was difficult to accurately determine because revertant numbers levelled off after only small increases above background levels (possibly because of inhibitors and/or toxicity). The residual aqueous layer was finally taken to dryness and resuspended in methanol. The methanol was then removed and both the residue and the methanol extract were evaporated and resuspended in water. About one-third of the mutagenic activity extracted into methanol. The resulting aqueous layer was then injected onto a C18-reverse phase HPLC column, and eluted with a water-methanol gradient.

Fractions were collected and assayed for mutagenic activity. When monitored at 254 nm a large fraction of the detected material eluted near the solvent front and a somewhat smaller amount of the material eluted at a later time. Nearly all of the mutagenic activity eluted near the solvent front. This result indicated that the mutagenic component(s) was very polar.

A cyanopropyl column was then employed as this stationary phase is more polar than C18 and should retain polar materials more efficiently. In a normal phase separation, with this column, the mutagenic component(s) was only slightly retained. Because of this result a different column was utilized in an effort to retain the mutagenic component(s). A strong anionic column was employed. Two peaks of mutagenic activity were observed. The first was only weakly retained and the second was quite strongly retained, indicating that it contained a negatively charged group. Attempts are currently underway to collect enough of the late-eluting peak for further characterization. One injection was also made onto a strong cationic exchange column to determine whether any of the components were retained on this column. A substantial fraction of the UV-absorbing material was retained on this column, suggesting that positively charged compounds also comprised a significant portion of this fraction. This was an analytical injection; scaling-up to analyze for mutagenic components will be attempted shortly (5).

The specific objective of a project on another grant is to determine the effects of smoking different types of cigarettes on cells of the respiratory tract in baboons. Specifically, the major target cell types for human lung cancer associated with cigarette smoking were investigated. Bronchial biopsies and pulmonary lavages were performed on baboons prior to smoking, after a four month

exposure to low tar/nicotine (University of Kentucky 1R4F cigarettes), and after cessation. The baboons are currently smoking medium tar/nicotine (University of Kentucky 1R31 cigarettes). No evidence of hemoptysis (pulmonary bleeding) was observed in the lavages obtained from the animals after smoking low tar/nicotine (11R4F) cigarettes for four months. Also, no gross evidence of cellular toxicity was observed in bronchial biopsy samples from the animals after exposure to low tar/nicotine cigarettes.

Comparison of covalently-linked cigarette smoke components to bronchial DNA (DNA adducts) is underway. The objectives of this study are to correlate the extent of inhalation with extent of DNA adduct formation, and to determine the persistence of specific smoking-related DNA adducts in the respiratory tissue. Bronchial biopsies were obtained from all animals prior to smoking, after four months exposure to low tar/nicotine cigarettes, and at several times after cessation. The same schedule will be followed with medium tar/nicotine cigarettes, high tar/nicotine cigarettes, and clove cigarettes in the near future. Bronchial biopsy samples will be obtained from the animals after smoking the four different types of cigarettes, and a qualitative comparison of the binding of tobacco-specific components to respiratory cell DNA will be performed to identify specific components that may be present in each cigarette type.

Blood samples are being collected from four of the baboons prior to smoking, four months after smoking, and two months after cessation to quantitate the effects of cigarette smoking on mutation of peripheral lymphocytes (resistance to 6-thioguanine). Plasma samples are being collected from all of the baboons at the same time points for analysis of plasma retinoid (beta carotene and retinol) levels. The objective of this study is to determine the effects of cigarette smoking on levels of agents that may afford protection against the development of lung cancer (8).

The primary objective of work under an interagency agreement with the Department of Energy is to identify and demonstrate chemical markers of environmental tobacco smoke (ETS) that may be used for source apportionment and for estimating exposure to ETS carcinogens. A secondary objective is to demonstrate the extraordinary potential of ion trap mass spectrometry (ITMS) for the facile measurement of ETS exposure markers in air and in physiological fluids.

The laboratory is building upon progress to date on the identification of ETS vapor phase chemical markers and on observations of the unique applicability of ITMS to ETS exposure measurements. Studies of ETS markers to date have involved the multicomponent quantitative determination of ETS vapor phase constituents and of ambient nicotine. Twenty-three vapor phase organic chemicals are determined by thermal desorption gas chromatographic analysis using simultaneous flame ionization and nitrogen-selective detection. Ambient nicotine has been determined either by bisulfite filter collection or resin trap collection followed by gas chromatographic analysis. Samples have been taken from both a controlled ETS atmosphere room and from common indoor air environments. They have found that isoprene, pyrrole, 3-picoline, 3-vinylpyridine, and selected nitriles are promising measures of exposure to ETS. Ethenyl pyridine (3-vinylpyridine) has similarly been identified as a promising ETS exposure measure by others. An especially important observation is that selected ETS vapor phase constituents are detectable at a site in a home very remote from the smoking location, while nicotine is undetectable at that site. Also important is the finding that

measurement of isopyrene in a normal restaurant environment predicts the concentrations of most other measured gas phase constituents rather well but that measurement of nicotine greatly underestimates those concentrations.

Recent studies employing ITMS have proven exceptionally promising. Nicotine can be determined by thermal desorption ITMS of collected air samples with excellent agreement with the results from standard methods. Analyses are performed in less than 10 minutes per sample and detection limits are less than 300 pg. A pilot study has recently been completed that also shows that it is possible to determine nicotine in urine with similar ease, speed, and sensitivity with no sample preparation.

These investigators are establishing the relative ratios of organic vapor phase constituents and identifying those constituents which best predict the quantities of the other constituents as functions of ETS concentration and age. Kentucky reference 1R4F cigarettes will be smoked under standard Federal Trade Commission (FTC) conditions with the sidestream smoke emitted directly into a 50 m³ ETS room to produce a test atmosphere of approximately 100 ug/m³ respirable suspended particulate (RSP) concentration. Multicomponent gas samples are taken for chromatographic analysis using a standard triple sorbent trap. Samples will be taken simultaneously for the determination of nicotine by bisulfite filter collection, for ethenylpyridine determination, and for the measurement of other candidate markers identified as work progresses. The concentrations of particulate matter and carbon monoxide will be monitored continuously during the experiments. The number of samples taken and the number of replicate experiments performed will be determined in consultation with the Oak Ridge National Laboratory (ORNL) Statistics Department.

In another task under this agreement the primary objective is to determine whether the chemical composition of RSP matter in ETS is such as to increase its potential carcinogenicity relative to that of mainstream smoke particulate matter. A secondary objective is to identify and test possible markers of exposure to ETS RSP.

The investigators are testing their hypothesis that changes in the composition of cigarette smoke particulate matter upon high dilution in ambient air are sufficient to increase its carcinogenicity relative to that of mainstream smoke particulate matter. If proven, this may help explain epidemiological evidence, suggesting a relationship between passive smoking and increased risk of lung cancer. This work is based on observation that cigarette smoke condensate, its chemical fractions, and selected constituents of cigarette smoke induce carcinogenesis in rat lung and tracheal implants at very low doses when incorporated in beeswax or other lipophilic matrices. It is also now wellknown that dilution of cigarette smoke to ambient concentrations results in an increased concentration of constituents being in the vapor phase. This suggests that the resulting particulate matter consists principally of the lower volatility, higher molecular weight constituents of smoke. The lower absolute exposure to cigarette smoke carcinogens encountered by passive smokers might be accompanied by a higher specific carcinogenicity of ETS particulate matter.

This issue is being addressed by generating and sampling controlled ETS environments and characterizing the resulting particulate matter. The physical and chemical properties of the particulate matter will be compared to that of

mainstream particulate matter. Properties of particular concern are lipophilicity, chemical class composition, aromaticity, and molecular weight distribution. Several individual constituents will also receive special attention. These include benzo(a)pyrene as a representative polycyclic aromatic hydrocarbon carcinogen, solanesol as a potential ETS RSP exposure marker, and aliphatic hydrocarbon waxes as matrix constituents.

Most of the required analytical methods are already in place or only require reestablishment. Aromaticity will be measured using nuclear magnetic resonance spectrometry, class composition and lipophilicity will be measured using high performance and column liquid chromatography, and molecular weight distribution will be measured by direct probe Fourier transformed infrared spectrometry (FTMS). The HPLC method for determining octanol-water partition coefficient measurements and microscale HPLC class fractionation methods require reestablishment and validation. Methods for the analysis of chemical class isolates are in place. These include gas chromatography/mass spectrometry, direct probe mass spectrometry, and gas chromatography/Fourier transformed infrared spectrometry.

The contractor anticipates making special use of FTMS and ITMS. They have demonstrated the utility of FTMS for the multicomponent analysis of smoke tar and for observing high molecular weight constituents in sidestream smoke particulate matter. The method requires no sample processing and is applicable to submilligram quantities of sample. Further, it is applicable to the direct analysis of material collected by tapered element oscillating microbalance used for RSP quantitation. They have also recently demonstrated ITMS to be uniquely applicable to the high sensitivity determination of preselected constituents in complex mixtures. ITMS will especially be tested for its applicability to rapid quantitative determination of solanesol or other possible RSP marker compounds.

The objective of other ongoing work is to provide support to ongoing NCI-sponsored activities at ORNL through the availability of a core facility, and the generation of data required by other parts of the program so that the physical facility and staff expertise can be maintained for response to high priority specialty needs of NCI. The contractor is beginning a study of the composition of the smoke of a new type of cigarette which is claimed to heat but not burn tobacco and the determination of sidestream smoke emission rates of vapor phase constituents in order to assess the range of potential inputs for ETS atmospheres being generated and/or examined by other parts of the program.

The Premier cigarette is a smoking device which is claimed by the manufacturer, R.J. Reynolds Co., to heat, rather than burn, tobacco. Extensive chemical compositional studies have been conducted on the aerosol which the Premier produces--all by the manufacturer. These data indicate that the major fraction of the particle phase of the smoke is comprised of glycercol, propylene glycol, and water. Nicotine and other flavor compounds comprise most of the remaining components. The objective of this work is to perform an independent multicomponent characterization of the particle phase and vapor phase of the Premier, with special attention given to any components previously unreported in the smoke (14).

There is increasing evidence that environmental tobacco smoke is hazardous to the health of nonsmokers. A need has been perceived for reliable dosimetry, and work in one grantee's laboratory is developing improved assays for exposure to tobacco

products. At present, measurement of concentrations of cotinine in plasma, saliva or urine is felt to be most sensitive and specific. As a prelude to studies of human exposure to environmental tobacco smoke, they have modified the analytical method for nicotine and cotinine to increase sensitivity for determining low levels in nonsmokers' plasma, which range from 0-5 ng/ml. This has been achieved by concentrating the final extract to a volume of about 20 μ l. The determination is carried out by capillary column gas chromatography using either nitrogen-phosphorus detection or selected ion monitoring mass spectrometry. The limit of sensitivity is about 0.2 ng/ml. Both nicotine and cotinine can be determined, although determination of nicotine concentrations in nonsmokers is confounded by the ubiquitous presence of nicotine in the environment which may interfere with the analysis. Analysis is underway for several hundred plasma samples from nonsmokers. The data are currently being analyzed.

