

91 annual report

Division Of

Cancer Etiology

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ANNUAL REPORT
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1990 through September 30, 1991

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

October 1, 1990 through September 30, 1991

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 transition to neoplasia receives particular emphasis. Investigations currently are focused upon defining the molecular structure and range of activity of the membrane permeabilizing and drug uptake enhancing cytokine, leukoregulin, with particular emphasis on cloning the leukoregulin gene for production of recombinant human leukoregulin.

The Somatic Cell Genetics Section studies involve 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 relevant model systems utilizing mammalian cells in conjunction with human papilloma-viruses, particularly HPV-16 and HPV-18 which belong to the subgroup of papillomaviruses associated with squamous cell carcinoma of the cervix.

Thus, it becomes possible to make meaningful contributions to multistage carcinogenesis. Immunological approaches are being used to down regulate HPV expression. Banding and nonisotopic in situ hybridization techniques are being combined with microcell hybridization for identifying chromosomes or

chromosome regions and ultimately genes for senescence or suppression of a variety of cancers. The latter includes the control and reversal of aberrant differentiation. The multistep carcinogenesis process proceeds through a series of physiological barriers that must be unblocked for a cell to progress toward malignancy. The primary question becomes what are the cellular and molecular mechanisms that govern multistep tumorigenesis? This laboratory is concerned in determining how the growth regulatory cell mechanism is overridden to produce the deregulation of malignancy. The ultimate goal is to define the oncogenes and suppressor genes associated with cervical cancer.

Because human populations are subjected repeatedly to both chemical and viral agents, it is logical to investigate the role of combinations of these agents in carcinogenesis. The process is extremely complex, involving diverse environmental and host factors. The nature of human cancer development, usually requiring several decades, suggests progression through multiple, distinct stages with opportunities for interaction of multiple factors required to overcome the physiological barriers controlling normal growth. Research has been focused on specific, ubiquitous viruses which are often considered the cause of cancers. The role of these viruses in cancer causation is controversial because often they are associated with non-malignant diseases. A morphologic transforming region of herpes simplex virus 2 (Bgl II-N) has been used. Although HPV is at center stage in terms of its relevance to cervical cancer, at one time herpes simplex virus (HSV) was considered important because a high incidence of herpes was found in genital lesions. In fact, a hypothesis was presented concerning the possible synergistic action between HSV 2 and HPV in the development of cervical cancer. A number of laboratories have claimed that HSV's major DNA binding protein is directly detectable by immunofluorescence assays in cervical and vulva tumor samples. Some support for a cocarcinogenesis hypothesis was provided by analysis of established cervical HPV lesions. The results with normal human genital epithelial cells differed from those obtained either in vitro or in vivo with animal cells; whereas the Bgl II-N fragment can induce transformation in hamster and mouse cells, it was ineffective in human cells neither causing transformation nor extension of the life span of control cells. However, when the same recombinant DNA is transfected into HPV immortal cells, a transformation occurs that converts non-tumorigenic, immortal cells into squamous carcinoma cells. The HPV was present but not rearranged and there was indication of the presence of HSV genome at the time that cells were injected into animals. Examination of tumor derived cultures as well as cultures that have been in continuous culture demonstrated that the HSV has disappeared. This conclusion is similar to the results observed with animal cells with the Bgl II-N genomic fragment or with animals that have been exposed to ultraviolet inactivated intact virus or the same genomic fragment. This demonstration of unstable integration of HSV has been referred to as hit and run. At this time there is no firm evidence to explain this phenomenon. There are, however, at least three possible explanations: 1) Bgl II-N may be toxic at high copy numbers because only low quantities of HSV DNA are detected as seen in our study; 2) the Bgl II-N may be an inhibitor inducing primarily lethal mutations resulting in a selection of cells that have only a few mutations; thus, herpes is said to act in a way similar to chemical carcinogens; and 3) because only the entire Bgl II-N is capable of producing the inhibitory effect, a protein or combination of proteins encoded by the sum of the Bgl II-N genome (38K, 55K, and 61K) might be responsible. Therefore, a number of subfragments of the Bgl II-N transforming region have been made.

These are referred to as xho fragments. Alone they are ineffective, but some are capable of converting HPV immortal but non-tumorigenic cells to cancer producing cells. Whether the tumors contain the Bgl II-N subfragments needs to be determined. Another approach involves determining whether activated oncogenes can convert immortalized human epithelial cells to malignant ones. The long term goal is to begin at the cell surface and to continue to the interactions within the nucleus.

Papilloma immortalized cells have the EGFR receptor family and express high levels of EGFR relative to normal cell. Current studies involve erbB because it is ligand independent and Δ NerbB-2 a truncated form of erbB-2. The HPV-16 immortalized cells transfected with Δ NerbB2 do produce tumors. Human papillomavirus type 18 (HPV-18) early proteins E6 and E7 have been implicated in maintenance of the malignant phenotype in cervical cancer. These viral oncoproteins are sufficient to immortalize normal genital epithelial cells and the immortal cells display dysplastic squamous differentiation resembling cervical intraepithelial neoplasia. To examine the molecular and cellular alterations induced by the HPV-18 E6 and E7 genes, high titer retroviruses were developed to efficiently transfer these genes efficiently to normal genital epithelial cells. The E6/E7 and E7 containing retroviruses induced a number of alterations of normal cells. Specifically these genes stimulated cell proliferation, reduced the requirement for bovine pituitary extract in the culture medium, delayed the onset of terminal differentiation in cells suspended in semisolid medium, and induced immortality. In contrast, when the retrovirus-infected cells were transplanted beneath a skin-muscle flap in nude mice well differentiated squamous epithelium were formed. Thus, expression of the HPV-18 E6/E7 and E7 genes alters cell growth and commitment to differentiation in vitro but does not directly induce aberrant squamous differentiation in vivo.

Normal genital epithelial cells possess an intracellular control mechanism directed against HPV gene transcription; however, cellular functions down-regulating HPV expression are absent in genital carcinoma cells, suggesting that this loss represents an important step in the development of cancer. Thus, a major goal has been to identify host factors that modulate HPV gene expression and to define the underlying molecular mechanisms. The beta transforming growth factors (TGF β s) are members of a family of polypeptides that modulate cell proliferation and gene expression in diverse cells. Thus, the effect of TGF β s on cell growth and HPV gene expression was determined in a series of immortal genital epithelial cell lines at different stages of malignant progression. TGF β 1 completely inhibited clonal growth in secondary cultures of normal genital epithelial cells derived from either foreskin or cervix. Different cell lines derived by transfection and immortalization of normal cells with HPV-16 DNA varied significantly in their response to TGF β 1, with some being more resistant than normal cells. Cell lines of late passage often exhibited a further increase in resistance. Two immortalized cell lines that were malignantly transformed after transfection with the v-Ha-ras oncogene or the HSV-2 DNA formed colonies in TGF β 1 containing medium with a frequency greater than the parental lines. Three cervical carcinoma-derived tumor lines grew clonally in medium containing TGF β 1. Thus, loss of responsiveness to TGF β 1 often precedes or accompanies malignant development in cultured genital epithelial cells.

The effect of TGF β 1 on the expression of the HPV-16 early genes E6 and E7 was examined in an HPV-16-immortalized cervical cell line that was partially resistant to TGF β 1 in clonal growth assays. TGF β 1 treatment for 24 hours markedly decreased levels of E6 and E7 protein expression. When cultures were maintained in the presence of TGF β 1, E7 expression remained low to undetectable for 48 hours, but expression was partially restored when cultures were switched to fresh medium. In contrast, E6 expression remained undetectable even after TGF β 1 was removed. TGF β 1 did not alter expression of involucrin, a marker of squamous differentiation in cervical epithelium. TGF β 1 regulates expression of a wide variety of cellular genes, and regulation may occur at multiple levels. These results are the first report that TGF β 1 inhibits transcription of viral genes.

RNA analyses were performed to further define the level at which TGF β 1 regulated papillomavirus gene expression. Exposure to TGF β 1 resulted in time-dependent reductions in both cell proliferation and steady-state levels of E6 and E7 RNAs. In addition, TGF β 1 induced the expression of its own RNA, thereby providing the potential to amplify and sustain inhibitory effects on HPV gene expression. Thus, these results suggest an autocrine function for TGF β 1 in down-regulating HPV gene expression in infected anogenital epithelium. Nuclear runoff experiments indicated that reductions in steady-state levels of HPV-16 RNAs by TGF β 1 and -2 were due to changes in transcription. Transcription of the HPV-16 E6 and E7 genes was dramatically inhibited by picomolar concentrations of TGF β 1 and -2 in immortalized cell lines derived from human cervical and foreskin epithelium. TGF β 1 and -2 increased transcription of RNAs for the laminin β 1 chain and β -actin. To determine whether the inhibition of virus gene expression by TGF β 1 might be further regulated by posttranscriptional mechanisms, the stability of HPV-16 early-gene transcripts was measured. When the hybridization intensity of the 1.7-kb RNA was plotted against time, its half-life was interpolated as 14.5 hours. Treatment with TGF β 1 did not alter its stability. Thus, alterations in HPV gene expression occurred mainly at the level of transcription.

Alterations in the ability of cells to synthesize or respond to TGF β s occur during the development of neoplasia, implying that these changes represent an important step in malignant progression. The biological significance of the ability of TGF β 1 to down-regulate papillomavirus early gene expression was examined with a series of cell lines at different stages of malignant progression. Treatment with TGF β 1 for 24 hours dramatically reduced steady-state levels of HPV-16 RNA in early-passage cultures of three different immortal lines. When the same cell lines were examined after extended maintenance in culture, down-regulation of HPV gene expression by TGF β 1 was often less pronounced but still detectable. HPV-16 E6/E7 RNA was decreased only minimally by TGF β 1 in the two cervical carcinoma cell lines; however, virus gene expression was down-regulated significantly in another tumor line. Furthermore, virus RNA expression decreased only slightly after TGF β 1 treatment of immortal cell lines that had been malignantly transformed with HSV-2 DNA or the V-Ha-ras oncogene. These results show a positive correlation between the ability of TGF β 1 to inhibit HPV gene expression and cell growth, suggesting that the two processes were related. Furthermore, they identify TGF β 1 as a potential negative regulator of growth and virus gene expression in premalignant cervical epithelial cells.

The cell mediated immune response influences the persistence or regression of HPV infections. Although the biological mechanisms of regression are incompletely understood, T lymphocytes and natural killer cells secrete a variety of lymphokines including leukoregulin and gamma interferon (IFN γ) that appear to modulate HPV infection. Clonal growth assays were used to assess whether leukoregulin and IFN γ inhibited proliferation of normal epithelial cells, HPV immortalized cells, or cervical carcinoma-derived lines. Leukoregulin and IFN γ completely inhibited clonal growth of secondary cultures of normal epithelial cells derived from either cervical or foreskin epithelium. Cell lines derived by transfection and immortalization of normal cells with recombinant HPV-16 DNA acquired resistance to leukoregulin and IFN γ as shown by their ability to form colonies at low frequency in the presence of these lymphokines. HPV immortalized cell lines malignantly transformed after transfection with the v-Ha-ras oncogene or HSV2 DNA formed colonies in medium containing leukoregulin or IFN γ with a frequency greater than the immortal cell lines from which they were derived. Two cervical carcinoma-derived tumor lines also developed partial resistance. Thus, growth inhibition by leukoregulin and IFN γ was reduced but not lost in malignantly transformed cervical cells. Treatment with IFN γ or leukoregulin for 48 hr decreased levels of the E6 and E7 proteins relative to untreated cells. Thus, lymphokines that inhibited clonal growth of immortalized cells also down-regulated expression of both HPV E6 and E7 proteins. The ability of leukoregulin and IFN γ to directly inhibit HPV oncoprotein expression and proliferation of immortal cells in vitro suggests that these lymphokines might also inhibit papilloma growth in vivo.

Because cells treated with leukoregulin and IFN γ were growth inhibited and displayed an altered morphology, several markers of squamous differentiation were assessed. None of the lymphokines induced expression of involucrin, a marker of squamous differentiation in normal cervical epithelia; however, the activity of epidermal transglutaminase, an enzyme that catalyzes formation of the cross-linked envelope in terminally differentiated squamous cells was increased significantly ($p < 0.01$) after treatment with IFN γ or leukoregulin. Both the membrane associated and soluble forms of epidermal transglutaminase were enhanced. Induction of transglutaminase activity was not reversible. Lymphokines also stimulated a small increase in cornified envelope formation, a characteristic of terminal differentiation in epidermal cells. These observations are consistent with the hypothesis that leukoregulin and IFN γ induced terminal differentiation in immortalized cervical cells.

Exposure to leukoregulin or IFN γ induced time dependent reductions in steady state levels of E6/E7 RNAs. This inhibition of HPV-16 E6/E7 RNAs was reversible when lymphokines were removed and cells allowed to recover in fresh medium for an additional 48 hr. CX16-2 cells expressed low levels of HLA class 1 RNA and lacked detectable class 2 RNA expression. Treatment with IFN γ or leukoregulin enhanced steady state levels of HLA class 1 RNA. HLA class 2 RNA was dramatically up-regulated by IFN γ and slightly induced by leukoregulin. In addition, expression of both class 1 and 2 RNAs remained elevated for an additional two days after IFN γ was removed from the medium. Class 1 and 2 histocompatibility antigens are cell surface proteins involved in immune recognition by T lymphocytes and antigen presentation to immune effector cells, respectively. Up-regulation of their expression on HPV immortalized cervical cells would enhance the cell mediated immune response directed

against these cells and would lead to their destruction. Leukoregulin and IFN γ directly induced multiple changes in cultured cervical cells including decreased expression of HPV transforming genes, growth inhibition, and enhanced expression of HLA cell surface antigens. These results suggest that leukoregulin and IFN γ may be particularly effective as therapeutic agents in the treatment of cervical or genital HPV infections.

Non-isotopic in situ hybridization was introduced for detecting and localizing specific nucleic acid sequences on interphase nuclei and metaphase chromosomes. After hybridization of nucleic acid probes labeled with non-isotopic reporter molecules, the hybridization signals were detected with conjugated fluorochromes resulting in a high resolution hybridization and lack of background. Non-isotopic hybridization using biotin labeled probes and avidin-conjugated fluorochrome was successfully used to localize relatively larger nucleic acid probe (over 2kb) sequences specific for centromeric or telomeric chromosomal regions as well as viral probes for mapping integration sites. Single copies of HPV or Epstein-Barr virus (EBV) were detected and localized on a cervical carcinoma cell line (C4-1) and on a Burkitt's lymphoma line, respectively. HPV-18 sequences were mapped on chromosome 8q21 and EBV sequences were localized on chromosome band 2 p13. In both cases the viral integration site overlapped with the location of a fragile site. In addition, EBV integration on chromosome 2 caused a distinct achromatic lesion that may have contributed to the development of this malignancy. The detection of single copy virus by non-radioactive hybridization has a practical significance as a further rapid approach for identifying viral sequences in malignancies associated with oncogenic viruses.

Immortalization of human keratinocytes after transfection with HPV-16 has been demonstrated to be a reproducible phenomenon that occurs with high frequency, independent of the genetic characteristics of the host cells. Only cell strains having HPV-16 sequences integrated into the cellular DNA become permanent lines, showing that genetic alterations caused by the viral DNA integration are necessary for the acquisition and maintenance of continuous growth. In situ localization of HPV-16 DNA in immortal cell lines showed the viral integration was responsible for the formation of chromosomal changes that persisted during the process of immortality. Significantly, HPV sequences in cervical carcinoma cell lines are integrated on both normal and abnormal chromosomes near fragile sites and proto-oncogenes. This phenomenon was demonstrated in vitro using a human exocervical epithelial cell line CX16-2, immortalized by transfection with HPV-16. Even though CX16-2 had an abnormal chromosomal constitution with several alterations common for solid tumors, it was not tumorigenic. Among these alterations a homogeneously staining region was identified on the long arm of chromosome 21. By in situ hybridization with HPV-16 DNA labeled with biotin and radioactively labeled nucleotides, viral sequences were observed at a site near ets-2 proto-oncogene. Therefore, the structure and expression of the ets-2 gene was examined. Compared to primary exocervical strains, ets-2 specific m-RNA levels were elevated in CX16-2 immortal cells and tumorigenic cells derived by further transfection with v-Ha-ras. Compared to primary exocervical strains, the increased ets-2 expression was not accompanied by structural gene alterations. Although nonisotopic in situ hybridization with biotinylated HPV-16 DNA allowed a precise localization of viral sequences at bands q22.2-22.3, the same band as ets-2 gene, molecular analysis showed that viral and

ets-2 sequences were distantly located. Linkage analysis by pulse field gel electrophoresis using several rare-cutting restriction enzymes failed to establish linkage between ets-2 and HPV-16, as the two probes did not cohybridize with common restriction fragments. Ets-2 proto-oncogene is implicated in cell proliferation and differentiation. Ets-2 increased expression is consistent with proto-oncogene activation caused by HPV integration in cervical carcinomas and suggests the importance of this alteration for the acquisition of cell immortality of CX16-2 cells. HPV may activate proto-oncogenes even from a distance, an event commonly involving retroviruses.

Chromatin alterations caused by the viral integration could influence the structure or activity of genes involved in cell growth regulation and tumorigenesis. Alteration in DNA replication pattern was identified by specific staining of HPV-16 integration sites after incubation of cells with 5-bromodeoxyuridine for 8 hrs before chromosome fixation. All integration sites exhibited late replication during the S phase at known heterochromatic blocks on chromosomes 1, 9 and 16, rich in highly repetitive sequences. This change in replication pattern could be a contributing factor to the formation of chromosomal rearrangements during the process of uncontrolled cell division. Incomplete chromatin condensation and recombination are consequences of the replication junction that flank late replication DNA and can explain the origin of structural alterations caused by HPV integration, particularly the formation of diffuse staining regions indicative of chromatin decondensation and homogeneously staining regions commonly associated with cellular gene amplification. In addition alteration in replication timing caused by viral integration may have consequences on cellular gene transcription. In human malignancies, abnormalities involving growth factor receptor genes appear most commonly to involve their overexpression, as reported for epidermal growth factor receptor or erbB-2 gene in a significant number of human epithelial malignancies. In human mammary tumors, a predictive role of erbB-2 gene amplification or overexpression, for a more aggressive disease course has been established. Two mammary cells neoplastically transformed in vitro by a 7-12 dimethylbenz(a)anthracene were examined for neu gene expression. Neu gene, the rat homolog of the human erbB-2, was neither amplified nor overexpressed, suggesting that this gene may not be involved in the transformation of rat mammary cells.

The gene that encodes a human cytosolic thyroid hormone binding protein (p58) was mapped by in situ hybridization to 15q24-25. This localization shows that the p58 gene is not linked to the L-type of pyruvate kinase, which is located on chromosome 1. The p58 gene was found to be activated in several forms of cancer. Current localization will permit us to assess the effect of alterations involving chromosome 15 on the structure and activity of the p58 gene in neoplasms or chromosome syndromes. In addition the finding that p58 is located at 15q24-25 raises the possibility that this gene may serve as a useful marker for Tay-Sachs disease. Hexosaminidase A, the enzyme lacking in patients with Tay-Sachs disease, is also located in the same chromosomal region (15q23-24) as the p58 gene.

Investigations within the Tumor Biology Section have focused on expression and cloning of the human leukoregulin gene encoding for the plasma membrane permeabilization and drug uptake activities characteristic of this cytokine. Continuing collaborative studies as part of CRADA CACR0043 with Genzyme Corp.,

indicate the rarity of leukoregulin expression and the extreme difficulty of obtaining sufficiently pure human leukoregulin for accurate protein sequencing. The latter suggested that a direct expression cloning system would be useful in isolating the gene encoding for leukoregulin. Calculations based upon the level of leukoregulin secretion by activated human lymphocytes indicated a minimum of 1 or 2 leukoregulin encoding regions should be present in a cDNA library from activated human lymphocytes. The similarity of protein profiles produced by the mRNA translates from paired activated and non-stimulated normal human peripheral blood lymphocyte samples supported the concept that if leukoregulin is a rare gene, a subtraction cDNA library would further enhance the probability of cloning the gene. Work is in progress on eight subtraction libraries, each being constructed from the mRNA from a single individual. A cDNA library of $0.5-1 \times 10^6$ plasmid inserts from a pool of mRNA obtained from the activated peripheral blood lymphocytes from a number of normal individuals at the NIH Blood Bank has been constructed and transfected into COS7 cells for analysis of the direct expression of leukoregulin membrane permeability activity in the proteins secreted by the transfected COS7 cells. The cDNA library has been divided into pools for transfection, each pool having 250 plasmid inserts from the cDNA library. In the first 1,200 COS7 cell transfected cultures, each culture representing one pool, about 1% of the transfectants express membrane permeabilizing activity. The positive pools are being further subdivided, transfected again, and analyzed to identify transfectants with a single insert for oligonucleotide sequencing to proceed with cloning of the leukoregulin gene and production of recombinant human leukoregulin.

The expression of leukoregulin in Xenopus oocytes injected with mRNA from RPMI 1788 human lymphocytes is also being examined. A cloned line of RPMI 1788 cells has been developed in the Laboratory which produces leukoregulin after cell stimulation of the cells with phorbol 12-myristate 13-acetate. The parental RPMI 1788 cell line was initially utilized by Genentech to isolate the gene encoding lymphotoxin. Isolation of the gene encoding for leukoregulin from RPMI 1788 cells will provide definitive evidence for the separate identities of the leukoregulin and lymphotoxin genes. It will also allow comparison of the leukoregulin oligonucleotide sequence with the sequence cloned from freshly activated normal human peripheral blood mononuclear leukocytes in the COS7 cell direct expression system.

Continuing evaluation of the biological action of leukoregulin has revealed several interesting new regulatory actions of this cytokine. Leukoregulin, while increasing membrane permeability, also protects tumor cells from complement mediated lysis. Treatment of K562 human leukemia cells with 1-5 units of leukoregulin for 60 minutes at 37° induces resistance to complement lysis. The leukoregulin induced state of complement resistance is transient and the cells recover within 4-6 hours unless exposed to leukoregulin for a second time. The protective action of leukoregulin is observed using both conventional ⁵¹Cr-release and trypan blue exclusion assays. The protein synthesis inhibitors puromycin and cyclohexamide and the protein kinase inhibitors tamoxifen, polymyxin B and W-7, each can block the ability of leukoregulin to induce the complement resistant state. This suggests that leukoregulin increases the capacity of K562 cells to down-regulate complement activation or to repair damage caused by complement activation, possibly by inducing synthesis of defense proteins and/or activation of protective protein kinases. As previously reported, leukoregulin increases the sensitivity of

K562 and other cells to lysis by NK and lymphocyte-activated killer lymphocytes. The current observations provide further support that target cells utilize different mechanisms to protect themselves against complement or cytotoxic lymphocytes.

In addition to its ability to modulate cation channel flux, membrane permeability and protein kinases in tumor cells, leukoregulin is able to modulate the metabolism of the major extracellular matrix components, collagen and glycosylaminoglycans, produced by dermal fibroblasts. Concentrations as small as 0.1 units leukoregulin/ml induce a dose-dependent decrease in collagen synthesis, demonstrated by decreased ³[H]-proline incorporation into collagenase-digestible protein, as early as 6 hours after the addition of the cytokine to human fibroblasts. Leukoregulin inhibits the synthesis of both type I and type III collagen, as measured by SDS-polyacrylamide gel electrophoresis and by specific radioimmunoassay. Neutralizing antibodies to interleukin-1 α , interleukin-1 β , tumor necrosis factor- α and interferon- γ fail to alter the action of leukoregulin on collagen synthesis indicating the specificity of the leukoregulin effect. Inhibition of collagen synthesis occurs concomitantly with increased secretion of prostaglandin E₂ and a transient rise in intracellular cyclic AMP content with a peak at 6 hours. Blocking prostaglandin synthesis with indomethacin does not counteract the inhibition of collagen synthesis demonstrating independence of this leukoregulin action from cyclooxygenase metabolites. Leukoregulin also stimulates glycosylaminoglycan production in a dose-dependent manner, affecting the synthesis of hyaluronic acid as the major fibroblast-derived extracellular glycosylaminoglycan. In addition, secretion of neutral proteases (collagenase, elastase, caseinase) is increased. These results demonstrate that leukoregulin has the ability to modulate both anabolic and catabolic functions of fibroblasts. Moreover, the induction of PGE₂ release by connective tissue cells also suggests that leukoregulin may have pro-inflammatory properties. This is the first demonstration for a potential role of this cytokine in the regulation of several functions of non-transformed, non-malignant cells that are central to tissue remodeling as well as to the inflammatory process. Leukoregulin may be a member of a family of leukocyte-derived cytokines which can control connective tissue formation or remodeling and that participate in a number of dermatological disorders in which leukocyte infiltrates are present including dermal fibrosis, keloid scars and scleroderma.

Because leukoregulin increases the sensitivity of tumor cells to NK and LAK lymphocyte cytotoxicity while concomitantly increasing the uptake of doxorubicin and other tumor inhibitory antibiotics, the question whether leukoregulin in combination with chemotherapeutic agents synergistically enhances the cytotoxicity of NK and LAK lymphocytes for HPV DNA containing cervical epithelial and carcinoma cells is being evaluated. Recent experiments with combinations of leukoregulin and cisplatin (CisPt) treatment of HPV-16 DNA-immortalized cervical epithelial cells demonstrate enhancement of target cell sensitivity to NK and to LAK lymphocyte cytotoxicity above that exhibited by the cervical cells treated with either agent alone. Although HPV-immortalized cervical epithelial cells and HPV-16 DNA positive cervical carcinoma cells are relatively resistant to NK lymphocytotoxicity, sensitivity to NK lymphocytotoxicity increases after one hour combination treatment with leukoregulin and CisPt. When the cytotoxicity over a range of NK lymphocyte to QGU target cells is expressed in lytic units (LU), one lytic unit being the

number of NK lymphocytes necessary to produce 30% target cell lysis, the LU/10⁶ LAK lymphocytes is <10 for medium and for CisPt treated target cells, >20 for leukoregulin treated cells, and >200 for the combination CisPt and leukoregulin treatment.

Even greater increases in cytotoxicity are observed with LAK lymphocytes where in a typical experiment the LU/10⁶ LAK lymphocytes for medium and CisPt treated QGU carcinoma cells are >200 compared to >1000 for the leukoregulin treatment and >3000 for the combination of CisPt and leukoregulin treatment. Similar enhancement of the sensitivity to LAK lymphocytotoxicity for HPV-immortalized and H-ras neoplastically transformed HPV-immortalized cervical epithelial cells is produced by combination CisPt and leukoregulin treatment. Cis-Pt alone increased the sensitivity of CX16.2 cells to LAK lysis 1.2-fold as indicated by the increase in LU_{20%}/10⁶ LAK cells but decreased the sensitivity of the PH1 and QGU cells 1.4- and 1.2-fold, respectively. Leukoregulin alone up-regulated the sensitivity of CX16.2 cells 1.4-fold, PH1 (HPV16 plus v-Ha-ras) cells 1.7-fold and QGU cells 2.5-fold. Combined leukoregulin and cisPt treatment produced a synergistic increase of 4.4-fold for the CX16.2 and QGU and 2.4-fold for the PH1 cells. γ -interferon alone or in combination with cisPt was less effective than leukoregulin and usually produced neither an additive nor a synergistic increase in target cell sensitivity when used in combination with cisPt. These results demonstrate that combination treatment with a cytokine and a chemotherapeutic agent has the potential to rapidly up- or down-regulate the sensitivity of HPV-16 positive dysplastic or neoplastic cervical epithelial cells to LAK lymphocyte cytotoxicity and that the addition of leukoregulin can overcome decrease target cell sensitivity to LAK lymphocyte killing produced by cis-Pt.

Most surprising is the observation that treatment of the HPV DNA immortalized cervical cells, the PH1 tumor cells and the QGU cervical carcinoma cells when treated for one hour with CisPt and γ -interferon, can lose their sensitivity to destruction by LAK lymphocytes. These are important observations as current combination chemotherapy with conventional drugs is not encouraging in terms of response rate, toxicity and other side effects. CisPt remains one of the drugs of choice in cervical cancer, alone or in combination with other chemotherapeutic agents. It is, therefore, important to determine the extent to which cytokines like leukoregulin and interferon up- or down-regulate the degree of NK and LAK lymphocyte cytotoxicity in the presence of anti-tumor chemotherapeutic agents. This is potentially a new therapeutic approach for the treatment of cervical dysplasia and neoplasia.

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

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PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT

Regulation of Stages of Carcinogenesis Induced by Chemical or Physical Agents

PRINCIPAL INVESTIGATOR

PI:	J.A. DiPaolo	Chief	LB	NCI
Others:	M. Chen	Expert	LB	NCI
	P. DeFiori	Visiting Scientist	LCMB	NCI
	N.C. Pop	Research Microbiologist	LB	NCI
	C.D. Woodworth	Senior Staff Fellow	LB	NCI

COOPERATING UNITS

Georgetown University, Dept. of Microbiology (L. Rosenthal); Georgetown University, Dept. of Radiation Medicine (V. Notario); University of Montreal, Dept. of Pathology, (A. Kessous-Elbaz)

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CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK

In vitro cell models for cervical cancer have been developed. Immortalized cell lines were obtained using recombinant human papillomavirus (HPV) associated with cancer. Squamous cell carcinomas were produced. Thus, it is possible to study the regulation of immortalization and the mechanisms for progression to malignancy. The possible importance of cofactors, other viruses, chemical carcinogens, and oncogenes for cervical cancer is being explored. Herpes simplex 2 studies have been extended utilizing subfragments of a subgenomic region of herpes. A number of cell lines containing a specific subfragment of the morphological transforming region (Bgl II N) have maintained the integrated HPV genome and the herpes fragment over 20 subpassages. Because infected cervical cells pass through premalignant stages, markers are needed to facilitate the recognition of malignancy. A number of spontaneous and experimentally induced carcinomas of the cervix were characterized by a cytoskeletal qualitative change. All tumors lacked keratin 14 which is found in normal and HPV immortalized cell lines. Therefore, it is possible that the loss of keratin 14 is a general marker for cervical cancer. The epidermal growth factor receptor family is being examined in detail because the epithelial cells have them. Foreskin immortalized by HPV-16 keratinocytes were converted to malignant cells that produced squamous cell carcinomas by delta Nerb B2. These cells continue to express the HPV sequences. Thus, this multistage model utilizing human cells indicates that in continuous expression of HPVs, property of spontaneous cancer is also found in cancers that are experimentally produced.

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

J. A. DiPaolo	Chief	LB	NCI
M. Chen	Expert	LB	NCI
P. DeFiori	Visiting Scientist	LCMB	NCI
N. C. Popescu	Res. Microbiologist	LB	NCI
C. D. Woodworth	Senior Staff Fellow	LB	NCI

Objectives:

This study investigates the progression through multiple, distinct stages required for neoplastic transformation of human and other cells relevant to the etiology and prevention of cancer. The specific objectives are: (1) to determine the molecular and cellular aspects of chemical, physical and biological interactions 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:

With procedures developed in this laboratory, genital epithelial cells derived from cervix and foreskin have been immortalized with a variety of human papillomaviral (HPV) DNA associated with invasive cancers. All these lines contained HPV DNA and expressed a number of viral messages, but none were tumorigenic. Because of the evidence linking HPV infections with cervical intraepithelial neoplasia and carcinomas, the question is whether synergism exists between papillomaviruses and other carcinogens. Genital epithelial cells, including normal and HPV immortalized, were treated with cofactors such as chemical carcinogens, biologicals and oncogenes related to growth factors. In addition markers relevant to progression were studied with emphasis on keratin changes that occur in the cervix with the onset of malignancy.

Major Findings:

Studies utilizing known chemical carcinogens in conjunction with recombinant HPV have been extended. Although the addition of carcinogen of various classifications have failed to induce the malignant state in cells containing HPV, the carcinogens were toxic regardless of whether relatively early or late passage of HPV immortalized cells were used. A comparison of cell lines that failed to produce tumors with those that did produce tumors as a result of viral transfection and spontaneous human cervical squamous cell carcinoma lines demonstrated the existence of a qualitative difference in keratin expression between tumor producing cells and normal cells derived from the exocervix. Keratin 14 expression determined by protein analysis and mRNA levels was dramatically down-regulated in the cervical squamous carcinoma lines, while keratin 5 (the co-ordinate partner) expression was not. A

similar down-regulation was noted in immortalized HPV cervical cells after tumorigenic transformation with recombinant v-Ha-ras DNA. In addition, neoplastic lines exhibited up-regulation of a series of keratins including some that are considered simple keratins. These results can be contrasted with those having tumor lines that were obtained by transfecting herpes simplex-2 into foreskin derived keratinocytes. With foreskin immortalized tumorigenic cells, no change has been observed thus far in keratin 14. Therefore, down-regulation of keratin 14 in tumorigenic cervical lines, in the absence of significant change in expression of K5, implies that the normal coordinate regulation K5 and K14 gene expression has been uncoupled. It should be possible to determine the probability of tumorigenicity of cervical lines by probing with a keratin 14-cDNA for expression at the RNA level.

A squamous cell antigen (TA-4), a glycoprotein of 48 kd, has been found to discriminate between keratinizing and non-keratinizing tumors and small cell carcinomas. Because women treated for invasive cervical cancer often have persistent or recurrent disease, markers are required to detect the presence of the disease. Markers are also required to monitor cell culture for immortality and progression to malignancy. A radioimmune assay utilizing TA-4 antigen that is used with serum was successfully adapted to use with tissue culture medium. The majority of immortal and tumorigenic cells responded.

The morphologic transforming region of herpes simplex virus-2 (mtr-2) has been successfully transfected into HPV immortalized keratinocytes. Cells injected had integrated HSV-2 sequences. The resulting tumors in nude mice have been diagnosed as squamous cell carcinomas. Although integrated HPV was found, herpes sequences were detectable. The mtr-2 had no effect on normal cells. The absence of herpes simplex in the cells has been postulated as being due to the toxicity of numerous proteins produced by mtr-2. Therefore, a series of additional constructs have been made that are subfragments of mtr-2. Some of these have produced tumors after having been transfected into HPV immortal cells. The cell lines after prolonged culturing do have integrated virus. Whether the tumors possess any herpes associated DNA will be determined.

A study is underway on the role of growth factors controlling genes in converting HPV immortal cells to malignancy as well as in complementing the E6 or E7 oncogenes that are relevant to HPV immortalization. Because the epidermal growth factor receptor family is relevant to epithelial cells, v-erbB and Δ NerbB2 have been transfected into HPV immortal cells. Thus far, one line has been converted to malignancy by Δ NerbB2.