This group and others reported that trans-3'-hydroxycotinine is a major metabolite of nicotine, with concentrations in urine generally exceeding those of cotinine. Consequently, 3'-hydroxycotinine is likely to be a good marker for human tobacco exposure. They have developed a GC/MS-selected ion monitoring assay, and have obtained data on urinary concentrations in smokers. In six subjects, concentrations of 3'-hydroxycotinine ranged from 1.2 to 6.5 μ g/ml (mean 3.8 μ g/ml). In these same subjects, cotinine concentrations ranged from 0.8 to 2.7 μ g/ml (mean 1.8 μ g/ml). Currently, they are working on improving the sensitivity of the assay for determination of plasma levels in pharmacokinetic studies (2).

SMOKING AND HEALTH

GRANTS ACTIVE DURING FY89

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. BARTSCH, Helmut World Hlth Org, Int'l Agcy Res on Cancer 1 R13 CA48683-01	Meeting on N-Nitroso Compounds Mycotoxins and Tobacco
2. BENOWITZ, Neal L. University of California, San Francisco 5 R01 CA32389-07	Nicotine and Tar Intake During Cigarette Smoking
3. BRADLOW, H. Leon Rockefeller University 5 R01 CA44458-02	Smoking and Estrogen Metabolism Related to Cancer Risk
4. FOILES, Peter American Health Foundation 5 R01 CA32391-06	Tobacco-Specific Nitrosamine: RIA for DNA-Adducts
5. GUTTENPLAN, Joseph B. New York University 5 R01 CA44986-02	Mutagenic Components in Smokeless Tobacco
6. HOFFMANN, Dietrich American Health Foundation 5 P01 CA29580-08	Experimental Tobacco Carcino- genesis
7. HOFFMANN, Dietrich American Health Foundation 1 R01 CA44161-01A1	Bioassay of Snuff for Carcino- genic Activity in Rats
8. MARSHALL, Milton V. Univ. of Texas Hlth Sci Ctr, San Antonio 5 R01 CA33069-06	Carcinogen Metabolism in the Cigarette Smoking
9. MARSHALL, Milton V. Univ. of Texas Hlth Sci Ctr, San Antonio 1 R01 CA49401-01	Tobacco Products and Oral Cavity Cancer
10. MICHL, Josef Health Science Center at Brooklyn 5 R01 CA22682-11	An In Vitro Model of Pancreas Carcinogenesis
11. POMERLEAU, Ovide F. University Of Michigan at Ann Arbor 5 R01 CA42730-04	The Role of Anxiety in Cigarette Smoking
12. SCHULLER, Hildegard M. University of Tennessee, Knoxville 5 R01 CA42829-03	Transplacental Carcinogenicity of NNK

13. SCHULLER, Hildegard M.
University of Tennessee, Knoxville
1 RO1 CA48014-01

Characterization of Induced
Neuroendocrine Lung Cancer

CONTRACT ACTIVE DURING FY89

Investigator/Institution/Contract Number

Title

14. GUERIN, Michael
Department of Energy
Y01-CP-60513

Collection, Separation and
Elucidation of the Components
of Cigarette Smoke and Smoke
Condensates.

SUMMARY REPORT

CHEMICAL RESEARCH RESOURCES

The Chemical Research Resources component of the Branch is composed of six resource contracts which endeavor to make available to the research community chemical carcinogens, metabolites and inhibitors which are of reference standard quality for the pursuit of chemical carcinogenesis studies. In order to identify and delineate the pathways of carcinogen metabolism, mode of activation and molecular mechanism(s) of action, a large number of well-characterized unlabeled and radiolabeled derivatives and metabolites are required for use as authentic reference standards and/or substrates. Very often the desired derivatives are not commercially available, and researchers who require these compounds are not usually equipped to carry out the necessary syntheses. The six contracts currently total \$1.60 million in FY89 dollars.

The CPCB contracts with Midwest Research Institute (MRI) and Chemsyn Science Laboratories (CSL) to operate repositories which supply standard reference grade compounds for cancer research. MRI operates the Carcinogen Reference Repository, which supplies unlabeled standards for cancer research. CSL operates the Radiolabeled Carcinogen Reference Repository, which supplies radiolabeled standards of PAHs, PAH metabolites, aromatic amine metabolites, radiolabeled retinoids and other compounds of interest. CSL also supplies unlabeled dilepoxide optical enantiomers.

The repositories, in order to effectively serve the carcinogenesis research community, are required to synthesize or procure chemical samples; to provide for their safe and stable storage until requested by users; to repackage to meet user needs and to ensure the uniformity of all samples; and to ship samples to users along with the appropriate analytical documentation, characterization data and safe handling instructions. All of these operations must be performed in a safe manner and in compliance with current Occupational Safety and Health Administration (OSHA), Nuclear Regulatory Commission (NRC) and Department of Transportation (DOT) requirements. In addition, the repositories maintain up-to-date inventory systems which make it possible to keep a running balance of all stocks and commitments and to project future needs.

Of importance comparable to that of the major goal of the program, the assurance of a supply of reliable research material, is the task of ensuring the safe handling of these dangerous materials at all steps in their processing, from the synthesis or receipt at the repositories to receipt by the user of a package which can be opened in complete confidence. The equipment and handling procedures used in this program have been selected to ensure the minimum hazard to personnel and the environment, conformance with all transportation regulations and complete safety to the user upon receipt of the materials.

Shipments made from the repositories are sent by commercial carrier air freight. Although most of the compounds stocked by the repositories are known carcinogens, some are not, and many may still be controversial, at least in regard to their effect on humans. However, to stock both carcinogens and noncarcinogens and to provide for different handling, packaging and shipping procedures for each seems to leave too many opportunities for error, leading to possible exposure, mislabeling, accidental release of carcinogens into the environment, and so forth. Therefore, all compounds entrusted to the repositories are regarded as potential

carcinogens and are handled, packaged and labeled accordingly. This is not necessarily meant to imply that the sample is a known carcinogen--only that it is intended for use in research involving chemical carcinogens, and unless the recipient has contrary information, it should be treated as a carcinogen.

The repositories provide data sheets on the compounds in stock, including chemical and physical properties, analytical data and hazard, storage and handling information.

The current inventory of the Chemical Carcinogen Reference Standard Repository consists of 647 chemicals, excluding in vitro samples. Nine new chemicals were added to the inventory this year. Twenty-nine samples of chemicals were also reprocured to replace depleted or decomposed stocks.

One hundred ninety-nine requests were received for chemicals other than fecapentaenes during the third year of the contract. One hundred seventy-two were received from organizations in the United States; 27 were from foreign organizations. One hundred ten requests came from academia, 46 from industry (including independent research organizations), and 43 from government facilities.

Six hundred sixty-nine aliquots of 156 chemicals were shipped during the third year of the contract. Over 190 chemicals were analyzed at least once during this period. Three chemicals were found to be unusable because excessive levels of impurities were detected. Ultraviolet/visible and nuclear magnetic resonance spectroscopy, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) were the most frequently used methods of analysis.

There are presently 313 PAHs, which represent 48.4% of the inventory. Five hundred fifty-four aliquots (83% of those shipped) were PAHs. Benzo(a)pyrenes were the most popular PAHs by far. Four hundred seven aliquots from 49 benzo(a)pyrenes, 70 aliquots of 32 benz(a)anthracenes, 3 aliquots of 2 benzofluoranthenes, and 74 aliquots of 35 other PAHs were shipped.

A new catalog was produced and distributed for the NCI Chemical Carcinogen Reference Standard Repositories. As part of this activity, the ordering process was revised to allow direct submission of orders to the respective repositories. Feedback on the repository services was requested from all users and received from over 100 individuals as part of catalog distribution.

Preparation of Volume 1 of the PAH handbook series was initiated. The format was determined after consultation with the NCI Project Officer, other NCI principal investigators and other resource contractors. Data collection is approximately 85% complete. Camera-ready copies have been produced for about 80% of the data collected.

Nine chemicals were procured to support NCI's in vitro testing program. Seventeen aliquots of chemicals have been sent to various toxicology laboratories for testing. Analytical reports for seven chemicals were submitted to NCI. The chemicals were procured from commercial vendors and custom synthesizers. Analytical data sheets were obtained from the supplier or analyses were performed to ascertain sample purity. Coded aliquots of the samples were provided to the toxicology contractors along with hazard summaries that allow for safe handling.

Two sets of nitrosamine standards were prepared, analyzed and shipped in support of the United States Department of Agriculture (USDA) nitrosamines in bacon program.

Approximately \$109,000 was invoiced to requestors this year from the repository at MRI. These charges represent cost recovery for packaging and shipping and a prorated cost for chemical synthesis. The funds received by the contractor are subtracted from the total monthly operation cost of the contract prior to invoicing the NCI (1).

There are four contractors who collaborate closely and who are involved in the synthesis of carcinogenic compounds and/or their metabolites for supply to the repository program. The four synthesis contractors develop suitable routes for the unequivocal organic synthesis of compounds designed by the NCI Project Officer, and they develop methods for production of adequate quantities of well-characterized compounds of high purity (generally greater than 98%). Compounds are analyzed by a meaningful combination of techniques to assess purity and confirm structure. These may include ultraviolet, fluorescence, and/or infrared spectrometry, nuclear magnetic resonance, mass spectrometry.

The major objective of two of the contractors is the synthesis and purification of NCI-selected nonlabeled and labeled (^3H , ^{14}C) polynuclear aromatic hydrocarbon derivatives of the following types: phenols; quinones; epoxides; dihydrodiols; alkyl and hydroxyalkyl substituted parent hydrocarbons; nitro-PAH derivatives; PAH-DNA adducts; and sulfate, glucuronide and glutathione conjugates. These derivatives are prepared by unequivocal methods to produce adequate quantities of well-characterized compounds of high purity ($\geq 98\%$) for distribution as metabolite standards through the NCI Chemical Carcinogen Reference Repository. Activities in support of the NCI Repository include the initial synthesis, maintenance of inventory through resynthesis, and shipments of compounds to authorized recipients of isotopically labeled PAH metabolites from a Radiochemical Repository at CSL.

The synthesis, purification and characterization of the following 15 compounds was completed during this report period at CSL: 3,4-Dihydrocyclopenta(c,d)pyrene; Cyclopenta(c,d)pyrene; ($\text{G-}^3\text{H}$)Cyclopenta(c,d)pyrene; Cyclopenta(c,d)pyren-3(4H)-one; (+)-trans-7,8-Dihydro(1,3- ^3H)benzo(a)pyrene-7,8-diol dibenzoate; r-7-t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro(1,3- ^3H)benzo(a)pyrene (anti); 7-Hydroxy(ring- $\text{G-}^3\text{H}$)benzo(a)pyrene; (+)-4,5-Dihydro($\text{G-}^3\text{H}$)benzo(a)pyrene-4,5-oxide; ($\text{G-}^3\text{H}$)Benz(a)anthracene; ($\text{G-}^3\text{H}$)Chrysene; r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro(7- ^{14}C)benzo(a)pyrene (anti); r-7,t-8-Dihydroxy-c-9,10-epoxy-7,8,9,10-tetrahydro(7- ^{14}C)benzo(a)pyrene (syn); r-7,t-8-Dihydroxy-c-9,10-epoxy-7,8,9,10-tetrahydro(1,3- ^3H)benzo(a)pyrene (syn); ($\text{G-}^3\text{H}$)Dibenzo(a,i)pyrene; ($\text{G-}^3\text{H}$)-Dibenzo(a,h)pyrene; and ($\text{G-}^3\text{H}$)Dibenz(a,c)anthracene.