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

Z01CP04673-20 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT

The Immunobiology of Carcinogenesis

PRINCIPAL INVESTIGATOR

PI:	C.H. Evans	Chief, Tumor Biology Section	LB	NCI
Others:	E.K. Farley	Guest Researcher	LB	NCI
	L. Liu	Visiting Fellow	LB	NCI
	L.J. Rezanka	IRTA	LB	NCI
	P.D. Baker	Microbiologist	LB	NCI
	A.C. Wilson	Chemist	LB	NCI

COOPERATING UNITS

J.L. Hooks, Chief, Immunology Section, LI, NEI; Department of Chemistry, Weizmann Inst. Sci. (Z. Fishelson), Genzyme Corporation (R. Douglas)

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

Characterization of leukoregulin, a cytokine first described in this project in 1983 with the unique ability to increase tumor cell plasma membrane permeability, is being pursued at both the structural and functional levels. Isolation of the gene encoding for the protein with plasma membrane permeability activity is being carried out by direct expression cloning in mammalian cells using clones from a cDNA library prepared from leukoregulin secreting normal human peripheral blood mononuclear leukocytes. More than 200,000 clones in pools of 250 clones have been examined for leukoregulin synthesis. Approximately 1.5% of the clone pools are able to express membrane permeability activity following plasmid transfection into COS7 cells. The actively expressing pools are being subdivided to isolate the leukoregulin cDNA clones to obtain the oligonucleotide sequence encoding leukoregulin and to produce recombinant human leukoregulin. Leukoregulin while increasing membrane permeability also protects tumor cells from complement mediated lysis. Leukoregulin induction of complement lysis is blocked by both inhibitors of protein synthesis and inhibitors of protein kinase C activity suggesting that complement resistance may be mediated through leukoregulin induction of protein kinase C stimulated complement resistance proteins. Leukoregulin also induces glycosylaminoglycan, collagenase and other protease synthesis and extracellular secretion which are important components in the extracellular matrix and may indicate a role for leukoregulin in immunological inflammatory connective tissue disorders. Production of recombinant leukoregulin will allow critical examination of whether the diverse functional properties of this cytokine are resident in a single molecule and further delineation of the intracellular pathways responsible for the functional activities of this cytokine.

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

C. H. Evans	Chief, Tumor Biology Section	LB	NCI
L. Liu	Visiting Fellow	LB	NCI
E. Farley	Guest Researcher	LB	NCI
P. D. Baker	Microbiologist	LB	NCI
A. C. Wilson	Chemist	LB	NCI
J. J. Hooks	Chief, Immunology Section	LI	NEI

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 preneoplastic 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 leukoregulin, a cytokine first described in this project in 1983 with the unique ability to increase tumor cell plasma membrane permeability, is being pursued at both the structural and functional levels. Isolation of the gene encoding for the protein with plasma membrane permeability activity is being carried out by direct expression cloning in mammalian cells using clones from a cDNA library prepared from leukoregulin secreting normal human peripheral blood mononuclear leukocytes under a Collaborative Research and Development Agreement (CACR0043) with Genzyme Corp. More than 200,000 clones in pools of 250 clones have been examined for leukoregulin synthesis. Approximately 1.5% of the clone pools are able to express membrane permeability activity following plasmid transfection into COS7 cells. The actively expressing pools are being subdivided to isolate the leukoregulin cDNA clones to obtain the oligonucleotide sequence encoding leukoregulin and to produce recombinant human leukoregulin.

Members of the NCI intramural staff are also investigating the expression of leukoregulin in *Xenopus* oocytes injected with mRNA from RPMI 1788 human lymphocytes. The RPMI 1788 cells are a cloned line developed in the Laboratory which produce leukoregulin following stimulation of the cells with phorbol diester. The parental RPMI 1788 cells are the same cells initially utilized by Genentech to isolate the gene encoding for lymphotoxin. Isolation of the gene encoding for leukoregulin from RPMI 1788 cells will provide definitive evidence for the separate identities of the leukoregulin and lymphotoxin genes and allow comparison of the leukoregulin oligonucleotide sequence with that obtained from freshly activated normal human peripheral blood mononuclear leukocytes.

Leukoregulin, while increasing membrane permeability, also protects tumor cells from complement mediated lysis. Treatment of K562 human leukemia cells with 1-5 units of leukoregulin for 60 minutes at 37° induces resistance to complement lysis. The leukoregulin induced state of complement resistance is transient and the cells recover within 4-6 hours unless exposed to leukoregulin for a second time. The protective action of leukoregulin is observed using both conventional ⁵¹Cr-release and trypan blue exclusion assays. The protein synthesis inhibitors puromycin and cyclohexamide and the protein kinase inhibitors tamoxifen, polymyxin B and W-7, each can block the ability of leukoregulin to induce the complement resistant state. This suggests that leukoregulin increases the capacity of K562 cells to down-regulate complement activation or to repair damage caused by complement activation, possibly by inducing synthesis of defense proteins and/or activation of protective protein kinases. As previously reported, leukoregulin increases the sensitivity of K562 and other cells to lysis by NK and lymphocyte-activated killer lymphocytes. The current observations provide further support that target cells utilize different mechanisms to protect themselves against complement or cytotoxic lymphocytes.

In addition to its ability to modulate cation channel flux, membrane permeability and protein kinases in tumor cells, leukoregulin is able to modulate the metabolism of the major extracellular matrix components, collagen and glycosylaminoglycans secreted by dermal fibroblasts. Concentrations as small as 0.1 units leukoregulin/ml induce a dose-dependent decrease in collagen synthesis, demonstrated by decreased ^3H -proline incorporation into collagenase-digestible protein, as early as 6 hours after the addition of the cytokine to human fibroblasts. Leukoregulin inhibits the synthesis of both type I and type III collagen, as measured by SDS-polyacrylamide gel electrophoresis and by specific radioimmunoassay. Neutralizing antibodies to interleukin- 1α , interleukin- 1β , tumor necrosis factor- α and interferon- γ fail to alter the action of leukoregulin on collagen synthesis indicating the specificity of the leukoregulin effect. Inhibition of collagen synthesis occurs concomitantly with increased secretion of prostaglandin E_2 and a transient rise in intracellular cyclic AMP content with a peak at 6 hours. Blocking prostaglandin synthesis with indomethacin does not counteract the inhibition of collagen synthesis demonstrating independence of this leukoregulin action from cyclooxygenase metabolites. Leukoregulin also stimulates glycosylaminoglycan production in a dose-dependent manner, affecting the synthesis of hyaluronic acid as the major fibroblast-derived extracellular glycosylaminoglycan. In addition secretion of neutral proteases (collagenase, elastase, caseinase) is increased. These findings demonstrate that leukoregulin has the ability to modulate both anabolic and catabolic functions of fibroblasts. Moreover, the induction of PGE_2 release by connective tissue cells also suggests that leukoregulin may have proinflammatory properties. This is the first demonstration for a potential role of this cytokine in the regulation of several functions of non-transformed nonmalignant cells that are central to tissue remodeling and to the inflammatory process. Leukoregulin may be a member of a family of leukocyte-derived cytokines which can control connective tissue formation or remodeling and that participate in a number of dermatological disorders in which leukocyte infiltrates are present including dermal fibrosis, keloid scars and scleroderma.

Publications:

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PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT

Chromosome Alterations and Proto-Oncogene Transposition in Carcinogenesis

PRINCIPAL INVESTIGATOR

PI:	N.C. Popescu	Research Microbiologist	LB	NCI
Others:	J.A. DiPaolo	Chief	LB	NCI
	S. Cheng	Research Chemist	LMB	NCI

COOPERATING UNITS

None

LAB/BRANCH

Laboratory of Biology

SECTION

Somatic Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1.8

PROFESSIONAL: 1

OTHER: 0.8

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK

Non-isotopic *in situ* hybridization is a powerful molecular approach for detecting and localizing specific nucleic acid sequences within interphase nuclei or chromosomes. Single viral copies were detected by fluorescent signals after hybridization with biotinylated virus DNA probes. This has a practical significance for detecting DNA and RNA viruses in malignant and premalignant lesions. On a cervical carcinoma cell line (C4-1), human papillomavirus-18 (HPV-18) sequences were localized at region 8q21 on an 8;12 rearranged chromosome. In a Burkitt's lymphoma cell line, Epstein-Barr virus sequences were mapped on chromosome 2p13 adjacent to a viral modification site. In both lines viral integration corresponded with the location of a fragile site. A nontumorigenic line (CX16-2) derived from exocervical epithelial cells transfected with recombinant HPV-16, harbor viral sequences on chromosome 2

near *ets-2* gene. The *ets-2* specific m-RNA level was elevated in the absence of structural gene alterations. However, no linkage between *ets-2* and HPV-16 sequences was established by pulse field gel electrophoresis using several rare-cutting restriction enzymes. Thus, HPV sequences can influence, from a distance, proto-oncogene expression. Several HPV-16 integration sites exhibited an aberrant late replication pattern. Incomplete chromatin condensation and recombination are consequences of the replication junction that flank late replicating DNA and can explain the origin of chromosomal changes associated with the cell's immortality. *Neu* proto-oncogene, the rat homolog of the human *erb-B-2*-gene, was neither amplified nor overexpressed in two rat mammary cell lines neoplastically transformed *in vitro* by a chemical carcinogen. A cDNA for the gene that encodes a human cytosolic thyroid hormone binding protein (p58) was mapped by *in situ* hybridization to 15q24-25. This localization may serve as a useful marker for Tay-Sachs disease and will permit assessment of the effect chromosome alterations involving this region have on human malignancies.

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
S. Cheng	Research Chemist	LMB	NCI

Objectives:

This project is directed toward understanding the role of chromosome alterations in malignant transformation. The localization of breakpoints on specific chromosome rearrangements or deletions and of proto-oncogenes 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). Molecular isotopic and nonisotopic in situ chromosome hybridization techniques and banding procedures 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. Molecular cytogenetic techniques are used in conjunction with Southern and Northern blot hybridization and pulse field gel electrophoresis.

Major Findings:

A nontumorigenic line (CX16-2) derived from exocervical epithelial cells transfected with recombinant human papillomavirus-16 (HPV-16) DNA had viral sequences integrated near the ets-2 gene. Compared to primary exocervical strains, ets-2 specific m-RNA levels were elevated in CX16-2 HPV transfected immortal cells and tumorigenic cells derived by transfection with v-Ha-ras. The increased ets-2 expression was not accompanied by structural gene alterations. By in situ hybridization HPV sequences were localized at 21q22.2-22.3, the same band as ets-2 gene. However, linkage analysis by pulse field gel electrophoresis using several rare-cutting restriction enzymes failed to establish linkage between ets-2 and HPV-16 because the two probes did not cohybridize with common restriction fragments. Ets-2 proto-oncogene is implicated in cell proliferation and differentiation. Ets-2 increased expression is consistent with proto-oncogene activation caused by HPV integration in cervical carcinomas suggesting the importance of this alteration for the acquisition of cell immortality.

Nonisotopic in situ hybridization was used to detect and localize HPV-18 sequences on the C4-1 line derived from a cervical carcinoma. Single copies LBHPV-18 were detected on both nuclei and metaphase chromosomes after hybridization with a biotinylated viral probe. This line has a near diploid

chromosome complement with several abnormal chromosomes. HPV-18 sequences were identified at a single chromosome site (8q21) on an abnormal chromosome originating from a translocation of the long arm of chromosomes 8 and 12. This localization offers the first example of a cervical carcinoma line with a single copy of integrated HPV.

Five foreskin-derived keratinocyte lines immortalized with HPV-16 DNA had chromosome translocations, achromatic lesions, partial chromosome duplications and homogeneously staining regions as a result of viral integration. Giemsa chromosome staining was used to distinguish the pattern of DNA replication at the first division post-treatment with 5-bromodeoxyuridine to determine the replication of HPV integration sites associated with structural alterations. All HPV integration sites exhibited an aberrant late replication most likely caused by the virus insertion into the cellular DNA.

A Burkitt's lymphoma cell line derived from a North American patient (NAB-2), was examined after in situ hybridization with biotinylated Epstein-Barr virus (EBV). The analysis of fluorescent signals of nuclei and chromosomes hybridized with a Bam HI "V" region of EBV, demonstrated that NAB-2 cells have a single copy virus integrated on the short arm of chromosome 2p13. The EBV integration at 2p13 overlaps with the location of a common fragile site induced by aphidicolin, rel proto-oncogene, and of a transforming growth factor gene. Virus insertion onto chromosomal DNA caused a stable modification site expressed as a distinctive achromatic region adjacent to the band 2p13. This chromatin alteration may represent a contributing factor to the development of this malignancy. EBV sequences at 2p13 provide a useful marker for further mapping in this region.

Two rat mammary cell lines, neoplastically transformed in vitro by a chemical carcinogen exhibited nonrandom structural alterations of chromosome 3 and trisomies of chromosomes X and 14. Chromosome 14 may carry neu proto-oncogene, the rat homolog of the human erb-B-2 gene. Neu gene was neither amplified nor overexpressed suggesting that this gene may not be involved in the transformation of rat mammary cells by chemical carcinogen.

A cDNA for the gene that encodes a human cytosolic thyroid hormone binding protein (p58) has been isolated and sequenced in the Laboratory of Molecular Biology. A full-length cDNA probe was used for in situ hybridization of normal human chromosomes and three sites of hybridization were identified; a major site at 15q24-25 and two other sites at 1p11-12 and 6p15.3. Using a 0.838-Kb fragment as a probe, hybridization was detected only at region 15q24-25 where the locus for p58 gene was assigned. P58 gene localization may be important for further studies with syndromes and neoplasms involving this region.

Publications:

DiPaolo JA, Bowden PE, Popescu NC, Woodworth CD. Transformation of human epithelial cells by recombinant human papillomavirus DNA associated with cervical cancer. In: Columbano A, Feo F, Pani P, eds. Chemical carcinogenesis: modulating factors in multistage chemical carcinogenesis. New York:Plenum Press. (In Press).

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Pash J, Popescu NC, Matocha M, Rapoport S, Bustin M. Chromosomal protein HMG14 gene maps to the Down syndrome region of human chromosome 21 and is over-expressed in mouse trisomy 16. Proc Natl Acad Sci USA 1990;87:3836-40.

Popescu NC, Cheng Y. Chromosomal localization of the gene for a human cytosolic thyroid hormone binding protein which is homologous to a subunit of pyruvate kinase, subtype 2. Som Cell Mol Genet 1990;16:593-8.

Popescu NC, Dahlberg JE, Ablashi DV, Monastier M, Bona C, DiPaolo JA, Hooper WC, Swan D. Oncogene expression and immunoglobulin synthesis in a North American Burkitt (NAB-2) lymphoma cell line with an 8;22 chromosome translocation. Oncogene Res 1990;5:295-303.

Popescu NC, Landsman D, Bustin M. Mapping the human gene coding for chromosomal protein HMG-17. Human Genet 1990;85:376-378.

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

Z01CP05552-04 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT

Lymphokine Modulation of Human Cervical Epithelial Cell Carcinogenesis

PRINCIPAL INVESTIGATOR

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

OPERATING UNITS

None

BRANCH

Laboratory of Biology

SECTION

Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 2.5	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK

As part of an ongoing evaluation of human papillomavirus (HPV)-immortalized cervical epithelial cells, the recently developed HPV/Hras and HPV positive cervical carcinomas are being studied in *in vitro* models of cytokine and chemotherapeutic drug modulation of target cell sensitivity to natural killer (NK) and to lymphokine activated killer (LAK) lymphocyte cytotoxicity. Treatment of HPV-16 positive cervical epithelial cells with cisplatin and γ -interferon or leukoregulin further influences the sensitivity of the cervical cells to LAK lymphocyte cytotoxicity. ^{51}Cr labeled HPV-16 immortalized CX16.2 cells, HPV-16 and H-ras sequentially transfected and tumorigenic PH1 cells and HPV-16 positive QGU cervical carcinoma cells were treated for one hour with either 2 units of leukoregulin/ml or with 100 units gamma-interferon/ml in the presence of 10 nM cisplatin. The cells were incubated with 7 day IL-2-stimulated LAK lymphocytes for 4 hours at lymphocyte:cervical cell ratios from 1 to 10:1 and the net release of ^{51}Cr was measured. Cisplatin alone increased the sensitivity of CX16.2 cells to LAK lysis 1.2-fold as indicated by the increase in $\text{LU}_{20\%}/10^6$ LAK cells, but decreased the sensitivity of the PH1 and QGU cells 1.4- and 1.2-fold, respectively. Leukoregulin alone up-regulated the sensitivity of CX16.2 cells 1.4-fold, PH1 cells 1.7-fold and QGU cells 2.5-fold. Combined leukoregulin and cisplatin treatment produced a synergistic increase of 4.4-fold for the CX16.2 and QGU and 2.4-fold for the PH1 cells. Gamma-interferon alone or in combination with cisplatin was less effective than leukoregulin and usually produced neither an additive nor a synergistic increase in target cell sensitivity when used in combination with cisplatin. These results demonstrate that combination treatment with a cytokine and a chemotherapeutic agent has the potential to rapidly up- or down-regulate the sensitivity of HPV-16 positive dysplastic or neoplastic cervical epithelial cells to LAK lymphocyte cytotoxicity and that the addition of leukoregulin can overcome decreased target cell sensitivity to LAK lymphocyte killing produced by cisplatin.

PROJECT DESCRIPTION

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

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

Objectives:

The overall objective of this project is to evaluate the susceptibility of human papillomavirus-immortalized human cervical epithelial cells at various stages of carcinogenesis to NK and to LAK cell cytotoxicity and to regulate these responses by modulating the target cell sensitivity with various cytokines and other growth and differentiation inducing agents. Because of the significance of chemotherapeutic drugs in cancer treatment regimens and because of the unsuccessful results with many of these drugs, especially in cervical cancer, we have asked whether combinations of cytokine/chemotherapeutic drug treatment will affect the sensitivity of HPV-16 positive cervical epithelial target cells to NK and to LAK.

Current evidence strongly suggests that HPV type 16 is involved in the etiology of cervical intraepithelial dysplasia and malignancy. In cervical cancers, HPV DNA is integrated into the cellular genome, while in premalignant and benign lesions, the DNA exists in an episomal form. Immunity against HPV infections and associated neoplasia was thought to be engineered by the classical cell-mediated effector arm of the host defense system. This was substantiated by the presence of T-cells and the absence of a significant number of B-cells in cervical metaplasia and in normal cervical epithelia. Although there is a general presence of T lymphocytes in normal cervical tissue, there is an overall absence of T cells in HPV infections and cervical intraepithelial neoplasia. NK cells are either almost totally absent or scattered throughout the transformation zone.

Previous studies in this laboratory have indicated that NK cells are ineffective against HPV-16 DNA-immortalized cervical cell lines in vitro. NK cells on the other hand may have an immunomodulatory function in that they produce biomodulators that regulate other effector cells, and modulate target cell sensitivity to these effectors. One of these effectors is the LAK cell system which, unlike NK, is very strongly reactive against the HPV cervical cell lines and an established HPV-16 DNA positive cervical carcinoma, QGU.

As part of the ongoing study of the HPV-16 DNA positive cervical cell sensitivity to NK and LAK and the modulation of the target cell sensitivity by cytokines, the present investigation has assessed the influence of HLA class I and II antigens and also the influence of combination leukoregulin and vincristine on target cell sensitivity to NK and to LAK.

Methods Employed:

Monolayer cultures of HPV-16 DNA-immortalized cervical epithelial cells were incubated with leukoregulin (2.0 u/ml), IFN γ (100 u/ml), IFN α (1000 u/ml), and TGF β (3ng/ml) for 1 and 48 hr. The cells were washed and then treated with undiluted EDTA (1X versene, 1:5000) to remove them from the culture dish. The cells were resuspended at 2×10^7 /ml in calcium chloride-free MCDB153LB media supplemented with the 0.1% sodium azide at 4°C. Fluorescein-labelled mouse monoclonal anti-HLA-A,B,C and anti-HLA-DR were used to detect and quantitate the class I and II antigens using a FACS analyzer (model # ANA-2513A, Becton Dickinson). For the cytotoxicity studies, ^{51}Cr labelled target cells were incubated with leukoregulin (2.5 u/ml) alone, cisplatin (10^{-10}M) alone and with a combination of these two agents for 1 hr, then mixed with NK or LAK effectors in a 4 hr chromium release assay. In addition to evaluating HLA surface antigen expression, northern blot analysis of cytokine-treated cells for HLA class I and II mRNA and HPV RNA were performed.

Major Findings:

Because leukoregulin increases the sensitivity of tumor cells to NK and LAK lymphocyte cytotoxicity while concomitantly increasing the uptake of doxorubicin and other tumor inhibitory antibiotics, we are evaluating whether leukoregulin in combination with chemotherapeutic agents synergistically enhances the cytotoxicity of NK and LAK lymphocytes for HPV DNA containing cervical epithelial and carcinoma cells. Recent experiments with combination leukoregulin and cisplatin (CisPt) treatment of HPV-16 DNA-immortalized cervical epithelial cells demonstrate enhancement of target cell sensitivity to NK and to LAK lymphocyte cytotoxicity above that exhibited by the cervical cells treated with either agent alone. Although HPV-immortalized cervical epithelial cells and HPV-16 DNA positive cervical carcinoma cells are relatively resistant to NK lymphocytotoxicity, sensitivity to NK lymphocytotoxicity increases after combination treatment with leukoregulin and CisPt for one hour. When the cytotoxicity over a range of NK lymphocyte to QGU target cells is expressed in lytic units (LU), one lytic unit being the number of NK lymphocytes necessary to produce 30% target cell lysis, the LU/ 10^6 LAK lymphocytes is <10 for medium and for CisPt treated target cells, >20 for leukoregulin treated cells, and >200 for the combination CisPt and leukoregulin treatment.

Even greater increases in cytotoxicity are observed with LAK lymphocytes where, in a typical experiment, the LU/ 10^6 LAK lymphocytes for medium and CisPt treated QGU carcinoma cells are >200 compared to >1000 for the leukoregulin treatment and >3000 for the combination of cisPt and leukoregulin treatment. Similar enhancement of the sensitivity to LAK lymphocytotoxicity for HPV-immortalized and H-ras neoplastically transformed HPV-immortalized cervical epithelial cells is produced by combination CisPt and leukoregulin treatment. CisPt alone increased the sensitivity of CX16.2 cells to LAK lysis 1.2-fold as indicated by the increase in LU $_{20\%}$ / 10^6 LAK cells but decreased the sensitivity of the PH1 and QGU cells 1.4- and 1.2-fold, respectively. Leukoregulin alone up-regulated the sensitivity of CX16.2 cells 1.4-fold, PH1 cells 1.7-fold and QGU cells 2.5-fold. Combined leukoregulin and cisPt treatment

produced a synergistic increase of 4.4-fold for the CX16.2 and QGU and 2.4-fold for the PH1 cells. γ -interferon alone or in combination with cisPt was less effective than leukoregulin and usually produced neither an additive nor a synergistic increase in target cell sensitivity when used in combination with cisPt. These results demonstrate that combination treatment with a cytokine and a chemotherapeutic agent has the potential to rapidly up- or down-regulate the sensitivity of HPV-16 positive dysplastic or neoplastic cervical epithelial cells to LAK lymphocyte cytotoxicity and that the addition of leukoregulin can overcome decreased target cell sensitivity to LAK lymphocyte killing produced by cisPt.

Most surprising is the observation that after treatment of the HPV DNA immortalized cervical cells for one hour with cisPt and λ -interferon, the PH1 tumor producing V-Ha-ras transfected HPV-16 HCX-2 cells and the QGU cervical carcinoma cells can lose their sensitivity to destruction by LAK lymphocytes. This is an important observation since current combination chemotherapy using conventional drugs is not encouraging in terms of response rate, toxicity and other side effects. CisPt remains one of the drugs of choice in cervical cancer, alone or in combination with other chemotherapeutic agents. Bonomi et al., however, found only a 22% response rate with combined cisPt and 5FU treatment in a phase II study in cervical cancer patients, although this combination of drugs produced an 88% response in squamous cell carcinoma of the head and neck. It is, therefore, important to determine the extent to which cytokines like leukoregulin and interferon up- or down-regulate the degree of NK and LAK lymphocyte cytotoxicity in the presence of anti-tumor chemotherapeutic agents. This is potentially a new therapeutic approach for the treatment of cervical dysplasia and neoplasia.

Publications:

DiPaolo JA, Woodworth CD, Furbert-Harris PM, Evans CH. Immunomodulation of HPV-16-immortalized exocervical epithelial cells. In: Gross E, Jablonska S, Pfister H, Stegner H-E, eds. Genital papillomavirus infections: advances in modern diagnosis and therapy. New York: Springer Verlag, 1990;283-99.

Evans CH, Furbert-Harris PM, Flugelman AA, Woodworth CD, DiPaolo JA. Synergistic regulation of LAK lymphocyte cytotoxicity by combination cytokine and cisplatin treatment. In: Goldstein, AL, ed. Proceedings first international conference on combination therapy. New York: Plenum Publishing (In Press).

Evans CH, Furbert-Harris PM, Woodworth CD, DiPaolo JA. Lymphokine modulation of HPV-16 transfected cervical epithelial cell sensitivity to natural killer lymphocyte immunologic destruction. In: Howley P, Broker T eds. Papillomaviruses: UCLA symposia on molecular and cellular biology, vol 124. New York: Alan R Liss, 1990;145-55.

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

Z01CP05625-02 LB

PERIOD COVERED

October 1, 1990 to September 30, 1991

TITLE OF PROJECT

Regulation of Cellular Gene Expression by Human Papillomaviruses

PRINCIPAL INVESTIGATOR

PI:	C.D. Woodworth	Senior Staff Fellow	LB	NCI
Others:	J.A. DiPaolo	Chief	LB	NCI
	M. Chen	Expert	LB	NCI
	H. Wang	Visiting Fellow	LB	NCI

COOPERATING UNITS

Dept. Biochemistry, Univ. Rochester School Med., Rochester, NY (L. Chow, T. Broker, S. Cheng), and Dept. Obstet. Gynecol., Albert Einstein Coll. Med. NY, NY (G. Romney)

LAB/BRANCH

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SECTION

Somatic Cells Genetics Section

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PROFESSIONAL: 2.5

OTHER: 1.0

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK

Studies have focused on the interaction between human papillomaviruses (HPV) and normal human genital epithelial cells. Specifically, 1) high-titer retroviruses were developed to examine the role of specific HPV genes in regulation of cellular growth and differentiation, and 2) the molecular mechanism by which transforming growth factors beta (TGF β s) 1 and 2 regulate HPV gene expression was investigated in genital epithelial cells at different stages of malignant progression. HPV E6 and E7 genes are implicated in maintenance of the malignant phenotype in cervical cancer. To examine whether E6 and E7 proteins directly regulate cell growth and differentiation genital keratinocytes were infected with recombinant retroviruses containing these genes alone or in combination. Retroviruses encoding the E6/E7 and E7 genes stimulated cell proliferation, reduced the requirement for bovine pituitary extract, delayed onset of terminal differentiation and induced immortality. However, these infected keratinocytes stratified and formed well-differentiated squamous epithelia when transplanted beneath a skin-muscle flap of nude mice. Thus, expression of HPV-18 E6 and E7 genes alters cell growth and commitment to differentiation but does not directly induce aberrant squamous differentiation in normal genital keratinocytes *in vivo*. TGF β s 1 and 2, polypeptides that regulate cellular growth and differentiation, reversibly inhibited expression of the HPV-16 E6 and E7 genes in several immortal genital epithelial cell lines. Loss of E6 and E7 protein expression followed a dramatic time- and dose-dependent decrease in E6 and E7 RNA levels and was accompanied by cessation of cell proliferation. TGF β s 1 and 2 also induced a six- to seven-fold increase in TGF β 1 RNA. Cells became partially resistant to the inhibitory effects of TGF β 1 on cell growth and HPV early gene expression after prolonged cultivation *in vitro* or after malignant transformation. Thus, TGF β 1 may function as an autocrine regulator of HPV gene expression in infected genital epithelial cells.

PROJECT DESCRIPTIONObjectives:

To investigate the interactions between normal human genital keratinocytes and HPV early gene products. Specifically to 1) examine the role of the HPV-18 E6, E6* and E7 genes in regulation of cell proliferation and squamous differentiation, and 2) identify the molecular mechanism by which TGF β s 1 and 2 regulate expression of the E6 and E7 genes in genital epithelial cells at various stages of malignant progression.

Methods Employed:

High-titer retroviruses containing specific HPV-18 early genes are produced by transfecting the appropriate recombinant vector DNAs into psi crip and psi cre packaging cell lines. Retroviruses are titered by assessing resistance of infected Hela cells to a coexpressed selectable marker (neomycin resistance) and all constructs are checked for gene rearrangement by Southern analysis. Expression of cellular and viral genes is measured at both the protein and RNA level using the techniques of immunoprecipitation and SDS polyacrylamide gel electrophoresis, Northern analysis, and nuclear runoff transcriptional analysis. Epithelial cells are isolated from cervical or foreskin tissue and cells are maintained in serum-free MCDB153-LB medium. For xenografts, confluent cell monolayers are removed from culture dishes by digestion with dispase and transplanted intact beneath a skin-muscle flap in athymic mice.

Major Findings:

High-titer retroviruses were used to transfer and express HPV-18 E6, E6*, and E7 genes alone or in combination in normal genital keratinocytes. Southern analyses showed that the HPV-18 genes were stably retained at an average of 1 copy per cell and they were actively expressed. The HPV-18 E6*/E7, E6/E7, and E7 constructs induced proliferation of normal cells, whereas the E6 gene or vector only were ineffective. E6*/E7, E6/E7, and E7 also reduced requirements for exogenous growth factors in the culture medium; in particular these cells grew well in medium with reduced bovine pituitary extract. Infected cells also had increased resistance to stimuli inducing terminal differentiation. Normal keratinocytes lost their ability to proliferate after being suspended in methylcellulose for 24 hours; but, cells infected with E6*/E7, E6/E7, or E7 could be recultured after suspension in methylcellulose for 48 hours. However, these same genes did not alter expression of involucrin or keratin 1 RNAs involved in normal squamous differentiation. Furthermore, when cells expressing E7 or E6/E7 were transplanted beneath a skin muscle flap in nude mice, they formed well-differentiated stratified squamous epithelia. Thus, direct expression of HPV-18 E6*, E6 and E7 alone or in combination does not stimulate dysplastic squamous differentiation in vivo.

Different cell lines derived by transfection and immortalization of normal cervical or foreskin cells with HPV16 DNA varied significantly in their response to TGF β 1. Some cell lines were more resistant than normal cells. Cell lines of late passage (>250 population doublings) often exhibited a further increase in resistance. Two immortalized cell lines that were

malignantly transformed after transfection with the v-Ha-ras oncogene or the herpes simplex virus type 2 BqIII-N fragment were also examined. Both cell lines formed colonies in TGF β 1-containing medium with a frequency greater than the parental lines. Two cervical carcinoma-derived tumor lines (QGU and SiHa) grew clonally in medium containing TGF β 1, although growth of a third line (QGH) was reduced significantly with respect to untreated cultures.

Treatment with TGF β 1 for 24 hours markedly decreased levels of HPV-16 E6 and E7 protein expression. When cultures were maintained in the presence of TGF β 1, E7 expression remained low to undetectable for 48 hours, but it was partially restored when cultures were switched to fresh medium. In contrast, E6 expression was not restored when TGF β 1 was removed from the medium. Exposure to TGF β 1 resulted in time-dependent reductions in both cell proliferation and steady-state levels of E6 and E7 RNAs. TGF β 1 also induced a time-dependent increase in TGF β 1 RNA that was maximal (sevenfold) after 24 hours. TGF β 1 and -2 were equally effective in reducing HPV-16 E6/E7 RNA levels after a 24 hour treatment. Both TGF β types inhibited virus RNA expression in a dose-dependent manner, and as little as 0.3 ng/ml was effective. Nuclear runoff transcription experiments indicated that reductions in steady-state levels of HPV-16 RNAs by TGF β 1 and -2 were due to inhibition of transcription. Transcription of HPV-16 RNA was reduced 90 to 80% in cells treated with either TGF β 1 or -2, respectively. This inhibition was selective because expression of other genes such as beta-actin or c-myc was not affected. To determine whether inhibition of virus gene expression by TGF β 1 might be further regulated by post transcriptional mechanisms, the stability of HPV-16 early-gene transcripts was measured in the presence or absence of TGF β 1. No changes in stability were detected; thus, TGF β regulation of HPV gene expression occurs predominantly at the level of transcription.

The biological significance of the ability of TGF β 1 to down-regulate papilloma-virus early gene expression was examined with a series of cell lines derived from human genital epithelium. Treatment with TGF β 1 for 24 hours dramatically reduced steady-state levels of HPV-16 RNA in early-passage cultures of four immortal lines. When the same cell lines were examined after extended maintenance in culture, down-regulation of HPV gene expression by TGF β 1 was often less pronounced but still detectable. HPV-16 E6/E7 RNA was decreased only minimally (56 to 76% of controls) by TGF β 1 in the 2 cervical carcinoma cell lines QGU and SiHa; however, virus gene expression was down-regulated significantly (80%) in another tumor line, QGH. Furthermore, virus RNA expression decreased only slightly after TGF β 1 treatment of immortal cell lines that had been malignantly transformed with the herpes simplex virus type two BqIII-N fragment or the v-Ha-ras oncogene. A direct correlation was observed between the basal level of involucrin expression in a particular cell line and its sensitivity to TGF β . Cell lines expressing higher levels of involucrin were more sensitive. Involucrin is a marker of squamous differentiation in normal cervix; thus, the susceptibility of immortal cells to TGF β is related to their level of differentiation. TGF β 1 also induced its own expression in most of the cell lines examined. Furthermore, some cell lines were susceptible to autoinduction of TGF β 1 RNA but were refractory to the effects of TGF β 1 on growth and HPV16 gene expression. Therefore, the resistance of these lines to TGF β 1 was not due solely to an absence of TGF β 1 receptors at the cell surface.

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- DiPaolo JA, Bowden PE, Popescu NC, Woodworth CD. Transformation of human epithelial cells by recombinant human papillomavirus DNA associated with cervical cancer. In: Columbano A, Feo F, and Pani P, eds. *Chemical carcinogenesis: modulating factors in multistage chemical carcinogenesis*. New York: Plenum Press (In Press).
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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, 1990 through September 30, 1991

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: Elevation of the medium [Ca^{2+}] of cultured keratinocytes induces terminal differentiation. The expression of differentiation-specific proteins is preceded by increases in intracellular free Ca^{2+} , inositol phosphate turnover, as well as increases in levels of phospholipase C and diacylglycerol. Protein kinase C is necessary for the induction and maintenance of Ca^{2+} -induced terminal differentiation. However, protein kinase C isozyme mRNA levels were not affected by an increase in the

concentration of extracellular Ca^{2+} . Neoplastic keratinocytes are defective in their terminal differentiation response to Ca^{2+} and to phorbol ester tumor promoters. The kinase inhibitor staurosporine induces terminal differentiation in neoplastic, as well as normal, keratinocytes. In grafts of papilloma cell lines to the backs of nude mice, treatment with staurosporine inhibits papilloma development. A cell culture model of initiated epidermis in which the growth of initiated keratinocytes is inhibited by coculture with normal keratinocytes was used to assay promoters and antipromoters. Several promoters for which protein kinase C is the receptor, as well as several non-phorbol ester-type promoters, allow growth of initiated foci in cocultures. Papillomas induced by introduction of an activated ras^{Ha} oncogene into dermal hair follicle cells could not be distinguished from papillomas induced by ras^{Ha} in cells prepared from epidermis. A procedure was developed for preparing hair follicle buds, which require a contribution from the dermis for hair development. The activated oncogenes ras^{Ha} and fos can cooperate to produce carcinomas in grafted keratinocytes. In the ras/fos carcinomas, levels of transcripts for the proteases transin and urokinase were increased compared to those seen in ras^{Ha} -induced papillomas. Inbred SENCAR mice, compared to outbred SENCARS, are more sensitive to promotion by 12-O-tetradecanoylphorbol-13-acetate, and to initiation by 7,12-dimethylbenz[a]anthracene and urethane, but not by N-methyl-N'-nitro-N-nitrosoguanidine.

Molecular Regulation of Epidermal-Specific Differentiation Products: To study epidermal differentiation and carcinogenesis, cDNA and genomic clones corresponding to the major proteins expressed in mouse epidermis have been isolated and characterized. These include the basal layer keratins K5 and K14, the suprabasal layer keratins K1 and K10, the hyperproliferative keratins K6 and K16, and K13, expressed in malignant tumors but not in benign tumors or normal epidermis. In addition, cDNA and genomic clones for filaggrin, the protein which organizes keratin filaments into larger bundles, and loricrin, the major precursor protein of the cornified envelope, have been isolated. Monospecific antibodies to the C terminal amino acid sequences, as well as nucleic acid probes to unique regions of the cDNAs, allow the study of the expression of these markers in normal epidermis, benign and malignant tumors, and cells in culture. Recombinant constructs of K1 and K10 coding sequences with heterologous promoters were transfected and expressed in mesenchymal cells and neoplastic keratinocytes. K1 and K10 filaments did not form in fibroblasts unless cytokeratins K5 and K14 were also expressed. K1 and K10 formed filaments in malignant keratinocytes which express K5 and K14 constitutively. Cells expressing K1 protein, but not K10 protein, continued to proliferate. An epidermal-specific regulatory region in a 12 kb fragment of the human K1 gene was localized in recombinant constructs with a reporter gene. A calcium-sensitive positive regulatory region was located 3' to the coding sequence. Deletion analysis identified several shorter elements in the terminal-1700 nucleotides of the 12 kb genomic clone. This regulatory region did not contain sequences responsive to retinoids. Samples of human skin from patients treated with retinoids showed marked changes in the expression of differentiation-related proteins within four days of exposure. Therefore, retinoid treatment of human skin is likely to be associated with functional changes in the epidermis.

Determinants for Susceptibility to Carcinogenesis: Cell lines made from papillomas resulting from treatment of SENCAR mice with

12-0-tetradecanoylphorbol-13-acetate (TPA) alone, without exogenous chemical initiation were found to express high levels of message for keratin 13, a keratin not normally expressed in the epidermis. The original tumors and cell lines were previously found not to have the additional XbaI restriction site in the *ras*^{H4} gene diagnostic for an A to T point mutation in the second base of codon 61, which leads to activation of the oncogene. We have now shown by direct sequencing of polymerase chain reaction products from DNA of the cell lines that there is an activating A to G mutation at the same position. Studies with the cell line SCR722, a presumed initiated cell line derived from adult SENCAR epidermal cells initiated in culture with N-methyl-N'-nitro-N-nitrosoguanidine, show that its parent produces carcinomas in skin grafts, while SCR722 produces normal skin. This suggests that SCR722 may be a model both for oncogene activation and tumor suppression. Staurosporine, widely used as an inhibitor of protein kinase C, at nmole levels appears to activate protein kinase C and induce terminal differentiation in SENCAR papilloma cell line SP-1. *In vivo*, treatment of grafted SP-1 cells with a single low dose of staurosporine can strongly or completely inhibit tumor formation.

Immunological Techniques to Study the Interaction of Carcinogens and Chemotherapeutic Agents with DNA: Antibodies specific for carcinogen-DNA adducts have been used to quantify DNA modification in biological samples substituted with polycyclic aromatic hydrocarbons (PAH), aromatic amines and cisplatin by quantitative immunoassays, immunohistochemistry, atomic absorbance spectrometry (AAS) and ³²P-postlabeling. Studies are being conducted to measure PAH-DNA adducts in blood cell DNA of coke oven workers, aluminum plant workers and subjects ingesting charcoal-broiled beef using a benzo[a]pyrene-DNA enzyme-linked immunosorbent assay (ELISA). The sensitivity of this assay has recently been improved 4- to 5-fold through the use of the DELFIA system with a Europium (fluorescent) end-point. Processing of aromatic amine-DNA adducts during chronic administration of either 2-acetylaminofluorene (AAF) or 4-aminobiphenyl (4-ABP) has been shown to be compound-, tissue- and sex-specific, and to have variable relationships with tumorigenesis in the same organs. A 370 base pair fragment is being used to study the effects of nucleotide composition on the localization of AAF-DNA adduct formation in livers of rats chronically-fed AAF. DNA adducts of ABP are being purified from human DNA by immunoaffinity chromatography and quantified by ³²P-postlabeling. The extent of cisplatin-DNA adduct formation in nucleated blood DNA of cancer patients (measured by cisplatin-DNA ELISA) has been positively correlated with disease response in breast, colon and ovarian cancer patients receiving platinum drug-based chemotherapy. The association with disease response did not hold for platinum-DNA adducts measured by AAS and was not statistically-significant for protocols (colon and breast) which showed poor overall response rates. Platinum-DNA adducts have been measured in tissues of a woman treated with platinum drugs for ovarian cancer during pregnancy. An antiserum specific for the anti-AIDS drug, 3'-azido-2',3'-dideoxythymidine (AZT) has been studied for cytotoxicity and DNA incorporation in human HL 60, hamster CHO and mouse NIH 3T3 cell lines. Incorporation of AZT into DNA has been demonstrated by radiolabeling and immunohistochemistry.

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.

The following major findings have been obtained in the past year:

1. Retinoids are necessary for skin tumor formation in female SENCAR mice by the two-stage and the complete tumorigenesis schedules. We have previously reported that vitamin A deficiency greatly inhibits mouse skin tumorigenesis by the two-stage schedule using 7, 12-dimethylbenz[a]anthracene (DMBA) as the initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as the tumor promoter. Retinoids at physiological concentration in the diet permitted tumor formation. We have obtained similar results in mice in which the tumorigenic response was induced by the complete tumorigenesis schedule using repeated topical applications of DMBA to the dorsal skin. In the two-stage system, excess retinoic acid enhanced carcinoma formation by over twofold. These data suggest caution in the use of retinoids as chemopreventive agents in humans.
2. Retinoids in cell growth, adhesion and tissue transglutaminase activity. We report that all-trans and 13-cis-retinoic acid (13-cis-RA) as well as the synthetic compound CH-55 enhance tissue transglutaminase activity as they increase NIH-3T3 cell adhesiveness. 4-Hydroxyphenylretinamide, with low activity in inducing attachment, lectin binding and growth inhibition, also fails to induce transglutaminase. Thyroxine, a compound with a response element common to RA, is inactive. The tumor promoter TPA, which increases adhesiveness with different kinetics than RA, failed to enhance transglutaminase. We conclude that retinoids with biological activity in inducing adhesion, inhibition of growth and increase of lectin binding, are also active in inducing transglutaminase activity.
3. The involvement of the extracellular matrix in differentiation of epithelial tissues. Mouse epidermal keratinocytes secrete an extracellular matrix composed of various attachment proteins, among which fibronectin, collagen and laminin are most prominent. We found that differentiation to mature keratinocytes greatly reduces cell adhesiveness to all substrates, including plastic, collagen, fibronectin and laminin. Attachment to laminin increased immediately after the differentiation stimulus (high calcium) was applied and then decreased sharply at the same time as keratin 1 expression became evident. Immunohistochemical monitoring of the laminin receptors, both of the integrin ($\alpha_3\beta_1$) and non-integrin type (LBP-37), indicated a loose cytoplasmic distribution in undifferentiated cells and an association with the plasma membranes in differentiated suprabasal cells. These data suggest that differentiation stimuli may well act through the extracellular matrix components and their cell surface receptors to permit a reorganization of the cytoskeleton and possibly provide the stimulus for the synthesis of terminal differentiation proteins (e.g., keratins).
4. Retinoids in tracheal epithelial cell differentiation. Using the condition of retinoid depletion, we have studied the histogenesis of squamous metaplasia in hamster trachea. Specific immunohistochemical

staining of basal cells was obtained in normal hamster trachea with antibody to keratin 5 (K5) in mild deficiency. These cells formed a continuous layer occupying the entirety of the basement membrane, thus marking the transition from normally pseudostratified to a stratified epithelium. Squamous metaplasia was found in severely vitamin A-deficient hamsters. All cell layers in the squamous lesion were positive for K5, but only the suprabasal layers showed positivity for K6 and K13. These findings are consistent with the basal cell being the main stem cell for the formation of squamous metaplasia. However, a contribution by mucous cells, which change the expression of their phenotype to squamous keratinizing, may also be a contributing factor.

Two biologically active retinoids, 13-cis RA and CH-55, were also active in stimulating cell adhesion and lectin binding to gp130. Structures which lacked activity in the adhesion response assay also failed to show an increase in lectin binding to gp 130. Although thyroid hormones are neither biosynthetically nor structurally related to RA, they have been suggested to activate gene expression through a common response element, so RA and the thyroid hormone may control overlapping biological events. Thyroxine had no effect on NIH-3T3 cell morphology or on the glycosylation of gp 130. This is in agreement with recent work that shows that thyroxine is unable to activate the retinoic acid responsive elements located in the 5'-noncoding region of the mouse laminin B1 gene. Therefore, we have demonstrated that biologically active retinoids alter growth characteristics, cell attachment properties and specific cell surface carbohydrate of specific glycoproteins.

MOLECULAR MECHANISMS OF TUMOR PROMOTION SECTION: The overall direction of the Molecular Mechanisms of Tumor Promotion Section is to understand the mechanisms underlying the initial events in tumor promotion. Particular emphasis is being directed to the factors responsible for heterogeneity in the patterns of response. Protein kinase C is the receptor for the phorbol esters, the best studied class of tumor promoters in the mouse skin model. The mechanisms of protein kinase C activators which fail to induce phorbol ester-like responses are being characterized at the cellular and whole animal levels. The patterns of response are being correlated with the selectivity of the agents for cloned protein kinase C isozymes. One class of compounds which is receiving particular attention is that of the short chain monoesters of 12-deoxyphorbol, exemplified by prostratin. Although prostratin is a weak protein kinase C activator, chronic treatment of mouse skin with prostratin blocks the inflammatory and hyperplastic response of skin to subsequent treatment with the strong tumor promoter phorbol 12-myristate 13-acetate. These properties predict that prostratin will function as an anti-promoter. Ingenol is being examined because it possesses all elements of the phorbol ester pharmacophore except for the presence of a hydrophobic domain. We find that ingenol still retains weak activity as a protein kinase C agonist. Although all tumor promoting phorbol esters are inflammatory, the converse is not true. We have found that resiniferatoxin, an ultra-inflammatory phorbol ester, acts through a distinct mechanism, stimulating unique receptors on sensory neurons involved in pain and neurogenic inflammation. We are continuing to characterize these receptors, developing strategies to clone them and screening for the existence of endogenous analogs which may act as the normal physiological mediators of this pathway.

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

PROJECT NUMBER

Z01CP04504-19 CCTP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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)

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SECTION

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INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

9.0

PROFESSIONAL:

6.0

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.)

Elevation of the medium $[Ca^{2+}]$ of cultured keratinocytes induces terminal differentiation. The expression of differentiation-specific proteins is preceded by increases in intracellular free Ca^{2+} , inositol phosphate turnover, as well as increases in levels of phospholipase C and diacylglycerol. Protein kinase C is necessary for the induction and maintenance of Ca^{2+} -induced terminal differentiation. However, protein kinase C isozyme mRNA levels were not affected by an increase in the concentration of extracellular Ca^{2+} . Neoplastic keratinocytes are defective in their terminal differentiation response to Ca^{2+} and to phorbol ester tumor promoters. The kinase inhibitor staurosporine induces terminal differentiation in neoplastic, as well as normal, keratinocytes. In grafts of papilloma cell lines to the backs of nude mice, treatment with staurosporine inhibits papilloma development. A cell culture model of initiated epidermis in which the growth of initiated keratinocytes is inhibited by coculture with normal keratinocytes was used to assay promoters and antipromoters. Several promoters for which protein kinase C is the receptor, as well as several non-phorbol ester-type promoters, allow growth of initiated foci in cocultures. Papillomas induced by introduction of an activated ras^{Ha} oncogene into dermal hair follicle cells could not be distinguished from papillomas induced by ras^{Ha} in cells prepared from epidermis. A procedure was developed for preparing hair follicle buds, which require a contribution from the dermis for hair development. The activated oncogenes ras^{Ha} and fos can cooperate to produce carcinomas in grafted keratinocytes. In the ras/fos carcinomas, levels of transcripts for the proteases transin and urokinase were increased compared to those seen in ras^{Ha} -induced papillomas. Inbred SENCAR mice, compared to outbred SENCARs, are more sensitive to promotion by 12-O-tetradecanoylphorbol-13-acetate, and to initiation by 7,12-dimethylbenz[a]anthracene and urethane, but not by N-methyl-N'-nitro-N-nitrosoguanidine.

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

S. Yuspa	Chief	LCCTP NCI
H. Hennings	Research Chemist	LCCTP NCI
J. Strickland	Research Chemist	LCCTP NCI
U. Lichti	Research Chemist	LCCTP NCI
A. Dlugosz	Biotech Fellow	LCCTP NCI
L. Li	Special Volunteer	LCCTP NCI
E. Lee	Guest Researcher	LCCTP NCI
W. Weinberg	IRTA Fellow	LCCTP NCI
A. Glick	Biotech Fellow	LCCTP NCI
M. Potter	Chief	LG 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:

Changes in levels of intracellular free calcium and inositol phosphate metabolism related to epidermal differentiation and neoplasia. Keratinocytes cultured from mouse epidermis respond to the level of Ca^{2+} in the medium by expressing markers of proliferation or terminal differentiation. A proliferating basal cell phenotype is selected with a medium [Ca^{2+}] of 0.05 mM, a spinous cell phenotype is seen with 0.12 mM Ca^{2+} , and cornification and development of squames occurs in 1.4 mM Ca^{2+} . Structural markers of keratinocyte differentiation, including keratins 1 and 10 (K1 and K10), loricrin and filaggrin, are induced by an increase in extracellular Ca^{2+} from 0.05 mM to >0.1 mM. A sustained, Ca^{2+} -dependent increase in intracellular free Ca^{2+} (Ca_i) is dependent on the [Ca^{2+}] in the medium and is associated with the induction of terminal differentiation and cornification in normal keratinocytes. In contrast, neoplastic keratinocytes do not terminally differentiate in response to elevated extracellular Ca^{2+} and experience only a transient increase in Ca_i .

The expression of differentiation-specific proteins is preceded by increases in inositol phosphate metabolism, as well as increases in levels of phospholipase C and diacylglycerol. The magnitude of Ca^{2+} -stimulated inositol phosphate turnover was dependent on the level of Ca^{2+} in the medium; increases in external [Ca^{2+}] from 0.05 mM to 0.12 mM or 1.4 mM result in a graded, sustained increase in inositol phosphates. Inositol-1,4,5-trisphosphate increased transiently while inositol-1,3,4-trisphosphate accumulated. In medium with 0.05 mM Ca^{2+} , the levels of Ca_i , inositol phosphates, and diacylglycerol were all 2- to 5-fold higher in neoplastic cells than in normal keratinocytes. Inositol phosphate metabolism was stimulated even further in neoplastic keratinocytes following an increase in extracellular [Ca^{2+}]. Thus, the turnover of inositol phosphates as well as a sustained increase in Ca_i contribute to the signals controlling expression of markers of differentiation. The very high inositol phosphate turnover and high diacylglycerol levels seen in neoplastic cells may inhibit the expression of differentiation markers.

Involvement of protein kinase C in keratinocyte differentiation and transformation. In cultured epidermal keratinocytes, protein kinase C (PKC) is necessary for both induction and maintenance of Ca^{2+} -mediated terminal differentiation. Keratinocytes pretreated with bryostatin to down-regulate PKC fail to respond to an increase of extracellular Ca^{2+} by expression of K1, K10, loricrin or filaggrin.

Since individual PKC isozymes appear to have unique functions, the isozyme pattern in a particular cell may determine the biological responsiveness to modulators of PKC activity. We have characterized the PKC isozyme mRNA profile by Northern blot analysis in normal and neoplastic keratinocytes. Both types of cells express mRNA encoding PKC alpha, delta, epsilon, zeta, and beta, but not PKC beta or gamma. In primary keratinocytes induced to differentiate by a 24-hour exposure to elevated extracellular Ca^{2+} , the PKC mRNA levels change very little. The PKC isozyme mRNA profile is similar in normal and neoplastic cells with the exception of PKC zeta, which is less

abundant in neoplastic cells. The PKC zeta transcripts in keratinocytes are about 200 nucleotides longer than in mouse brain, suggesting tissue-specific processing.

The tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA) selectively induces the terminal differentiation of normal keratinocytes without inducing differentiation in neoplastic keratinocytes, a property that may be critical to its action as a promoter. The kinase inhibitor staurosporine induces cell cornification and maturation in neoplastic as well as normal keratinocytes. Thus, staurosporine could be an anticarcinogen, and has been shown to inhibit development of papillomas when a papilloma cell line was grafted to the backs of nude mice in a reconstituted epidermis (see Project #Z01CP05178-09 CCTP). These activities of staurosporine, as well as its reported activity as a promoter and an inhibitor of TPA-induced promotion, have led us to study its mechanism of action. Staurosporine unexpectedly induces several responses typical of PKC activators, including cornification, induction of ornithine decarboxylase activity, inhibition of ^{125}I -EGF binding, and accumulation of *c-fos* mRNA. Inhibition of ^{125}I -EGF binding and cornification induced by either TPA or staurosporine are blocked by bryostatin pretreatment, implicating PKC in these responses. However, staurosporine does not induce phosphorylation of a 40kD putative PKC substrate and blocks its phosphorylation by TPA. Thus, the mechanism of action of staurosporine is complex, and may involve activation of certain PKC isozymes and inhibition of others.

Cell culture model for initiated epidermis to study tumor promotion and antipromotion. The focal growth of initiated mouse keratinocytes (line 308) is suppressed by coculture with normal keratinocytes. The inhibition is Ca^{2+} -dependent, epidermal cell-specific, and requires cell contact between normal and initiated cells. Treatment of cocultures with TPA allows growth of initiated cell colonies, an effect inhibited by the antipromoters retinoic acid, fluocinolone acetonide and bryostatin. We validated this keratinocyte coculture model by testing a number of promoters or potential promoters of TPA-type (those with PKC as receptor) or non-TPA-type, and, in mechanistic studies, compared our results with those reported in fibroblast cocultures. Only TPA-type promoters have been reported to be active in fibroblast cocultures. Teleocidin, 12-0-retinoylphorbol-13-acetate, mezerein and aplysiatoxin, TPA-type promoters, are as active as TPA in allowing focal growth of 308 cells. The endogenous activator of PKC, diacylglycerol, was also active, indicating that PKC is likely to be involved in the mechanism of action of these compounds. The kinase inhibitor staurosporine inhibited TPA-induced focus formation when given along with TPA. However, other mechanisms not involving PKC are likely since the non-TPA-type promoters okadaic acid, benzoyl peroxide, staurosporine and thapsigargin are also active in the assay. Okadaic acid affects phosphorylation by inhibiting protein phosphatases and thapsigargin elevates intracellular Ca^{2+} by inhibiting the endoplasmic reticulum Ca^{2+} pump. Thapsigargin and TPA exhibited a strong synergistic effect when given together. Synergism between these two agents as promoters is now being tested in an initiation-promotion experiment in mice.

Hair follicle cells as potential target cells in skin carcinogenesis.
Introduction of an activated *ras*^{H₉₉} oncogene into primary cultures of mouse

epidermal keratinocytes results in cells that produce papillomas when grafted to the backs of nude mice (see Project #Z01CP05178-09 CCTP). When cells prepared from dermal hair follicles were used as recipients, the papillomas that developed could not be distinguished from those using ras^{Ha}-transfected epidermal cells with regard to antibody staining for keratins 1, 6, 10, 13 and 14, filaggrin, and gamma-glutamyltranspeptidase histochemistry. No difference was observed in the frequency of malignant conversion, suggesting that the cell of origin alone is not responsible for the frequency of conversion of papillomas to carcinomas.

Primary mouse keratinocyte preparations from newborn mice, when grafted to nude mice, produce haired grafts, indicating the presence of all cell types required for hair follicle formation in vivo. Histological examination of trypsin-split skin from newborn mice shows that immature hair follicle buds remain associated with the epidermis and become part of the keratinocyte preparation. By centrifugations through Ficoll, hair follicle buds were prepared devoid of single cells, but containing some clumps of granular cells. Purified buds did not give rise to haired grafts unless grafted together with a dermal cell preparation, presumably containing the dermal papilla cells, an integral component of the mature hair follicle. These hair follicle buds, containing epithelial stem cells, provide a relatively pure epithelial component for use in studies of hair follicle development and the role of hair follicle cells in epidermal carcinogenesis. In monolayer culture, the buds plate at a higher efficiency than single keratinocytes (60% versus 25%) and continue proliferation for a longer time period. In collagen matrix culture, hair follicle buds undergo squamous differentiation within 2-4 days, a process that can be delayed by coculture with dermis-derived cells.

Role of specific oncogenes and proto-oncogenes in malignant conversion. Co-infection of primary mouse keratinocytes with defective retroviruses containing the oncogenes v-fos and v-ras^{Ha} results in cells with a malignant phenotype, which produce carcinomas when grafted to nude mice. The introduction of v-fos alone produces normal-appearing skin when grafted, while v-ras^{Ha}-containing keratinocytes produce papillomas in grafts. We are examining the basis for this cooperation between fos and ras^{Ha} by examining protease gene expression in carcinomas containing v-fos and v-ras^{Ha}. In the 6 fos/ras carcinomas examined, the levels of transin and urokinase mRNAs were both elevated compared to the levels in 4 ras^{Ha}-induced papillomas.

Expression of both fos and ras has been shown to be regulated by the transcription factor AP-1, the product of c-jun. In order to study the role of AP-1 in multistage carcinogenesis, we have transfected the v-jun oncogene into the papilloma cell lines 308 and SP-1 and isolated clones that express the gene. These lines are currently being tested in skin grafts to see whether jun can cooperate with ras^{Ha} to produce the malignant phenotype.

Studies of mechanisms of initiation, promotion and antipromotion in mouse skin. Treatment of cultures of normal or initiated keratinocytes with the Ca^{2+} ionophore ionomycin induces a prolonged elevation of intracellular Ca^{2+} followed by the induction of markers of terminal differentiation in both cell types. The kinase inhibitor staurosporine induced terminal differentiation in

initiated keratinocyte cell lines, and inhibited papilloma development when neoplastic keratinocytes were grafted to nude mice (See Project #Z01CP05178-09 CCTP). Extrapolation of these results to the mouse skin carcinogenesis model suggested that treatment with ionomycin or staurosporine might induce terminal differentiation of initiated cells in initiation-promotion experiments. Initiated mice were treated topically with either ionomycin or staurosporine before promotion was begun to see whether fewer tumors developed in the mice treated with the potential inhibitors. Although papilloma development was delayed for 3-4 weeks by ionomycin, the final incidence of papillomas and carcinomas was not changed. With topical staurosporine, neither the latent period nor the tumor incidence was affected. Based on the promising cell culture studies, we are continuing to pursue the use of ionomycin and staurosporine as anticarcinogenic agents and will be testing additional doses, treatment schedules, and methods of delivery.

Dr. Michael Potter (NCI, Division of Cancer Biology, Diagnosis, and Centers, Laboratory of Genetics) has developed 3 inbred lines of SENCAR mice for studies of the genetic basis for susceptibility to initiation and promotion. In a preliminary experiment, we have determined the sensitivity to skin carcinogenesis of one of these lines 3-4 generations prior to its becoming inbred. The results are compared to those in outbred SENCAR mice. With doses of the initiator 7,12-dimethylbenz[a]anthracene (DMBA) between 1 and 5 µg, more than 5 times as many papillomas developed in the inbred mice. A dose of 0.1 µg of DMBA was non-initiating in outbred SENCARs, but induced more than 2 papillomas per mouse after TPA promotion of the inbred mice. The inbred mice are about 5-fold more sensitive to urethane as initiator, but inbreds and outbreds were equally sensitive to initiation by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). When the dose of the promoter TPA was varied between 0.5 and 2 µg per weekly application, the inbreds developed 3-5 times more papillomas than the outbreds. In this ongoing experiment, more carcinomas are developing in the groups with a higher incidence of papillomas. The inbred SENCAR mice, with high sensitivity to initiation by some, but not all carcinogens, and increased sensitivity to promotion by TPA, promise to be useful in future studies of the mechanisms of susceptibility to tumor development.

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Patents:

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

PROJECT NUMBER

Z01CP04798-21 CCTP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Retinoids in Differentiation and Neoplasia

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

PI:	L. M. De Luca	Research Chemist	LCCTP NCI
OTHERS:	F. Lancillotti	Guest Researcher	LCCTP NCI
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	Li-Chuan Chen	IRTA	LCCTP NCI
	K. Kosa	Visiting Fellow	LCCTP NCI
	S. Ross	Biologist	LCCTP NCI
	N. Darwiche	Biologist	LCCTP NCI

COOPERATING UNITS (if any)

NIDR, NIH (Y. Yamada, H. Kleinman); NCI, NIH (M. Sobel); Biocon, Rockville, MD (Linda Sly)

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

9

PROFESSIONAL:

6

OTHER:

3

CHECK APPROPRIATE BOX(ES)

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

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

(1) Our work has shown that retinoid depletion inhibits skin tumorigenesis in female SENCAR mice in the two-stage model, using 7,12-dimethylbenz[a]anthracene (DMBA) as the initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as the tumor promoter. We asked whether tumor formation by the complete tumorigenesis schedule also requires retinoic acid (RA). At week 9, 15% of mice fed the RA-containing diet had developed skin tumors compared to 0% of mice fed the vitamin A-deficient diet. A tumorigenic response was observed upon switching the diet to the RA-containing one. We conclude that dietary retinoids are necessary for skin tumor formation in female SENCAR mice both by the two-stage and by the complete tumorigenesis schedules. (2) We have studied the distribution of a laminin binding protein of 37 kd (LBP) in normal mouse skin and in skin papillomas and carcinomas. In cultured keratinocytes, LBP is mainly cytoplasmic in basal cells and is found in the particulate fraction in differentiating cells. In vivo, LBP was found localized to the plasma membrane of differentiating epidermal cells mainly in the spinous and granular layers. A similar pattern was found in hyperplastic skin and in benign tumors either induced chemically or formed by grafting keratinocytes transfected with the v-ras^{Ha} oncogene. In carcinomas LBP immunostaining was generally reduced and predominantly cytoplasmic. The data suggest a specific function for cell surface LBP in normal skin differentiation and an altered function in tumors. (3) RA induces differentiation of embryonal carcinoma cells (F9) in an irreversible manner. It also increases the production of extracellular matrix. Therefore, we investigated the effects of RA on F9 cell attachment to plastic, collagen and laminin. RA treatment enhanced F9 cell attachment to all three substrates. Moreover, laminin biosynthesis was markedly increased by RA, consistent with the finding by others that an RA-response element is present in the promoter sequence of the laminin gene. (4) We have characterized keratin gene expression under conditions of vitamin A-deficiency in hamster tracheal epithelium.

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

L. M. De Luca	Research Chemist	LCCTP NCI
F. Lancillotti	Guest Researcher	LCCTP NCI
T. Tennenbaum	Guest Researcher	LCCTP NCI
Li-Chuan Chen	IRTA Fellow	LCCTP NCI
K. Kosa	Visiting Fellow	LCCTP NCI
S. Ross	Biologist	LCCTP NCI
N. Darwiche	Biologist	LCCTP NCI

Objectives:

1. To study the effect of dietary retinoid status on mouse skin tumorigenesis.
2. To study the effect of retinoids on the cell surface.
3. To test the hypothesis that the interaction of epithelial cells with the extracellular matrix is an important modulator of differentiation.
4. To study modulation of keratin gene expression by retinoid status in hamster tracheal epithelium.

Methods Employed:

1. To study the effect of dietary retinoids status on mouse skin tumorigenesis.

For the two-stage tumorigenesis schedules each mouse received a single topical application of 20 µg of DMBA at 3 weeks of age followed by 20 topical applications of TPA, (2.0 µg per dose). For the complete tumorigenesis schedule mice received 20 topical applications of DMBA at 51 µg/wk/mouse from week 3 through week 22.

Mice were fed a vitamin A-deficient diet. Some of these mice were switched to an RA-containing (3 µg/g diet) diet and a control group was maintained on the RA-diet for life. In addition SENCAR mice were also maintained on purina blocks to compare tumor incidence (independent of retinoid status) between the purified and the purina diets.

2. To study the effect of retinoids on the cell surface.

Attachment assay: cells (1×10^4) were plated in 96 well tissue culture clusters (Costar) at day one; culture media were changed to those containing solvent control (usually 0.1% DMSO) and/or various retinoids at 10^{-7} M. At day four, plates were washed with Ca^{++} and Mg^{++} -free PBS, 50 µl 0.025% trypsin and 0.01% EDTA was added in each well for 5 minutes; plates

were tapped until most of the cells in the control group were detached. The cells remaining attached were rinsed twice with PBS. Staining and absorbance readings were performed to assess cell numbers.

Tissue transglutaminase assay: tissue transglutaminase activity was measured according to the methods described with minor modifications.

3. To test the hypothesis that the interaction of epithelial cells with the extracellular matrix is an important modulation of differentiation. Expression of extracellular matrix components (laminin, fibronectin and collagen) and their receptors (integrin and non-integrin types) is monitored by biochemical (western blot analysis) and immunohistochemical methods utilizing specific antibodies. Squamous epidermoid differentiation is monitored by antibodies developed against specific keratins, in particular keratin 1 (K1), keratin 5 (K5) and keratin 6 (K6).

Major Findings:

1. Effect of retinoid status on mouse skin tumorigenesis by the two stage and complete tumorigenesis schedules. In two experiments we found that retinoic acid is required for papilloma formation in the skin of female SENCAR mice. In the absence of dietary retinoids skin tumors failed to form by either the two-stage or the complete schedule and upon administration of physiological amounts of retinoic acid in the diet, skin tumors became visible within a week. Retinyl-palmitate and β -carotene were also effective in permitting tumor formation, albeit to a lesser extent than retinoic acid.

Excessive doses of retinoic acid (30 μ g per gram of diet) did not have any significant effect on tumor formation compared to physiological doses (3 μ g per gram of diet) or the normal laboratory chow diet. Carcinoma formation, on the other hand, increased by two- to threefold in the excess RA group. These findings warrant caution in the use of excess RA as chemopreventive agents.

2. Effects of retinoic acid on the cell surface and transglutaminase activity. Retinoic acid induced tissue transglutaminase activity in a dose dependent manner in the range from 1 nM to 10 μ M. Treatment of NIH-3T3 cells with as low as 1 nM RA for 48 hours also caused an increase in transglutaminase activity compared with DMSO control. 10 μ M RA induced transglutaminase activity about 4 times above control levels. 1 μ M RA caused a detectable increase in transglutaminase activity by 4 hours and a 48 hour treatment increased transglutaminase activity about 3 times above control levels.

We examined the effect of the following compounds on tissue transglutaminase activity: RA, 13-cis-RA, CH-55, 4-hydroxyphenylretinamide, thyroxine and TPA. Of these, 13-cis-RA, an isomer of RA and CH-55, a synthetic compound, showed the same effect as RA on NIH-3T3 cell adhesion. On the other hand, 4-HPR, a derivative of RA, and thyroxine which shares a common response element with RA, showed less

effect on NIH-3T3 cell adhesion. TPA also induced NIH-3T3 cell adhesion; however, the effect is much faster than RA.

Our data also show that, of the six compounds tested, 1 μM 13-cis-RA and 1 μM CH-55 have a similar effect to RA on inducing transglutaminase activity. However, 1 μM 4-HPR, 1 μM Thy and 0.1 μM TPA failed to induce transglutaminase activity.

These results parallel the data of the retinoid effect on adhesion reported in our previous work. RA, 13-cis-RA and CH-55 increased NIH-3T3 cell adhesiveness to a similar degree; however, 4-HPR and Thy showed much smaller effects. Although TPA also induced NIH cell adhesiveness, dose dependence and time course studies showed different kinetics from RA, which suggested a different mechanism. The parallel effects of retinoids in inducing NIH-3T3 cell adhesion and transglutaminase activity suggests that transglutaminase may be involved in the process of cell adhesion.

3. To test the hypothesis that the interaction of epithelial cells with the extracellular matrix is an important modulator of differentiation. Mouse epidermal keratinocytes produce an extra-cellular matrix (ECM) when cultured on plastic surfaces. We have found that the differentiation process as monitored by keratin 1 (K1) production is accelerated when the freshly harvested keratinocytes are plated on ECM-coated dishes. In particular, we find that induction of keratinocyte differentiation by Ca^{2+} is followed by a decreased adhesiveness to fibronectin, collagen and laminin. Contact of undifferentiated epidermal keratinocytes with laminin appears necessary for the differentiation response as monitored by K1 production. Laminin and its receptors appear to play a key role in the interaction of undifferentiated keratinocytes with the ECM and in providing the signal for the cells to differentiate.

Laminin receptors of the integrin and non-integrin type appear to have a regulatory function in differentiation and their subcellular distribution is altered during differentiation and tumorigenesis. The laminin binding protein of 37,000 daltons was monitored histochemically and found to be mostly in undifferentiated basal cells. In differentiated cells, on the other hand, LBP 37 becomes associated mainly with the plasma membranes. In papillomas LBP 37 was mostly cytoplasmic in dividing cells and associated with the plasma membrane in differentiated cells. A reduced histochemical staining and a cytoplasmic diffuse localization was observed in carcinomas. Similar results were obtained with the integrin $\alpha_3\beta_1$. The $\alpha_6\beta_4$ was found localized to the basal surface in normal skin and in papillomas. In carcinomas, there was an increase, and more diffuse expression throughout.

4. To study modulation of keratin gene expression by the retinoid status in hamster tracheal epithelium. The tracheal epithelium *in vivo* and in cell culture responds to vitamin A deficiency by loss of the mucociliary phenotype, and formation of a squamoid epithelium. We followed the establishment of the squamous phenotype in cell culture by keratin immunoblot analysis and by histochemical staining using antibodies against

specific mouse keratins. By these procedures keratin 5 (K5) was found exclusively localized within basal cells of either retinoid-sufficient or early depleted hamster tracheas. The first alteration observed during histogenesis of squamous metaplasia was that K5-positive basal cells occupied the entirety of the basal membrane, thus marking the transition from a pseudostratified to a stratified epithelium. No squamoid lesions were observed at this stage of retinoid depletion. At later stages, however, squamous cells and squamous metaplasia formed. These metaplastic lesions contained several cell layers (5-10), all of which expressed K5. Distinctively, K6 was confined to the upper layers. Similar to K6, K13 was also found to be expressed suprabasally and only in squamous tracheas. These data are consistent with the basal cells playing a major role in the formation of the squamous lesions but also suggest the possibility that suprabasal multipotential cells may be directed towards the mucous secretion or keratinizing pathways depending on the availability of dietary retinoids.