CSL also maintains a radiorepository for the NCI which presently has an inventory of 65 radiolabeled (^3H , ^{14}C) polynuclear aromatic hydrocarbons and their metabolites. A portion of the synthesis, packaging and shipping costs is recovered from radiorepository users by a payback mechanism. During this report period, a total of 110 radiolabeled compounds were delivered in 67 shipments to 53 different investigators at a total billed cost of approximately \$37,241 (6).

The other contractor preparing derivatives of polycyclic aromatic hydrocarbons is American Health Foundation. Their focus during this report period has been on the synthesis of authentic DNA-adducts of the benzofluoranthene series for use in the

³²P-postlabeling assay. They have prepared three diol epoxides for synthesis of the corresponding N^c-deoxyguanosyl-3'-phosphate adducts. The diol epoxides are: fluoranthene-2,3-diol-1,10a-epoxide; benz[b]fluranthene-9,10-diol-11,12-epoxide; and benz[c]phenanthrene-3,4-diol-1,2-epoxide. Other completed adducts are: 4-(N^c-deoxyguanosyl-3'-phosphate)-1,2,3-trihydroxy-1,2,3,4-tetrahydrochrysene; 4-(N^c-deoxyguanosyl-3'-phosphate)-1,2,3-trihydroxy-6-methyl-1,2,3,4-tetrahydrochrysene. These standards represent the first authentic standards for the Randerath procedure.

Two contractors prepare selected chemical carcinogens for the repository program. The goals of these contracts include the resynthesis, purification and characterization of selected metabolites (e.g., quinones, dihydrodiols, epoxides, diol-epoxides and phenols) of PAH and the synthesis of selected aromatic amines, steroid derivatives, nitrosamines, physiologically active natural products and other parent polynuclear aromatic hydrocarbons. The products include both unlabeled and labeled (³H and ¹⁴C) compounds at a purity level of > 98%. These derivatives are characterized by a meaningful combination of analytical techniques including TLC, IR, UV, NMR, GC, melting point, boiling point and elemental analysis.

Most of the PAH derivatives have been previously prepared by other NCI contractors who have developed the synthesis routes, characterized the products, determined their relative stabilities and maximized product yields. The resyntheses of these compounds on these contracts utilize these proven procedures to produce well-characterized compounds of high purity to resupply the depleted stores of the Repository. Other compounds which were required by the Repository, but for which there was no previously reported synthesis, were prepared in small scale exploratory runs designed to develop the synthesis route and to determine maximum yields; the required quantity of material was generated in a production run(s).

On one of the contracts, again at CSL, the synthesis, purification and characterization of the following 11 compounds has been completed: (+)-r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; Benzo(a)pyrene-9-ol; Benzo(a)pyrene-9-sulfate, postassium salt; Benzo(a)pyrene-3,6-dione; (+)-r-7,t-8,9,10-Tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene; (+)-r-7,t-8,9,c,10-Tetrahydroxy-7,8,9,10-tetrahydrobenzo(a)pyrene; (+)-r-7,t-8-Dihydroxy-c-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; Benzo(a)pyrene-4,5-dione; Benzo(a)pyrene-6-ol; (+)-7,8-Dihydrobenzo(a)pyrene-7,8-epoxide; and Benzo(a)pyrene-2-ol.

The synthesis, resynthesis or repurification of the following seven compounds are in progress: Benzo(a)pyrene-1,6-dione; Benzo(a)pyrene-2-ol; Methidiumpropyl-EDTA (MPE); Benzo(a)pyrene-4-ol; (+)-3,4-Dihydrocyclopenta(c,d)pyrene-3,4-epoxide; Benzo(a)pyrene-5-ol; and Benzo(a)pyrene-8-ol (5).

The other contractor, SRI International (4), has completed synthesis of 130 mg of fecapentaene-1; fecapentaene-14 (20 mg); ³H-fecapentaene-12 (1 mCi); as well as the resynthesis of 11 PAH derivatives: trans-3,4-dihydroxy-3,4-dihydro-7,12-dimethyl-benz(a)anthracene; trans-3,4-dihydroxy-anti-1,2-epoxybenz(a)anthracene; trans-3,4-dihydroxy-3,4-dihydrodibenz(a,h)anthracene; trans-1,2-dihydroxy-1,2-dihydrochrysene; benzo(a)pyrene-7,10-dione; indeno(1,2,3-cd)pyrene-1,2-epoxide; benzo(a)pyrene-1-0-sulfate; and PAH glucuronides (4).

RESEARCH RESOURCES
CONTRACTS ACTIVE DURING FY89

<u>Investigator/Institution/Contract Number</u>	<u>Title</u>
1. GRAVES, Steven Midwest Research Institute NO1-CP-51012	Chemical Carcinogen Reference Standard Repository
2. HECHT, Stephen S. American Health Foundation NO1-CP-61041	Synthesis of Derivatives of Polynuclear Aromatic Hydrocarbons
3. PETRAZZUOLO, Gary Technical Resources, Inc. NO1-CM-57658	Working Group on Neoplasms and Related Disorders in Fishes
4. REIST, Elmer J. SRI International NO1-CP-71108	Synthesis of Selected Chemical Carcinogens
5. RUEHLE, Paul H. Eagle-Picher Industries, Inc. NO1-CP-71007	Synthesis of Selected Chemical Carcinogens
6. WILEY, James C. Eagle-Picher Industries, Inc. NO1-CP-61037	Synthesis of Derivatives of Poly- nuclear Aromatic Hydrocarbons

ANNUAL REPORT OF
THE RADIATION EFFECTS BRANCH
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

The Radiation Effects Branch (REB), established in response to Public Law 95-622, plans, directs and administers a program consisting of grants and contracts investigating the means by which exposure to ionizing and non-ionizing radiations, particularly at low doses or dose rates, leads to molecular and cellular events and processes resulting in mutagenesis, cell transformation, and carcinogenesis, and the associated dose-effect relationships; directs and administers selected epidemiological studies investigating the effects of radiation exposure in humans; provides a broad spectrum of information, advice, and consultation to scientists and to institutional science management officials relative to the National Institutes of Health (NIH) and the National Cancer Institute (NCI) funding and scientific review policies and procedures, preparation of grant applications, and choice of funding instruments; maintains contact with other Federal agencies and institutions and with the broader relevant scientific community to identify new and needed research in, and related to, the fields of radiation mechanisms and effects; provides NCI management with recommendations concerning funding needs, priorities, and strategies for the support of relevant research areas consistent with the current state of development of individual research elements and the promise of new initiatives; provides information, advice, and guidance to NCI management and staff on radiation-related issues; implements the mandates of Public Law 97-414, Section 7(a); and represents the Department of Health and Human Services on the Science Panel of the Committee on Interagency Radiation Research and Policy Coordination, which is located within the Office of Science and Technology Policy, Office of the President.

The extramural activities of the Branch are accomplished through contractual agreements with universities and other Federal agencies, and through traditional individual research grants (R01), conference grants (R13), first independent research support and transition (FIRST) awards (R29), new investigator research grants (R23), program project grants (P01), small business innovative research (SBIR) grants (43/44), outstanding investigator grant (OIG) awards (R35), methods to extend research in time (MERIT) awards (R37), and academic research enhancement awards (AREA) (R15). At present the Branch administers over 110 extramural research activities with an annual budget in excess of 17 million dollars (Tables I and II). The program consists of two broad categories of research: mechanisms of radiation damage and repair, and radiation carcinogenesis. In addition, the NIH and the NCI have assigned to the Branch responsibility for the implementation of sections of two Public Laws addressing radiation-related issues emanating from Congressional policy concerns.

Section 7(a) of Public Law 97-414, the Orphan Drug Act, requires the Secretary to conduct scientific research and prepare analyses necessary to develop valid and credible (1) assessments of the risks of thyroid cancer that are associated with thyroid doses of Iodine-131, (2) methods to estimate the thyroid doses of Iodine-131 that are received by individuals from nuclear weapons fallout, and

(3) assessments of the exposure to Iodine-131 that the American people received from the Nevada atmospheric nuclear bomb tests. A working committee consisting of relevant expertise within and outside of the government, including foreign nationals, has been formed and is addressing these issues. The committee is organized into three task groups addressing the risk of thyroid cancer per unit dose of Iodine-131 to the thyroid, the dose of Iodine-131 to the thyroid per unit of exposure to Iodine-131, and the development and verification of models to estimate the exposure of the American people to Iodine-131 resulting from radioactive fallout associated with atmospheric nuclear weapons tests at the Nevada Test Site. A number of meetings of the task groups and a meeting of the committee have been held during this year. In particular, considerable effort has been expended with respect to the required exposure and dose reassessments; these are being carried out via interagency agreements and with the assistance of staff expertise acquired for this purpose. A comprehensive final report will not be available for some time, although activities associated with items (2) and (3) above should be complete during the next reporting period, with final reports to be submitted thereafter.

Reviews and analyses of fallout source terms and available monitoring data for each of the atmospheric tests have been completed. In addition, meteorological conditions at multiple altitudes at the time of each test, together with wind and precipitation patterns across the United States during the days the fallout cloud was over United States territory, have been reconstructed. These data form the bases upon which statistical interpolation and extrapolation of fallout levels (i.e., ground surface levels) of Iodine-131 have been made for each of the 3,071 counties within the continental United States for each of the tests.

Since the primary exposure of the public to Iodine-131 was via the consumption of milk produced by grazing cows, milk production and distribution patterns, by county, were reconstructed. This methodology resulted in estimates of Iodine-131 concentrations in milk in each county and included the consideration of such factors as the location and times of the year when dairy herds were on pasture in relation to the date of each test and the areas from which milk was mixed. These estimated Iodine-131 concentrations in milk, combined with demographic information and individual consumption rates, will permit an estimate of Iodine-131 exposure for residents of each county as a function of age and sex. Completion of exposure estimates is anticipated to be complete within approximately two years.

The Mechanisms of Radiation Damage and Repair program includes, but is not limited to, studies on molecular and cellular changes resulting from exposure to ionizing and non-ionizing, principally ultraviolet, radiation, DNA damage and repair following radiation exposure, the hypermutability, mutagenesis, and malignant transformation of exposed cells, mechanisms of tumor promotion, and mutagenicity-carcinogenicity relationships following exposure to radiation.

The Radiation Carcinogenesis program addresses the effects of exposure to ionizing and non-ionizing, principally ultraviolet, radiation, including, for example, the role of oncogenes, the identification of molecular markers unique to cells transformed by radiation, the role of cofactors and systemic mediators in radiation-induced carcinogenesis, the effect of dose rate and linear energy transfer on radiation-induced effects, dose-effect relationships, interspecies comparisons, cocarcinogenesis, the incidence of selected diseases as they may relate to exposure from radioactive fallout, and synthesis of radiobiological

data in the assessment of risk and the establishment of appropriate radiation protection practices.

TABLE I
 RADIATION EFFECTS BRANCH
 (Extramural Activities - FY 1989 - Estimated)

	<u>No. of Contracts/Grants</u>	<u>\$ (Millions)</u>
Research Contracts	3	0.33
Research Grants	111	17.17
Traditional Research Grants (R01) (89 grants; \$12.64 million)		
Conference Grants (R13) (3 grants; \$0.02 million)		
FIRST Awards (R29) (7 grants; \$0.66 million)		
New Investigator Research Grants (R23) (1 grant; \$0.04 million)		
Program Project Grants (P01) (1 grant; \$1.70 million)		
Small Business Grants (R43/44) (1 grant; \$0.05 million)		
Outstanding Investigator Grants (R35) (2 grants; \$0.79 million)		
MERIT Awards (R37) (6 grants; \$1.22 million)		
AREA Grants (R15) (1 grant; \$0.05 million)		
TOTAL	114	17.50

TABLE II
 RADIATION EFFECTS BRANCH
 (Contracts and Grants Active During FY 1989)

	FY 89 (Estimated)			
	CONTRACTS		GRANTS	
	No. of Contracts	\$ (Millions)	No. of Grants	\$ (Millions)
Radiation Mechanisms and Carcinogenesis	2	0.33	106	16.81
Office of the Chief	<u>1</u>	<u>0.00</u>	<u>5</u>	<u>0.36</u>
TOTAL	3	0.33	111	17.17

Research activities are concerned with a wide variety of radiation effects including mechanisms of damage and repair of DNA by ionizing and non-ionizing radiation, and radiation carcinogenesis. The majority of the 111 grants (88) support investigations relating to mechanisms of radiation damage and repair of cellular DNA, 63 of which investigate the effects of exposure to ionizing radiation and 25 of which study the consequences of exposure to ultraviolet (UV) radiation, microwaves and ultrasound. Seventeen grants and two contracts fund studies in radiation carcinogenesis; research addressing radiation risks and the compilation and assessment of information is supported by six grants; and one contract supports environmental studies related to Public Law 97-414, Section 7(a).

Mechanisms of Radiation DNA Damage and Repair: The Radiation Effects Branch provides substantial support for basic research related to mechanisms of DNA damage and repair, mutagenesis and malignant transformation by radiation. This information is essential for providing both insight into the mechanisms of carcinogenesis and the rationale for the carcinogenic effects seen following exposure to radiation. Such studies contribute to the evaluation of risks to individuals and to populations and thereby assist in providing a data base upon which radiation standards might be developed.

A. Ionizing Radiation: Gradually over the past few years the sharp focus of research on the nucleotide excision repair mechanism in eukaryotes has broadened to include the important areas of alkyl transferase, recombination repair, and cell cycle-dependent mechanisms that are essential to maintain the integrity of the cellular genome. It is now becoming apparent that DNA replication and repair of damage are complex integrated processes and that their interdependence is at least partly a result of the complexity of chromatin structure. An additional logistic complication arises from the necessity of DNA replication, transcribing DNA to RNA for protein synthesis, and cellular regulation. These processes result in opening the structure of DNA, which alters the DNA reactivity and increases susceptibility to damage. An investigator in the Radiation Effects Branch program who is studying gamma-radiation-induced DNA damage in mammalian cells has found that both damage and repair are modulated by chromatin structure. Irradiation of isolated chromatin results in the formation of many cross-links between histone proteins and DNA. However, in intact cells few histones are cross-linked to DNA but many nuclear matrix proteins are cross-linked. Further, although the same dose-response for DNA-protein cross-links is observed for both isolated chromatin and intact cells, the yield of cross-links saturates much earlier in the intact cells. Experiments stabilizing the nuclear matrix structure with copper ions indicate that most of the nuclear DNA is protected from DNA-protein cross-link formation by cellular scavengers and structural features of natural (condensed), as opposed to isolated (expanded), chromatin. Enhanced yields of DNA-protein cross-links appear to result from excess radicals generated through Fenton-type reactions at sites stabilized by multivalent metal ions. This work suggests that hydrogen peroxide is much more important than previously considered in understanding ionizing radiation damage of DNA, and that knowledge of the microdistribution of damage in chromatin and the nuclear matrix is essential for understanding cellular radiosensitivity.

By far, most environmental (as opposed to experimental) damage to DNA is oxidative in nature for both chemicals and non-ionizing radiation, as is essentially all damage from ionizing radiation. The repair response of cells to this damage is very complex, involving various multienzyme processes both for

repair of damaged DNA and for protection against oxidants. An investigator working with bacteria has focused on the regulation of protein in response to different kinds of oxidative stress. Two dimensional polyacrylamide gel analysis of the proteins produced in these bacteria indicates two general responses to low-level oxidative stress; one response is obtained with hydrogen peroxide and another with redox-cycling agents. A set of 35 polypeptides is switched on by exposure to micromolar concentrations of hydrogen peroxide. Of these 35 polypeptides, only eight are controlled by a previously described gene (oxyR) known to be involved in regulating this cellular response. Low levels of redox-cycling agents (which generate superoxide within the cell) activate the synthesis of an additional >35 polypeptides, plus all of the hydrogen peroxide-inducible proteins. This investigator has studied the control of these oxidation-inducible proteins by isolating regulatory mutants. Several important strains that were isolated have mutations at a gene locus named soxR, located near the well-studied uvrA gene which facilitates cloning the soxR gene. This gene appears to encode a positive regulator of a superoxide response, and the soxR-constitutive mutants have increased resistance to various oxidative damaging agents including the redox-cycling quinones and organic peroxides. Two dimensional gel analysis of these constitutive mutants demonstrates increased levels of 12 proteins that otherwise require induction by menadione or paraquat. Among these 12 proteins several enzyme activities have been found including the DNA repair enzyme endonuclease IV, Mn-superoxide dismutase, glucose-6-phosphate dehydrogenase and NADPH-dependent diaphorase which reduces menadione. This work illustrates both the multilayered defenses needed to cope with oxygen radical stress and the possible use of these multi-drug-resistant mutants to better understand resistance to chemotherapeutic drugs.

Another investigator studying cellular response pathways in bacteria has concentrated on repair endonucleases. Deficiencies in this group of enzymes have been implicated in certain human disease conditions including xeroderma pigmentosum. There are now at least nine of these enzymes which are specific for oxidized bases, the last two of which have just now been identified, characterized and purified. These last two, endonucleases VIII and IX, appear to serve as backup enzymes for the well-characterized endonucleases III and IV and exonuclease III. These enzymes are responsible for repair of such potentially lethal oxidation products as urea glycoside and thymine glycol. The discovery and elucidation of the structural specificities of these various enzymes is important because human cells have enzymes remarkably similar to and often identical with the bacterial enzymes; this knowledge from bacterial studies greatly facilitates the analogous work required to understand DNA repair deficiencies and limitations in human cells.

One of the forms of DNA damage induced by x-irradiation in human cells is base-free sites. An investigator now studying the enzymes that recognize and repair such damaged sites has recently discovered that a particular magnesium-dependent purified mammalian apurinic endonuclease from this group recognizes only a subset of the lesions which were previously assumed to be equivalent damaged sites. This indicates that the remainder of such sites are recognized and repair-initiated by other enzymatic processes and that recognition of the surrounding DNA base sequence may be important for some as yet unknown reason.

Many investigators have attempted to identify the specific damage produced in DNA by ionizing radiation and the cellular responses that give rise to the biological

end points of lethality, mutagenesis and recombination. This effort is important because lethality precludes inducing cancer, and recombination repair can greatly reduce mutagenesis and cancer induction in mammalian cells. Thus, information from this project could lead to techniques that permit evaluation of individual susceptibility to cancer. An investigator has now constructed a plasmid vector to measure single strand and double strand DNA breaks and the plasmid also has been adapted to measure survival, mutation frequency (lac^+ to lac^-), and the frequency of gene recombination following gamma irradiation. He found a much higher frequency of recombination than mutagenesis after irradiation in aqueous solution. The recombination was dependent on the rec A gene, but the level of survival was independent of this gene. The lesions giving rise to recombination were caused by indirect action of aqueous free radicals. Since lethal events are known to be associated with direct double strand breaks, these results indicate that one significant class of lesions resulting from ionizing radiation can possibly be minimized or prevented from inducing cancer by optimizing the recombination process.

Plutonium has been an essential component of the nation's nuclear weapons since shortly after its discovery, and its use for this and other purposes has led to its production since that time. Because of the inventories of this very useful but potentially carcinogenic radioactive substance, it behooves the Radiation Effects Branch to consider possible approaches to minimize or reverse untoward effects of an accidental exposure of people to plutonium or other radioactive elements. With these ends in view, an investigator is designing, synthesizing and testing multidentate chelating agents for general decorporation of internally deposited heavy metal radiotoxins. The agents being developed have properties that allow them to be orally administered. The chemical model used for the conceptual design of new compounds is that of the Food and Drug Administration approved chelator, DTPA (diethylenetriaminepentaacetic acid). These agents are derived from triethylenetetramine to which a straight alkyl group with 8 to 16 carbon atoms has been attached. Following exhaustive carboxy methylation of the remaining amino-nitrogen, partially lipophilic octadentate agents are produced. These compounds have chemical properties that allow them to penetrate bilipid cellular membranes and form a strong chelate with transuranium elements. Current *in vitro* test results indicate that extensive *in vivo* testing is warranted. The significance of this work is that this group of chelators appears to have greatly improved pharmacological properties for the removal of metals and radionuclides from the body. Another investigator has now demonstrated in life-span experiments with dogs that the prolonged use of Zn-DTPA, which accelerates excretion of actinide elements from the body, is apparently safe and that such treatment markedly decreases the risk of radionuclide-induced bone and liver cancer associated with body burdens of Plutonium-239 or Americium-241.

B. Ultraviolet Radiation: The Radiation Effects Branch is interested in and concerned with ultraviolet radiation because it is a potent carcinogen that pervades the environment. Further, it interacts with other carcinogens and agents in complex ways which can result in either enhancement or inhibition of cancer induction and progression. An important goal of the Radiation Effects Program is to understand these complexities in order to identify, quantify and minimize the deleterious effects of ultraviolet radiation.

The possibility of ozone depletion in the upper atmosphere caused by the production and release of gaseous contaminants is of increasing concern because it could result in a substantial increase of ultraviolet radiation reaching the

earth's surface. Thus, questions regarding the extent of the increase and the wavelengths that are responsible for effects on cancer induction and progression are likely to be of increasing importance as time progresses and as the extent and intensity of exposure to ultraviolet light increases. An investigator working on this problem of increased ultraviolet radiation due to ozone depletion has completed a determination of pyrimidine dimer formation in human skin in situ as a function of wavelength dependence. After the resulting absolute action spectrum has been convoluted with solar emission spectra for conditions representative of today's stratospheric ozone concentration, and also convoluted for other fixed levels of ozone concentration, the extent to which pyrimidine dimer formation increases can be determined. From these data, the correlation between pyrimidine dimer formation and ozone concentration can be calculated, showing, for example, that a 50% ozone depletion would result in a 2.5-fold increase in the dimer yield. Estimates of the potential increase in human skin cancer resulting from such an increase in DNA damage can be made based upon published epidemiologic data. For example, such an increase in dimer yield would be expected to elevate the rate of skin cancer incidence for white males living in Seattle, WA, to approximately that which now occurs for white males in Albuquerque, NM. This would be a 3-fold increase of nonmelanoma skin cancer and a 50% increase in skin melanoma. These estimates, of course, assume that solar induction of cancer is virtually all proportional to, and a function of, those factors that cause pyrimidine dimers, including, most particularly, the effect of the ozone absorption spectrum on dimer formation.