Since RA increases fibroblast attachment to laminin, we investigated the response of laminin binding proteins (LBP) to vitamin A deficiency in hamster tracheas. By immunofluorescent staining using an antibody against LBP-37, we found that normal tracheal cells in vivo stain heavily in the apical portion of the epithelium and in the submucosal glands. The squamous metaplastic epithelium of RA-deficient tracheas, however, failed to show any reactivity. Immunoblot analysis of extracts from cultured tracheal epithelial cells showed that vitamin A deficiency causes a major decrease in the expression of the LBP-37 KD starting at day four of culture. These data also suggest that retinoic acid may be necessary for the expression of physiological interactions between the components of the basal membrane and their cell surface receptors.

Publications:

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

PROJECT NUMBER

201CP05177-10 CCTP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Visiting Associate	LCCTP NCI
	B. Schoket	Visiting Scientist	LCCTP NCI
	H. Shamkhani	Biotechnology Fellow	LCCTP NCI
	S. Yuspa	Chief	LCCTP NCI
	E. Reed	Senior Investigator	MB NCI
	A. Weston	Visiting Associate	LHC NCI
	N. Rothman	Staff Fellow	EEB NCI

COOPERATING UNITS (if any) NCTR, Jefferson, AR (F. A. Beland); Johns Hopkins University, Baltimore, MD (P. Strickland); Albert Einstein College of Medicine, New York, NY (C. Runowicz).

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

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In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

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.)

Antibodies specific for carcinogen-DNA adducts have been used to quantify DNA modification in biological samples substituted with polycyclic aromatic hydrocarbons (PAH), aromatic amines and cisplatin by quantitative immunoassays, immunohistochemistry, atomic absorbance spectrometry (AAS) and ³²P-postlabeling. Studies are being conducted to measure PAH-DNA adducts in blood cell DNA of coke oven workers, aluminum plant workers and subjects ingesting charbroiled beef using a benzo[a]pyrene-DNA enzyme-linked immunosorbent assay (ELISA). The sensitivity of this assay has recently been improved 4- to 5-fold through the use of the DELFIA system with a Europium (fluorescent) end-point. Processing of aromatic amine-DNA adducts during chronic administration of either 2-acetylaminofluorene (AAF) or 4-aminobiphenyl (4-ABP) has been shown to be compound-, tissue- and sex-specific, and to have variable relationships with tumorigenesis in the same organs. A 370 base pair fragment is being used to study the effects of nucleotide composition on the localization of AAF-DNA adduct formation in livers of rats chronically-fed AAF. DNA adducts of ABP are being purified from human DNA by immunoaffinity chromatography and quantified by ³²P-postlabeling. The extent of cisplatin-DNA adduct formation in nucleated blood DNA of cancer patients (measured by cisplatin-DNA ELISA) has been positively correlated with disease response in breast, colon and ovarian cancer patients receiving platinum drug-based chemotherapy. The association with disease response did not hold for platinum-DNA adducts measured by AAS and was not statistically-significant for protocols (colon and breast) which showed poor overall response rates. Platinum-DNA adducts have been measured in tissues of a woman treated with platinum drugs for ovarian cancer during pregnancy. An antiserum specific for the anti-AIDS drug, 3'-azido-2',3'-dideoxythymidine (AZT) has been studied for cytotoxicity and DNA incorporation in human HL 60, hamster CHO and mouse NIH 3T3 cell lines. Incorporation of AZT into DNA has been demonstrated by radiolabeling and immunohistochemistry.

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

M. C. Poirier	Research Chemist	LCCTP NCI
O. Olivero	Visiting Associate	LCCTP NCI
B. Schoket	Visiting Scientist	LCCTP NCI
H. Shamkhani	Biotechnology Fellow	LCCTP NCI
S. Yuspa	Chief	LCCTP NCI
E. Reed	Senior Investigator	MB NCI
A. Weston	Visiting Associate	LHC NCI
N. Rothman	Staff Fellow	EEB NCI

Objectives:

To study interactions between DNA and chemicals. This program is focused on the quantitative and qualitative analysis of DNA adduct formation and removal in cultured cells and tissues from xenobiotic-exposed animals and humans. The data are correlated with the biological consequences of drug exposure, including cell transformation, tumorigenesis, clinical response and specific toxicities. The major areas of intensive investigation have focused on polycyclic aromatic hydrocarbons, aromatic amines and platinum-ammine compounds.

Methods Employed:

Chemical synthesis of radiolabeled and unlabeled DNA adducts and modified DNA samples. Purification of adducts by column chromatography, and determination of adduct concentration by UV spectrum or AAS. Eliciting polyclonal antisera using DNA adducts or modified DNA samples covalently or electrostatically coupled to protein carriers. Characterization of polyclonal antisera and assay of biological samples by competitive radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) using colorimetric and fluorescent end points (including Europium). Immunohistochemical localization of DNA adducts in whole tissue, cultured cells, chromosome spreads and DNA using immunofluorescence with or without additional markers, and immunoelectron microscopy with colloidal gold. Exposure of cultured cells, mice and rats to chemical carcinogens and chemotherapeutic agents under chronic or acute conditions of treatment. Preparation of DNA from cultured cells, animal tissues and human tissues by either CsCl₂ buoyant density gradient centrifugation, phenol extraction or proteinase K digestion. Immunoaffinity column preparation (IAC) by coupling of IgG to Sepharose, and utilization of these columns to purify adducts of a particular chemical class from hydrolyzed DNA samples of various origins. Atomic absorbance spectroscopy (AAS) of platinum-containing DNA samples.

Major Findings:

A pilot study, designed by Drs. P. Strickland and N. Rothman (Johns Hopkins University and NCI) showed that blood cell DNA adducts, measurable by the

(BP)-DNA ELISA, increased in 2 of 4 individuals during charbroiled beef ingestion. In order to extend these observations approximately 20 volunteers will have abstained from charbroiled food for 1 month prior to 6 days of eating well-cooked hamburgers and will then abstain again for one month. Blood samples will be obtained before, during and after the start of beef ingestion. DNA, prepared by Dr. Strickland, will be assayed for PAH-DNA adducts by BP-DNA ELISA using the newly-established DELFIA system with a fluorescent Europium end point. This application of the ELISA gives a 4- to 5-fold increase in sensitivity as compared to the methyl-umbelliferone end point previously used routinely.

The BP-DNA-ELISA (with Europium end point) is also being used to measure PAH-DNA adducts in blood cell DNA from aluminum plant workers and coke oven workers. Dr. Bernadette Schoket, a Visiting Scientist, has brought DNA samples from Hungarian aluminum plant workers who are at increased risk of lung and bladder cancer. Monitoring is being carried out on individuals at two plants having different levels of exposure. Most of the samples have already been assayed by ³²P-postlabeling and a comparison will be made with ELISA. In collaboration with Dr. Paul Strickland (Johns Hopkins U.) blood has been collected from 527 male coke oven workers in Italy and the extracted DNA will be assayed to correlate ELISA results with exposure, lung pathology and eventual tumor incidence.

We are continuing studies, performed in collaboration with Dr. F. A. Beland (NCTR), of DNA adduct processing during chronic carcinogen administration of aromatic amines. DNA adduct formation and tumorigenesis are being compared in livers and bladders of mice ingesting 2-acetylaminofluorene and 4-aminobiphenyl. Patterns observed to date are very different for these two aromatic amines even though the metabolism of both compounds is similar. Chronic feeding studies in the rat are focused on adduct formation and removal in various sub-fractions of DNA from livers of rats fed AAF. The most recent of these has demonstrated that a 370 base pair fragment, containing a reiterated sequence that comprises approximately 2% of the total genome, has the same adduct removal kinetics as the whole liver DNA. This fragment will be subjected to sequencing and the location of "hot spots" for adduct formation determined.

Pilot studies, performed in collaboration with Dr. A. Weston (Laboratory of Human Carcinogenesis, NCI), have demonstrated aromatic amine-DNA adducts in DNA from human placenta and human lung prepared by immunoaffinity chromatography (IAC). The antiserum used was elicited against guanosin-8-yl-acetylaminofluorene but is also specific for guanosin-8-yl-aminobiphenyl. These studies are in progress and suggest that a highly sensitive assay can be obtained by combining IAC purification with ³²P-postlabeling.

An ELISA developed with cisplatin-DNA antisera is being used to quantitate adducts in DNA extracted from nucleated peripheral blood cells of cancer patients receiving platinum drug therapy (in collaboration with Dr. E. Reed, Medicine Branch, Division of Cancer Treatment, NCI). In order to extend previous observations, which showed a significant correlation between ELISA-measurable cisplatin-DNA adducts and favorable disease response in testicular

and ovarian cancer patients, we have obtained blood cell DNA from ovarian, breast and colon cancer patients who failed previous chemotherapy. Approximately 300 blood samples from 67 individuals were assayed by cisplatin-DNA ELISA, and about 200 of these were also assayed by atomic absorbance spectrometry (AAS). Overall patients with the highest adduct levels determined by ELISA also experienced the most favorable clinical outcomes, but this relationship did not hold for total platinum bound to DNA that was measured by AAS. For the ovarian cancer patients a 60% rate of complete plus partial response (CR + PR) was observed and there was a statistically significant correlation between high adduct formation (measured by ELISA) and favorable response. For the colon (20% CR + PR) and breast (11% CR + PR) cancer groups, patients with high adduct levels had the best clinical outcome but the association was not statistically significant. These studies suggest that the correlation with adduct formation is not so strong in protocols that have a high failure rate.

Recently placenta and blood cell DNA have become available from a woman treated with platinum drugs for ovarian cancer discovered during her pregnancy. This collaboration was initiated by Dr. Carolyn Runowitz (Albert Einstein College of Medicine). Preliminary assays are positive for the presence of cisplatin-DNA adducts measured by ELISA in all of the tissues tested.

During the past year Dr. Ofelia Olivero (Visiting Associate) has studied the cytotoxicity and incorporation of the anti-AIDS drug 3'-azido-2',3'-dideoxythymidine (AZT) into the DNA of human (HL 60), hamster (CHO) and mouse (NIH 3T3) cells. HPLC analysis of digested DNA from these cell lines showed radiolabel from ³H-AZT co-chromatographing with thymidine and AZT. Immunohistochemical localization of AZT in metaphase chromosome spreads from these cells showed a telomeric localization for high concentrations of the drug. Future studies will focus on the long-term incorporation of AZT into DNA of bone marrow, vagina and other tissues of chronically-exposed rodents. Tissues which are sites for either tumorigenesis or toxicity induced by this drug will be examined by radioimmunoassay and immunohistochemistry.

Publications:

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

PROJECT NUMBER

Z01CP05178-10 CCTP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

COOPERATING UNITS (If any)

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In Vitro Pathogenesis Section

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.7

PROFESSIONAL:

1.7

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.)

Cell lines made from papillomas resulting from treatment of SENCAR mice with 12-O-tetradecanoylphorbol-13-acetate (TPA) alone, without exogenous chemical initiation were found to express high levels of message for keratin 13, a keratin not normally expressed in the epidermis. The original tumors and cell lines were previously found not to have the additional XbaI restriction site in the *ras*^{Ha} gene diagnostic for an A to T point mutation in the second base of codon 61, which leads to activation of the oncogene. We have now shown by direct sequencing of polymerase chain reaction products from DNA of the cell lines that there is an activating A to G mutation at the same position. Studies with the cell line SCR722, a presumed initiated cell line derived from adult SENCAR epidermal cells initiated in culture with N-methyl-N'-nitro-N-nitrosoguanidine, show that its parent produces carcinomas in skin grafts, while SCR722 produces normal skin. This suggests that SCR722 may be a model both for oncogene activation and tumor suppression. Staurosporine, widely used as an inhibitor of protein kinase C, at nmole levels appears to activate protein kinase C and induce terminal differentiation in SENCAR papilloma cell line SP-1. *In vivo*, treatment of grafted SP-1 cells with a single low dose of staurosporine can strongly or completely inhibit tumor formation.

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

J. E. Strickland	Research Chemist	LCCTP NCI
S. H. Yuspa	Chief	LCCTP NCI
H. Hennings	Research Chemist	LCCTP NCI
C. Sutter	Research Scientist	German Cancer Research Center, Heidelberg

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. Papillomas were removed, minced, and cells were dissociated by treatment with collagenase, followed by trypsin, and cells were cultured in medium with low $[Ca^{2+}]$. Specific cloned genes are introduced into cultured cells by infection with retroviral vectors defective in replication or by co-transfection of cultured cells with the neomycin resistance gene and cloned genes of interest and selection for transfectants by survival in G418. Southern blot DNA analysis and Northern blot RNA analysis are used to demonstrate presence of the exogenous DNA in the target cells and expression of the appropriate mRNAs, respectively. Polymerase chain reaction, using appropriate oligonucleotide primers, is used to amplify DNAs, which are sequenced to discover mutations in genes of interest. Western immunoblot protein analysis and immunohistochemical staining techniques are used to demonstrate the presence of the proteins coded for by the inserted exogenous genes. A grafting system has been developed in athymic nude mice to determine the biological potential of the various cells generated in culture. Grafting produces 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:

A small number of papillomas develop in SENCAR mice by treatment with the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) alone, without exogenous chemical initiation. We had previously obtained 10 such papillomas and one carcinoma resulting from the same protocol and had developed cell lines from several of them. We examined the DNA from these tumors and cell lines for the presence of an A to T transversion in codon 61 of the *ras*^{Ha} gene, which had been reported to occur in a high percentage of such tumors. An additional XbaI restriction site is created by this mutation. By XbaI restriction analysis, only the 12 kb normal *ras*^{Ha} gene was found, with no 8 or 4 kb fragments as seen in positive controls with known codon 61 A to T mutations. However, we recently found high expression of keratin 13 in these cell lines, a keratin not present in normal epidermis and thought by some to be associated with an activated *ras*^{Ha} gene. Expression of this keratin was not regulated by retinoic acid or Ca²⁺. Using PCR, we sequenced the codon 12 and codon 61 areas of the DNA from these cell lines, where activating mutations are frequently located, and found that, while all lines had normal codon 12 sequences, all also had an A to G transition in the second nucleotide of codon 61. This mutation is not detectible by XbaI restriction analysis but changes the codon from CAA to CGA, leading to a substitution of Arg for the normal Gln in the *ras*^{Ha} gene and leads to activation. DNA is currently being analyzed to determine whether the mutation arose in culture or was present in the original tumors.

Cell line SCR722 was developed from adult SENCAR epidermal cells initiated in culture with the directly acting carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The parent line was made by selection for survival in medium with 0.5 mM Ca²⁺, and SCR722 was produced by two subsequent ring clonings. SCR722 cells produced normal skin when grafted to nude mice. SCR722 cells are tetraploid, while its parent cell line, SCR7, was diploid. SCR7 is no longer available, but when SCR72, the intermediate line between SCR7 and SCR722, which has both diploid and tetraploid populations, was grafted, the result was a carcinoma. These results, along with earlier observations, suggest that SCR722 may be a useful model both for oncogene activation and for tumor suppression.

Staurosporine, a reported inhibitor of protein kinase C, induces terminal differentiation in cultured SP-1 cells, which were derived from SENCAR papillomas. These initiated cells are resistant to the induction of terminal differentiation by phorbol esters. These results suggested that staurosporine treatment may provide a mechanism to kill initiated cells. We confirmed this hypothesis by grafting SP-1 cells to nude mice and treating the grafts with staurosporine. A single treatment with a low dose of staurosporine (0.025 nmoles is optimal) was sufficient to prevent or strongly inhibit tumor formation.

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

PROJECT NUMBER

Z01CP05270-10 CCTP

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October 1, 1990 to September 30, 1991

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

Molecular Mechanism of Action of Phorbol Ester Tumor Promoters

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

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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.)

The overall direction of the Molecular Mechanisms of Tumor Promotion Section is to understand the mechanisms underlying the initial events in tumor promotion. Particular emphasis is being directed to the factors responsible for heterogeneity in the patterns of response. Protein kinase C is the receptor for the phorbol esters, the best studied class of tumor promoters in the mouse skin model. The mechanisms of protein kinase C activators which fail to induce phorbol ester-like responses are being characterized at the cellular and whole animal levels. The patterns of response are being correlated with the selectivity of the agents for cloned protein kinase C isozymes. One class of compounds which is receiving particular attention is that of the short chain monoesters of 12-deoxyphorbol, exemplified by prostratin. Although prostratin is a weak protein kinase C activator, chronic treatment of mouse skin with prostratin blocks the inflammatory and hyperplastic response of skin to subsequent treatment with the strong tumor promoter phorbol 12-myristate 13-acetate. These properties predict that prostratin will function as an anti-promoter. Ingenol is being examined because it possesses all elements of the phorbol ester pharmacophore except for the presence of a hydrophobic domain. We find that ingenol still retains weak activity as a protein kinase C agonist. Although all tumor promoting phorbol esters are inflammatory, the converse is not true. We have found that resiniferatoxin, an ultra-inflammatory phorbol ester, acts through a distinct mechanism, stimulating unique receptors on sensory neurons involved in pain and neurogenic inflammation. We are continuing to characterize these receptors, developing strategies to clone them and screening for the existence of endogenous analogs which may act as the normal physiological mediators of this pathway.

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

P. M. Blumberg	Research Chemist	LCCTP	NCI
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A. Szallasi	Visiting Associate	LCCTP	NCI
C. Hasler	IRTA Fellow	LCCTP	NCI
M. Kazanietz	Visiting Fellow	LCCTP	NCI
M.R. Boyd	Chief	LDDR	NCI
J.H. Cardellina II	Research Chemist	LDDR	NCI
H. Mischak	Visiting Fellow	LG	NCI
F. Mushinski	Research Biologist	LG	NCI

Objectives:

The initial events in the mechanism of action of tumor promoters are being characterized. Particular emphasis is being directed at factors contributing to heterogeneity of response to the phorbol ester tumor promoters and related compounds. Specific aims include the following: 1) clarify structural determinants for binding to protein kinase C; 2) characterize at the biological level the effects of novel natural products acting through protein kinase C; 3) evaluate the role of protein kinase C proteolysis, translocation, and isozyme heterogeneity in the divergent biological actions of protein kinase C activators; 4) characterize receptors for resiniferatoxin and their endogenous analogs; 5) determine the role of the diverse elements of inflammation in hyperplasia and tumor promotion.

Methods Employed:

The Molecular Mechanisms of Tumor Promotion Section uses a broad range of techniques to fulfill its research mission. Analogs of compounds of interest are synthesized and radiolabeled. Ligand binding assays are performed on membrane preparations and purified receptors. Biochemical responses are monitored on treated cultured cells and on intact mouse skin. Physiological responses are measured in mice and rats. Cloned receptor sequences are expressed in COS cells or in a baculovirus expression system, and receptors of interest are purified. The overall approach is to develop whatever methodologies are needed to explore our specific research questions rather than to restrict our approaches to those methodologies already in use in the laboratory.

Major Findings:

The studies on 12-deoxyphorbol derivatives represent one very promising direction in our overall effort to understand the basis for heterogeneity in

response to protein kinase C activators. The projected impact of this program is that current knowledge cannot explain why various phorbol esters differ in their promoting activity, cannot predict the biological consequences of activation of the protein kinase C arm of signal transduction by oncogenes, and offers no strategies for design of selective therapeutic agents having protein kinase C as a target. By identifying protein kinase C agonists which have different biological consequences from typical phorbol esters and by using these agents to dissect differential control of the protein kinase C pathway, we hope to resolve each of these issues.

Our analysis of prostratin (12-deoxyphorbol 13-acetate) began as part of a collaborative effort with the Division of Cancer Treatment (DCT) to clarify the potential toxicity of the compound, which had been identified by them in a screen of natural products possessing anti-HIV activity. We found that prostratin was a protein kinase C agonist with weak activity. In mouse skin, it induced acute phorbol ester responses but failed to cause hyperplasia either upon single or chronic treatment. These results suggested the model that prostratin could activate protein kinase C but had a superimposed inhibitory effect. We confirmed this interpretation by demonstrating that prostratin pretreatment blocked the hyperplasia and inflammation induced by phorbol 12-myristate 13-acetate. The dose response data for inhibition indicate at least two targets differing by 100-fold in affinity for prostratin. We have prepared and examined other 12-deoxyphorbol derivatives. 12-deoxyphorbol 13-phenylacetate is 100-fold more potent than prostratin. It also blocks acute hyperplasia, but it is less effective for blocking chronic hyperplasia.

Current biological efforts are directed at characterizing the effects of the 12-deoxyphorbol derivatives on various measures of inflammation, on cytokine production and responsiveness, and correlating these effects with hyperplasia. We can demonstrate, for example, the inhibition of neutrophil infiltration under specific conditions where we retain a hyperplastic response, thus arguing that this aspect of inflammation is not involved in hyperplasia. Biochemical analysis focuses on isotype specific stimulation, translocation, and down regulation. In collaborative studies with DCT, we are evaluating our prediction that prostratin is an anti-promoter, testing combinations of prostratin and phorbol 12-myristate 13-acetate in CD-1 mice.

Ingenol represents a second example of a compound evaluated because of potential selective action. Ingenol possesses all of the functionalities identified by computer modeling of the phorbol ester pharmacophore with the exception of a hydrophobic domain. Our hypothesis is that ingenol might therefore bind to protein kinase C but fail to induce its stabilization at the membrane. To the degree that membrane stabilization might be important in biological activity, ingenol might thus antagonize some phorbol ester responses. Contrary to the accepted view in the field, we demonstrated that ingenol could activate protein kinase C. Further, it induced morphological change and ornithine decarboxylase activity in keratinocytes, inhibited epidermal growth factor binding and arachidonic acid release in C3H10T1/2 cells, and blocked intercellular communication in rat liver epithelial cells. We have not found, so far, systems in which ingenol shows selective activity.

Other collaborations directed at clarifying protein kinase C structure activity relations involve the groups of Pettit at Arizona State University, Wender at Stanford, and Marquez in DCT, NCI. With Dr. Pettit's group we are examining the binding potency of synthetic and naturally occurring bryostatin derivatives. Interestingly, we find that neristatin, which is deficient in one element of the phorbol ester pharmacophore, the free C-26 hydroxyl group, still retains binding activity, albeit with a 100-fold loss of potency. A similar loss of potency had been observed previously for a different modification at the same group in the case of epi-bryostatin. Dr. Wender's group has accomplished the total synthesis of phorbol. With Dr. Wender's group we are exploring the role of those functional groups on the phorbol ring system that are not modified in the naturally occurring phorbol variants. With Dr. Marquez we are seeking to define the active conformation of sn-1,2-diacylglycerol, the endogenous analog of the phorbol esters, by examining the relative activities of conformationally constrained ring system analogs of diacylglycerol.

Our pharmacological studies are paralleled by biochemical efforts to define the nature of the binding interaction with protein kinase C and the subsequent activation response. We find a marked difference between the concentrations of phorbol derivatives which bind to protein kinase C and those which cause irreversible insertion of the enzyme into the membrane. These differences suggest the existence of a second low affinity site of interaction. We are testing the hypothesis that phorbol derivatives may differ in their relative potencies for reversible and irreversible activation of protein kinase C. Preliminary evidence suggests that prostratin, for example, may be less effective at inducing insertion than would be expected based on its binding activity. The studies are being carried out with purified protein kinase C isozymes. For those protein kinase C isozymes which are expressed at low levels normally, we are using cloned enzymes expressed in a baculovirus system.

Resiniferatoxin was one of the first phorbol related diterpenes with unusual biological properties for which we were able to identify the basis of the difference in behavior. We showed that resiniferatoxin acted through a mechanism distinct from protein kinase C. Resiniferatoxin functioned as an ultrapotent analog of capsaicin and could be used to demonstrate for the first time the existence of a capsaicin receptor. We have identified a strategy for cloning the receptor which appears to be feasible and are now in the process of trying to clone the receptor. The existence of a high affinity binding site for a xenobiotic suggests that there may be an endogenous ligand which normally interacts at that site. Efforts are underway to assay for such ligands by competition for resiniferatoxin binding to its receptor.

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Patents:

Blumberg PM, Szallasi Z. US Patent 07/681,679: Inhibitors of Protein Kinase C Function, April 8, 1991.

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

PROJECT NUMBER

Z01CP05445-07 CCTP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	C. A. Huff	Howard Hughes Fellow	LCCTP NCI
	T. Kartasova	Visiting Fellow	LCCTP NCI
	U. Lichti	Research Chemist	LCCTP NCI

COOPERATING UNITS (if any)

Baylor College of Med., Houston, TX (D. Roop); 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, MD 20892

TOTAL MAN-YEARS:

3.2

PROFESSIONAL:

3.2

OTHER:

none

CHECK APPROPRIATE BOX(ES)

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

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

To study epidermal differentiation and carcinogenesis, cDNA and genomic clones corresponding to the major proteins expressed in mouse epidermis have been isolated and characterized. These include the basal layer keratins K5 and K14, the suprabasal layer keratins K1 and K10, the hyperproliferative keratins K6 and K16, and K13, expressed in malignant tumors but not in benign tumors or normal epidermis. In addition, cDNA and genomic clones for filaggrin, the protein which organizes keratin filaments into larger bundles, and loricrin, the major precursor protein of the cornified envelope, have been isolated. Monospecific antibodies to the C terminal amino acid sequences, as well as nucleic acid probes to unique regions of the cDNAs, allow the study of the expression of these markers in normal epidermis, benign and malignant tumors, and cells in culture. Recombinant constructs of K1 and K10 coding sequences with heterologous promoters were transfected and expressed in mesenchymal cells and neoplastic keratinocytes. K1 and K10 filaments did not form in fibroblasts unless cytokeratins K5 and K14 were also expressed. K1 and K10 formed filaments in malignant keratinocytes which express K5 and K14 constitutively. Cells expressing K1 protein, but not K10 protein, continued to proliferate. An epidermal-specific regulatory region in a 12 kb fragment of the human K1 gene was localized in recombinant constructs with a reporter gene. A calcium-sensitive positive regulatory region was located 3' to the coding sequence. Deletion analysis identified several shorter elements in the terminal-1700 nucleotides of the 12 kb genomic clone. This regulatory region did not contain sequences responsive to retinoids. Samples of human skin from patients treated with retinoids showed marked changes in the expression of differentiation-related proteins within four days of exposure. Therefore, retinoid treatment of human skin is likely to be associated with functional changes in the epidermis.

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

S. H. Yuspa	Chief	LCCTP NCI
D. Rosenthal	Biotechnology Fellow	LCCTP NCI
C. A. Huff	Howard Hughes Fellow	LCCTP NCI
T. Kartasova	Visiting Fellow	LCCTP NCI
U. Lichti	Research Chemist	LCCTP NCI

Objectives:

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 mechanisms 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 gill 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. Transfection is accomplished by a modification of the calcium-phosphate precipitation method. Chloramphenicol acetyltransferase is assayed from cell lysates by a scintillation diffusion method.

Major Findings:

Formation of K1 and K10 filaments in mesenchymal cells requires the presence of other cytokeratins. Expression of two differentiation-specific keratins of skin, K1 and K10, is down-regulated during progression of squamous papilloma to malignancy. To study the relationship of expression of the K1/K10 pair and malignant conversion, active copies of mouse K1 and K10 genes were introduced into different cell lines.

Three questions have been asked: 1) whether the K1/K10 pair differs from other keratin pairs in its ability to form a filament network; 2) whether formation of the K1/K10 filament network is compatible with cell proliferation; 3) whether the keratin network is a dynamic interchangeable structure.

The complete K1 gene was constructed, using three overlapping genomic clones. The K10 gene was completed from two overlapping cDNA clones. Three different promoters: SV40-early, mouse metallothionein I (MT-I) and human cytomegalovirus promoter (CMV) were placed upstream of the K1 and K10 protein coding sequence to ensure a cell-type independent expression.

The K1 and K10 constructs were introduced into cell lines using the calcium phosphate precipitation method. Recipient cell lines were NIH 3T3 fibroblasts, the 308 papilloma cell line and SLC-1 carcinoma cell line. Both transient (24 - 72 hours after transfection) and long-term expression (in stable transfectants) of K1 and K10 proteins as well as their ability to form a filament network was analyzed. This was done by immunohistochemistry, Northern and Western blot analyses.

Expression of the K1 and K10 proteins can be detected in NIH 3T3 fibroblasts by immunofluorescence when both constitutive (SV40-early and CMV), or inducible (MT-I), promoters drive the transcription of the K1 and K10 genes. The K1 and K10 proteins expressed alone or together in NIH 3T3 fibroblasts did not form a filament network and stained with fluorescent antibodies as bright amorphous aggregates in the cytoplasm. On Western blots the K1 and K10 specific antibodies detected proteins of the appropriate size in the cytoskeletal extracts of NIH 3T3 cells stably transfected with the K1 and K10 constructs. The K1/K10 filament network could be "rescued" by fusing a NIH 3T3 line expressing the K1 and K10 proteins with 308 papilloma or SLC-1 carcinoma cell lines. Sixteen hours after fusion, the K1 and K10 filaments could be detected in the heterokaryons by immunohistochemistry. These filaments were co-localized with the endogenous K5/K14 network. Essentially the same observations were made when two NIH 3T3 lines, one expressing the K1/K10 pair and another expressing the K5/K14 pair, were fused.

Expression of K1 protein but not K10 protein is compatible with proliferation in tumor cells. The K1 protein transiently expressed in 308 and SLC-1 cell lines integrated into the preexisting keratin network and its expression was compatible with DNA synthesis. This was shown by double immunostaining with BrdU- and K1-specific antibodies. The number of K1-positive SLC-1 cells was found to be 4-5 times greater at confluence than in synchronized cells. This indicates that the K1 integration into the preexisting network may be cell cycle-dependent. In contrast, most of the 308 and SLC-1 cells expressing the K10 protein were negative for BrdU staining. Moreover, the K10 protein was frequently detected as dots in the cytoplasm of both 308 and SLC-1 cells. Analysis of K1 expression in the 2-3 week old transfectants revealed a heterogeneity among different cells within the same clones. Several SLC-1 cell lines stably transfected with the K1 construct showed consistently variable low levels of the K1 protein. These lines lost their ability to make the K1 protein *in vivo* upon grafting to nude mice. Long-term K10 expression led to a collapse of the endogenous K5/K14 network. All of the isolated K10 transfectants were negative for K10 protein as judged by immunostaining. The K10 protein could only be detected in the cells of an NIH 3T3 (K1/K10)/SLC-1 hybrid kept at confluence for a week. Thus, the integration of the K10 into the preexisting network also may be cell cycle dependent.

The K1 protein may replace the K5 protein in the endogenous K5/K14 network of the transiently transfected SLC-1 cells. In the SLC-1 cell line stably transfected with the K1 construct, the cells positive for K1 were also positive for K5. The fate of K6 in this clone as well as in transient K1 transfectants is presently under investigation.

Isolation of an epidermal-specific, calcium-inducible regulatory region controlling the human keratin K1 gene. Keratins K1 and K10 are induced at the RNA level to form the vast majority of the intermediate filament network in the suprabasal cells of epidermis. This laboratory has shown that in cultured mouse keratinocytes, keratins K1 and K10 can also be induced at the transcriptional level in response to an increase in Ca^{2+} in the culture medium from 0.05 mM to >0.10 mM; a cycloheximide-sensitive factor is involved in this induction. The Ca^{2+} -response of K1 can be suppressed by a variety of agents which block terminal differentiation of keratinocytes. In an attempt to define DNA regulatory elements that mediate the response of the K1 gene to these positive and negative modulators of differentiation, we have cloned regions spanning the 5' and 3' flanking sequences, coding regions, and introns from both the mouse and human K1 genes into vectors containing the chloramphenicol acetyltransferase (CAT) reporter gene. A 4.4 kb region located 3' to the human K1 gene stimulated CAT activity in primary mouse keratinocytes in response to elevated levels of Ca^{2+} in the medium. The 4.4 kb fragment is also active in human epidermal cells, but inactive in NIH 3T3 cells and primary mouse fibroblasts, indicating cell-type and perhaps epidermal specificity. Finally, activity was not suppressed by several agents that block terminal differentiation and endogenous K1 expression, suggesting that the effects of these negative modulators are mediated independently by other regulatory elements.

The 3' regulatory region of human keratin K1 is comprised of a complex array of genetic regulatory elements. The genetic elements within the 4.4 kb regulatory region of the human K1 gene (HK1) have been further defined by deletion analysis. Most of the information for calcium inducibility as well as epidermal specificity resides within the distal 1.7 kb of the 3' regulatory region, beginning 2.6 kb downstream from the last exon of HK1. Most further deletions within this region resulted in the partial or complete loss of activity. However, the removal of the terminal 207 bp of the fragment resulted in a strong increase in activity, although this construct was still stimulated by calcium. Unlike the parent 4.4 kb or 1.7 kb constructs, this 3' deletion construct was expressed in cell types other than keratinocytes, including NIH 3T3 cells; this indicates a role for the 207 bp region in epidermal specificity. Further deletion analysis demonstrated that two elements located 900 bp apart function synergistically to confer calcium responsiveness. Finally, DNase I protection experiments show sequence-specific binding of a keratinocyte nuclear regulatory factor(s) to at least two small regions within the 5'-most regulatory element.

Retinoids cause extensive changes in proteins expressed in human skin. We have previously shown in this laboratory that chronic topical application of retinoic acid (RA) alters the expression of several markers of epidermal differentiation in photo-aged skin. This reprogramming of differentiation may

indicate that the barrier function of the skin to uv irradiation has been compromised. In order to determine whether these changes can be induced by acute topical RA treatment, cutaneous biopsies from 10 subjects treated under occlusion for four days, and from 19 subjects treated nightly for 16 weeks were examined histologically and immunocytochemically. Acute application of RA causes epidermal thickening (9 of 10 samples), stratum granulosum thickening (7/10), parakeratosis (4/10), a marked increase in the number of cell layers expressing epidermal transglutaminase (7/10), and focal expression of two keratins, K6 (8/10) and K13 (2/10), not normally expressed in the epidermis, consistent with the changes that we have previously observed with chronic treatment. In addition, three other markers of differentiation, involucrin, filaggrin and loricrin, were altered in samples from both acute and chronic treatment. An increased number of cell layers expressed both involucrin and filaggrin in a majority of biopsies from both the acute (7/10) and chronic (14/19) treatment groups. Loricrin, however, demonstrated a different pattern of expression between the acute and chronic groups. In the acute group, loricrin expression was significantly reduced or absent in some regions of the epidermis (5/10). In contrast, none of the samples from the chronic group showed any reduction, while most showed an increased number of cell layers expressing loricrin (12/19). As in the case of the chronic treatment group, the pattern of expression of three major epidermal differentiation products, keratins K1, K10, and K14, were not significantly altered in any of the 10 sets of acute samples, although there was a slight reduction in the detection of K10 in two of the acute samples. Thus, acute topical RA treatment under occlusion causes substantial changes in the epidermis, and mimics most, but not all of the effects of chronic treatment.

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

October 1, 1990 through September 30, 1991

The present activities of the Laboratory of Chemoprevention are devoted largely 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. Our current activities 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 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 and bone healing. In addition to studies on TGF-beta, the Laboratory maintains an active interest in the subject of chemoprevention, particularly the use of retinoids, tamoxifen, and other steroid-like agents for the prevention of epithelial cancer.

Mammalian cells express three distinct TGF-beta isoforms, called TGF-beta's 1, 2, and 3. There is already substantial evidence of differential control of expression of these three TGF-beta isoforms both in vitro and in vivo. We have obtained insight into the molecular mechanisms of transcriptional control of TGF-beta expression in mammalian cells by comparative analyses of the promoter regions of the genes for TGF-beta's 1, 2, and 3. The 5' flanking regions of the three genes are distinctly different. The promoters for the TGF-beta2 and 3 genes contain TATAA boxes just upstream of the start sites, whereas initiation of transcription of the TGF-beta1 gene is thought to result from a cluster of SP1 binding sites. Selective expression may result from the use of AP-1 sites in the TGF-beta1 promoter, whereas both TGF-beta2 and 3 promoters contain CRE and AP-2 sites which suggest responsiveness to cyclic AMP. Correlating with the observed high levels of expression of TGF-beta3 in muscle, it has been found that the TGF-beta 3 promoter is selectively regulated during the differentiation of myoblasts into myotubes through novel sites. Recent studies demonstrate that while several oncogenes such as jun, fos, src, abl, and ras, selectively activate TGF-beta1 expression through its AP-1 sites, the product of the tumor suppressor gene, Rb1, activates expression of all three TGF-beta promoters through retinoblastoma response element (RCE) sites. This finding suggests that a general mechanism of growth regulation by RB, a nuclear protein, might involve control of the expression of a secreted peptide which acts to control growth via its interaction with cell membrane receptors.

Although each of the three mammalian TGF-beta isoforms shows 65-85% homology to each other, the amino acid sequence of any particular isoform is conserved greater than 98% between different mammalian species and greater than 95% between mammalian, avian, and amphibian species. This strongly suggests that the mature peptides will have certain unique biological activities. Only TGF-beta's 1 and 2 have been purified from natural sources. So that we might be able to compare the activities of all three isoforms, we developed a system for expression of recombinant TGF-beta3. Moreover, to begin to explore exactly which regions of the molecule are responsible for isoform-specific binding and activity, we have used this same expression system to express specific recombinant chimeric TGF-beta's in which regions of the amino acid sequence of TGF-beta's 1, 2, and 3 are substituted for each other in the mature peptide. Each of these recombinant TGF-beta's has been purified to homogeneity using a sequence of high pressure liquid chromatography steps. The availability of TGF-beta3 has permitted us to raise antibodies against this peptide which can now be used to detect its presence in a variety of experimental systems. Moreover, selective effects of TGF-beta3 have been identified in inhibition of the growth of hematopoietic cells, in migration of fetal fibroblasts, and in inhibiting the survival of certain neuronal cells. Using the chimeric TGF-beta constructs we have been able to deduce that the middle third of the TGF-beta molecule is sufficient to confer isoform-specific biological activity. In a related aspect of this project, we are exploring the effects of the different TGF-beta isoforms on protection of cardiac myocytes from damage resulting from the action of growth factors such as interleukin-1 and tumor necrosis factor-alpha.

In further studies, we have characterized rodent and avian TGF-betas in terms of chemistry, biology, and molecular biology of each of the component members and to understand their mechanisms of expression. The emphasis has been on continuing our investigation of the expression of the TGF-beta isoforms in the rat and chicken using rat and chicken TGF-beta cDNA homologs that have been previously cloned. Rat TGF-beta1 cDNAs corresponding to the 2.4 and 1.9 Kb TGF-beta1 mRNAs have been cloned by polymerase chain reaction (PCR) amplification of reverse-transcribed mRNAs extracted from adult rat liver and adult rat heart following experimental myocardial infarction, respectively. The 1.9 Kb transcript level has been shown to be significantly higher in infarcted heart tissue than in normal heart tissue, suggesting an important role for this mRNA species in response to injury. In addition, cDNA probes and antibodies for TGF-betas 1, 2 and 3 were used to study expression of these different isoforms in normal liver and during rat liver regeneration following partial hepatectomy. Expression of all three TGF-beta mRNAs increased following hepatectomy, and all three isoforms were shown to inhibit DNA synthesis in cultured hepatocytes, suggesting that the different TGF-beta isoforms may function in an inhibitory mechanism that is activated following liver injury. In addition, cDNA probes and antibodies for TGF-betas 1, 2, 3 and 4 were used to study expression of the different TGF-beta isoforms in cultured chicken embryo chondrocytes and myocytes, as well as in developing cartilage and heart tissues and also in the developing chicken embryonic nervous system. RNA Northern blot analysis using TGF-beta cDNA probes and immunohistochemical staining using TGF-beta antibodies suggest that chicken TGF-betas 2, 3 and 4 mRNAs and proteins are co-expressed in chicken cartilage and heart. Similarly, RNA analysis and immunohistochemical staining has shown

expression of TGF-betas 2, 3 and 4 in the chicken embryonic nervous system. In situ hybridization is being used to identify cell-specific localization of chicken TGF-beta mRNAs in the developing chicken nervous system.

Under normal physiological conditions, TGF-betas are synthesized predominantly in biologically latent forms. We have purified recombinant latent TGF-beta and radioiodinated it for use in pharmacokinetic studies. We have demonstrated that the latent complex has a much longer plasma half-life in rats than active TGF-beta1, and a very different tissue distribution. This suggests that latent TGF-beta1 may be the form of choice for clinical use. We have further shown that certain members of the steroid hormone superfamily can induce the secretion of active, rather than latent, TGF-beta1 in specific target tissues, and we propose that this local induction of an endogenous inhibitor of cell growth by steroids could be exploited to develop a new pharmacology of cancer prevention or treatment. In addition, the possibility that active TGF-beta might interact with its cognate receptor intracellularly was investigated by the use of recombinant DNA techniques to add an endoplasmic reticulum retention sequence on to both active and latent TGF-beta. The results indicated that addition of any C-terminal extension to the TGF-beta1 molecule interferes with biosynthetic processing and destroys biological activity. Thus, strategies to develop antagonists or superagonists of TGF-beta should avoid modification of this region.

Changes that may occur in the localization of immunoreactive TGF-beta isoforms in pathological situations as compared to normal conditions have been investigated using specific peptide antibodies to TGF-beta proteins and the avidin-biotin peroxidase staining technique. For example, we have found that conditions which raise plasma renin activity, such as dehydration, cause an increase in TGF-beta immunostaining in the juxtaglomerular apparatus and certain arterioles in the mouse kidney. In childhood tumors, TGF-betas 1 and 3 are present in rhabdomyosarcomas and undifferentiated neuroblastomas, while TGF-beta2 is not observed. The roles of TGF-betas in vascular injury and in mediating cardioprotection are also being evaluated. Treatment of rats with endotoxin causes a striking decrease in immunoreactive TGF-beta in the vascular smooth muscle, while TGF-beta expression seems to increase following balloon injury of the aorta or carotid artery. Previous studies have shown that the localization of TGF-beta changes following myocardial infarction in rats and application of exogenous TGF-beta may limit tissue damage following ischemia. Isolated perfused hearts are being damaged by anoxia, cytokines and heat shock so that changes in cardiac function and expression of TGF-beta, matrix proteins and other cytokines can be examined. These studies will be extended to include the addition of exogenous TGF-beta during or following tissue damage to determine which parameters it modulates. Additionally, studies on effects of TGF-beta on expression of growth factors, proteases, gap junction proteins, cytokines and matrix proteins in primary cultures of neonatal rat cardiac fibroblasts and myocytes are beginning. In primary cultures of neonatal rat astrocytes, TGF-betas have been found to antagonize the mitogenic effects of basic fibroblast growth factor, but to synergize with it to increase expression of collagen mRNA. TGF-beta is present in astrocytes in vivo and may play a role in reactive astrocytosis.

Specific polyclonal antisera in rabbits against synthetic peptides from the mature region and latency associated protein (LAP) region of TGF-betas 1 through 5, alternative spliced TGF-beta2 LAP and the TGF-beta1 "modulator"

associated protein have been developed. These antisera have a broad spectrum of specificities and are useful in one or more of the following assays for TGF-betas: Western immunoblots, immunoprecipitation of metabolically labeled material, radioimmunoassays, and immunohistochemical staining. However, none of these antibodies are useful in neutralization of TGF-beta bioactivity. With the recent availability of purified recombinant chicken TGF-beta3 (rcTGF-beta3), reactivity patterns previously generated against TGF-beta peptides have been characterized on Western blots. An antibody to amino acids 50-60 of TGF-beta3 reacts with purified rcTGF-beta3 but not with porcine TGF-betas (pTGF-betas) 1 and 2. We have also developed polyclonal antisera against native pTGF-betas 1 and 2 in rabbits and turkeys and against rcTGF-beta3 in rabbits. Antisera against pTGF-betas 1 and 2 generated in both species were proven useful in neutralizing the biological activity of TGF-betas 1 and 2, and collectively have allowed the specific detection and/or quantitation of TGF-beta1 and TGF-beta2 by radioimmunoassays, Western immunoblots, immunoprecipitation of metabolically labeled material, radioreceptor assays, growth inhibition assays and sandwich enzyme-linked immunosorbent assays, but not immunohistochemical staining of TGF-betas. Although antisera generated against native rcTGF-beta3 neutralized the biological activity of rcTGF-beta3 and immunoprecipitated ¹²⁵I-rcTGF-beta3, anti-rcTGF-beta3 was unable to neutralize TGF-beta3 secreted by rat skeletal myotubes and neonatal cardiac myocytes. Among the various mentioned assays for quantitation of TGF-betas 1 and 2 the SELISAs are the method of choice for specific, sensitive, precise and rapid measurements of TGF-betas 1 and 2 in biological fluids. The development of a SELISA for TGF-beta3 using antibodies against native rcTGF-beta3 is in progress.

A major new project that has been started in the Laboratory during the past year is an attempt to establish an intensive effort directed at the chemoprevention of prostate cancer. Prostate cancer is the second cause of cancer death in the male population. The lack of progress in treatment and management of this disease is hampered by inadequate experimental animal models. The purpose of this new project is to develop and establish an efficient and reproducible animal model system for inducing prostate carcinogenesis; analyze the molecular and cellular events following the development of prostate cancer and to identify agents that prevent and/or suppress prostate cancer incidence. We have initiated induction of prostate carcinogenesis by initiation with N-methyl-nitrosourea (NMU) and promotion with testosterone propionate (TP) in Lobund/Wistar rats of varying age ranging from 2-4 months old. We have partially succeeded in growing primary cultures of normal rat prostate epithelium for 3 weeks in chemically defined media supplemented with exogenous nutrients and growth promoting substances. Using 3 month old rats, we have shown increased levels of TGF-beta1 and beta3 message and protein in the prostate gland at 3-6 days following castration. This effect was reversed by treatment with TP. In collaboration with Dr. Morris Pollard, Lobund Laboratory, we have demonstrated for the first time decreased incidence of prostate cancer in NMU/TP-treated animals fed with N-(4-Hydroxyphenyl)retinamide. We thus have established an experimental basis for further studies of prevention of prostate cancer. In the coming year, such studies will be pursued intensively, both at the molecular level, as well as in whole animals, with the practical goal of developing a clinically acceptable agent for chemoprevention of cancer in men at high risk.

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

PROJECT NUMBER
 Z01CP05051-13 LC

PERIOD COVERED
 October 1, 1990 to September 30, 1991

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 B. Roberts	Staff Scientist	LC	NCI
Others:	Nanette B. Roche	Biologist	LC	NCI
	Su Wen Qian	Visiting Fellow	LC	NCI
	Jim Burmester	IRTA Fellow	LC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
 Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION
 NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.2	2.2	1.0

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 (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.)
 Three distinct isoforms of TGF-beta are expressed in mammals. Although each of these isoforms shows 65-85% homology to each other, the amino acid sequence of any particular isoform is conserved greater than 98% between mammalian, avian, and amphibian species. This strongly suggests that the mature peptides will have certain unique biological activities. Only TGF-beta's 1, and 2 have been purified from natural sources. So that we might be able to compare the activities of all three isoforms, we developed a system for expression of recombinant TGF-beta 3. Moreover, to begin to explore exactly which regions of the molecule are responsible for isoform-specific binding and activity, we have used this same expression system to express specific recombinant chimeric TGF-beta's in which regions of the amino acid sequence of TGF-beta's 1, 2, and 3 are substituted for each other in the mature peptide. Each of these recombinant TGF-beta's has been purified to homogeneity using a sequence of high pressure liquid chromatography steps. The availability of TGF-beta 3 has permitted us to raise antibodies against this peptide which can now be used to detect its presence in a variety of experimental systems. Moreover, selective effects of TGF-beta 3 have been identified in inhibition of the growth of hematopoietic cells, in migration of fetal fibroblasts, and in inhibiting the survival of certain neuronal cells. Using the chimeric TGF-beta constructs, we have been able to deduce that the middle third of the TGF-beta molecule is sufficient to confer isoform-specific biological activity. In a related aspect of this project, we are exploring the effects of the different TGF-beta isoforms on protection of cardiac myocytes from damage resulting from the action of growth factors such as interleukin-1 and tumor necrosis factor-alpha.

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

Anita B. Roberts	Staff Scientist	LC	NCI
Nanette B. Roche	Biologist	LC	NCI
Su Wen Qian	Visiting Fellow	LC	NCI
Jim Burmester	IRTA Fellow	LC	NCI

Objectives:

This project is directed toward four goals: 1) to purify to homogeneity recombinant TGF- β proteins expressed under control of strong promoters and to positively identify these proteins in terms of their amino acid sequence, or by immunoreactivity, when that is possible; 2) to construct novel chimeric TGF- β s with the purpose of identifying specific regions of the amino acid sequence responsible for selective activities of the isoforms; 3) to characterize fully the biological activity of the different isoforms and chimeras of TGF- β in a spectrum of both *in vitro* and *in vivo* assays, and 4) to address the mechanism of the protective effect of TGF- β in cardiac myocytes.

Methods Employed:

Methods for extraction of TGF- β -like peptides from tissues, cells, and conditioned medium include acid-ethanol and absorption to solid phase supports. Methods for purification are based on HPLC chromatography and include size exclusion chromatography, ion-exchange chromatography, and a variety of reverse-phase systems. Narrowbore HPLC technology is employed for purification and concentration of small quantities of proteins. Purity of the proteins is assessed by analytical and preparative SDS-PAGE analysis; electrophoretically separated proteins are transferred to Immobilon paper for amino acid sequencing. Standard recombinant technology is utilized for the construction of chimeric TGF- β 's and for their expression in NIH3T3 cells under control of the metallothionein promoter. Methods have been developed for the reproducible culture of neonatal cardiac myocytes prepared on Percoll density gradients.

Major Findings:

We have purified substantial quantities of recombinant TGF- β 3 and have used this peptide to explore whether it might have selective biological activities compared to TGF- β 's 1 and 2. Thus far studies demonstrate that this peptide is the most potent of the three isoforms in inhibition of the growth of myeloid cells. Moreover, it behaves differently than TGF- β 's 1 and 2 in inhibiting the migration of fetal fibroblasts in response to a novel protein which promotes their migration. We continue to explore different biological activities both *in vivo* and *in vitro* to better understand the distinctive properties of the different isoforms.

TGF- β 's 1 and 3 are over 100-fold more potent in inhibiting the growth of large vessel endothelial cells than is TGF- β 2. To try to identify which regions of the TGF- β molecule are responsible for this selectivity, we constructed a chimeric TGF- β in which the middle 50 amino acids of TGF- β 2 are substituted by the corresponding region of TGF- β 1. This chimeric TGF- β has been expressed in NIH3T3 cells, purified to homogeneity, and characterized both on the basis of N-terminal amino acid sequence analysis and on the basis of Western blot analysis using antibodies raised against specific epitopes of TGF- β 's 1 and 2. Surprisingly, this chimeric TGF- β 2.1.2 behaves indistinguishably from TGF- β 1 in the endothelial cell assay system. We are now making additional chimeric TGF- β 's in which only limited portions of this 50 amino acid region are substituted to attempt to identify if there are specific amino acids which can be shown to confer isoform-specificity upon the molecule.

We are using cultured primary cardiac myocytes to begin to understand the mechanism of cardioprotection by TGF- β that has been observed both in vivo and in vitro in the perfused heart. Initial observations indicate that TGF- β can maintain the regular beating rate of the cultured cells in the presence of agents such as interleukin-1 β and tumor necrosis factor- α which, by themselves, perturb the normal rhythmic beating pattern of the cells. This effect is observed only when the cardiac myocytes are cultured in the presence of cardiac fibroblasts, suggesting an interaction of these two cell types. We are examining whether transport or calcium or the energy metabolism of the cells is involved in this response.

Publications:

Jampel HD, Roche N, Stark WJ, Roberts AB. Transforming growth factor-B in human aqueous humor. *Current Eye Res* 1990; 9:963-9.

Joyce ME, Roberts AB, Sporn MB, Bolander ME. Transforming growth factor-B and the initiation of chondrogenesis and osteogenesis in the rat femur. *J Cell Biol* 1990;110:2195-207.

Qian SW, Kondaiah P, Casscells W, Roberts AB, Sporn MB. A second messenger RNA species of transforming growth factor β 1 in infarcted rat heart. *Cell Regul* 1991;2:241-9.

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Roberts AB, Kim S-J, Kondaiah P, Jakowlew SB, Denhez F, Glick AB, Geiser AG, Watanabe S, Noma T, Lechleider R, Sporn MB. Transcriptional control of expression of the TGF-B's. *Ann NY Acad Sci* 1990;593:43-50.

Roberts AB, Kim SJ, Noma T, Glick AB, Lafyatis R, Lechleider R, Jakowlew S, Geiser A, O'Reilly MA, Danielpour D, Sporn MB. Multiple forms of TGF-B: differential expression and distinct promoters. Ciba Foundation, Symposium No. 157 1991;157:7-28.

Roberts AB, Kondaiah P, Rosa F, Watanabe S, Good P, Danielpour D, Roche NS, Rebbert ML, Dawid IB, Sporn MB. Mesoderm induction in Xenopus laevis distinguishes between the various TGF-B isoforms. Growth Factors 1990;3:277-86.

Sporn MB, Roberts AB. Interactions of retinoids and transforming growth factor-B (TGF-B) in regulation of cell differentiation and proliferation. Mol Endocrinol 1991;5:3-7.

Sporn MB, Roberts AB. TGF-B: problems and prospects. Cell Regul 1990;1:875-82.

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

PROJECT NUMBER

Z01CP05398-08 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Function and Regulation 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 Scientist	LC	NCI
Others:	Thomas S. Winokur	Sr. Staff Fellow	LC	NCI
	Robin Hollands	Special Volunteer	LC	NCI

COOPERATING UNITS (if any) Genentech, Inc., San Francisco, CA (A. Levinson);
 Univ. of Cambridge, Cambridge, U.K. (A. Colletta)

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

1.5

OTHER:

1.0

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

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

Under normal physiological conditions, TGF-betas are synthesized predominantly in biologically latent forms. We have purified recombinant latent TGF-beta and radioiodinated it for use in pharmacokinetic studies. We have demonstrated that the latent complex has a much longer plasma half-life in rats than active TGF-beta, and a very different tissue distribution. This suggests that latent TGF-beta may be the form of choice for clinical use. We have further shown that certain members of the steroid hormone superfamily can induce the secretion of active, rather than latent, TGF-beta in specific target tissues, and we propose that this local induction of an endogenous inhibitor of cell growth by steroids could be exploited to develop a new pharmacology of cancer prevention or treatment. In addition, the possibility that active TGF-beta might interact with its cognate receptor intracellularly was investigated by the use of recombinant DNA techniques to add an endoplasmic reticulum retention sequence on to both active and latent TGF-beta. The results indicated that addition of any C-terminal extension to the TGF-beta molecule interferes with biosynthetic processing and destroys biological activity. Thus strategies to develop antagonists or super-antagonists of TGF-beta should avoid modification of this region.

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

Lalage M. Wakefield	Visiting Scientist	LC	NCI
Thomas Winokur	Senior Staff Fellow	LC	NCI
Robin Hollands	Special Volunteer	LC	NCI

Objectives:

The purpose of this project is to examine the role of TGF-Bs in the control of normal cell growth and in the process of malignant transformation. Particular emphasis is being given to understanding the regulation of the bioavailability of TGF-Bs through synthesis and activation of biologically latent complexes, and the effect of members of the steroid hormone superfamily on these processes.

Methods Employed:

Quantitation and characterization of latent and active TGF-B species using a combination of specific radioreceptor assay, radioimmunoassay, sandwich ELISA assay and growth inhibition bioassay. Protein purification techniques using FPLC technology. Generation and affinity purification of specific polyclonal antisera to synthetic peptides and their use in immunoblotting, immunohistochemistry and radioimmunoassays. Standard techniques of recombinant DNA technology, including subcloning, PCR, DNA sequencing, Northern blots. Tissue culture using established cell lines. Standard techniques of animal anaesthesia and surgery.

Major Findings:

Pharmacokinetics of latent TGF-B1. We purified the TGF-B1 latency-associated protein (LAP) from recombinant conditioned medium, and recombined it with radioiodinated active TGF-B1 to reform the latent complex. This was injected into rats and the pharmacokinetics were compared with that of the active TGF-B. The latent complex was found to have a greatly extended plasma half-life (109 min.) compared with active TGF-B which was rapidly cleared through the liver (half-life of <3 min.). While active TGF-B became concentrated in the liver, spleen, kidney and particularly the lungs, latent TGF-B was distributed at low levels to all perfused organs. Latent TGF-B in the circulation appeared to be protected from degradation, whereas active TGF-B was very rapidly degraded. However, after delivery to the tissues, both active and latent TGF-B appeared to be degraded at similar rates. These results confirm that one role of the latent TGF-B complex may be to protect the TGF-B from rapid clearance and degradation, and to alter its organ distribution. From a clinical standpoint, the longer halflife may make latent TGF-B1 the form of choice for systemic administration.

Induction of active TGF-B by steroids. Breast tumor cells in culture are strongly growth-inhibited by a novel synthetic progestin, gestodene. We have shown that treatment of the breast tumor cell lines T47D, MCF-7, MDA-MB-231 and BT20 caused a 3- to 90-fold induction of TGF-B1, and that the growth inhibitory effect of gestodene on these cell lines could be partially reversed by anti-TGF-B antibodies. This suggests that the inhibitory effect of gestodene is due in part

to induction of TGF- β . The effect was shown to be specific for breast cancer lines, and correlated with levels of expression of a novel gestodene binding protein that was only expressed in malignant breast cells and not in normal breast or malignancies of other tissues. Other progestins did not show this effect, indicating the classical progestin receptor was not involved. The induction of TGF- β 1 did not involve changes in mRNA levels, suggesting a translational or post-translational effect. Furthermore, essentially all the TGF- β induced by gestodene was secreted in the biologically active form, not the more common latent form. The exquisite tissue specificity of the response suggests that gestodene may have potential as a chemopreventive agent for breast cancer. A similar experimental approach was employed to show that antiestrogens can induce the secretion of active TGF- β 1 from human embryonic fibroblasts which do not express estrogen receptors. These were chosen to model the fetal-like phenotype of breast tumor stromal cells. The results provided a rationalization for the clinical observation that women with breast tumors that have no detectable estrogen receptors nevertheless respond to antiestrogen therapy.

Mutagenesis of TGF- β 1 by addition of a C-terminal extension. Recombinant expression constructs were designed to test the hypothesis that one role of the latent complex is to prevent premature interaction of active TGF- β with its cognate receptor inside the cell. A SEKDEL endoplasmic reticulum retention sequence was added by the polymerase chain reaction to TGF- β 1 coding sequences specifying either the latent or the active forms of TGF- β . It was demonstrated that addition of this or an irrelevant control sequence interfered with the biosynthetic processing of TGF- β by preventing glycosylation and biosynthetic cleavage, and decreasing the efficiency of dimerization. The resulting molecules were secreted but had no biological activity. The results indicate that correct folding of the highly conserved C-terminus is necessary for proper processing to occur, and that mutagenesis strategies should avoid this region of the molecule.

Publications:

Colletta AA, Wakefield LM, Howell FV, Danielpour DD, Baum M, Sporn MB. The growth inhibition of breast cancer cells by a novel synthetic progestin involves the induction of transforming growth factor- β . *J Clin Invest* 1991;87:277-83.

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

PROJECT NUMBER

Z01CP05550-04 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Localization of TGF-Beta in Tissues and Its Effects on Gene Expression

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:	Thomas S. Winokur	Sr. Staff Fellow	LC	NCI
	Bryan K. McCune	Biotechnology Fellow	LC	NCI
	Larry T. Mullen	Biological Technician	LC	NCI
	Theresa K. Chen	Biologist	LC	NCI

COOPERATING UNITS (if any) Dept. of Anatomy & Cell Biology, Univ. of Marburg, West Germany (K. Unsicker); Laboratory of Developmental Biology and Anomalies, NIDR (P. Klotman); Cardiology Branch, NHLBI (W. Cascells)

LAB/BRANCH

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INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.2

PROFESSIONAL:

2.2

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Changes that may occur in the localization of immunoreactive TGF- β isoforms in pathological situations as compared to normal conditions are being investigated using specific peptide antibodies to TGF- β proteins and the avidin-biotin peroxidase staining technique. For example, we have found that conditions which raise plasma renin activity, such as dehydration, cause an increase in TGF- β immunostaining in the juxtaglomerular apparatus and certain arterioles in the mouse kidney. In childhood tumors, TGF- β s 1 and 3 are present in rhabdomyosarcomas and undifferentiated neuroblastomas, while TGF- β 2 is not observed. The roles of TGF- β s in vascular injury and in mediating cardioprotection are also being evaluated. Treatment of rats with endotoxin causes a striking decrease in immunoreactive TGF- β in the vascular smooth muscle, while TGF- β expression seems to increase following balloon injury of the aorta or carotid artery. Previous studies have shown that the localization of TGF- β changes following myocardial infarction in rats and application of exogenous TGF- β may limit tissue damage following ischemia. Isolated perfused hearts are being damaged by anoxia, cytokines and heat shock so that changes in cardiac function and expression of TGF- β , matrix proteins and other cytokines can be examined. These studies will be extended to include the addition of exogenous TGF- β during or following tissue damage to determine which parameters it modulates. Additionally, studies on effects of TGF- β on expression of growth factors, proteases, gap junction proteins, cytokines and matrix proteins in primary cultures of neonatal rat cardiac fibroblasts and myocytes are beginning. In primary cultures of neonatal rat astrocytes, TGF- β s have been found to antagonize the mitogenic effects of basic fibroblast growth factor, and to synergize with it to increase expression of collagen mRNA. TGF- β is present in astrocytes in vivo and may play a role in reactive astrocytosis.

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

Kathleen C. Flanders	Sr. Staff Fellow	LC	NCI
Thomas S. Winokur	Sr. Staff Fellow	LC	NCI
Bryan K. McCune	Biotechnology Fellow	LC	NCI
Larry T. Mullen	Biological Technician	LC	NCI
Theresa K. Chen	Biologist	LC	NCI

Objectives:

TGF-Bs have been localized to a number of normal tissues both during embryogenesis and in the adult. The localization of the three mammalian TGF-B isoforms in pathological situations is being examined. Differences in TGF-B expression between these conditions and the normal tissue may suggest functions of specific isoforms of TGF-B in these disease states. Also, TGF-Bs may have different functions in normal tissue and tissues damaged by drugs, mechanical injury or ischemia. The roles of TGF-Bs in several organ systems are being evaluated by examining its effects on primary cell cultures, as well as organ perfusion systems.

Methods Employed:

Polyclonal antibodies raised in rabbits to peptide sequences of the various TGF-Bs have been affinity purified and used to localize proteins on sections of paraffin-embedded tissue using the avidin-biotin peroxidase technique. RNA prepared from tissues and cultured cells is being analyzed by Northern blotting for expression of TGF-Bs, as well as other growth factors, extracellular matrix proteins and proteases. Primary cell cultures are being set up by standard procedures involving tissue dissociation and isolation of specific cell types by differential plating or centrifugation. Retrograde perfusion of rat hearts, as well as a working heart model, are being established.

Major Findings:

Localization of TGF-B Isoforms in Pathological Conditions. In collaboration with Dr. Maria Tsokos (Laboratory of Pathology, NCI), we are completing an immunohistochemical survey of TGF-B in "small round cell" tumors of childhood. TGF-Bs 1 and 3 are regularly present in cases of embryonal and alveolar rhabdomyosarcoma, but absent in almost all undifferentiated neuroblastomas. Accumulations of TGF-Bs 1 and 3 are observed in differentiating cells of ganglioneuroblastoma, but the results are variable in primitive neuroectodermal tumors and Ewing's sarcoma. Immunoreactive TGF-B2 is not observed in any cases. The results suggest different roles for TGF-B in neuroblastoma and rhabdomyosarcoma.

TGF-B in the kidney is being studied in collaboration with Dr. Paul Klotman's group. Conditions which raise plasma renin activity, such as dehydration and angiotensin converting enzyme inhibitor therapy, cause a marked increase in

TGF-B2 immunostaining in the juxtaglomerular apparatus and certain arterioles of the mouse kidney. Antirenin antibodies demonstrate colocalization of renin and TGF-B2 in treated animals. The results suggest a role for TGF-B in control of the renin/angiotensin system. Studies are in progress to further characterize this phenomenon.

Effects of TGF-B in the Nervous System. Using immunohistochemical techniques, TGF-Bs 2 and 3 have been detected in neurons and astrocytes of embryonic and adult rodents. We have begun to determine the effects of TGF-Bs on primary cultures of these cells. When survival of cultures of chick embryonic ciliary ganglia is potentiated by a tissue extract, addition of TGF-Bs 2 or 3 inhibit this survival in a dose dependent manner. TGF-B1 has no effect. In primary cultures of rat neonatal astrocytes, TGF-Bs 2 and 3 inhibit the mitogenic effects of basic fibroblast growth factor (bFGF) on astrocytes, while TGF-B1 does not alter mitogenesis. We have also demonstrated that astrocytes produce all 3 isoforms of TGF-B and that in astrocytes TGF-B3 is the only isoform that shows autoinduction. TGF-Bs alone do not increase extracellular matrix production in astrocytes as they do in many other cell types, but a combination of bFGF and TGF-Bs do increase collagen synthesis. The combined effects of bFGF and TGF-B in regulation of astrocyte growth and matrix production suggest a role for these growth factors in reactive astrocytosis.

TGF-B in the Cardiovascular System. Previous immunohistochemical studies show the expression of all 3 isoforms of TGF-B in rodent embryonic and adult heart and the staining pattern changes following ischemia. Preliminary results from other laboratories suggest that TGF-B treatment may decrease post-ischemic tissue damage. In order to investigate the mechanisms of this protective effect and to examine the effects of TGF-B and other growth factors on normal myocardium, we have set up an isolated perfused heart system. Hearts can be damaged by ischemia, cytokines, or drugs and the potential protective effects of TGF-B can be evaluated by looking at changes in cardiac function, as well as production of growth factors, extracellular matrix proteins, proteases and oxygen free radicals. These studies are being complemented by examining effects of TGF-B on primary cultures of neonatal rat cardiac fibroblasts and myocytes where initial results indicate that TGF-B is capable of regulating its own expression in these cells.

Alterations in expression of TGF-B in the vasculature are also being investigated. Preliminary studies of animals administered purified endotoxin show a global decrease in immunoreactive TGF-Bs 1, 2, and 3, which is especially striking in the vascular smooth muscle. In contrast, intravascular balloon injury of the aorta and carotid artery induces an increase in TGF-B expression above normal levels. There is a suggestion that the aortic injury shows a downregulation of TGF-B within the first week following injury, whereas the carotid injury, which remains incompletely repaired, continues to express high levels of TGF-B.

Publications:

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Flanders KC, Cissel DS, Jakowlew SB, Roberts AB, Watanabe S, Danielpour D, Sporn MB. Immunohistochemical localization of TGF- β s 2 and 3 in the nervous system. *Ann NY Acad Sci* 1990;593:338-9.

Flanders KC, Cissel DS, Mullen LT, Sporn MB, Roberts AB. Antibodies to transforming growth factor- β 2 peptides: specific detection of TGF- β 2 in immunoassays. *Growth Factors* 1990;3:45-52.

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Glick AB, McCune BK, Abdulkarem N, Flanders KC, Smith JM, Sporn MB. Complex regulation of TGF- β expression by retinoic acid in the vitamin A-deficient rat. *Development (In Press)*.

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

PROJECT NUMBER

Z01CP05617-03 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Characterization of the Promoters of TGF-beta's 1, 2, and 3

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:	Seong-Jin Kim	Visiting Scientist	LC	NCI
	Klaus Busam	Guest Researcher	LC	NCI
	Andrew Geiser	IRTA Fellow	LC	NCI
	Michael O'Reilly	Staff Fellow	LC	NCI
	Renee Webbink	Special Volunteer	LC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5.3

PROFESSIONAL:

4.3

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Mammalian cells express three distinct TGF-beta isoforms, called TGF-beta's 1, 2, and 3. There is already substantial evidence of differential control of expression of these three TGF-beta isoforms both in vitro and in vivo. The purpose of this project is to gain insight into the molecular mechanisms of transcriptional control of TGF-beta expression in mammalian cells by comparative analyses of the promoter regions of the genes for TGF-beta's 1, 2, and 3. The 5' flanking regions of the three genes are distinctly different. The promoters for the TGF-beta 2 and 3 genes contain TATAA boxes just upstream of the start sites, whereas initiation of transcription of the TGF-beta 1 gene is thought to result from a cluster of SP1 binding sites. Selective expression may result from the use of AP-1 sites in the TGF-beta 1 promoter, whereas both the TGF-beta 2 and 3 promoters contain CRE and AP-2 sites which suggest responsiveness to cyclic AMP. Correlating with the observed high levels of expression of TGF-beta 3 in muscle, it has been found that the TGF-beta 3 promoter is selectively regulated during the differentiation of myoblasts into myotubes through novel sites. Recent studies demonstrate that while several oncogenes such as jun, fos, src, abl, and ras, selectively activate TGF-beta 1 expression through its AP-1 sites, the products of the tumor suppressor gene, Rb1, activates expression of all three TGF-beta promoters through retinoblastoma response elements (RCE) sites. This finding suggests that a general mechanism of growth regulation by RB, a nuclear protein, might involve control of the expression of a secreted peptide which acts to control growth via its interaction with cell-membrane receptors.

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

Anita B. Roberts	Staff Scientist	LC	NCI
Seong-Jin Kim	Visiting Scientist	LC	NCI
Klaus Busam	Guest Researcher	LC	NCI
Andrew Geiser	IRTA Fellow	LC	NCI
Su Wen Qian	Visiting Fellow	LC	NCI
Michael O'Reilly	Staff Fellow	LC	NCI
Renee Webbink	Special Volunteer	LC	NCI

Objectives:

The objects of this project are 1) to identify and characterize regulatory regions in the promoters of the TGF- β 1, TGF- β 2 and TGF- β 3 genes; 2) to identify the specific transcription factors which bind to identified regulatory regions of the promoters; 3) to examine the transcriptional regulation and the post-translational modifications of transcription factors by TGF- β 1; 4) to determine the regions of the TGF- β promoters regulated by various oncogenes; 5) to characterize regions in the promoters responsible for tissue-specific expression of the TGF- β isoforms, and 6) to determine at a molecular level the mechanisms whereby TGF- β inhibits cell growth or controls differentiation.

Methods Employed:

Methods are those basic to molecular biology including standard DNA cloning techniques, oligonucleotide synthesis, dideoxynucleotide (chain termination) sequencing of single- and double-stranded DNA clones, tissue culture techniques, transfer of DNA into mammalian cells, northern blot analysis of mRNA expression, transcriptional analysis by S1 nuclease, RNase protection, and primer extension, nuclear run-on transcription analysis, chloramphenicol acetyl transferase (CAT) assays, transgenic mice technologies-pronuclear DNA injections, gel retardation analysis of transcription factor binding, DNase protection assays for transcription factor binding, purification of TGF- β promoter-specific transcription factors by sequence-specific affinity chromatography, site-directed mutagenesis using M13 vectors, or a polymerase chain reaction (PCR) method, in vitro, and in vivo phosphorylation of transcription factors.