It is nearly universally accepted that damage to, or a change in, DNA is a prerequisite for malignant transformation. This concept has stimulated great interest in the repair of DNA damage. Damage to DNA resulting from exposure to ionizing radiation generally has been thought to be qualitatively different from that resulting from exposure to ultraviolet radiation, with each type of damage being repaired by separate cellular processes. These concepts were strengthened by studies with chemical carcinogens and mutagens which seemed to fall into two groups mimicking either the ionizing or the ultraviolet radiation damage and repair processes. It now appears that the damaging process is largely a quantitative difference and that the molecular and cellular repair responses are remarkably integrated, although they appear to be more complex than was previously thought. For example, an investigator has compared the induction of single strand DNA breaks in human cells by short, middle and long wavelength ultraviolet light (UVC, UVB, UVA, respectively) and blue light (434 nanometers) with single strand breaks obtained after exposure to x-rays, gamma rays and fission neutrons. Although DNA strand breaks generally are associated with ionizing radiation and usually are not associated with ultraviolet light, this investigator found that the ultraviolet radiation (UVB and UVA), and even the blue light, produced many more DNA strand breaks than did the more energetic ionizing radiations when compared on a per lethal dose basis. Thus, even though ionizing radiation is more damaging than ultraviolet or blue light when compared on a per quantum basis, the latter is potentially more dangerous because far more quanta of the UVB, UVA, and blue light reach the earth's surface. This work is particularly relevant because these wavelengths in the solar spectrum are those associated with carcinogenesis.

Other important similarities between DNA damage caused by ionizing radiation and that caused by solar radiation at the earth's surface also are emerging. Studies of DNA damage induced by ultraviolet light have concentrated, until recently, almost exclusively on the short wavelength UVC (which does not reach the surface

of the earth) that damages DNA after direct absorption of the energy, while DNA damage by ionizing radiation is generally believed to result largely from the production of reactive oxygen species by the radiolysis of water. A recent study has raised questions regarding these presumed qualitative differences and has shown that the hydroxyl radical (OH), the main reactive species produced by ionizing radiation, also is abundantly produced in cells by the UVB, UVA, and blue portion of the solar spectrum. These hydroxyl radicals do not come directly from water, but are a result of the photosensitization of non-DNA chemicals in the cells which absorb the light.

In another study, when the investigator irradiated human cells with monochromatic 365 nanometer UVA, initial yields of DNA strand breaks were three times greater in repair-deficient xeroderma pigmentosum cells than were the strand breaks observed for the same dose to repair-proficient normal cells. At doses which result in the same number of DNA strand breaks in repair-proficient and -deficient cells, repair is rapid and complete in the repair-proficient cells, whereas 25% of the strand breaks in the xeroderma pigmentosum cells are not repaired. In addition, results showed that strand breaks induced by UVA are not all identical to those which are induced by hydrogen peroxide, thereby suggesting that, contrary to speculations by many investigators, not all of the strand breaks are produced by hydrogen peroxide. These results illustrate additional complexities in the seemingly simple and facile cellular process of repairing single strand breaks.

A very strong causal relationship between the defective repair of ultraviolet radiation damage to DNA and carcinogenesis in the clinical syndrome, xeroderma pigmentosum, has been developed over many years. Nonetheless, the actual molecular defects (there are at least nine complementation groups) of this complex syndrome still are not understood. For example, two prominent and competing hypotheses to explain the deficiency in complementation group A are: (1) the enzyme(s) for incising the DNA near the damaged site is defective or missing, and (2) the process by which the incising enzyme(s) reach the damaged site through the tertiary DNA structure is defective. An investigator has now cloned and characterized a gene which confers enhanced survival to cells from a patient with xeroderma pigmentosum, complementation group A, following exposure to ultraviolet light. The gene was cloned by using a 10.6 S size fraction of cDNA libraries from the heterogynous non-affected mother's cells and isolated by a competition hybridization technique developed by the investigator. The competitive hybridization enriched the DNA fraction expressed by cells from the mother in the DNA which was not expressed by cells from the affected child, thus greatly increasing the probability of cloning the missing or altered DNA related to xeroderma pigmentosum. Using an appropriately constructed expression vector, pCV108, the child's cells were transfected with six different clones, and ultraviolet light-resistant colonies were isolated. With these resistant cell lines indicating that the cloned DNA reverses the group A defect, the investigator is now in a position to critically evaluate the two competing hypotheses regarding the defect in this complementation group.

To date there have been two generally accepted mechanisms for pyrimidine dimer repair. In the first mechanism, photoreactivation, an enzyme uses visible blue light energy to simply reverse the ultraviolet light damage. The second method, nucleotide excision repair, entails a complex process by means of which the dimer is excised, the resulting gap is filled by repair synthesis, and the broken DNA strands rejoined by a ligase. Recent studies have provided evidence that a third

mechanism is used by mammalian cells to remove dimers in the special case where two dimers are close together on opposite strands of the DNA. This repair mode is very rapid, independent of excision repair capacity, depends on semi-conservative synthesis, and appears to be a result of translesion synthesis, which is a mechanism whereby the cell can replicate the damaged strands without leaving gaps in the daughter strands opposite the damaged sites. It also appears that neither homologous nor nonhomologous recombination is involved, again adding further complexity to DNA repair with yet another mechanism by which the cell copes with pyrimidine dimers.

The Radiation Effects Branch also has a limited number of grants investigating biological effects resulting from exposure to radiations other than ionizing and ultraviolet. For example, results from one study indicate that mouse fibroblasts exposed in vitro to high power pulsed ultrasound at an intensity found at the focal spot of a typical diagnostic transducer either alone or in conjunction with x-irradiation exposure did not affect cell transformation. This indicates that ultrasound under these conditions is neither a complete carcinogen, nor an initiator or cocarcinogen with x-rays. Another study investigating microwave irradiation of mouse fibroblasts at 2.45 GHz found that this radiation did not transform the cells. However, in the presence of a promoting agent (TPA) a small excess of transformants was found, indicating the possibility that, under these conditions, microwaves can be a very weak initiator. These results will have to be carefully confirmed because of the logistic problems involved with large in vitro transformation experiments.

Radiation Carcinogenesis: This program includes a diversity of investigations that focus on basic studies on the molecular mechanisms of radiogenic cell transformation and cancer as well as research that is oriented towards practical radiation protection and epidemiological applications. Within its scope of activities, the program includes the carcinogenic effects resulting from exposure to (1) ionizing and non-ionizing radiations, (2) high-LET and low-LET forms of ionizing radiations, and (3) combinations of various forms of radiation or radiation plus chemical carcinogens. Many of the projects included within this program also have components involving work on DNA damage and repair, and mutagenesis, because of the linkage between these phenomena and cancer. Major program areas include studies of the molecular mechanisms of radiation-induced cell transformation, including the role of DNA-damage and repair; the role of specific oncogenes and other radiation-specific genes as possible markers for radiogenic cancer; and the significance and influence of dose-rate and type of radiation in determining effects in molecules, cells and whole animals.

The molecular mechanisms that underly oncogenic transformation induced by ionizing radiation are not well understood and may differ in fundamental aspects from transformation induced by chemical carcinogens. One project that involves a comprehensive comparison of the molecular effects induced by ionizing radiation in terms of DNA damage, mutagenesis and cell transformation, showed that the expression of mutations was delayed far beyond the comparable expression times for known chemical mutagens. Depending on the specific type of mutation (i.e., a recessive mutation at a defined locus, or a lethal mutation), expression of the mutation continued for up to 10-30 population doublings of survivors and progeny of survivors following the initial radiation exposure. Such a prolonged delay in the expression of mutations in mammalian cells is uncommon and may not occur following exposures to chemical carcinogens which act initially as mutagens. In other results obtained by the same investigator, it was shown that following

exposure to x-rays the transformation phenotype could be transfected with DNA from primary foci (mouse C3H10T1/2 cells) to both primary and secondary recipients (NIH/3T3 cells). When DNA extracted from the primary foci and from the primary and secondary transfectants was analyzed for amplifications and rearrangements of known oncogenes associated with chemically induced transformation, none were found. This data is consistent with the possible existence of unidentified oncogenes as components in malignant cell transformation induced by ionizing radiation.

Mechanistic differences in the way mammalian cells respond to damage induced by exposure to high-LET ionizing radiation vs. that caused by low-LET ionizing radiation, non-ionizing radiation or chemical agents, is an area of considerable interest to the scientific community. In one project, cultured murine cells were exposed to alpha particles at varying doses and dose-rates; endpoints included cell killing, large-scale chromosomal aberrations, sister chromatid exchanges, and molecular changes to DNA. Preliminary results suggest that the cellular repair processes that result in increased survival and lead to a reduction in chromosomal aberrations with confluent holding following exposure to x-rays do not operate or are relatively inactive in cells exposed to alpha particles. The data also indicated that low doses of high-LET radiation caused a much higher frequency of large-scale molecular effects to DNA than did the irradiation of cells with x-rays. These investigations help in understanding the effect of dose and dose rate as factors in DNA damage induced by high-LET radiation, and they are of interest to the Radiation Effects Branch because it is increasingly evident that possibly a large number of individuals in the general population are exposed to densely ionizing radiation in doses that may represent a health risk due to exposure to radon and its decay radionuclides from natural sources in the environment.

The transfer of human genes for the repair of DNA damage from exposure to ultraviolet radiation has been difficult to achieve in the laboratory. However, other strategies are beginning to yield results that may elucidate the repair of ultraviolet radiation damage in human cells. One investigator has been able to affect the transfection of human xeroderma pigmentosum type A (XPA) cells with foreign chromosomal DNA from the marsupial, Potorous tridactylis. As a result, XPA clones resistant to ultraviolet radiation were obtained, demonstrating the transfer of higher eukaryotic genes capable of restoring excision DNA repair into human cells. The marsupial genes are now being isolated for studies at the molecular level. This work provides a promising approach for studies on genetic organization and enzymology of DNA repair in higher cells, and it can be viewed as a technique with the potential for gene therapy in humans.

It is important to find and identify the oncogenes induced by ultraviolet radiation in order to (a) obtain a greater understanding of the enzymatic and genetic mechanisms involved in this type of malignant transformation, and (b) provide potentially useful markers for the detection of skin cancer. In work done on the same project cited above (i.e., transfection of marsupial DNA repair genes into human cells), putative oncogenes from human cells initiated with ultraviolet radiation were identified by the transformation of normal human keratinocytes to anchorage-independent growth (i.e., a phenotype associated with malignant transformation). This work is a preliminary step in the attempt to identify the oncogenes involved in ultraviolet light-induced malignant transformation and to determine whether there is a single oncogene or multiple oncogenes associated with malignant melanoma in humans. Similarly, it may also

be possible to extend this approach to comparisons of other oncogenes that may be associated with skin cancer induced by agents other than ultraviolet light and of oncogenes from origins other than skin.

Leukemia is recognized as a radiation-induced effect in humans and in animals following exposure to ionizing radiations, and efforts are being made to identify and clarify the genetic components associated with the susceptibility of the mouse to x-ray-induced leukemia. Thus far, results from one study have shown the ril-1 locus on chromosome 15 to be a critical genetic determinant of susceptibility to leukemia induced by x-rays. The region of this locus has been mapped with the identification of numerous RFLPs in the immediate vicinity of the locus, a preliminary molecular map of the region has been constructed, and a high expressor cell line has been established for use in subsequent insertional mutagenesis efforts. In addition, numerous congenic mouse strains have been identified which have different alleles of this gene and therefore can be studied for susceptibility to leukemia. This work, drawing heavily on both molecular and classical mammalian genetics to study a genetic locus, may have its counterpart in other mammals, including humans.