Major Findings:

Due to its promoter being characterized first, more is known about regulation of TGF- β 1 transcription than that of TGF- β 's 2 and 3. The AP-1 sites, located in both the first and second promoters, regulate transcription of TGF- β 1 by several different oncogenes including jun and fos which bind to the site as a heterodimer. Regulation of TGF- β 1 expression in myeloid cells by the oncogenes src and abl is also mediated by these sites. Upregulation of TGF- β 1 transcription by ras appears to be mediated by these sites as well as other regions of the promoter.

Recent evidence indicates that AP-1 sites may also be the targets of activation of TGF- β 1 promoter activity and TGF- β 1 secretion by the retrovirus, human T lymphotropic virus type I (HTLV-1). Freshly isolated ATL cells and HTLV-I infected T-cell lines show increased levels of expression of TGF- β 1 mRNA and secrete increased levels of TGF- β 1 protein. The Tax protein encoded by HTLV-I, a potent transcriptional activator of its own LTR promoter and of other cellular genes, has been shown to activate transcription of both the first and second promoters of TGF- β 1 through their AP-1 sites. In Tax transgenic mice, expression of TGF- β 1 mRNA in different tissues correlates quite closely with that of the Tax gene and levels of TGF- β 1 protein are selectively increased in those tissues such as salivary gland and muscle which also express high levels of Tax. In two cell lines derived from these mice, treatment with TGF- β increases expression of Tax demonstrating that TGF- β and Tax can regulate each other's expression. These data suggest that the increased production of TGF- β 1 by ATL cells may be related to transactivation of the TGF- β 1 promoters by the HTLV-I Tax protein and that TGF- β 1 may be important in the pathogenesis associated with the immunosuppression characteristic of this disease.

Initial characterization of the 5' flanking sequences for the TGF- β 2 and TGF- β 3 genes has shown significant differences between regulation of those genes and TGF- β 1. Whereas the TGF- β 1 promoter has no TATAA box and is regulated principally by AP-1 sites, the TGF- β 2 promoter has only one consensus AP-1 site which does not confer phorbol ester responsiveness, and the TGF- β 3 promoter has no such sites. In contrast, both the TGF- β 2 and TGF- β 3 promoters contain TATAA boxes immediately upstream of the transcriptional start sites. More detailed analysis of the TGF- β 3 promoter shows that mutation of the CRE site abolishes transcription from all promoter constructs. Two distinct upstream regions of the TGF- β 3 promoter are responsible for the high basal level of expression of TGF- β 3 in myocytes and for the increase in expression which accompanies differentiation of these cells into myotubes. In addition, a distal repeated TCCC motif near the AP-2 site has been found to be necessary for the activity of the upstream sequences in both myocytes and myotubes.

In terms of the negative regulation of the growth of many epithelial and lymphoid cells by TGF- β , interest has focussed on possible mechanistic connections between the product of the suppressor gene, Rb-1, and TGF- β . Analysis of the promoters for TGF- β 's 1, 2, and 3 demonstrates the presence of sites similar to a retinoblastoma responsive element, RCE, identified in the c-fos promoter. These sites can mediate RB control of the TGF- β 1 promoter. Expression of the c-fos, c-myc, and TGF- β 1 promoters is coordinately upregulated or downregulated by RB depending on the cell type. Gel retardation studies demonstrate binding of specific cellular proteins to the TGF- β 1 RCE, but clearly show that RB, itself, does not bind and must act indirectly. The ability of RB to upregulate expression of TGF- β in certain cells suggests that RB might be able to control growth of RB-negative cells in trans by inducing secretion of a growth inhibitory peptide.

Publications:

Birchenall-Roberts MC, Ruscetti FW, Kasper J, Lee H-D., Friedman R, Geiser A, Sporn MB, Roberts AB, Kim SJ. Transcriptional regulation of the transforming growth factor- β 1 promoter by v-src gene products is mediated through the AP-1 complex. *Mol Cell Biol* 1990;10:4978-83.

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Lafyatis R, Lechleider R, Kim S-J, Jakowlew S, Roberts AB, Sporn MB. Structural and functional characterization of the transforming growth factor- β 3 promoter: a cAMP responsive element regulates basal and induced transcription. *J Biol Chem* 1990;265:19128-36.

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

PROJECT NUMBER

Z01CP05622-02 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecular Identification of TGF-Beta mRNAs

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

PI:	Sonia B. Jakowlew	Sr. Staff Fellow	LC	NCI
Others:	Su Wen Qian	Visiting Fellow	LC	NCI
	Jeremy Cubert	Biologist	LC	NCI

COOPERATING UNITS (if any) Pathology Dept., Brown University, Providence, RI (N. Fausto, J. Mead, and J. Wu)

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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 unreduced type. Do not exceed the space provided.)

The purpose of this project is to characterize rodent and avian TGF-Bs in terms of chemistry, biology, and molecular biology of each of the component members and to understand their mechanisms of expression. The emphasis has been on continuing our investigation of the expression of the TGF-B isoforms in the rat and chicken using rat and chicken TGF-B cDNA homologs that have been previously cloned. Rat TGF-B1 cDNAs corresponding to the 2.4 and 1.9 Kb TGF-B1 mRNAs have been cloned by polymerase chain reaction (PCR) amplification of reverse-transcribed mRNAs extracted from adult rat liver and adult rat heart following experimental myocardial infarction, respectively. The 1.9 Kb transcript level has been shown to be significantly higher in infarcted heart tissue than in normal heart tissue, suggesting an important role for this mRNA species in response to injury. In addition, cDNA probes and antibodies for TGF-Bs 1, 2 and 3 were used to study expression of these different isoforms in normal liver and during rat liver regeneration following partial hepatectomy. Expression of all three TGF-B mRNAs increased following hepatectomy, and all three isoforms were shown to inhibit DNA synthesis in cultured hepatocytes, suggesting that the different TGF-B isoforms may function in an inhibitory mechanism that is activated following liver injury. In addition, cDNA probes and antibodies for TGF-Bs 1, 2, 3 and 4 were used to study expression of the different TGF-B isoforms in cultured chicken embryo chondrocytes and myocytes, as well as in developing cartilage and heart tissues and also in the developing chicken embryonic nervous system. RNA Northern blot analysis using TGF-B cDNA probes and immunohistochemical staining using TGF-B antibodies suggest that chicken TGF-Bs 2, 3 and 4 mRNAs and proteins are co-expressed in chicken cartilage and heart. Similarly, RNA analysis and immunohistochemical staining has shown expression of TGF-Bs 2, 3 and 4 in the chicken embryonic nervous system. In situ hybridization is being used to identify cell-specific localization of chicken TGF-B mRNAs in the developing chicken nervous system.

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

Sonia B. Jakowlew	Sr. Staff Fellow	LC	NCI
Su Wen Qian	Visiting Fellow	LC	NCI
Jeremy Cubert	Biologist	LC	NCI

Objectives:

This project is directed toward three goals: 1) identifying and cloning mammalian and avian homologs of members of the TGF-B family and characterizing their specific patterns of expression; 2) determining the range of biological activities of these TGF-Bs, and 3) correlating TGF-B mRNA expression with TGF-B protein expression using in situ hybridization and immunohistochemical staining techniques.

Methods Employed:

Standard methods are utilized such as basic recombinant DNA technology for the cloning, propagation, and sequencing of recombinant plasmids, preparation of RNA and analysis by RNA Northern blots, and construction and screening of cDNA libraries. In addition, polymerase chain reaction (PCR) methodology is being used for the cloning of homologs of the various TGF-Bs in other species. In situ hybridization and immunohistochemical staining techniques are being used to localize TGF-B mRNAs and proteins.

Major Findings:

As a continuation of our interest in cloning homologs of the various TGF-B isoforms in other species, we have cloned a second TGF-B1 cDNA in rodents encoded by a 1.9 Kb mRNA. TGF-B1 has been shown to be predominantly encoded by a 2.4 Kb mRNA in most tissues in the rat. An additional transcript of 1.9 Kb has been detected in rat heart after experimental myocardial infarction caused by ligation of the left coronary artery. This transcript is significantly higher in infarcted heart tissue than in normal heart tissue, suggesting an important role for this mRNA species in response to injury. Structural characterization of the 1.9 Kb mRNA showed that it included the entire coding sequence present in the 2.4 Kb TGF-B1 mRNA, but also contained an additional nonhomologous 3'-untranslated region. The junction between the shared and unique 3' sequence in the 1.9 Kb mRNA occurred only two nucleotides before the proposed polyadenylation site of the rat TGF-B1 2.4 Kb mRNA. The unique 3'-untranslated region and the deduced shortened 5'-untranslated region in the 1.9 Kb TGF-B1 mRNA suggest differential transcription and translational regulatory mechanisms under conditions of injury.

As an extension of our interest in expression of TGF-B in the rat, expression of TGF-Bs 1, 2 and 3 was studied in normal liver and during liver regeneration after partial hepatectomy in the rat to determine whether each of these isoforms might be involved in hepatocyte growth in vivo. Expression of the mRNAs for all three TGF-B isoforms increased in the regenerating liver, but whereas TGF-B1 mRNA

expression remained elevated for several days, the levels of expression of TGF- β s 2 and 3 mRNAs increased transiently, but returned to normal before the major wave of hepatocyte DNA synthesis. Immunohistochemical staining analysis showed a similar distribution of all three TGF- β s in normal and regenerating livers; however, in both tissues, the level of expression of TGF- β 1 was about 10-fold higher than that of TGF- β 2 as determined by sandwich ELISA. All three TGF- β mRNAs are restricted to liver nonparenchymal cells. All three TGF- β isoforms inhibited DNA synthesis induced *in vitro* by epidermal growth factor in hepatocytes from normal and regenerating livers with approximately the same kinetics. Hepatocytes from regenerating livers were capable of activating latent TGF- β 1 complexes *in vitro*, whereas normal hepatocytes were not. This suggests that the different TGF- β isoforms may function in an inhibitory paracrine mechanism that is activated during liver regeneration.

cDNA probes and antibodies for TGF- β s 1, 2, 3 and 4 were used to study the expression of these different TGF- β isoforms in cultured chicken embryo chondrocytes and cardiac myocytes, as well as in developing cartilage and heart tissues. TGF- β s 2, 3 and 4 mRNAs, but not TGF- β 1 mRNA, were detected in cultured chondrocytes and myocytes. Immunoprecipitation studies demonstrated expression of TGF- β in both the conditioned media and cell lysates of metabolically-labelled chondrocyte and myocyte cell cultures. Immunohistochemical staining of cultured chondrocytes and myocytes and of cartilage and heart tissues of developing chicken embryos with antibodies specific for each TGF- β isoform showed immunoreactive TGF- β s 1, 2, 3 and 4. Our results demonstrate coordinate expression of these four TGF- β isoforms in chicken embryo chondrocytes and myocytes.

cDNA probes and antibodies for TGF- β s 1-4 were also used to study expression of the different TGF- β isoforms in the developing embryonic chicken nervous system. TGF- β s 2, 3 and 4 mRNAs, but not TGF- β 1 mRNA, were detected by day 4 in the embryonic brain. Expression of TGF- β s 2 and 3 mRNAs increased with age, while expression of TGF- β 4 mRNA was independent of age in 4- to 16-day-old embryonic brains. We have also used immunohistochemical staining techniques to show expression of immunoreactive TGF- β s 2, 3 and 4 proteins in the nervous system during embryonic chicken development. Significant expression of TGF- β s 2, 3 and 4 was detected as early as stage 4 of incubation (definitive primitive streak). At neurulation, all three germ layers showed significant immunohistochemical staining for TGF- β s 2, 3 and 4.

Publications:

Jakowlew SB, Dillard PJ, Winokur TS, Flanders KC, Sporn MB, Roberts AB. Expression of transforming growth factor- β s 1-4 in chicken embryo chondrocytes and myocytes. *Dev Biol* 1990;143:135-48.

Jakowlew SB, Mead JE, Danielpour D, Wu J, Roberts AB, Fausto N. Transforming growth factor- β isoforms in rat liver regeneration: messenger RNA expression and activation of latent TGF- β complexes by hepatocytes. *Cell Regul* (In Press).

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Z01CP05622-02 LC

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

PROJECT NUMBER

Z01CP05624-02 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Development & Application of Antibodies Specific for Different Isoforms of TGF- β

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:	David Danielpour	Guest Researcher	LC	NCI
	Lisa V. Cook	Guest Researcher	LC	NCI
	Ahlke Heydemann	Biological Lab Tech	LC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

1.5

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.)

Specific polyclonal antisera in rabbits against synthetic peptides from the mature region and latency associated protein (LAP) region of TGF-Bs 1 through 5, alternative spliced TGF-B2 LAP and the TGF-B1 "modulator" associated protein have been developed. These antisera have a broad spectrum of specificities and are useful in one or more of the following assays for TGF-Bs: Western immunoblots, immunoprecipitation of metabolically labeled material, radioimmunoassays, and immunohistochemical staining. However, none of these antibodies are useful in neutralization of TGF-B bioactivity. With the recent availability of purified recombinant chicken TGF-B3 (rcTGF-B3), reactivity patterns of previously generated antibodies against TGF-B peptides have been characterized on Western blots. An antibody to amino acids 50-60 of TGF-B3 react with purified rcTGF-B3, but not with porcine TGF-Bs (pTGF-Bs) 1 and 2. We have also developed polyclonal antisera against native pTGF-Bs 1 and 2 in rabbits and turkeys and against rcTGF-B3 in rabbits. Antisera against pTGF-Bs 1 and 2 generated in both species were proven useful in neutralizing the biological activity of TGF-Bs 1 and 2, and collectively have allowed the specific detection and/or quantitation of TGF-B1 and TGF-B2 by radioimmunoassays, Western immunoblots, immunoprecipitation of metabolically labeled material, radioreceptor assays, growth inhibition assays and sandwich enzyme-linked immunosorbent assays, but not immunohistochemical staining of TGF-Bs. Although antisera generated against native rcTGF-B3 neutralized the biological activity of rcTGF-B3 and immunoprecipitated ¹²⁵I-rcTGF-B3, anti-rcTGF-B3 was unable to neutralize TGF-B3 secreted by rat skeletal myotubes and neonatal cardiac myocytes. Among the various mentioned assays for quantitation of TGF-Bs 1 and 2 the SELISAs are the method of choice for specific, sensitive, precise and rapid measurements of TGF-Bs 1 and 2 in biological fluids. The development of a SELISA for TGF-B3 using antibodies against native rcTGF-B3 is in progress.

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

Kathleen C. Flanders	Sr. Staff Fellow	LC	NCI
David Danielpour	Guest Researcher	LC	NCI
Lisa V. Cook	Biologist	LC	NCI
Ahlke Heydemann	Biological Lab Tech	LC	NCI

Objectives:

1) To generate polyclonal antibodies to peptides corresponding to various regions of all isoforms of TGF- β s including their precursor regions. 2) To develop polyclonal antibodies against native TGF- β s that can be used to specifically neutralize, immunoprecipitate and immunoblot each of the five known TGF- β s. 3) To develop rapid and sensitive radioimmunoassays for TGF- β s. 4) To develop sensitive radioimmunoassays and sandwich ELISAs that can specifically measure each of the five TGF- β s in complex biological fluids.

Methods Employed:

1) Production of region specific polyclonal antibodies in rabbits, 2) affinity purification of antisera, 3) ELISA to titer antisera, 4) immunoblotting, 5) immunoprecipitation of 125 I-TGF-Bs and radiolabelled cell media, 6) TGF- β receptor binding assay, 7) production of neutralizing antibodies against TGF- β s 1, 2 and 3 in rabbits and turkeys, 8) TGF- β growth inhibition assay, 9) TGF- β 1, TGF- β 2 and TGF-B3 radioimmunoassays, 10) TGF- β 1, TGF- β 2 and TGF- β 3 sandwich ELISAs.

Major Results:

Peptide Antibodies: With the availability of purified recombinant chicken TGF- β 3 (rcTGF-B3) in the past year, reactivity patterns of previously generated peptide antibodies have been characterized on Western blots. An antibody to amino acids 50-60 of TGF- β 3 reacts with purified rcTGF- β 3, but not with purified porcine TGF- β s (pTGF-Bs) 1 or 2. As detected on a nonreducing Western blot, purified rcTGF- β 3 migrates with a slightly higher molecular weight than pTGF- β s 1 or 2. Antibodies raised to larger peptides of TGF- β 1 (amino acids 1-30) or TGF- β 2 (amino acids 50-75) show some cross reactivity with rcTGF- β 3 on Western blots where the proteins are completely denatured.

Sequence analysis of human proTGF- β 2 cDNAs identified two distinct putative preproTGF- β 2 proteins, which differ by 28 amino acids in the amino-terminal precursor region. Alignment of human genomic DNA with these cDNAs revealed that this difference was due to alternative splicing of the second exon. The functional significance that this insertion imparts to the proTGF- β 2 protein, as well as the relative tissue distribution and humoral regulation of these two

isoforms is not known. In an effort to clarify the biological differences in the two proTGF- β 2 isoforms, we have generated site-specific antibodies against both the 28 amino acid insertion and common epitopes in both proTGF- β 2 isoforms. These antibodies recognize proTGF- β 2 by Western blot analysis. Future studies will be to identify tissue distribution of these two proTGF- β 2 isoforms by immunohistochemistry and to determine whether the expression of these isoforms is independently regulated in vitro and in vivo.

Generation of TGF- β neutralizing antibodies in turkey and rabbits and their application: To enhance immunogenicity of TGF- β s, rabbits were immunized and boosted with either native >96% pure pTGF- β 1 or pTGF- β 2 together with KLH, soybean trypsin inhibitor and ovalbumin. Remarkably, all of these animals developed TGF- β neutralizing antibodies. Final antisera titers from rabbits immunized against TGF- β 2 were 50- to 100-fold greater than from rabbits immunized against TGF- β 1. Antisera from both rabbits immunized against TGF- β 2 neutralized the receptor binding and biological activity of pTGF- β 2, but not pTGF- β 1 or rcTGF- β 3. This is in contrast to all rabbits immunized against TGF- β 1, which developed antisera that blocked receptor binding and biological activity of pTGF- β 1, pTGF- β 2 and rcTGF- β 3. Although rabbit anti-TGF- β 2 neutralized TGF- β 2 and immunoprecipitated TGF- β 2 with complete specificity, on Western blots these antisera did not distinguish between TGF- β 1 or TGF- β 2. Turkeys were immunized and boosted with the above method and gave better titers with less than half as much antigen than before. Titers of antisera against TGF- β 2 in turkeys were considerably lower than antisera against TGF- β 1. Unlike rabbit antisera, on Western blots turkey antisera detected only the TGF- β isoform the antisera was raised against.

Recently, we have immunized two rabbits against rcTGF- β 3. Both rabbits developed antisera that were able to neutralize 125 I-rcTGF- β 3 receptor binding and the biological activity of rcTGF- β 3. However, anti-rcTGF- β 3 even at saturating concentrations did not neutralize any TGF- β activity in media conditioned by either differentiated C₂C₁₂ skeletal myotubes or primary cultures of neonatal rat cardiac myocytes, both of which express high levels of TGF- β 3 mRNA and secrete a TGF- β activity that is not neutralized by either anti-TGF- β s 1 or 2. These data are suggestive of an important difference between the post-translational modification of the recombinant and natural forms of TGF- β 3. Complete verification of this will require the availability of purified natural TGF- β 3. Anti-TGF- β 3 immunoprecipitated 125 I-rcTGF- β 3, as well as 125 I-pTGF- β 2, but did not immunoprecipitate 125 I-pTGF- β 1. Antibodies raised to pTGF- β 2 do not immunoprecipitate 125 I-rcTGF- β 3. The endogenous putative TGF- β 3 in medium from metabolically labeled rat neonatal cardiac myocytes was immunoprecipitated with anti-rcTGF- β 3 once it had been precleared of TGF- β 2 by immunoprecipitation with rabbit anti-TGF- β 2 IgG. This endogenous TGF- β 3 migrated with pTGF- β s 1 and 2 on SDS-polyacrylamide gels and had an apparent molecular weight slightly less than that of rcTGF- β 3, further supporting the possibility of a difference in the post-translation modification of these mature forms of TGF- β 3.

Among a variety of different assays for measuring TGF- β s developed using turkey, rabbit or both turkey and rabbit neutralizing antibodies, our results support that sandwich enzyme-linked immunosorbent assays (SELISAs) are the method of choice for rapid, specific, precise and sensitive methods for the measurements of TGF- β s 1 and 2 in biological fluids. We have been using these assays for several important studies on the regulation of the expression of TGF- β 1 and TGF- β 2 by various modulators such as retinoic acid, growth factors, steroid hormones, and steroid hormone antagonists. These assays have also been used to study the localization of TGF- β s in tissues and within cells. A major difficulty in the extended use of the TGF- β 1 SELISA is the limited availability of rabbit anti-TGF- β 1 antisera, which give relatively low titers. For this reason we are attempting to enhance the immunogenicity of TGF- β 1. One promising approach in progress is coimmunization of rabbits with pTGF- β s 1 and 2. Several attempts at developing a SELISA for TGF- β 3s using antibodies against synthetic peptides of TGF- β 3 or recombinant chicken and human TGF- β 3 have not been successful. Further work on development of a TGF- β 3 SELISA is in progress.

Publications:

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Danielpour D, Kim KY, Dart LL, Watanabe S, Roberts AB, Sporn MB. Evidence for differential regulation of TGF- β 1 and TGF- β 2 expression by sandwich enzyme-linked immunosorbent assays. *Ann NY Acad of Sci.* 1990;593:300-3.

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Flanders KC, Cissel DS, Mullen LT, Danielpour D, Sporn MB, Roberts AB. Antibodies to transforming growth factor- β 2 peptides: specific detection of TGF- β in immunoassays. *Growth Factors* 1990;3:45-52.

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Heine UI, Burmester JK, Flanders KC, Danielpour D, Munoz EF, Roberts AB, Sporn MB. Localization of transforming growth factor- β 1 in mitochondria of murine heart and liver (In Press).

MacKay K, Kondaiah P, Danielpour D, Austin III HA, Brown PD. Expression of transforming growth factor- β 1 and β 2 in rat glomeruli. *Kidney Int* 1990;38:1095-100.

Roberts AB, Kondaiah P, Frederic R, Watanabe S, Good P, Danielpour D, Nanette SR, Rebbert ML, Dawid IB, Sporn MB. Mesoderm induction in Xenopus laevis distinguishes between the various TGF- β isoforms. Growth Factors 1990;3:277-86.

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

PROJECT NUMBER

Z01CP05661-01 LC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Mechanism of Prostate Carcinogenesis and Chemoprevention by Retinoids

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

PI:	Mario A. Anzano	Expert	LC	NCI
Others:	Kenji Kadomatsu	Visiting Fellow	LC	NCI
	Joseph M. Smith	Biologist	LC	NCI

COOPERATING UNITS (if any)

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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.)

Prostate cancer is the second cause of cancer death in the male population. The lack of progress in treatment and management of this disease is hampered by inadequate experimental animal models. The purpose of this project is to develop and establish an efficient and reproducible animal model system for inducing prostate carcinogenesis; analyze the molecular and cellular events following the development of prostate cancer and to identify agents that prevent and/or suppress prostate cancer incidence. We have initiated induction of prostate carcinogenesis by initiation with N-methyl-nitrosourea (NMU) and promotion with testosterone propionate (TP) in Lobund/Wistar rats of varying age ranging from 2-4 months old. We have partially succeeded in growing primary cultures of normal rat prostate epithelium for 3 weeks in chemically defined media supplemented with exogenous nutrients and growth promoting substances. Using 3 month old rats, we have shown increased levels of TGF- β 1 and β 3 message and protein at 3-6 days following castration. This effect was reversed by treatment with TP. In collaboration with Dr. Morris Pollard, Lobund Laboratory, we have demonstrated for the first time decreased incidence of prostate cancer in MNU/TP treated animals fed with N-(4-Hydroxyphenyl)retinamide.

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

Mario A. Anzano	Expert Scientist	LC	NCI
Kenji Kadomatsu	Visiting Fellow	LC	NCI
Joseph M. Smith	Biologist	LC	NCI

Objectives:

The objectives of this project are: 1) to develop an efficient and reproducible animal model for prostate carcinogenesis; 2) to characterize the molecular and cellular changes in growth factor (TGF- β , FGF), oncogene (ras mutation) and oncogene (RB, p53, DCC) expression following the onset of prostatic cancer development; 3) to generate primary cultures and cell lines of rat prostate epithelium and fibroblast from Lobund/Wistar rats at various times following MNU/TP treatment, and 4) to identify chemopreventive agents such as retinoids and steroids against prostate cancer.

Methods Employed:

Methods for induction of prostatic carcinogenesis involve initiation with a single dose of NMU (30 mg/Kg) and promotion with TP (45 mg/rat) in Lobund/Wistar rats. Growth factor, oncogene and suppressor gene expression will be analyzed using standard molecular biology techniques including northern blot hybridization of mRNA, in situ hybridization, polymerase chain reaction (PCR) methodology and cellular biology techniques such as radioimmunoassay, tissue culture of primary and established prostate epithelial and mesenchymal cells, immortalization of primary prostate epithelial cells by retroviral transfection and standard protocols for handling and surgery of laboratory animals.

Major Findings:

TGF- β expression in castrated rats. We have shown increased TGF- β 1 and β 3 mRNA and protein levels following castration of Lobund/Wistar rats. Using established human prostate cell lines, we have observed that cells that are androgen responsive (LNCap) have undetectable levels of TGF- β 1 message, whereas cells that are androgen insensitive (PC-3 and DU-145) show high levels of TGF- β 1 message indicating negative regulation of TGF- β 1 expression by androgens. Using immunochemistry, we have observed high levels of TGF- β 1 and β 3 staining in castrated rats after 4 days, especially in areas of prostate epithelium showing apoptotic bodies.

Chemoprevention of prostate cancer by N-(4-Hydroxyphenyl)retinamide. In collaboration with Dr. Morris Pollard, Lobund Laboratory, Univ. of Notre Dame, we have demonstrated suppression of primary prostate

cancer and metastatic tumor incidence in MNU/TP treated rats for 7 months following initiation of prostate carcinogenesis when fed with diets containing 1.0 mole of 4-HPR/Kg diet.

Publication:

Morris P, Luckert PH, Sporn MB. Prevention of primary prostate cancer in Lobund/Wistar rats by N-(4-Hydroxyphenyl)retinamide. Cancer Res (In Press).

ANNUAL REPORT OF

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

October 1, 1990 through September 30, 1991

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.

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

The Developmental Biology Working Group and its projects on the roles of oncogenes in pathogenesis of naturally-occurring tumors in humans and in chemically induced tumors in rodents was administratively transferred from the Perinatal Carcinogenesis Section, and the Primate Research Working Group was transferred to that Section this year.

The role of ras (especially K-ras) and of neu in chemically induced tumors in rodents continues to be investigated in efforts to distinguish between activating mutations that are the direct result of chemical reactions of carcinogenic agents with DNA encoding these specific genes, and stochastic events that occur independently of the inducing carcinogen during tumor progression. Studies on rat renal mesenchymal tumors have been especially informative in this regard. Extrapolation of activating mutational events in experimental tumors to human cancers that involve activation of the corresponding human oncogene; human pediatric renal tumors, as well as mucinous carcinoma of the ovary, endometrial carcinoma of the uterus, the "intestinal" variant of human gastric carcinomas and prostatic carcinoma are currently under investigation.

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

The effects of carcinogens encountered during the perinatal period are modulated by (1) the capacity of perinatal tissues to activate the chemicals and (2) postnatal tumor promotive influences, among other factors. With regard to activation, attention is currently being focused on formation of DNA adducts by benzo[a]pyrene (BP) in the placenta and fetal tissues of patas monkeys, assayed by ³²P-postlabeling (with Dr. L.J. Lu); and on effects of the arylamine food mutagen, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). IQ is activated metabolically by cytochrome P450IA2, with several other isozymes contributing to detoxification. A study of transplacental carcinogenesis by IQ in mice and hamsters is ongoing. Preliminary to investigation of ontogeny of IQ metabolism as related to tumorigenesis, the effects of IQ as an enzyme inducer is being studied in mice. Significant increases in a cytochrome P450IA1 enzyme activity and decreases in total cytochrome P450 in C57BL/6 mice were noted. Study of tumor promotive influences has concentrated on the actions of retained congeners of polychlorinated biphenyls (PCBs), after a single dose of the mixture Aroclor 1254, and of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). A preliminary study of the effects of restriction in dietary fat after PCB treatment showed that weight loss increased toxicity and increased level of induction of a specific cytochrome P450 isoform associated with tumor promotion, presumably due to release of stored PCB congeners from fat depots. A single dose of TCDD had persistent, organ-specific inducing effects, with lung being more sensitive than liver to continued induction after low doses of TCDD.

Ethanol, while at best a weak carcinogen in animal models, increases human risk of cancer at several sites. Elucidation of the mechanism by which it does so would not only illuminate this particular public health issue but also provide insight into the more general question of the impact of interaction between environmental chemicals on cancer risk. Current efforts focus on the effect of ethanol on carcinogen metabolism and clearance in mice. In collaboration with Dr. P.G. Forkert, cytochrome P450IIE1, which activates environmental nitrosamines such as N-nitrosodimethylamine (NDMA), was found to be induced 2- to 7-fold by ethanol in drinking water or liquid diet, as indicated by enzyme assay and Western immunoblotting with a specific monoclonal antibody. Immunohistochemical staining showed uniform staining in centrilobular hepatocytes. Ethanol competitively inhibits, as well as induces, P450 activity toward NDMA, and this was investigated systematically in a toxicokinetic study. Doses of 10-20% ethanol given i.g. before i.v. doses of 1-10 mg/kg NDMA had large negative effects on clearance parameters, up to 25-fold. Even more striking inhibition was seen with oral co-administration, up to 250-fold. Finally, ethanol in the drinking water was found to increase a UDP-glucuronosyltransferase participating in clearance of polycyclic aromatic hydrocarbons.

Long-term observation of transplacentally carcinogen-exposed animals of the species Erythrocebus patas and Macaca fascicularis for tumor development, and concurrent short-term investigations of aspects of carcinogen metabolism, with biochemical parameters as an endpoint, continue. Continuing studies on

immunodeficiency virus-associated antigens are now reported. Preliminary characterization of the pleiotropic response of patas monkeys to hepatic enzyme induction by phenobarbital (PB) has been accomplished. This primate species was previously shown to be susceptible to promotion of hepatocellular carcinogenesis by PB. Initial experiments in PB-treated monkeys suggest the existence of a pleiotropic induction effect involving CYP2B and 3A, epoxide hydrolase, aldehyde dehydrogenase and other enzymes that is comparable to the response previously characterized in rodents.

In a study of in vivo interactions of important environmental carcinogens and toxicants, both ethanol and isopropanol have been found to have profound effects on the metabolism of N-nitrosodimethylamine (NDMA) in male patas monkeys, leading to much reduced blood clearance and increased urinary excretion of the carcinogen by several orders of magnitude. This has important implications for the human, since the cytochrome P450 involved, IIE1, has similar biochemical properties in humans and patas monkeys.

Investigation of the in vivo metabolism and toxicokinetics of the tobacco-specific nitrosamine carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in a female patas monkey is revealing rapid clearance of low doses and extensive α -carbon hydroxylation (activating metabolism) with the possibility of high adduction ratio in the animal. A new metabolite has been observed; identification is in progress.

In an ongoing study of the diol-epoxide adduct of the environmental polycyclic aromatic hydrocarbon carcinogen benzo[a]pyrene in patas fetal and placental tissues, level of adduct formation has been found to relate in part to placental/fetal weight ratio, suggesting important protective functions of the placenta. Adducts disappeared almost completely from DNA of placenta and fetal tissues over a 50-day time course, while total DNA content decreased only slightly, indicating effective repair processes in these tissues.

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.

The mechanisms by which cadmium and certain other metals induce cancer are currently under study at various levels. On the level of the whole animal, association of oral cadmium exposure and neoplasia of the rat prostate was confirmed, an important advance in support of cadmium as a factor in human prostatic cancer. Dietary zinc deficiency was found to either enhance or suppress cadmium carcinogenesis depending on the route of exposure to cadmium and/or the specific target tissue. The F344 rat was found to be particularly susceptible to sarcoma at the site of cadmium injection, indicating a genetic basis of susceptibility to tumor formation even at this target site. On the cellular and molecular level, it was found that cadmium is clearly capable of producing profound DNA damage, including frequent DNA single strand breakage. Pretreatments, such as zinc, which prevent cadmium carcinogenesis in certain tissues also prevent these genotoxic effects of cadmium. In testicular interstitial cells, several studies were directed at identifying factors involved in chemically-induced or genetically-based resistance. Testicular interstitial cells from strains of mice resistant to the effects of cadmium on the testes showed less cadmium uptake and

more efflux than cells from susceptible mice. On the molecular level, it was determined that the testicular metallothionein gene does not play a major role in tolerance to cadmium carcinogenesis induced by pretreatment with zinc, as no differences in metallothionein mRNA levels were detected in tolerant cells. These findings support the concept that unresponsiveness to induction of the metallothionein gene is a consistent finding in target tissues of cadmium carcinogenesis.

Studies on the mechanism of action of the carcinogenic element nickel, carried out in this Section, have been administratively transferred this year to the Chemistry Section.

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.

Nucleophile/NO complexes have been found to have a number of interesting biological activities. Members of this compound class are potent vasorelaxants both *in vivo* and *in vitro*. One of them is a good inhibitor of platelet aggregation. Several have the ability to damage DNA in a suitably oxidizing environment, with the corollary property of being mutagenic in Salmonella typhimurium. The biological activity is correlated to a greater or lesser extent with the rate and extent of spontaneous nitric oxide release these complexes engender. The nucleophile/NO complexes should be useful both as research tools and for possible drug design strategies.

Oxidation of unsymmetrically deuteriated N-nitrosodimethylamine by acetone-induced rat liver microsomes was found to proceed with little or no regioselectivity with respect to enzymatic attack on the syn versus anti methyl groups, in contrast to the high stereospecificity expected from the action of most enzymes. Acetylation of the potent bacterial mutagen, fecapentaene-12, was shown to improve its solubility in dimethylsulfoxide, allowing the compound to be tested in higher molar doses than in previous carcinogenicity studies with the underivatized material; despite very high exposures on repeated skin painting of this material in SENCAR mice, no increase in tumor incidence was observed. This was true whether or not Vitamin E was added to the test agent to prevent atmospheric oxidation during application. The results are inconsistent with the conclusion that the potently mutagenic fecapentaenes are mammalian carcinogens.