The mechanisms by which ionizing radiation can affect promotion are being investigated in cultured cells by studying the effects of low-dose x-irradiation on the activity of membrane-associated protein kinase C (PKC). Protein kinase C is known to be associated with growth regulation and tumor promotion. It was shown that activators of PKC enhanced radiation-induced transformation in cultured murine C3H10T1/2 cells in the manner of a promoter, suggesting that PKC plays a direct role in the promotion of x-ray-induced transformation. A protease inhibitor (abbreviated TPCK) that inhibits PKC activity also inhibited x-ray-induced transformation, again indicating a role for this enzyme in the x-ray-induced transformation process. This work suggests that factors that alter the activity of PKC (and possibly other components of mammalian growth regulation) may be useful in defining the pathways associated with radiation-induced transformation, and may provide a possible means for blocking promotion of radiation-induced transformation.

Hormonal effects on radiation-induced mammary cancer were studied in the mouse. Monodispersed mammary cells exposed *in vitro* to x-rays were transplanted into subcutaneous fat pads of syngeneic recipients. The subsequent development of mammary tumors in female mice from the population of "clonogenic" survivors was highly dependent on high ratios of prolactin to glucocorticoid hormones. Prolactin favors ductal differentiation in proliferating clonogens, shortens the latency period for tumorigenesis, and enhances the survival of the radiation exposed clonogens. Conversely, glucocorticoids favor the differentiation of stem cells into alveolar structures, which do not readily become malignant in the mouse. This study illustrates the importance of systemic factors associated with cell differentiation and hormonal function that may be essential to the development of radiogenic mammary cancer in the mouse, and raises similar questions about the influence of hormones and hormonal ratios on the etiology of radiation-induced human breast cancer.

The effects on the carcinogenic process in humans or animals following exposure to combinations of radiation (e.g., x-rays and ultraviolet light) or radiation plus chemical agents are poorly understood. Additivity would imply independent effects, such as might be expected with DNA repair enzymes that act independently of other cellular or systemic effects, while non-additivity would imply

synergistic or antagonistic responses which may be mediated indirectly through defense mechanisms associated with complex systems (e.g., immune system, hormonal ratios) of the intact animal. Possibly the most common situation involving such cocarcinogenicity in humans is simultaneous or sequential exposure to ultraviolet light and to chemical agents potentially capable of inducing cancer. In one project, the effect of exposure to ultraviolet light (UVB) on the carcinogenicity of known polycyclic aromatic hydrocarbon chemical carcinogens is being studied in the mouse. The carcinogen, 7,12-dimethylbenz(c)anthracene (DMBA), was applied to the ventral skin of the animal in order to initiate tumorigenesis. Subsequent irradiation of the dorsal skin with ultraviolet light (UVB) did not interfere with the initiation of tumorigenesis by DMBA, but did interfere with the promotion of tumorigenesis using a phorbol ester as the promoter. Since the location of the UVB-exposed skin was different from that of DMBA application, the inhibitory effect was thought to be indirect and mediated by the animal. Because other studies indicated that UVB is immunosuppressive, it was thought that this indirect effect might be mediated in the skin through the induction of activated vitamin D3 synthesis. However, it was found that chronic application of activated vitamin D3 to the dorsal mouse skin in an attempt to duplicate the effect of UVB on promotion of DMBA-initiated skin did not produce the same inhibitory effect on promotion as did UVB exposure alone. Therefore, the effect of UVB on the promotion of DMBA carcinogenesis was apparently not solely via induction of vitamin D3. Conversely, when benzo(a)pyrene was applied ventrally to the mouse skin, dorsal irradiation with UVB enhanced carcinogenesis. Thus, UVB irradiation of mouse skin apparently had different effects on the susceptibility of the animal to chemical carcinogenesis, depending on the carcinogen. While the mechanism(s) is not yet clear for the different effects of UVB on the tumorigenicity of these chemical carcinogens, the findings strongly suggest that systemic effects induced by UVB may be a major determinant of the susceptibility of the animal to potentially carcinogenic chemical agents. To the extent that mouse skin can serve as a model for humans, the results obtained in this work broaden the possible role of UV radiation in modulating human cancers where chemicals are involved. In another project, the interaction between low-LET gamma rays and the chemical carcinogen, N-methyl-N-nitrosoguanidine (MNNG), in cultured mouse cells (C3H10T1/2) was investigated. The preliminary data indicate that oncogenic transformation and mutation frequencies for a dose of 1 Gy gamma irradiation plus graded levels of MNNG are nearly additive. This apparent additivity is in contrast to the apparent non-additive effects observed when the mouse skin is exposed to UVB plus the chemical carcinogen, DMBA, as discussed above. These projects illustrate the importance both of cellular studies and of animal models in understanding cellular susceptibility to oncogenic transformation and DNA damage in the intact organism, as well as the influence of systemic effects that can modulate tumorigenesis within tissues and organs.

In addition to being a carcinogen and cocarcinogen, ultraviolet light also has the capacity to induce melanogenesis, one of the body's major protective mechanisms against photocarcinogenesis by non-ionizing radiation. Elucidation of the cellular mechanisms that underlie melanogenesis may permit the use of such naturally occurring mechanisms to give enhanced protection against the carcinogenic effects of ultraviolet light. Investigators are studying the mechanism(s) of melanogenesis using human epidermal melanocytes in culture. Results have shown that a diacylglycerol (DAG) analog (1-oleoyl-2-acetyl-glycerol) stimulates melanogenesis without affecting cell growth; it also was demonstrated that the stimulation of melanogenesis could be blocked by protein

kinase C (PKC) inhibitors. This observation suggests that human melanogenesis is controlled more directly by the DAG-PKC pathway than by the cyclic AMP pathway as is thought to be the case for murine cells. This work both indicates the need for careful molecular analyses in order to compare results obtained from animals with those from humans, and suggests that it may be possible to stimulate the protective tanning of human skin without exposure to potentially carcinogenic ultraviolet radiation.

Comparative studies with rodents and dogs have been carried out in order to provide the information required for extrapolating and scaling the effects of radiation damage in animals to humans. Current work is describing the effects of radiation on life shortening of the beagle dog. Study variables include dose rate, total dose, type of exposure (single, fractionated, protracted, or duration-of-life), sex and age at exposure. One part of the study is measuring the relative importance of dose rate and total dose on the late effects of protracted whole-body gamma irradiation on beagle dogs at several dose rates. The results of these studies indicate that (a) there is no evidence for a dose rate effect, (b) death from tumors is proportional to total dose, (c) time of fatal tumor onset is shortened by increased total dose, and (d) increased total dose increases the number of nonfatal tumors. The final results of this comprehensive study may provide a better basis than is currently available for using interspecies data for predicting the late effects of protracted whole body irradiation in man.

Radiation-induced lymphomas in the mouse possess a high molecular-weight glycoprotein oncofetal antigen (OFA). An investigator has demonstrated that OFA is conserved in mouse and in man, and is found only in early to mid-gestation fetuses; it is not detected in term fetal or normal tissues. The OFA appears to promote some degree of cross-protective immunity against malignant lymphomas when used as an immunogen in post-irradiated animals, suggesting that OFAs may play a role in host-mediated resistance in the mouse. The OFA is but one (along with the pattern of sulfate modification of thyroglobulin, expression of the transin gene in murine cells, and possibly certain oncogenes) of several possible molecular markers for radiogenic cancers being investigated within the Radiation Effects Branch program.

The identification of molecular markers associated with exposure to ionizing radiation was also the objective of a study which showed that amplified and rearranged c-myc oncogenes are associated with advanced skin tumors in the rat. Both the copy number and the frequency of rearrangement of the c-myc oncogene were correlated with tumor size. However, amplification of c-myc was not seen in the early stages of tumor development, suggesting that this oncogene is not a precursor marker for radiogenic cancer; nevertheless, it is not yet clear whether or not there is a selective growth advantage for those cells with rearranged and high copy numbers of the c-myc oncogene.

In a related study, at least three different non-ras, dominant transforming genes were found in x-ray-induced squamous cell carcinomas in the mouse skin. The data suggest that oncogene activation did not result from a direct effect of the ionizing radiation, but, rather, as the result of cellular repair of the initial damage to cellular DNA. This adds to the evidence that ionizing radiations elicit a delayed set of molecular responses to damaged DNA within cells surviving the initial exposure, and which eventually lead to transformation. Other

evidence obtained in this study suggests that there are differences in DNA repair and gene expression between mouse skin tumors induced by ionizing radiation (x-rays) and mouse skin tumors induced by the polyaromatic hydrocarbon, dimethylbenzanthracene (DMBA). In radiation-induced benign papillomas, RNA transcripts for the transin gene (a metalloproteinase related to the human stromelysin) were associated with initiation following exposure to x-rays. Conversely, benign papillomas induced by DMBA showed no transin mRNA. Moreover, radiation-induced papillomas containing transin demonstrated a higher conversion rate to malignant squamous cell carcinomas than did the chemically-induced papillomas. In addition, transin gene expression was undetectable in non-invasive, basal cell carcinomas initiated by ionizing radiations. From these results the transin gene appears to be a possible specific early molecular marker for radiation-induced malignant squamous cell carcinomas in mouse skin.

Other investigators are also attempting to ascertain if there are correlations between molecular endpoints and radiogenic cancer. For example, squamous cell carcinomas of the head and neck are known to be heterogeneous in their responsiveness to radiation therapy. One study is investigating possible correlations between oncogene transcripts, or the expression of other genes, and this radiation sensitivity. A highly resistant human cell line (SQ20B) was found to have high expression of mRNA for the raf oncogene, suggesting a role for this oncogene in the radiation resistance of this human tumor cell line. On the other hand, ADP-ribosylation was significantly elevated in radiation-sensitive strains of Ewing's sarcoma cell line, possibly indicating a correlation between ADP-ribosylation and the high degree of radiosensitivity demonstrated in this type of human cancer. In related work done on a separate project, the raf oncogene has been shown to be functionally important in maintaining the radioresistance of the SQ20B cell line. This investigator showed that transfection of the radiation resistant SQ20B human cell line could be accomplished with genetically engineered plasmids containing part or all of the raf oncogene in either the sense or antisense orientation relative to mRNA transcription. Cells transfected with the raf gene in the "normal" or sense orientation showed the same or increased tumorigenicity after implantation into the nude mouse. However, the same human cells transfected with plasmids carrying the raf gene in the antisense orientation showed substantially less tumorigenicity in the nude mouse, suggesting that antisense mRNA from the raf oncogene blocked expression of the endogenous raf oncogene in the SQ20B cell line. Further efforts are characterizing the radiation resistance of SQ20B and other mammalian cell lines carrying normal or altered raf oncogenes. This system, using cloned oncogenes illustrates a powerful approach from molecular genetics that can be used to probe for a functional role of individual genes involved in radiogenic cancer.