Nitric oxide (NO) has been reacted with a variety of nucleophiles to form adducts of widely differing properties, extending our understanding of the electrophilic behavior of NO. Many of these compounds have shown both vasorelaxant activity in isolated rabbit aorta and *in vivo* hypotensive action on intravenous injection in rats. The *in vitro* potency was strongly correlated with the amount of nitric oxide these compounds were independently shown to release on spontaneous decomposition in physiological buffers, indicating that compounds in this series are of predictable potency based on physicochemical data. The most active of these

agents proved comparable in potency to the clinical vasodilators, sodium nitroprusside and nitroglycerin. Duration of action in the in vivo experiments was most prolonged using a slow-release compound, the spermine/NO complex. The nucleophile/NO adducts have been derivatized by attaching alkyl groups or metal ion centers to either or both of the oxygen atoms; the resulting complexes have interesting properties as potential prodrugs. Other tests have shown the diethylamine/NO complex to be an inhibitor of platelet aggregation rivaling aspirin in potency. The results show that the nucleophile/NO complexes should be very useful tools for probing the involvement of nitric oxide in a variety of biomedical applications. Nitric oxide has also been shown to be capable of deaminating nucleic acids and their constituent units at pH 7.4 in the presence of air. NO-releasing compounds, including the nucleophile/NO complexes mentioned above and nitroglycerin, have been shown to induce point mutations in bacteria; nearly all the cases identified so far have been GC to AT transitions. The in vivo significance of these genotoxic deamination reactions is currently being evaluated.

Molecular mechanisms of nickel (Ni) genotoxicity were studied. The working hypothesis tested assumes that Ni derivatives initiate tumors through active oxygen species. Increased contents of a DNA oxidation product, 8-hydroxy-2'-deoxyguanosine (8-OHdG) were found in DNA isolated from NRK-52 and NIH 3T3 cells and from kidneys of mice exposed to Ni(II). Moreover, the magnitude of 8-OH-dG increase by Ni was greatest in kidneys of BALB/c mice, i.e., mice which were also more susceptible to renal lipid peroxidation (LPO) by Ni than C3H, B6C3F1, and C57BL mice. No such concurrence was observed in NRK-52 and NIH 3T3 cells indicating that LPO and nucleobase oxidation might constitute two independent phenomena. Our hypothesis was also tested on pure 2'-deoxyguanosine (dG), DNA, and nuclear chromatin isolated from the human-derived K562/S cell line. Three major discoveries were made in these systems: (1) activation of hydrogen peroxide by Ni is facilitated by L-histidine (His), a principal in vivo Ni carrier, and by tetraglycine (TG), a model Ni-binding peptide; (2) Ni + H₂O₂ modifies all 4 DNA bases; besides 8-OH-dG, 10 more potentially mutagenic products were identified; (3) nuclear proteins enhance Ni-catalyzed attack of H₂O₂ or O₂ on DNA. The relatively high sensitivity of guanine to attack by Ni-mediated oxygen radicals is consistent with correspondingly strong complex formation between Ni and dG. It is also consistent with growing evidence that G:C base pairs are the major site of point mutation in the K-ras oncogene isolated from Ni-induced renal tumors in rats (see Project Z01CP05399). Further, both TG and L-histidine (His) enhanced DNA-protein cross-linking in kidneys of rats injected with Ni, a phenomenon characteristic for oxygen radical attack on cell nuclei. Studies on the mechanisms of yet another genotoxic effect by Ni subsulfide (Ni₃S₂), deamination of 5-methyl-2'-deoxycytidine (5MedC), disclosed that the deamination is caused by O₂ activated through auto-oxidation of the sulfur moiety of Ni₃S₂. Hence, sulfur is capable of increasing Ni genotoxicity. This may account for the uniquely high carcinogenicity of Ni₃S₂ compared with other Ni derivatives.

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 cellular and biological mechanisms for tumor promotion in nonsquamous epithelium of rodents is under investigation. In select rodent renal and hepatic tumor promotion model systems, it was discovered that tumor promoters may enhance carcinogenesis by selectively stimulating the growth and progression of preneoplastic foci into benign or malignant tumors. Barbital sodium, a nephrotoxin and human pharmaceutical agent, was found to promote renal tubular epithelial carcinogenesis by targeting preneoplastic dysplastic renal tubular lesions. Use of N-bis(2-hydroxypropyl)nitrosamine, streptozotocin or potassium bromate as renal tubular cell tumor initiators produced preneoplastic dysplastic renal epithelial lesions. Barbital sodium, a renal tubular epithelial tumor promoter, enhanced renal carcinogenesis after either of the initiators.

Studies continue to further characterize the ability of phenobarbital (PB) and certain other compounds, including polychlorinated biphenyls (PCB) to induce a specific pleiotropic effect which includes the induction of cytochrome P450IIB1 (CYP2B1) and to promote liver tumorigenesis in diethylnitrosamine (NDEA)-initiated animals. Specifically, A) We looked at the ability of a variety of compounds to induce this pleiotropic response and to promote NDEA-initiated tumorigenesis in B6D2F1 mice. As observed previously with F344 rats, we found a strong correlation between potency as an inducer and potency as a promoter of hepatic adenomas, carcinomas, and hepatoblastomas. Certain chemicals including 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), pentobarbital and ethylphenylhydantoin showed striking species-dependent differences in ability to induce CYP2B1 and promote hepatocarcinogenesis. B) In preliminary studies, we examined the ability of Aroclor 1254 (a mixture of PCBs) to induce various cytochromes P450 (IA, IIB) in various rodent species. While all rodents showed a PCB dose-dependent increase in P450 activities, there were striking differences in sensitivity among species (rat greater than mouse greater than *Reithrodontomys fulvescens*). C) In a series of inbred rat strains we observed induction of a pleiotropic effect (CYP2B, 3A, epoxide hydrolase) in all strains, although there were minor variations. D) Initial experiments in PB-treated monkeys imply a similar pleiotropic effect to that observed in rodents (i.e., induction of CYP2B, 3A, epoxide hydrolase, aldehyde dehydrogenase).

Vimentin metaplasia, a newly discovered pathologic lesion, was found to be associated with preneoplastic and neoplastic rat and human renal lesions. The vimentin phenotype, usually reserved for mesenchymal tissues, was discovered in renal lesions of rats exposed to various renal epithelial carcinogens and tumor promoters. Similar lesions were found by others and by ourselves in human renal lesions. The mechanisms responsible for this unusual phenomenon are being studied.

A novel model to study myoepithelial biology and carcinogenesis was discovered using mammary tumors induced in mice by 7,12-dimethylbenz[a]anthracene. The tumors were transplanted and cultivated in vitro. The tumors represent the first valid model of myoepithelial carcinogenesis in any species. An animal model for pulmonary bronchiolar carcinogenesis were discovered and developed. Using N-nitroso-(2-chloroethyl)urea, a spectrum of pulmonary hyperplastic and neoplastic bronchiolar cell lesions were induced. Tumor cells expressed antigens found within normal Clara cells. This new experimental system represents a novel model for studying the biology and origin of these tumors.

A new giant cell enteritis was discovered in rhesus monkeys infected with SIV/Delta. Monkeys had diarrhea and inflammatory intestinal lesions with

multinucleated syncytial giant cells expressing SIV antigens. Several infected rhesus monkeys also developed B cell lymphomas in association with marked lymphoid B cell hyperplasia. One monkey had a pancreatic adenocarcinoma. These unusual findings mimic certain unusual aspects of human lentiviral infection and its outcomes.

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

PROJECT NUMBER

Z01CP04542-19 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	R. W. Nims	Chemist	LCC	NCI
	R. A. Lubet	Expert	LCC	NCI
	D. E. Devor	Biologist	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
	J. M. Rice	Chief	LCC	NCI

COOPERATING UNITS (if any) Program Resources, Inc./DynCorp., Frederick, MD (C. Jones, J. R. Henneman); ABL-Basic Research Program, Frederick, MD (C. Michejda, M.B. Kroeger-Koepke); Rutgers Univ., Piscataway, NJ (C.S. Yang)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21702-1201

TOTAL MAN-YEARS:

3

PROFESSIONAL:

2.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

Oxidation of unsymmetrically deuterated N-nitrosodimethylamine by acetone-induced rat liver microsomes was found to proceed with little or no regioselectivity with respect to enzymatic attack on the syn versus anti methyl groups, in contrast to the high stereospecificity expected from the action of most enzymes. Acetylation of the potent bacterial mutagen, fecapentaene-12, was shown to improve its solubility in dimethylsulfoxide, allowing the compound to be tested in higher molar doses than in previous carcinogenicity studies with the underivatized material; despite very high exposures on repeated skin painting of this material in SENCAR mice, no increase in tumor incidence was observed. This was true whether or not Vitamin E was added to the test agent to prevent atmospheric oxidation during application. The results are inconsistent with the conclusion that the potently mutagenic fecapentaenes are mammalian carcinogens.

PROJECT DESCRIPTION

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

L. K. Keefer	Chief, Chemistry Section	LCC	NCI
R. W. Nims	Chemist	LCC	NCI
R. A. Lubet	Expert	LCC	NCI
D. E. Devor	Biologist	LCC	NCI
J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
J. M. Rice	Chief	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 exposure to the carcinogenic effects of nitrosamine; (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:

Though much of the resource base previously devoted to this project has been temporarily diverted to the Section's studies on the chemistry and biology of nitric oxide, progress has nevertheless continued. In the fecapentaene area, earlier results had shown that acetylation of the hydroxyl groups in fecapentaene-12 greatly improved its solubility in dimethylsulfoxide and that vitamin E (α -tocopherol) retarded atmospheric oxidation of this sensitive substrate. These findings were incorporated into the experimental design in a further attempt to probe the possible mammalian carcinogenicity of this potent bacterial mutagen family. However, fecapentaene-12 diacetate yielded no evidence of tumor-inducing potential on skin painting in SENCAR mice with or without the copresence of vitamin E, even at high dose levels. We conclude that the fecapentaenes do not warrant further research attention in this laboratory.

Regarding the chemistry and biology of nitrosamines, *N*-nitrosodimethylamine (NDMA) was synthesized in a form containing isotopically distinguishable syn and anti methyl groups, with the methyl group syn to the nitroso oxygen being deuterated in one preparation and the anti methyl group being fully deuterated in the other isotopic variant. This rendered the two methyl groups chemically distinguishable, allowing tests of the regioselectivity of the nitrosamine-metabolizing enzymes to be conducted. These tests have now been completed in collaboration with Drs. C.S. Yang, M.B. Kroeger-Koepke, and C.J. Michejda. The nitrosamine solutions were

incubated with acetone-induced rat liver microsomes and cofactors. The rates of metabolism of both substrates, though similar to each other, were very different from those of the fully deuterated or completely undeuterated nitrosamine molecules. These kinetic deuterium isotope effect data point to a lack of regioselectivity on the part of the nitrosamine-metabolizing enzymes, which are apparently able to oxidize either the syn or the anti methyl group with approximately equal facility. The kinetic results were confirmed using formaldehyde trapping experiments, in which this principal oxidative metabolite of NDMA was trapped as the dimedone adduct and analyzed by mass spectrometry; the deuterium content of the formaldehyde was approximately equal for both the syn and trideuterated substrates, and again different from the outcome with the fully deuterated NDMA. Thus, in contrast to results with many enzymes, which are stereospecific in their action, NDMA-demethylase displays little regioselectivity in its action.

Publications:

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Devor DE, Henneman JR, Keefer LK, Logsdon DL, Rice JM, Streeter AJ, Ward JM. Carcinogenicity study of fecapentaene-12 diacetate on skin painting in SENCAR mice. *Cancer Lett* 1991;56:11-5.

Ho MYK, Keefer LK, Chen TK. Determination of 3-methyl-4,5-dihydro-1,2,3-oxadiazolium ion, a putative nitrosamine metabolite, by ion-pair chromatography with electrochemical detection. *Anal Chim Acta* 1990;232:397-400.

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Streeter AJ, Nims RW, Sheffels PR, Hrabie JA, Ohannesian L, Heur Y-H, Mico BA, Keefer LK. Deuterium isotope effect on the toxicokinetics of monomethylamine in the rat. *Drug Metab Dispos* 1990;18:447-52.

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Sullivan BP, Meyer TJ, Stershic MT, Keefer LK. Acceleration of N-nitrosation reactions by electrophiles. In: O'Neill IK, Chen JS, Bartsch H, eds. Relevance to human cancer of nitroso compounds, tobacco, and mycotoxins. Lyon: IARC Sci Publ 1991;370-4.

von Hofe E, Schmerold I, Nims RW, Keefer LK, Reist EJ, Kleihues P. β -Deuteration of N-nitrosoethylmethylamine causes a shift in DNA methylation from rat liver to esophagus. Carcinogenesis 1991;12:545-9.

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

PROJECT NUMBER

Z01CP04582-16 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Mechanisms of Nickel Carcinogenesis

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

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	J. M. Ward	Chief, Tumor Pathology & Pathogenesis Sect.	LCC	NCI
	A. K. Datta	Intramural Research Training Awardee	LCC	NCI
	M. Misra	Visiting Fellow	LCC	NCI
	A. O. Perantoni	Staff Fellow, Develop. Biology Working Group	LCC	NCI
	S. L. North	Biologist	LCC	NCI

COOPERATING UNITS (if any) Program Resources, Inc./DynCorp, Frederick, MD (B. Diwan); Data Mgmt. Systems, Inc., Frederick, MD (C. Riggs, M.J. Fivash); ABL-Basic Research Prog., Frederick, MD (L. Hernandez); NIST, Gaithersburg, MD (M. Dizdaroglu)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

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Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 21702-1201

TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

2.5

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.)

Molecular mechanisms of nickel (Ni) genotoxicity were studied. The working hypothesis tested assumes that Ni derivatives initiate tumors through active oxygen species. We found increased contents of a DNA oxidation product, 8-hydroxy-2'-deoxyguanosine (8-OHdG) in DNA isolated from NRK-52 and NIH 3T3 cells and from kidneys of mice exposed to Ni(II). Moreover, the magnitude of 8-OH-dG increase by Ni was greatest in kidneys of BALB/c mice, i.e., mice which were also more susceptible to renal lipid peroxidation (LPO) by Ni than C3H, B6C3F1, and C57BL mice. No such concurrence was observed in NRK-52 and NIH 3T3 cells indicating that LPO and nucleobase oxidation might constitute two independent phenomena. Our hypothesis was also tested on pure 2'-deoxyguanosine (dG), DNA, and nuclear chromatin isolated from the human-derived K562/S cell line. Three major discoveries were made in these systems: (1) activation of hydrogen peroxide by Ni is facilitated by L-histidine (His), a principal *in vivo* Ni carrier, and by tetraglycine (TG), a model Ni-binding peptide; (2) Ni + H2O2 modifies all 4 DNA bases; besides 8-OH-dG, 10 more potentially mutagenic products were identified; (3) nuclear proteins enhance Ni-catalyzed attack of H2O2 or O2 on DNA. The relatively high sensitivity of guanine to attack by Ni-mediated oxygen radicals is consistent with the correspondingly strong complex formation between Ni and dG. It is also consistent with growing evidence that G:C base pairs are the major site of point mutation in the K-ras oncogene isolated from Ni-induced renal tumors in rats (see Project Z01CP05399). Further, both TG and His enhanced DNA-protein cross-linking in kidneys of rats injected with Ni, a phenomenon characteristic for oxygen radical attack on cell nuclei. Our studies on the mechanisms of yet another genotoxic effect by Ni subsulfide (Ni3S2), deamination of 5-methyl-2'-deoxycytidine (5MedC), disclosed that the deamination is caused by O2 activated through autooxidation of the sulfur moiety of Ni3S2. Hence, sulfur is capable of increasing Ni genotoxicity. This may account for the uniquely high carcinogenicity of Ni3S2 compared with other Ni derivatives.

PROJECT DESCRIPTION

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

K. S. Kasprzak	Visiting Scientist	LCC	NCI
J. M. Rice	Chief	LCC	NCI
J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
L. K. Keefer	Chief, Chemistry Section	LCC	NCI
A. K. Datta	Intramural Research Training Awardee	LCC	NCI
M. Misra	Visiting Fellow	LCC	NCI
A. O. Perantoni	Staff Fellow, Develop. Biology Working Group	LCC	NCI
S. L. North	Biologist	LCC	NCI

Objectives:

To study mechanisms of nickel carcinogenesis by investigating a hypothesis that nickel initiates tumors by damaging cellular genetic material through reactions with oxygen species activated by the Ni(III)/Ni(II) redox couple. This may result in DNA strand breaks, DNA-protein and interprotein cross-linking and/or modification of DNA bases. Both types of lesions may lead to erratic DNA replication (mutations) and gene expression, and thus facilitate cell transformation and development of tumors.

Major Findings:

Testing of the above hypothesis has been continued in cell-free systems, in cultured NRK-52 and NIH 3T3 cells, and in mice of different strains. Increased contents of a major DNA oxidation product, 8-hydroxy-2'-deoxyguanosine (8-OHdG) were found in DNA isolated from the cells and from kidneys of mice exposed to soluble Ni(II) salts. Thus, our original finding of Ni-enhanced 8-OH-dG production in rats was confirmed in other species. Moreover, in mice, the magnitude of 8-OH-dG increase by Ni was greatest in kidneys of BALB/c mice, i.e., mice which were also more susceptible to Ni-caused renal lipid peroxidation (LPO) than C3H, B6C3F1, and C57BL mice. No such concurrence was observed in NRK-52 and NIH 3T3 cells. This indicates that lipid peroxides may not necessarily damage DNA and that LPO and nucleobase oxidation constitute independent phenomena. Our hypothesis was also tested on pure 2'-deoxyguanosine (dG), calf thymus DNA, and nuclear chromatin isolated from the human-derived K562/S (leukemia) cell line. Three major discoveries were made in these systems: (1) activation of hydrogen peroxide (H₂O₂) by Ni to attack dG (either free or DNA-bound) is facilitated by L-histidine (His) which is the principal Ni carrier in cells and tissues and by tetraglycine (TG), a model Ni-binding peptide; (2) Ni + H₂O₂, acting on nuclear chromatin modifies all four nucleobases; besides 8-OH-dG, ten more different, potentially mutagenic products were identified including one, 2-hydroxyadenine, which has not been described before; and (3) nuclear proteins enhance Ni-catalyzed attack of H₂O₂ or O₂ on DNA. The effect of His on DNA oxidation was complex. In the absence of Ni, His completely prevented dG oxidation to 8-OH-dG with ascorbate + H₂O₂; this inhibition could be attenuated and even turned into enhancement by addition of increasing amounts of Ni. Further increase of 8-OH-dG production could be achieved by withdrawing ascorbate from the system. The relatively highest sensitivity of guanine among the nucleobases to Ni-mediated oxygen radicals' attack, found in the present experiments, is consistent with our most recent finding of correspondingly stronger chemical interaction of Ni

with dG than with the remaining DNA bases. The latter also concurs with further evidence that guanine is the major site of point mutation in the K-ras oncogene isolated from Ni-induced renal tumors in rats (compare Project Z01CP05399-08). The in vitro experiments were followed by in vivo tests in rats: i.v. administration of either TG or His enhanced DNA-protein cross-linking in kidneys of rats injected with a single i.v. Ni dose. Formation of such cross-links is characteristic for oxygen radical attack on cell nuclei. It proved that the oxidative phenomena observed with Ni + His or TG in vitro can be reproduced in animals, most likely with participation of endogenous H₂O₂, other peroxides, and/or O₂. The active oxygen species which interact with Ni at target cells for Ni carcinogenesis do not originate from phagocytes. A bioassay in rats injected i.m. with Ni₃S₂ plus an inflammatory agent, M. bovis cell walls, did not enhance Ni carcinogenesis. On the contrary, M. bovis greatly enhanced phagocytosis of Ni₃S₂ particles and inhibited tumor formation. This finding, once again, indicated the very complex character of Ni carcinogenesis involving, among other factors, the cellular immune defenses.

The nature of yet another genotoxic effect mediated by nickel subsulfide (Ni₃S₂) and its metabolite Ni(II)sulfite, i.e., deamination of 5-methyl-2'-deoxycytidine, has been further explored in a cell-free in vitro model. The deamination was found to take place only in the presence of oxygen. This effect is produced by oxygen activated during autooxidation of the sulfide and sulfite moieties of Ni₃S₂. Thus, sulfur in Ni₃S₂ appears to be capable of increasing the genotoxic potential of Ni. This, in turn, may explain the exceptionally high carcinogenicity of Ni₃S₂ compared with other Ni derivatives and, perhaps, carcinogenic activity of sulfides of other transition metals.

Publications:

Datta AK, Riggs CW, Fivash MJ, Kasprzak KS. Mechanisms of nickel carcinogenesis. Interaction of Ni(II) with 2'-deoxynucleosides and 2'-deoxynucleotides. Chem Biol Interact (In Press).

Kasprzak KS. Metal interactions in nickel, cadmium, and lead carcinogenesis. In: Foulkes EC., ed. Biological effects of heavy metals, vol. 2, metal carcinogenesis. Boca Raton: CRC Press, 1990;173-89.

Kasprzak KS, Ward JM. Prevention of nickel subsulfide carcinogenesis by local administration of Mycobacterium bovis antigen in male F344/NCr rats. Toxicology 1991;67:97-104.

Misra M, Rodriguez RE, Kasprzak, KS. Nickel-induced lipid peroxidation in the rat: correlation with nickel effect on antioxidant defense systems. Toxicology 1990;64:1-17.

Misra M, Rodriguez RE, North SL, Kasprzak KS. Nickel-induced lipid peroxidation in kidneys of different mouse strains and its relation to nickel effects on antioxidant systems. Toxicol Lett (In Press)

Nakerdien Z, Kasprzak KS, Rao G, Halliwell B, Dizdaroglu M. Nickel(II)- and cobalt(II)-dependent damage by hydrogen peroxide to the DNA bases in isolated human chromatin. Cancer Res (In Press).

Rodriguez RE, Misra M, Kasprzak KS. Effects of nickel on catalase activity in vitro and in vivo. Toxicology 1990;63:45-52.

Rodriguez RE, Misra M, North SL, Kasprzak KS. Nickel-induced lipid peroxidation in the liver of different strains of mice and its relation to nickel effects on antioxidant systems. Toxicol Lett (In Press).

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

PROJECT NUMBER

Z01CP05092-13 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Supervisory Research Biologist	LCC	NCI
	R. A. Lubet	Expert	LCC	NCI

COOPERATING UNITS (if any) SEMA, Inc., Rockville, MD (R. Bradbury); U. Texas, Galveston, TX (L.J. Lu); Oak Ridge Associated Universities, Oak Ridge, TN (N. Clapp); Temple U, Philadelphia, PA (G. Harrington); SK&F Labs., King of Prussia, PA (C. Gombar)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section, Primate Research Working Group

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

This project continues to involve long-term observation of transplacentally carcinogen-exposed animals of the species Erythrocebus patas and Macaca fascicularis for tumor development, and concurrent short-term investigations of aspects of carcinogen metabolism, with biochemical parameters as an endpoint. Continuing studies on immunodeficiency virus-associated antigens are now reported in project Z01CP05301. Preliminary characterization of the pleiotropic response of patas monkeys to hepatic enzyme induction by phenobarbital (PB) has been accomplished. This primate species was previously shown to be susceptible to promotion of hepatocellular carcinogenesis by PB. Initial experiments in PB-treated monkeys suggest the existence of a pleiotropic induction effect involving CYP2B and 3A, epoxide hydrolase, aldehyde dehydrogenase and other enzymes that in is comparable to the response previously characterized in rodents (see project Z01CP05299).

PROJECT DESCRIPTION

Names, 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	Supervisory Research Biologist	LCC	NCI
R. A. Lubet	Expert	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:

Levels of various drug metabolizing enzymes were quantified in control or PB-treated patas and cynomolgus monkeys. Preliminary evidence using primarily enzymatic techniques implies the induction of cytochromes P450IIB, IIC, and IIIA epoxide hydrolase and a form of ALDH that turns over propionaldehyde (NAD⁺ cofactor). These results imply a similar pleiotropic response in both rodents and nonhuman primates. These studies are the most comprehensive studies of induction in primates. This is the first demonstration of induction of CYP2C10 (mephenytoin) and cytosolic ALDH1 in primates. These results may help to explain the tumor promoting effects of phenobarbital in patas monkeys, as well as shed light on the effects of phenobarbital on drug/drug interactions, ethanol metabolism, etc.

Analysis has continued of the reduced blood clearance and increased urinary excretion of N-nitrosodimethylamine (NDMA) in patas monkeys by exposure to ethanol or isopropanol. Low concentrations of NDMA, an important environmental carcinogen, are metabolized in rodent and human liver by cytochrome P450IIE1, whose activity is competitively inhibited by ethanol (EtOH). In rodents, co-administration of ethanol with NDMA results in increased tumorigenicity in peripheral organs, which is related to reduced first-pass hepatic extraction. To test this concept in a primate model, we measured the effects of ethanol co-treatment on the toxicokinetics of NDMA in male patas monkeys. Ethanol, 1.2 g/kg, given orally before i.v. NDMA (1 mg/kg) or concurrently with i.g. NDMA, resulted in a 10- to 20-fold increase in area under the curve (AUC) and mean residence time (MRT) for NDMA. Isopropanol, 3.2 g/kg, 24 hrs. before treatment with NDMA, also increased AUC and MRT (about 5-fold for both parameters), in spite of the ability of this treatment to induce NDMA demethylase 5-fold in patas liver. Its effect was associated with persistence of isopropanol and/or acetone, both P450IIE1 inhibitors, in the blood, overriding any enzyme induction effect. Ethanol and isopropanol pretreatment also changed the percent of dose of NDMA excreted in the urine, which increased by 3 orders of magnitude with respect to controls. These results show that ethanol and isopropanol greatly increase NDMA systemic exposure to extrahepatic organs.

In a new effort, the toxicokinetics of the potent tobacco-specific nitrosamine carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), has been followed

in young adult patas female monkeys, using radiolabeled substrate, to establish a manipulatable primate model for the qualitative and quantitative distribution and metabolism of NNK by humans. After injection of approximately 1 mCi of tritiated NNK, disappearance of parent compound and formation and excretion of metabolites was analyzed by collaborators at the American Health Foundation in New York. The half-time in the blood was found to be 10-15 min., and the compound was extensively α -hydroxylated (activating metabolism), with excretion of the products in the urine. High levels of a new metabolite, not previously observed in rodents, were found in both blood and urine, and identification of this is in progress. This metabolite was also observed after treatment of the monkey with a smoker's dose of NNK (0.1 ug/kg). After both doses, a high percentage of the label was not recovered from the monkey, raising the possibility of high levels of adduction of the chemical in vivo in primates. These preliminary findings indicate that the patas monkey will be an interesting, useful, and novel model for study of environmental, cancer-related nitrosamines.

Study continues on the formation and repair of DNA-diol-epoxide adducts of benzo[a]pyrene (BP) in placentas and fetal tissues of patas monkeys. Adducts formed after treatment of pregnant monkeys with 50 mg/kg BP on gestation day 100 decreased over 50 days, in both placentas and fetal tissues, to 3-8% of the levels observed 24 hrs after treatment. Over the same time period the weight of the tissues increased by factors of 2-3, while average DNA content dropped by at least 30% in all tissues but lung. Therefore, the rapid loss in adducts was attributable to repair rather than DNA dilution. This is the first evidence that primate fetal tissues and placenta are capable of repair of this important promutagenic DNA adduct of an environmental carcinogen. Individual variations in adduct levels between particular fetuses is also being analyzed, and indications are seen that low adduct levels are associated with a high placental/fetal weight ratio, implicating the protective function of the physical and biochemical barrier presented by the placenta.

Publication:

Gombar CT, Harrington GW, Pylypiw HM, Anderson LM, Palmer AE, Rice JM, Magee PN, Burak ES. Interspecies scaling of the pharmacokinetics of N-nitrosodimethylamine. *Cancer Res* 1990;50:4366-70.

CONTRACT IN SUPPORT OF THIS PROJECT

SEMA, Inc., N01-CP-15657Title: Resources for Transplacental Carcinogenesis and Tumor Promotion in Old World MonkeysCurrent Annual Level: \$543,957Man Years: 0.5Objectives:

This contract provides animal care and technical support for closed colonies of patas and cynomolgus monkeys totaling 185 animals. A breeding colony of patas monkeys is maintained by this contract. 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.

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

PROJECT NUMBER

Z01CP05093-13 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

COOPERATING UNITS (if any) Program Resources, Inc./DynCorp., Frederick, MD (B. Diwan); Univ. Colorado, Denver CO (B. Beckwith)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

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INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

1.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

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

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

Currently, major emphasis is focussed on the role of stable, diffusible mediators of differentiation in the mammalian kidney. Growth and branching of the ureteric bud has been shown not to require cell-to-cell contact with metanephrogenic mesenchyme. Matrices and factors secreted by this mesenchyme may mediate these activities in vivo.

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:

Renal tubulogenesis occurs when the branching ureteric bud induces metanephrogenic mesenchyme to form primordial nephrons at branch termini. This process begins on day 13 of gestation in rats, and tubulogenesis cannot proceed in the absence of induction. We have evaluated growth factors and matrix components for ability to replace an inductive tissue and induce tubulogenesis in isolated metanephrogenic mesenchyme from 13-gestation-day rat renal rudiments. Three essential components have been identified that promote tubulogenesis in culture: type 4 collagen or laminin, epidermal growth factor, and pituitary extract. In combination, these factors in a basal medium induced tubulogenesis in 36/36 metanephric mesenchymal masses. Compaction was apparent within 24 hr, and tubular structures first arose at 5 days and developed maximally by 9 days in culture. Deletion of any of the 3 components eliminated the inductive ability of the culture conditions. In addition, replacement of pituitary extract with fetal bovine serum severely limited tubulogenesis. These data support the idea that paracrine factors and not necessarily direct cell-cell contact mediate differentiation in the kidney.

The growth and differentiation of the epithelium in many tissues is mediated by interactions with the adjacent mesenchyme, but the mechanisms responsible remain undefined. To identify the factors involved in the growth and branching morphogenesis of ureteric bud, which is the collecting duct anlagen, buds from 13-gestation-day rat embryos were separated from the metanephrogenic mesenchyme and

explanted to culture dishes coated with gelled type I collagen in a defined medium. Under these conditions, buds attached to the substrate and grew out without indication of cell crisis or senescence. When buds were instead suspended in gelled type I collagen, branching morphogenesis was observed despite the absence of mesenchyme. Since growth occurred much more slowly in culture than expected, culture conditions were varied in attempts to accelerate the process. Despite extensive screening of matrices and growth factors, only epidermal and endothelial cell growth factors stimulated growth to a significant extent. Transforming growth factor- β , on the other hand, was a potent inhibitor of growth. Homogenates from tumors that caricature metanephrogenic mesenchyme were highly mitogenic for bud cells and, thus, will be a source of material for characterizing regulatory factors involved in renal growth. These studies show that growth and branching morphogenesis of the ureteric bud do not require direct cell-cell interactions with the metanephrogenic mesenchyme and that matrices and factors secreted by the mesenchyme may mediate these activities in vivo.

Somatic cells of the developing Syrian hamster were isolated after single transplacental exposure to N-nitrosoethylurea (NEU). NEU decomposes rapidly at physiological pH and therefore acts as a pulse when given i.v. or i.p. in aqueous solution. Mutants were detected by standard in vitro genetic tests using either diphtheria toxin or 6-thioguanine as selective agents. When three litters per timepoint were used, the highest induced mutant frequencies were detected in litters treated on days 8 and 9 of gestation. When larger numbers of litters were tested (10-15 litters or >100 fetuses per time point), thus increasing the number of potential target cells, maximum mutant frequencies became apparent on treatment days 6-7. This stage, immediately post-implantation, in early embryogenesis is also the most sensitive to embryo-lethal damage, while sensitivity to induction of tumors is maximal during later (fetal) development.

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

PROJECT NUMBER

Z01CP05299-10 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
	K. Dragnev	Visiting Fellow	LCC	NCI
	R. W. Nims	Chemist	LCC	NCI

COOPERATING UNITS (if any)

Program Resources, Inc./DynCorp., Frederick, MD (B.A. Diwan, C.R. Jones); Vanderbilt U., Nashville, TN (F.P. Guengerich); Hoffmann-LaRoche, Nutley, NJ (R.M. McClain); Rutgers U., Piscataway, NJ (P. Thomas); Oklahoma State Univ, Stillwater, OK (K. McBee)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Chemistry Section

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TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

2.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

We have undertaken studies to further characterize the ability of phenobarbital (PB) and certain other compounds, including polychlorinated biphenyls (PCB), to induce a specific pleiotropic effect which includes the induction of cytochrome P450IIB1 (CYP2B1) and to promote liver tumorigenesis in diethylnitrosamine (NDEA)-initiated animals. Specifically, A) We looked at the ability of a variety of compounds to induce this pleiotropic response and to promote NDEA-initiated tumorigenesis in B6D2F1 mice. As observed previously with F344 rats, we found a strong correlation between potency as an inducer and potency as a promoter of hepatic adenomas, carcinomas, and hepatoblastomas. Certain chemicals including 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), pentobarbital and ethylphenylhydantoin showed striking species-dependent differences in ability to induce CYP2B1 and promote hepatocarcinogenesis. B) In preliminary studies, we examined the ability of Aroclor 1254 (a mixture of PCBs) to induce various cytochromes P450 (IA, IIB) in various rodent species (see also project Z01CP05352). While all rodents showed a PCB dose-dependent increase in P450 activities, there were striking differences in sensitivity among species (rat greater than mouse greater than *Reithrodontomys fulvescens*). C) In a series of inbred rat strains we observed induction of a pleiotropic effect (CYP2B, 3A, epoxide hydrolase) in all strains, although there were minor variations. D) Initial experiments in PB-treated monkeys imply a similar pleiotropic effect to that observed in rodents (i.e., induction of CYP2B, 3A, epoxide hydrolase, aldehyde dehydrogenase) (cf. project Z01CP05092).

PROJECT DESCRIPTION

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

R. A. Lubet	Expert	LCC NCI
J. M. Rice	Chief	LCC NCI
J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC NCI
K. Dragnev	Visiting Fellow	LCC NCI
R. W. Nims	Chemist	LCC NCI

Objectives:

To determine mechanisms by which a variety of compounds which induce various cytochromes P450 (especially CYP2B and CYP1A) promote tumorigenesis in liver, thyroid, and other tissues employing biochemical, genetic, and chemical techniques. A) Characterization of species (mouse vs. rat) differences in the biochemical response to PB-type inducers (barbiturates, hydantoins, TCPOBOP), and their relationship to tumor promotion/carcinogenesis in the respective species. This work showed that there was a strong relationship between induction of CYP2B and tumor promotion within a particular species (rat or mouse) and helped to explain striking species-dependent differences in susceptibility to tumor promotion by these chemicals (e.g., ethylphenylhydantoin [EPH], TCPOBOP). B) Characterization of profound differences in the response to induction of CYP1A and CYP2B by Aroclor 1254 (a mixture of PCBs) in F344 rats, B6C3F1 mice, and a feral mouse (Reithrodontomys fulvescens). These studies indicate the feasibility of these animals both as potential biomonitors of environmental exposure to various contaminants as well as the use of induction of certain forms of P450 as predictors of the potential toxic and carcinogenic effects of PCBs in these species. C) Further characterization of the pleiotropic response induced by various tumor promoters which are inducers of CYP2B1. These studies involved characterization of various inbred rat strains as well as the induction response of a cytosolic class III aldehyde dehydrogenase (propionaldehyde, NAD).