The linkage between epidemiology and molecular biology also is evident in a study of approximately 5000 people who received radiation treatment for benign conditions of the head and neck during childhood, and who are being followed in order to detect the development of any secondary cancers. During the past year, a study was completed on the use of thyroid hormone therapy for patients who developed benign thyroid nodules. It was found that radiation-associated benign nodules recurred frequently, but at a rate that was similar to that reported for non-irradiated patients. The treatment with thyroid hormone decreased the risk of benign recurrences, particularly in women, but not the risk of cancer. It was concluded that any patient with a history of radiation treatment who developed a thyroid nodule should be treated with thyroid hormone medication. Evidence that familial factors independent of known risk factors (female sex, younger age at

irradiation, and higher exposure dose) also contributed to the susceptibility of individuals to radiation-induced thyroid cancer was obtained. Laboratory studies provided evidence that the thyroid-specific glycoprotein, thyroglobulin, obtained from cancerous thyroid tissues was different from that produced by normal thyroid tissue with respect to both the level and the position of covalently attached sulfate. The differences, although subtle, may have implications with respect to the identification of molecular markers of human thyroid cancer. The epidemiologic component of the study (carried out in collaboration with the Radiation Epidemiology Branch of the NCI) has nearly completed the acquisition of organ-specific data for the thyroid gland. This data will be used in new analyses of both the dose-response curve and the cancer risk factors associated with this type of x-ray treatment.

An indicator of genetic predisposition to radiogenic cancer in individual humans may be associated with abnormal responses of somatic cells to radiation-induced DNA damage. In order to test this hypothesis, an investigator is measuring DNA damage induced by x-irradiation of skin fibroblasts from 24 clinically normal individuals (i.e., without obvious defects in their capacity for DNA repair), using the loss of cellular capacity for colony formation as the endpoint for toxicity. The results showed considerable intrinsic variability in radiosensitivity and in the ability to repair DNA damage within a population of apparently normal human cells. The variability among these individuals was not related to passage level, cloning efficiency, or to the sex of the donor. In a parallel study, fibroblasts from ten other normal individuals were irradiated with x-rays and subsequently analyzed for chromosomal aberrations in the G2 phase of the cell cycle (following a G1 phase block after irradiation). While generally in agreement with the results of the larger study, the variability of aberrations observed among the cells from the ten individuals was considered to be too high to permit the use of aberrations in G2 phase cells as a means to detect radiosensitive persons among the general population. The same technique also was used in an attempt to detect ataxia telangiectasia (AT) heterozygotes. Although the results were suggestive in that cells from the AT heterozygous carriers were significantly more sensitive to ionizing radiation than were normal cells, and less sensitive than were cells from homozygous AT patients, the variability among individuals was considered to be too great for its use as a reliable method for the detection of prospective heterozygote AT carriers in the general population.

The incidence of leukemia and of thyroid disease in Utah is being assessed in relation to fallout from atmospheric nuclear weapons tests conducted at the Nevada Test Site between 1951 and 1962. A leukemia case-control study consists of 1,195 cases and 6,159 matched controls. The exposure of each person to fallout, primarily Cesium-137, was estimated based upon each persons location(s) of residence since the time of the tests and upon age at that time, lifestyle and occupation(s). The thyroid study consists of a follow-up of 4,819 children who were examined for thyroid disease during the 1960s as a part of a Public Health Service study. Of that number, 3,122 were located and available for re-examination of the thyroid during the 1980s as part of this study. Because the thyroid concentrates iodine and because radioactive iodine in fallout is consumed by grazing cows and concentrated in and transferred through milk, the primary exposure pathway was the consumption of milk containing the radioiodine isotopes in milk, particularly Iodine-131. Fallout patterns of Iodine-131 associated with each atmospheric nuclear test were estimated, together with dairy herd grazing and consumption patterns, milk processing and mixing procedures in

use at that time, and milk distribution patterns. This information, combined with the milk consumption habits and residence history of each individual obtained through personal interviews, permitted an estimation of the Iodine-131 received by each of the 3,122 cohort subjects. Known metabolic parameters of iodine were then applied in order to estimate the radiation dose to the thyroid for each individual. Relationships between this dose and the clinical detection of thyroid disease currently are being analyzed, as are possible correlations between dose to the bone marrow from fallout and leukemia. The completion of these analyses, together with a final report, is not expected for approximately two years.

The Radiation Effects Branch also provides grant support to several national and international advisory bodies which analyze and disseminate information concerning, and provide guidance on matters pertaining to, occupational and public radiation protection issues: the National Council on Radiation Protection and Measurements (NCRP), the International Commission on Radiation Units and Measurements (ICRU), and the International Commission on Radiological Protection (ICRP). During the year, the ICRU adopted and published two reports on 1) "Determination of Dose Equivalents from External Radiation Sources - Part 2" and 2) "Tissue Substitutes in Radiation Dosimetry and Measurement." The NCRP published seven reports during FY 1989 on the following subjects: 1) "Comparative Carcinogenicity of Ionizing Radiation and Chemicals," 2) "Measurement of Radon and Radon Daughters in Air," 3) "Guidance on Radiation Received in Space Activities," 4) "Quality Assurance for Diagnostic Imaging Equipment," 5) "Exposure of the U.S. Population from Diagnostic Medical Radiation," 6) "Exposure of the U.S. Population from Occupational Radiation," and 7) "Medical X-Ray Electron Beam and Gamma Ray Protection for Energies Up to 50 MeV - Equipment Design Performance and Use."

RADIATION EFFECTS
GRANTS ACTIVE DURING FY89

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ALDERFER, James L. New York State Department of Health 2 R01 CA 39027-05	Effects of Light on Nucleic Acids
2. APOSHIAN, Vasken H. University of Arizona 9 R01 CA 49252-03	Polonium-210 Radiation and Muta- genesis
3. ASHMAN, Charles R. University of Chicago 5 R01 CA 45336-02	Specificity of Ionizing Radiation Mutagenesis
4. BALCER-KUBICZEK, Elizabeth K. University of Maryland at Baltimore 5 R23 CA 32729-06	Oncogenesis from Low Dose-Rate Irradiation
5. BALCER-KUBICZEK, Elizabeth K. University of Maryland at Baltimore 5 R01 CA 42820-02	Mechanism of Microwave Carcinogenesis in Vitro
6. BALCER-KUBICZEK, Elizabeth K. University of Maryland at Baltimore 1 R01 CA 50629-01	Neoplastic Transformation by Fission Neutrons and Gamma Rays: Dose-Rate Effect
7. BARROWS, Louis R. University of Utah 7 R01 CA 41561-03	Repair of X-Ray-Induced DNA Damage: Genetic Basis
8. BASES, Robert E. Yeshiva University 2 R01 CA 36492-05	X-Ray Damage and Repair of Primate Cell Alpha-DNA Sequences
9. BEDFORD, Joel S. Colorado State University 5 R01 CA 18023-15	Dose and Time Factors in Cellular Radiosensitivity
10. BEDFORD, Joel S. Colorado State University 1 R01 CA49501	Radiation Cytogenetics
11. BENJAMIN, Stephen A. Colorado State University 5 R01 CA 36456-04	Prenatal Thymic Radiation Injury and Immune Development

12. BERNHARD, William A.
University of Rochester
5 R01 CA 32546-13
Solid State Radiation Chemistry
of Nucleic Acid Bases
13. BOWDEN, George T.
University of Arizona
5 R01 CA 42239-03
Radiation-Induced Skin Tumors
and Oncogene Activation
14. BOX, Harold C.
Roswell Park Memorial Institute
5 R37 CA 25027-22
Transfer Mechanisms in
Irradiated Biological Systems
15. BOX, Harold C.
Roswell Park Memorial Institute
1 R01 CA 46838-02
Analysis of Radiation Damage
in DNA
16. BRENT, Thomas P.
St. Jude Children's Research Hospital
5 R01 CA 14799-15
Enzymes and Reactions for
Repair of DNA in Human Cells
17. BROOKS, Anton L.
Lovelace Biomed & Env. Res. Inst.
1 R01 CA44590
Radiation-Induced Chromosome
Damage in Vitro
18. CALOS, Michele P.
Stanford University
5 R01 CA 45365-02
LacI Shuttle Vectors for Study
of Ionizing Radiation
19. CARDIFF, Robert D.
University of California, Davis
5 R01 CA 36493-05
Radiation Activation of Oncogenes
20. CHEN, David
University of California
1 R01 CA 50519-01
Molecular Cloning of a Human
Radiation Repair Gene
21. CLAYCAMP, Gregg H.
University of Iowa
5 R01 CA 43324-04A1
Radiation Biochemistry of DNA
Base Damage
22. CLIFTON, Kelly H.
University of Wisconsin, Madison
2 R37 CA 13881-17
Radiation In Vitro - Mammary
Neoplasia
23. CLIFTON, Kelly H.
University of Wisconsin, Madison
1 R13 CA 49740
Cell Transformation and Radio-
genic Cancer
24. COGGIN, Joseph H., Jr.
University of South Alabama
5 R01 CA 39698-04
Role of Oncofetal Antigens in
Radiation Carcinogenesis

25. COOPER, Priscilla K. Inducible Responses to Carcino-
University of California, Berkeley genic DNA Damage
5 R01 CA 32986-06
26. CORNFORTH, Michael N. Cytogenetic Effects Pertaining to
Univ. of California/Los Alamos Natl. Lab. Low Doses of Radiation
5 R29 CA 45141-02
27. DEMPLÉ, Bruce Oxidative DNA Damage: Repair
Harvard University and Cellular Responses
5 R01 CA 37831-04
28. DOETSCH, Paul W. Repair of Oxidative and
Emory University Radiation-Induced DNA Damage in
5 R01 CA 42607-04 Human Cells
29. DRITSCHILO, Anatoly Molecular Studies of Radiation
Georgetown University Resistant Tumor Cells
5 R01 CA 45408-02
30. ELKIND, Mortimer M. Radiation Transformation and Its
Colorado State University Modulation by Chemicals
5 R37 CA 41483-03
31. ELKIND, Mortimer M. Radiobiology of Lethality,
Colorado State University Mutation, & Transformation
5 R35 CA 47497-02
32. ESSIGMANN, John M. Genetic Effects of Ionizing
Massachusetts Inst. of Technology Radiation
5 R01 CA 33821-07
33. EVANS, Helen H. Mutants and Altered Radioresponse
Case Western Reserve University to Cells and Tumors
5 R37 CA 15901-15
34. EWING, David Lethal Damage from O2 and OH
Hahnemann Univ. School of Medicine in Irradiated Cells
5 R01 CA 28932-06
35. EWING, David Mechanisms of Damage in
Hahnemann Univ. School of Medicine Irradiated Cells
5 R01 CA 30921-03
36. FRY, R. J. Michael Radiation Environments and
University of Tennessee Effects in Space
1 R13 CA 48803-01
37. FRY, R. J. Michael Low Dose Radiation - Basis
University of Tennessee of Risk Assessment
1 R13 CA 49108-01

38. GARTE, Seymour J.
New York Univ. Medical Center
5 R01 CA 43199-03
Oncogene Activation in
Radiation Carcinogenesis
39. GEARD, Charles R.
Columbia University
1 R01 CA49672
Short-Range Radiations: Lesions
and Chromosomal Changes
40. GENSLER, Helen L.
University of Arizona
5 R29 CA 44504-03
UV Modulation of Chemical
Carcinogenesis
41. GILCHREST, Barbara A.
Tufts University
5 R01 CA 45687-02
Retinoids and UV-Induced
Melanogenesis
42. GLICKMAN, Barry
York University, Canada
5 R01 CA 45498-02
Radiation-Induced Mutation in
Mammalian Cells
43. GRIFFITHS, T. Daniel
Northern Illinois University
5 R01 CA 32579-08
DNA Replication after Insult
with UV
44. GRIGGS, Henry G.
John Brown University
2 R01 CA 18809-13
Ultraviolet and Ionizing
Radiation Damage
45. GUERNSEY, Duane L.
University of Iowa
5 R01 CA 36483-05
X-Irradiation-Induced Oncogene
in Mouse Embryo Cells
46. HALL, Eric J.
Columbia University
2 P01 CA 12536-18
The Effects of Small Doses of
Radiation
47. HARRISON, George H.
University of Maryland
5 R01 CA 40223-04
Ultrasound and Malignant
Transformation In Vitro
48. HENNER, William D.
Dana-Farber Cancer Institute
5 R01 CA 35767-05
Ionizing Radiation-Induced DNA
Damage and Repair
49. HILL, Colin
Univ. of Southern California
2 R01 CA 42808-04
Neutron Energy: Dose Protraction
Effect on Transformation
50. HUBERMAN, Eliezer
University of Chicago
5 R01 CA 33974-06
Mutation-Transformation: Neutron
Damage and Repair

51. HUMPHREY, Ronald M.
Univ. of Texas System Cancer Center
5 R01 CA 04484-31
DNA Repair and Recovery in
the Mammalian Cell Cycle
52. HUMPHREY, Ronald M.
Univ. of Texas System Cancer Center
5 R01 CA 24540-09
The Importance of DNA Damage
and Repair for Cell Survival
53. ILIAKIS, George
Thomas Jefferson University
2 R01 CA 42026-04A1
Has Cellular Repair a Common
Molecular Base?
54. ILIAKIS, George
Thomas Jefferson University
1 R01 CA 45557-02
Radiosensitization by BrdUrd/
IdUrd: Cellular and Molecular
Effects
55. JORGENSEN, Timothy J.
Georgetown Univ. Med. Ctr.
1 R29 CA 48716
Biochemistry of Radiation-
Induced DNA Strand Breaks
56. KANTOR, George J.
Wright State University
1 R01 CA49411
Domain-Specific DNA Excision
Repair in Human Cells
57. KASID, Usha N.
Georgetown University
1 R29 CA 46641-02
RAF Oncogene Analysis and
Radiation Resistant Tumor
Cells
58. KENNEDY, Ann R.
University of Pennsylvania
5 R01 CA 34680-07
Hormones, Radiation, and
Malignant Transformation
59. KOCH, Cameron
University of Pennsylvania
1 R01 CA 49498
Mechanisms of Cellular and
Molecular Sensitivity
60. KOVAL, Thomas M.
George Washington University
5 R01 CA 34158-09
Insect Cells: A Basis for
Radioresistance
61. LANGE, Christopher S.
Downstate Medical Center
3 R01 CA 39045-03
Radiosensitivity Prognosis Based
on DNA Repair Assay
62. LEITH, John
Brown University
1 R01 CA 50350
Tumor Bed Effect: Influence on
Growth Factor Expression in Cell
63. LIBER, Howard L.
Harvard School of Public Health
1 R01 CA 49696
Ionizing Radiation Mutagenesis
in Human Cells

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| 64. LITTLE, John B.
Harvard University
5 R01 CA 34037-06 | Radiation Mutagenesis in Human
Cells |
| 65. LITTLE, John B.
Harvard University
5 R35 CA 47542-02 | Effects of Radiation on
Mammalian Cells |
| 66. MARGULIES, Lola
New York Medical College
5 R01 CA 35580-06 | Ionizing Radiation and
Transposon Mobility |
| 67. MERUELO, Daniel
New York University
5 R01 CA 35482-05 | Reverse Genetics of a Leukemia
Susceptibility Locus |
| 68. Miller, Richard C.
Columbia University
5 R01 CA 37967 | Oncogenic Transformation and
High LET Radiations |
| 69. MILLER, Scott C.
University of Utah
5 R01 CA 47659-02 | Occupational Risk Reduction
by Radiotoxin Chelation |
| 70. NAIRN, Rodney S.
Univ. of Texas System Cancer Center
5 R01 CA 36361-06 | Repair and Recombination in
Radiation-Sensitive Cells |
| 71. NELSON, William H.
Georgia State University
2 R01 CA 36810-04 | Radiation Chemistry of Purines
in the Solid State |
| 72. NORDLUND, Thomas M.
Univ. of Rochester Medical Center
5 R01 CA 41368-03 | DNA Damage Studied by Ultrafast
Spectroscopy |
| 73. OLEINICK, Nancy L.
Case Western Reserve University
5 R01 CA 15378-16 | Radiation-Induced Modifications
in Protein Synthesis |
| 74. PEAK, Meyrick J.
University of Chicago
5 R01 CA 34492-05 | Solar UV Damage in Human Cells |
| 75. PEAK, Meyrick J.
University of Chicago
5 R01 CA 37848-05 | Biological Effects of Solar-UV-
Generated Oxygen Species |
| 76. PIEPKORN, Michael W.
University of Utah
5 R29 CA 41591-04 | Glycosaminoglycans of Skin
Tumors |

77. PRAKASH, Satya
University of Rochester
5 R01 CA35035-07
Excision Repair of UV-Irradiated DNA in Yeast
78. PRAKASH, Satya
University of Rochester
5 R01 CA 41261-04
Repair of UV-Irradiated DNA: Excision Genes of Yeast
79. RAABE, Otto G.
University of California, Davis
1 R01 CA46296-01
Cancer and Injury Risk Assessment for Radionuclides
80. RALEIGH, James A.
Cross Cancer Institute
1 R01 CA 46548-01
Molecular Radiobiology of Nucleic Acids
81. RAMANATHAN, Brinda
New Mexico Highlands University
5 R23 CA 43079-04
Nuclear Protein Modifications in UV-Damaged Human Cells
82. REDPATH, John L.
University of California, Irvine
2 R01 CA 39312-04
Radiobiological Studies of Human Hybrid Cell Lines
83. REYNOLDS, Richard J.
Univ. of California/Los Alamos Natl. Lab.
5 R01 CA 42390-04
Radiation Damage of Eukaryotic DNA and Its Repair
84. RINALDY, Augustinus
Vanderbilt University
2 R01 CA 43769-03
Molecular Cloning of Human DNA Repair Gene(s)
85. ROSENSTEIN, Barry
Brown University
2 R01 CA 45078
Repair of Solar UV-Induced DNA Damages
86. SCHNEIDER, Arthur B.
Michael Reese Hospital & Med. Ctr.
5 R01 CA 21518-12
Radiation-Induced Thyroid Cancer
87. SEVILLA, Michael D.
Oakland University
5 R01 CA 45424-02
Radiation-Induced Lipid & Sulfhydryl Autoxidation
88. SHADLEY, Jeffrey D.
University of Chicago
1 R01 CA 49181
Inducible Repair Response in Human Lymphocytes
89. SINCLAIR, Warren K.
National Council on Radiation Protection
5 R01 CA 18001-22
Radiation Protection and Measurements

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| 90. | SMITH, Hylton
Int'l. Comm. on Rad. Protection
5 R01 CA 30163-08 | Recommendations on Radiological
Protection |
| 91. | SMITH, Kendric C.
Stanford University
5 R01 CA 06437-27 | Molecular Basis of Radiation
Lethality |
| 92. | SMITH, Kendric C.
Stanford University
5 R01 CA 33738-07 | Ionizing Radiation Mutagenesis
in Escherichia coli |
| 93. | STAMATO, Thomas D.
Wistar Institute
5 R01 CA 45277-03 | Isolation of Radiation-
Sensitive Mammalian Cell
Mutants |
| 94. | STAMATO, Thomas D.
Wistar Institute
5 R01 CA 48636-02 | Poly(ADP-Ribose) and Repair
of Radiation-Induced Damage |
| 95. | SUMMERS, William C.
Yale University
5 R01 CA 45300-02 | Molecular Basis of DNA Damage
by Ionizing Radiation |
| 96. | SUTHERLAND, Betsy
Associated Univ./Brookhaven Natl. Lab.
5 R01 CA 23096-11 | DNA Repair: Human and E. coli
Photoreactivating Enzymes |
| 97. | TAYLOR, GLENN N.
University of Utah
5 R01 CA 28314-09 | Reducing Cancer Risk by
Radionuclide Chelation |
| 98. | TAYLOR, William D.
Pennsylvania State Univ.
5 R01 CA 44658-02A1 | Mutagenic & Recombinogenic
Effects of Gamma Rays |
| 99. | TAYLOR, Yvonne C.
Washington Univ. School of Medicine
1 R29 CA 47855-01 | Chromatin Conformation and
PLD Repair |
| 100. | THONNARD, Norbert
Atom Sciences, Inc.
1 R43 CA 50021-01 | Sensitive Personnel Dosi-
metry of 3 kev to 20 MeV
Neutrons |
| 101. | UHLENHOPP, Elliott L.
Grinnell College
1 R15 CA 47656-01 | DNA Damage and Repair |
| 102. | ULLRICH, Robert L.
University of Texas, Galveston
5 R01 CA 43322-05 | Carcinogenic Interactions of
Radiation and Chemicals |

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| 103. | VERMA, Surendra
Tufts University
5 R01 CA 36195-05 | Membrane Composition and
Radiation Damage |
| 104. | WALDREN, Charles A.
Colorado State University
2 R01 CA 36447-04 | Cell Genetic Damage at Low
Doses and Dose Rates |
| 105. | WALLACE, Susan S.
University of Vermont
5 R37 CA 33657-09 | Repair of DNA Damage Induced by
Ionizing Radiation |
| 106. | WARD, John F.
Univ. of California, San Diego
5 R37 CA 26279-10 | Mechanisms in Shouldered
Survival Curves |
| 107. | WARD, John F.
Univ. of California, San Diego
1 R01 CA 46295-01A1 | Studies of Biologically
Significant Damage in DNA |
| 108. | WILLIAMS, Jerry R.
Johns Hopkins University
5 R01 CA 39543-05 | X-Ray Induction of Cellular
Hypersensitivity |
| 109. | YASUI, Linda S.
University of Utah
5 R29 CA 45011-02 | Cytotoxicity of ¹²⁵ I Decay
Produced Lesions in Chromatin |
| 110. | ZAIN, Sayeeda B.
University of Rochester
5 R01 CA 36432-06 | Oncogenes, Oncogene Products in
Radiation-Induced Tumors |
| 111. | ZAIN, Sayeeda B.
University of Rochester
5 R01 CA 46625-02 | C-abl Oncogene in Radiation-
Induced Thyroid Carcinoma |

CONTRACTS ACTIVE DURING FY 89

<u>Investigator/Institution/Contract Number</u>	<u>Title</u>
112. FRITZ, Thomas E. Department of Energy Y01-CP-50503	Late Effects of Protracted Irradiation in Dogs
113. HOFFMAN, F. Owen Department of Energy Y01-CP-60504	Pasture Grass Interception and Retention of Iodine-131
114. STEVENS, Walter University of Utah N01-C0-23917	Assessment of Leukemia and Thyroid Disease in Relation to Fallout in Utah

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44

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