Major Findings:

Interspecies Comparisons

A. Species Differences (Mouse vs. Rat) with Respect to the Tumor Promoting Effects of Various CYP2B1-Type Inducers.

Over the last few years we have demonstrated a strong relationship between the abilities of a variety of compounds (barbiturates, hydantoins, oxazolinediones) to induce CYP2B1 and to promote NDEA-initiated liver and thyroid tumorigenesis in male F344 rats. In the present studies, we have expanded these investigations to examine the liver tumor promoting effects (using adenomas, carcinomas, and hepatoblastomas as endpoints) in NDEA-initiated D2B6F1 mice of a variety of CYP2B1-type inducers (barbiturates, hydantoins, TCPOBOP). A strong correlation was found between the induction of CYP2B1 and tumor promoting activity by these various agents ($r > 0.9$, $P < 0.01$) in both rats and mice. However, there were major differences in the ability of these compounds to induce CYP2B1 and to promote in the two species. First, TCPOBOP is perhaps the most potent inducer and promoter/carcinogen we have observed

in the mouse, yet this compound is relatively weak in both respects in the rat. Second, EPH and PTB are strong inducers and promoters in the rat yet are relatively weak at either function in the mouse. In summary, we have expanded our relationship between CYP2B1 induction and tumor promotion to a second species and have found that the induction of the pleiotropic effect associated with CYP2B1 explains certain striking species-dependent differences which we have observed in response to various chemicals (TCPOBOP, EPH, PTB). Given the wide variety of pharmacologic effects of the agents employed, it argues that tumor promotion is, itself, directly related to induction of the pleiotropic effect. In fact, this basic mechanism is likely to explain much of the tumor promotion data generated in this laboratory examining a wide variety of drugs to which humans are exposed.

Promoting effects of two environmental contaminants, Aroclor and DDT and various PB-type enzyme inducers on development of hepatoblastomas were compared in D2B6F1 male mice. Five-week-old mice were given a single i.p. injection of 90 mg NDEA/kg body weight. Beginning two weeks later, they were given a diet containing Aroclor (350 ppm or 175 ppm), DDT (300 ppm), PB (500 ppm) or equimolar doses of pentobarbital (PTB), ethylphenylhydantoin (EPH), diethylhydantoin (DEH) or monoethylbarbituric acid (MEB) for 53 weeks or 3 mg TCPOBOP/kg i.p. every 2 weeks for 16 weeks. No significant differences were observed in the incidences of hepatocellular tumors between NDEA.PB and NDEA.Aroclor, NDEA.DDT or NDEA.TCPOBOP groups. DDT promoted hepatoblastomas in 27% of NDEA-initiated mice. The incidence of hepatoblastomas in the NDEA.PB group was 72% and the NDEA.TCPOBOP group was 90%, while it was 33% in low dose and only 9% in high dose Aroclor-treated animals. EPH and PTB, strong inducers of CYP2B1 in rats, were weak inducers of this activity in mice and weak promoters of hepatoblastoma (2/29 and 3/28, respectively) in this model. Non-inducers of CYP2B1, DEH and MEB were totally ineffective. TCPOBOP, a strong inducer of CYP2B1 in mice, alone induced hepatoblastomas in 21/30 mice.

In contrast to a low incidence of hepatoblastomas in Aroclor-promoted groups, several of these animals developed lesions resembling benign and malignant cholangiocellular neoplasms within hepatocellular tumors. Although such lesions, i.e., dysplasias, cystadenomas, and adenocarcinomas, were also found in some mice given Aroclor alone, the incidence and severity of these lesions were significantly ($P < 0.05$) greater in NDEA.Aroclor groups. Thus, PB and TCPOBOP were more effective than DDT or Aroclor in stimulating progression of hepatocellular neoplasms to hepatoblastomas, while only Aroclor was effective in promoting the development of benign and malignant cholangiocellular-like lesions within hepatocellular neoplasms. Thus, genetic and epigenetic factors appear to modify both the incidence and phenotype of neoplasms seen during mouse hepatocarcinogenesis.

B. Species Dependent Differences with Respect to Induction of CYP1A and CYP2B by Aroclor 1254.

The PCB are a group of persistent and pervasive environmental contaminants which cause a wide variety of biologic/toxicologic effects (induction of drug metabolizing enzymes, immunotoxicity, carcinogenesis). In fact, data in the mouse would imply that most or all of these various biological effects may be associated with specific interactions of the individual PCBs with a high-affinity cytosolic receptor (Ah receptor). The present experiments examined the ability of graded dietary concentrations of Aroclor 1254 primarily to induce specific CYP1A (associated with induction by the Ah receptor) and CYP2B (the major cytochrome induced by PB-type

inducers). Induction was examined in laboratory-treated male and female F344/NCr rats and male B6C3F1 mice and in feral mice (Reithrodontomys fulvescens) of both sexes exposed environmentally to Aroclor. These studies showed that (A) there is a dose and time-dependent increase in hepatic PCB burden and induced CYP1A levels; (B) levels of CYP1A are increased at lower levels of PCBs than levels of CYP2B1. This occurs despite the fact that virtually all of the accumulated PCBs are either pure PB-type inducers or mixed (PB-type and TCDD-type) inducers; (C) there is a strong correlation between hepatic PCB burdens and levels of CYP1A activity in all species; and (D) there are striking species differences in sensitivity to induction by Aroclor. In general, sensitivity decreases in the following order: rat >> mouse > Reithrodontomys fulvescens. These studies bring out two potentially important predictions: (1) feral rats should be the most sensitive species with which to monitor environmental PCB exposure; (2) rats should be more susceptible to PCB-induced promotion/tumorigenesis and potentially other toxic manifestations than mice. Such a hypothesis could be tested experimentally and might be extrapolated to other species as well.

Publications:

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Lubet RA, Kouri RE, Curren RA, Putman DL, Schechtman LM. Induction of mutagenesis and transformation in BALB/c-3T3 clone A31-1 cells by diverse chemical carcinogens. *Environ Mol Mutagen* 1990;16:13-20.

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

PROJECT NUMBER

Z01CP05301-10 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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 & Pathogenesis Section	LCC	NCI
Others:	S. Rehm	Visiting Scientist	LCC	NCI
	R. Benveniste	Medical Officer	LVC	NCI
	Y. Kurata	Visiting Fellow	LCC	NCI
	A. O. Williams	Visiting Scientist	LEP	NCI

COOPERATING UNITS (if any) Program Resources, Inc./DynCorp., Frederick, MD (B. Diwan); W. Alton Jones Cell Science Center, Lake Placid, NY (J.L. Stevens); NIMH, Bethesda, MD (L. Elden, J. Lendvay)

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Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

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TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

2.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

A new pathological lesion, vimentin metaplasia, was discovered to be associated with the preneoplastic and neoplastic phenotype of renal tubular epithelial lesions in F344 rat kidney. The transition to the vimentin phenotype appeared to be a prerequisite for neoplastic transformation by several genotoxic carcinogens and tumor promoters. A novel mouse lung tumor model was discovered and developed. N-nitrosobis(2-chloroethyl)urea was found to induce hyperplastic, preneoplastic and neoplastic lesions arising within bronchioles. Tumors of similar cellular origin are found in human lung. This new model could play a role in the study of the etiology, pathogenesis, and therapy of comparable human tumors. An animal model for the study of myoepithelial tumors of epithelial tissues was found and described. Mouse mammary tumors with significant myoepithelial components were induced in B6D2F1 and D2B6F1 mice by 7,12-dimethylbenz[a]anthracene. Transplantable myoepithelial tumors were established and provided an important model for studying myoepithelial differentiation and biology. Rhesus monkeys infected with SIV/Delta developed a unique giant cell enteritis, not previously described. Also, B cell lymphomas and a pancreatic carcinoma were found in these monkeys. The lymphomas were always associated with marked lymphoid hyperplasia.

PROJECT DESCRIPTION

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

J. M. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
S. Rehm	Visiting Scientist	LCC	NCI
R. Benveniste	Medical Officer	LVC	NCI
Y. Kurata	Visiting Fellow	LCC	NCI
A. O. Williams	Visiting Scientist	LEP	NCI

Objectives:

To study and characterize the biology and pathology of selective naturally-occurring and experimentally-induced tumors of laboratory animals; to develop animal models for the study of human tumors and associated diseases; and to develop methods, especially immunohistochemistry, utilizing experimental animals to aid in understanding the causes, natural history, and prevention of human cancer and related diseases.

Major Findings:

A new pathological lesion, vimentin metaplasia, which usually precedes renal epithelial neoplasia, has been discovered for the first time in any species in our rat renal tumor models. Vimentin is normally expressed in mesenchymal cells of many types in the various tissues. We found that tubule cells in the precursor lesion of renal adenoma and carcinoma, tubular dysplasia, expresses vimentin. The renal tubular cell tumors induced by carcinogens also express vimentin. The vimentin phenotype for preneoplastic and neoplastic renal epithelial lesions suggests dedifferentiation of renal epithelium. Preliminary research with rat fetal tissues indicate, however, that rat fetal renal epithelium does not express vimentin, but rat embryonal renal mesenchyme does. The exact mechanism responsible for metaplasia to this phenotype in adult kidney and renal preneoplastic and neoplastic lesions remains to be found. Our rat models may provide important research tools for studying the origin and biology of rat and human tumors. We are also obtaining preneoplastic and precancerous human renal tubular cell lesions for study and comparison with the rat model, since some human renal carcinomas were recently found to express vimentin.

A novel mouse lung tumor model for induction of bronchiolar cell neoplasms by systemic application of a carcinogen was discovered and studied morphologically. *N*-nitrosobis(2-chloroethyl)urea, applied topically to the skin, was found to induce bronchiolar cell hyperplasia, metaplasia and various tumors consisting of the different cell types populating the conducting airways. Tumors included squamous cell carcinomas, adenocarcinomas, ciliated and mucinous tumors, and glandular or pleomorphic lesions consisting of cells immunoreactive for Clara cell antigen. Tumor growth pattern was either nodular or, alternatively, the neoplastic tissue was frequently disseminated throughout the parenchyma starting from multicentric peribronchiolar foci. A unique cell type, with large eosinophilic globules and associated eosinophilic crystals was seen lining airways or forming hyperplastic and neoplastic lesions. This is the first mouse model of bronchiolar cell neoplasia

without alveolar type II cell involvement and requiring not direct but systemic carcinogen application.

Neoplasms of humans with important myoepithelial components develop, in particular, from salivary glands and the breast. They often show a wide array of morphological features including the formation of cartilage which is attributed to metaplasia of the myoepithelium. Therefore, studies with our new mouse mammary gland myoepithelial carcinoma model were initiated. Tumors were induced in mice by gavage of 7,12-dimethylbenz[a]anthracene (DMBA) in B6D2F1 and D2B6F1 mice and transplanted consecutively to susceptible animals. Ten transplanted tumors were established and will be maintained for a maximum of several passages to explore the complete morphologic differentiation capacity of these tumors. Early results indicate that for the first time, we will be able to show in an experimental model that myoepithelial tumors indeed have inherent capability to form cartilage and bone, or change to a trabecular/comedo-type pattern with glycogen-containing clear cells, or to a poorly differentiated spindle/mesenchymal tumor cell type. Furthermore, metastases in regional lymph nodes and lungs have been observed that are rarely seen in the original host. From two transplantation lines, tissue cultures were established. At present we are cloning cell lines to establish pure myoepithelial cell lines for the first time in any species. The experiments may provide valuable insights to the nature and biology of myoepithelial cells that express ambivalent features of mesenchymal and epithelial tissues, their differentiation capabilities, and their role in the origin of tumor cells.

For epithelial tissues it has clearly been shown that the process of cancer development usually involves a multistep process. Histologically, lesions change from hyperplasia or preneoplasia to a benign tumor, a semi-malignant process without metastases, to ultimately a neoplasm metastasizing to lymph nodes and lung or other organs. Such progressive events, however, have not been documented for mesenchymal tumors. In a recent experiment, mice treated with DMBA by gavage developed a progressive sequence of vascular lesions ranging from simple, small ectasias to cavernous hemangiomas with thrombi, and finally to invasive, poorly differentiated hemangiosarcomas. Organs most frequently involved included liver, uterus, small intestine, and pancreas. These results clearly demonstrate that similar progressive carcinogenic steps may occur in tumors of mesenchymal origin.

The origin and nature of mouse hepatoblastomas was investigated (see also project Z01CP05299). These tumors arise within mouse hepatocellular tumors in D2B6F1 mice. They are comparable to the small cell form in humans. We studied the expression of various hepatocyte and biliary gene products in tumor cells. Tumor cells expressed keratin and albumen but lacked alpha-fetoprotein. Tumors often arose within hepatic plates of hepatocellular adenomas and carcinomas. Hepatoblastomas seemed to differentiate from hepatocytes in the earliest lesions. We are attempting to demonstrate the activity of hepatocyte-specific gene expression within tumor cells by in situ hybridization and hepatocyte-specific gene products by immunohistochemistry. A transplantable hepatoblastoma tumor cell line is in its first passage.

Our research on the pathogenesis of lentiviral infections in humans and monkeys has revealed important new information on target cells and tissues of viral infection. In collaboration with Dr. A.O. Williams, we have found that although PCR amplification of formalin fixed Kaposi's sarcomas of Americans and Africans revealed

the presence of the HIV-1 viral genome, antigen could only be found in inflammatory cells (macrophages) of tumor-associated inflammatory lesions and within regional lymph nodes. Monocyte-like cells within blood vessels expressed HIV-1 antigens even in a few cases from the early 1970's prior to the AIDS pandemic. Likewise, rhesus monkeys infected with SIV-Delta strain revealed viral antigens in monocyte-like cells within blood vessels and in dendritic cells in lymph nodes. For the first time in monkeys, we found a clinical diarrhea and giant cell enteritis with viral antigen in giant macrophage (syncytial) cells in the lamina propria of the intestine. Neoplasms (B cell lymphomas and a pancreatic carcinoma) developed in SIV-Delta infected monkeys. Tumor cells did not express viral antigens. The lymphomas were all associated with marked B cell follicular hyperplasia in lymphoid tissues.

Publications:

- Gibson W, McNally LK, Benveniste RE, Ward JM. Evidence that HIV-1 gag presursor shares antigenic sites with the major capsid protein of human cytomegalovirus. *Virology* 1990;175:595-9.
- Goering PL, Rehm S. Inhibition of liver, kidney, and erythrocyte δ -aminolevulinic acid dehydratase (porphobilinogen synthase) by gallium in the rat. *Environ Res* 1990;53:135-51.
- Jones TC, Ward JM, Mohr U, Hunt RD, eds. Hemopoietic system - monographs on pathology of laboratory animals. New York: Springer-Verlag 1990;336.
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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05303-10 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	C. M. Weghorst	Intramural Research Training Awardee	LCC	NCI
	Y. Kurata	Visiting Fellow	LCC	NCI

COOPERATING UNITS (if any) Program Resources, Inc./DynCorp., Frederick, MD (B. Diwan, R. Kantor)

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Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

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TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

2.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

The cellular mechanisms responsible for the tumor promoting effects of renal and hepatic tumor promoters are under investigation in rodents. We previously established that the renal tumor promoter and former human drug, barbital sodium, stimulates the growth of preneoplastic renal tubular cell foci initiated by genotoxic carcinogens (see also project Z01CP05299). Enhancement of these foci to progress to adenoma and carcinoma is a major effect of barbital sodium. The relationship between the target organ toxicity of the barbital, renal tubular hyperplasias and neoplasms, is under study. Barbital, itself, was found to be a relatively "weak" carcinogen for rat kidney. In the rat liver, using naturally-occurring or carcinogen-initiated glutathione S-transferase, placental form (GSTP) single immunoreactive hepatocytes as putative initiated cells, the nongenotoxic hepatocarcinogen, di(2-ethylhexyl)phthalate (DEHP), a peroxisomal proliferator, could not promote these cells to develop into hepatic 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
C. M. Weghorst	Intramural Research Training Awardee	LCC	NCI
Y. Kurata	Visiting Fellow	LCC	NCI

Objectives: To determine cellular and biologic mechanisms responsible for the progression of carcinogenesis in non-squamous epithelium.

Major Findings:

The mechanisms of tumor promotion and carcinogenesis by nongenotoxic renal epithelial-specific chemicals was studied in F344 rats. Evidence was obtained that renal tubular epithelial tumor promoters act via growth stimulation of preneoplastic populations in renal tubules. These preneoplastic populations were found to possess characteristics of dysplastic epithelium and could be differentiated from other renal tubule populations. Tumor promotion was manifested as increased numbers and sizes of renal tubular epithelial tumors. Dysplastic lesions occurred in various portions of the nephron. Their location was dependent in part on the tumor initiating agent. We used streptozotocin, a pharmaceutical agent; potassium bromate, a bread additive; or N-bis(2-hydroxypropyl)nitrosamine as initiating agents for renal tubular epithelium. Barbitol sodium was found to be a classical renal tumor promoting agent. Barbitol also induced renal tumors in low incidence after long periods of exposure.

Potassium bromate, commonly used in the manufacture of bread, is genotoxic in most in vitro assay systems and is a renal epithelial carcinogen in rats. Because it was genotoxic in in vitro assays, it was assumed to potentially possess initiating activity for rat renal tubular epithelium. Rats received a single i.p. injection of potassium bromate at 300 mg/kg at 6 weeks of age. Two weeks later, they received the renal tumor promoting agent, barbitol sodium, in the diet. No tumor initiating activity for this dosage of potassium bromate was detected even after 78 weeks of the experiment. The numbers of preneoplastic or neoplastic renal tubular epithelial lesions were not increased in number or size in rats exposed to potassium bromate and barbitol sodium.

Our model of the putative single initiated cell, the GSTP-immunoreactive hepatocyte in liver of the aging F344/NCr rat, was used to determine potential mechanisms of carcinogenesis by di(2-ethylhexyl)phthalate (DEHP). DEHP is a nongenotoxic rodent hepatocarcinogen and peroxisomal proliferator. We have shown that it is a hepatic tumor promotor in mouse liver after carcinogen initiation. It does not promote rat hepatocarcinogenesis after carcinogen initiation nor spontaneous mouse hepatocarcinogenesis. We exposed aging F344 rats to DEHP in the diet for periods of up to 9 months to determine if spontaneous putative preneoplastic GSTP-immunoreactive hepatocytes or basophilic foci could be promoted. Rats were sacrificed at various time intervals to study the fate of the single GSTP-reactive hepatocytes and foci after DEHP exposure. By 9 months, we could not find evidence for promotion of these cells or any foci into tumors. These studies suggest that DEHP is not a "carcinogen" because of its promotion of spontaneous initiated cells or foci.

One potential mechanism by which nongenotoxic carcinogens enhance hepatic tumorigenesis may involve the selective expansion of certain subpopulations of "initiated" cells within the liver by the tumor promoting compounds. Recently, we have shown that not all initiated cell populations are responsive to two known mouse liver tumor promoters. Utilizing the 15-day-old, N-nitrosodiethylamine (NDEA)-initiated C3H/HeNcr male mouse, preneoplastic hepatocellular foci were apparent by 15 weeks of age. At this time, animals received either phenobarbital (PB), di(2-ethylhexyl)phthalate (DEHP), or no treatment for 2 days to 24 weeks of exposure. The foci in the NDEA-only group increased in numbers and progressed to large adenomas over the 24-week period. In contrast, the number of grossly visible adenomas was significantly reduced with PB and DEHP administration after 9 and 13 weeks. However, by 17 and 24 weeks of exposure, animals exposed to PB or DEHP exhibited increased numbers of gross liver tumors compared to animals given NDEA only. When cell turnover was evaluated in individual preneoplastic foci from 15 to 18 weeks of age, foci exposed to PB demonstrated increased levels only at 2 days compared to foci in animals given NDEA only, and subsequently returned to basal levels. In contrast, levels of cell turnover in DEHP-treated foci were significantly reduced for at least 3 weeks. These studies suggest that the majority of foci induced by NDEA, which were endogenously promoted, were not sensitive to the promoting effects of PB. However, latent initiated hepatocytes generated at 15 days of age with NDEA, which were apparently not sensitive to the endogenous promoters, were susceptible to PB's promoting stimuli. Likewise, DEHP-susceptible initiated cells were promoted with chronic DEHP administration, while the NDEA-only foci regressed. The data also suggests potentially different mechanisms of focus regression.

Publications:

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

PROJECT NUMBER

Z01CP05352-09 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Chief, Perinatal Carcinogenesis Section	LCC	NCI
Others:	J.M. Rice	Chief	LCC	NCI
	L. Beebe	Intramural Research Training Awardee	LCC	NCI
	P. Nerurkar	Visiting Fellow	LCC	NCI
	S. S. Park	Expert	LMC	NCI
	H. V. Gelboin	Chief	LMC	NCI
	S. Thorgeirsson	Chief	LEC	NCI
	E. Snyderwine	Senior Staff Fellow	LEC	NCI

COOPERATING UNITS (if any)

Program Resources, Inc./DynCorp, Frederick, MD (H. Issaq); Pathology Associates, Frederick, MD (R. Kovatch); American Health Foundation, Valhalla, NY (S. Hecht, D. Hoffman); U. Texas, Galveston, TX (L.J. Lu); U. Chicago, Chicago, IL (R.G. Harvey); Sema, Inc., Rockville, MD (T. Moskal, J. Phillips)

LAB/BRANCH

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SECTION

Perinatal Carcinogenesis Section

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TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

2.5

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 effects of carcinogens encountered during the perinatal period are modulated by (1) the capacity of perinatal tissues to activate the chemicals and (2) postnatal tumor promotive influences, among other factors. With regard to activation, attention is currently being focused on formation of DNA adducts by benzo[a]pyrene (BP) in the placenta and fetal tissues of patas monkeys, assayed by 32P-postlabeling (with Dr. L.J. Lu, see Project Z01CP05092); and on effects of the arylamine food mutagen, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). IQ is activated metabolically by cytochrome P450IA2, with several other isozymes contributing to detoxification. A study of transplacental carcinogenesis by IQ in mice and hamsters is ongoing. Preliminary to investigation of ontogeny of IQ metabolism as related to tumorigenesis, the effects of IQ as an enzyme inducer is being studied in mice. Significant increases in a cytochrome P450IA1 enzyme activity and decreases in total cytochrome P450 in C57BL/6 mice were noted. Study of tumor promotive influences has concentrated on the actions of retained congeners of polychlorinated biphenyls (PCBs), after a single dose of the mixture Aroclor 1254, and of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). A preliminary study of the effects of restriction in dietary fat after PCB treatment showed that weight loss increased toxicity and increased level of induction of a specific cytochrome P450 isoform associated with tumor promotion, presumably due to release of stored PCB congeners from fat depots (see also project Z01CP05299). A single dose of TCDD had persistent, organ-specific inducing effects, with lung being more sensitive than liver to continued induction after low doses of TCDD.

PROJECT DESCRIPTION

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

L. M. Anderson	Chief, Perinatal Carcinogenesis Section	LCC	NCI
J. M. Rice	Chief	LCC	NCI
L. Beebe	Intramural Research Training Awardee	LCC	NCI
P. Nerurkar	Visiting Fellow	LCC	NCI
S. S. Park	Expert	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI
S. Thorgeirsson	Chief	LEC	NCI
E. Snyderwine	Senior Staff Fellow	LEC	NCI

Objectives:

To establish and characterize the tumor-initiating effects of environmental chemicals during the perinatal period, or the modulation of tumor initiation by such chemicals; to investigate postnatal influences on the development of perinatally-initiated neoplasms.

Major Findings:

The arylamine food mutagen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) is a carcinogen in adult rodents but has not been studied for effects on perinatal animals. A test of the transplacental effects of IQ, as related to genotype at the Ah locus, is in progress. A key question in this, and for evaluation of its carcinogenicity in general, is the extent to which IQ may induce its own metabolism, with activation by cytochrome P450IA2 and detoxification by several other isoforms. Mice differing in genotype at the Ah locus (C57BL/6, Ah-inducible, DBA/2, Ah-noninducible) were treated with 20-50 mg/kg IQ and their livers assessed for levels of cytochromes P450IA1, IA2, and IIB1. Preliminary results indicate that IQ may induce P450IA1 and IA2 up to 2-fold, depending on strain and age.

In the postnatal period, among the chemicals that are commonly encountered by infants are chlorinated hydrocarbons, which are retained in body fat but readily transferred into breast milk. These chemicals, including polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), may act as tumor promoters. In a study of the effects of mobilization of stored PCB congeners from body fat, Swiss mice were given a single dose of the PCB mixture Aroclor 1254 and then placed on normal (6%), low fat (0.4%) or high fat (12%) diets. As indicated by measurement of total cytochrome P450 and an enzyme assay specific for cytochrome P450IIB1, benzyloxyresorufin-0-dealkylase (BenzROD), loss of weight by the mice on the special diets, particularly the low fat diet, was associated with increase in liver enzymes, as well as enhanced toxicity. After 1 week, BenzROD was 8-fold higher in the Aroclor-low fat group than in the Aroclor-normal diet mice.

Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) after a single dose of 5 or 50 nmole/kg in liver and lung were studied with total cytochrome P450, BenzROD and ethoxyresorufin-0-dealkylase (EROD, an activity of cytochrome P450IA1) as biochemical endpoints and confirmed by Western immunoblotting. Greatest effects were seen for EROD, which after 12 weeks was still elevated above controls in both

organs at both doses. At the lower dose, this activity and the amount of P450 seen on blots was more persistently elevated in lung than in liver. This specific effect of TCDD on lung is interesting in light of recent epidemiology implicating TCDD in human lung carcinogenesis.

Publications:

Anderson LM, Beebe LE, Fox SD, Issaq HJ, Kovatch RM. Promotion of mouse lung tumors by bioaccumulated polychlorinated aromatic hydrocarbons. *Exp Lung Res* 1991;17:455-71.

Anderson LM, Fox SD, Dixon D, Beebe LE, Issaq HJ. Long-term persistence of polychlorinated biphenyl congeners in blood and liver and elevation of liver aminopyrine demethylase activity after a single high dose of Aroclor 1254 to mice. *Environ Toxicol Chem* 1991;10:681-90.

Anderson LM, Jones AB, Kovatch RM. Effect of pretreatment with β -naphthoflavone on tumorigenesis by N-nitrosoethylurea in five mouse strains. *Cancer Lett* 1990;52:91-4.

Anderson LM, Jones AB, Riggs CW. Long-term (imprinting) effects of transplacental treatment of mice with 3-methylcholanthrene or β -naphthoflavone on hepatic metabolism of 3-methylcholanthrene. *Pharmacol Toxicol* (In Press).

Beebe LE, Fox SD, Issaq HJ, Anderson LM. Biological and biochemical effects of retained polyhalogenated hydrocarbons. *Environ Toxicol Chem* 1991;10:757-63.

Beebe LE, Park SS, Anderson LM. Differential enzyme induction of mouse liver and lung following a single low or high dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Biochem Toxicol* 1990;5:211-9.

Chauhan DP, Miller MS, Owens IS, Anderson LM. Gene expression, ontogeny and transplacental induction of hepatic UDP-glucuronosyl transferase activity in mice. *Develop Pharmacol Therapeut* (In Press).

Lu L-J, Harvey RG, Lee H, Baxter JR, Anderson LM. Age-, tissue-, and Ah genotype-dependent differences in the binding of 3-methylcholanthrene and its metabolite(s) to mouse DNA. *Cancer Res* 1990;50:4239-47.

Miller MS, Jones AB, Anderson LM. The formation of 3-methylcholanthrene-initiated lung tumors correlates with induction of cytochrome P450IA1 by the carcinogen in fetal but not adult mice. *Toxicol Appl Pharmacol* 1990;104:234-45.

Miller MS, Jones AB, Chauhan DP, Anderson LM. Role of the maternal environment in susceptibility to transplacentally-induced chemical carcinogenesis in mouse fetuses. *Carcinogenesis* 1990;11:1979-89.

Miller MS, Jones AB, Chauhan DP, Park SS, Anderson LM. Induction of cytochrome P-450 IA1 in fetal rat liver by a single dose of 3-methylcholanthrene. *Biochem Biophys Res Comm* 1991;176:280-7.

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

PROJECT NUMBER

Z01CP05353-09 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

COOPERATING UNITS (if any)

Temple U., Philadelphia PA (G. Harrington, P.N. Magee); Smith Kline Labs, King of Prussia, PA (C. Gombar); SEMA, Inc., Rockville, MD (T. Moskal, J. Phillips); Pathology Assoc., Frederick, MD (R. Kovatch); Queen's U., Ontario, Canada (P.G. Forkert)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

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.)

Ethanol, while at best a weak carcinogen in animal models, increases human risk of cancer at several sites. Elucidation of the mechanism by which it does so would not only illuminate this particular public health issue but also provide insight into the more general question of the impact of interaction between environmental chemicals on cancer risk. Current efforts focus on the effect of ethanol on carcinogen metabolism and clearance in mice. In collaboration with Dr. P.G. Forkert, cytochrome P450IIE1, which activates environmental nitrosamines such as N-nitrosodimethylamine (NDMA), was found to be induced 2- to 7-fold by ethanol in drinking water or liquid diet, as indicated by enzyme assay and Western immunoblotting with a specific monoclonal antibody. Immunohistochemical staining showed uniform staining in centrilobular hepatocytes. Ethanol competitively inhibits, as well as induces, P450 activity toward NDMA, and this was investigated systematically in a toxicokinetic study. Doses of 10-20% ethanol given i.g. before i.v. doses of 1-10 mg/kg NDMA had large negative effects on clearance parameters, up to 25-fold. Even more striking inhibition was seen with oral co-administration, up to 250-fold. Finally, ethanol in the drinking water was found to increase a UDP-glucuronosyltransferase participating in clearance of polycyclic aromatic hydrocarbons.

PROJECT DESCRIPTION

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

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

Objectives:

To study the interaction between environmental chemicals and the interplay between carcinogenic and noncarcinogenic agents, resulting in a modulation of carcinogenesis; specifically, to characterize the influence of ethanol on tumorigenesis by nitrosamines and polycyclic aromatic hydrocarbons.

Major Findings:

Ethanol is at best a weak carcinogen in animal models, but alcoholic beverage use impacts in a major way on human risk for cancer at several target sites. A possible mechanism by which it does so is through modulation of the action of active genotoxic carcinogens, as by influencing their activation or detoxification. We have studied these possibilities with mouse models.

Ethanol in drinking water or liquid diet of Swiss mice caused an increase of cytochrome P450IIE1. This P450 metabolizes several important environmental volatile nitrosamines, including N-nitrosodimethylamine (NDMA) (with Dr. P.G. Forkert). With liquid diet the effect increased progressively during 3 weeks, for a 7-fold induction, as measured by Western blot immunoassay with monoclonal antibody 1-98 to IIE1. These results were correlated with increased immunohistochemical stain, uniformly distributed in centrilobular hepatocytes. The progressive increase in enzyme over a period of weeks in mice given high ethanol dosage is a particularly interesting and human-relevant new finding.

The competitive inhibition of NDMA metabolism by ethanol was also studied, with measurement of toxicokinetic parameters for clearance of 1, 5, or 10 mg/kg NDMA. A bolus i.g. dose of 20% ethanol given before i.v. NDMA caused a 16- to 23-fold decrease in clearance and concomitant increase in area-under-the curve (AUC) and mean residence time. At 10% ethanol there was a 3- to 25-fold effect. Given together with NDMA i.g., 10% ethanol reduced clearance and increased AUC of a 1 mg/kg dose by 240-fold. Thus, the amount of NDMA being delivered to posthepatic targets would be greatly increased under these conditions.

Ethanol may also influence the metabolism of other types of chemical carcinogens. At a concentration of 15% in the drinking water it caused a significant doubling in UDP-glucuronosyltransferase (UDGPT) activity toward p-nitrophenol; this effect was not additive with a similar inductive action of 3-methylcholanthrene. Western immunoblotting revealed an increase of similar magnitude in one of the three prominent UDGPT isoforms. In this case, the selective induction might tend to reduce the impact of high molecular weight carcinogens.

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

PROJECT NUMBER

Z01CP05399-08 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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	Adjunct Scientist (Special Volunteer)	LCC	NCI
	C. D. Reed	Senior Health Services Officer	LCC	NCI

COOPERATING UNITS (if any) Program Resources, Inc./DynCorp., Frederick, MD (G. Buzard); Osaka U. Med. School, Osaka, Japan (M. Inoue, O. Tanizawa, T. Nomora); Kagawa Medical School, Kagawa, Japan (H. Miki, M. Ohmori); U. Colorado, Denver, CO (B. Beckwith)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Office of the Chief, Developmental Biology Working Group

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

1.0

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 role of ras (especially K-ras) and of neu in chemically induced tumors in rodents continues to be investigated in efforts to distinguish between activating mutations that are the direct result of chemical reactions of carcinogenic agents with DNA encoding these specific genes, and stochastic events that occur independently of the inducing carcinogen during tumor progression. Studies on rat renal mesenchymal tumors have been especially informative in this regard. A second theme has been extrapolation of activating mutational events in experimental tumors to human cancers that involve activation of the corresponding human oncogene; human pediatric renal tumor, as well as mucinous carcinoma of the ovary, endometrial carcinoma of the uterus, the "intestinal" variant of human gastric carcinomas and prostatic carcinoma are currently under investigation.

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	Adjunct Scientist (Special Volunteer)	LCC	NCI
C. D. Reed	Senior Health Services Officer	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:

Renal mesenchymal tumors were induced in high incidence in male F344 rats by a single injection of nickel subsulfide (Ni_3S_2) alone or Ni_3S_2 plus iron (Fe^0) (Kasprzak et al., Proc. AACR, 31:127, 1990). The tumors appeared comparable in histogenesis to renal mesenchymal tumors induced by nitrosamines but were more pleomorphic and had a greater tendency to metastasize. Selective oligonucleotide hybridization analysis and direct sequencing of polymerase chain reaction (PCR) amplified K-ras gene sequences confirmed that 7/9 primary tumors induced with Ni_3S_2 and Fe^0 and 1/13 primary tumors induced with Ni_3S_2 alone contained exclusively GGT-GTT activating mutations in codon 12, in contrast to comparable N-nitrosomethylurea-induced tumors that are reported to contain GGT-GAT. Sequencing revealed no mutations within other codons, 13 and 59-61, that can result in K-ras activation. The K-ras codon 12 activation pathway appears important but not exclusive for Ni_3S_2 carcinogenesis in this tissue.

In rats, a single transplacental exposure to N-ethylnitrosourea (ENU) induces schwannomas that characteristically (43/45, 96%) contain a specific T-A transversion within the transmembrane domain of the neu oncogene, as detected by selective oligonucleotide hybridization. We now report that this same mutation has also been found in 2 of 3 schwannomas induced transplacentally by ENU in mice and in 46 of 54 schwannomas in Syrian golden hamsters, as determined by direct genomic sequencing of PCR-amplified schwannoma DNA. Only 50% of rat schwannomas (5/10) induced transplacentally by 7,12-dimethylbenz[a]anthracene (DMBA) contained this activating mutation. Direct partial sequencing of neu from negative tumors in the rat has thus far revealed no novel mutations elsewhere in the transmembrane domain. These transfection-negative tumors may contain neu activated by a novel mechanism outside the transmembrane domain, or an entirely different transforming gene.

The frequency of spontaneous liver tumorigenesis in aging mice is strain dependent. Untreated C3H mice demonstrate a tumor incidence of 30-50% by 2 yrs, while C57BL/6 mice have < 5% incidence. B6C3F1 mice (C57BL/6 x C3H) exhibit an intermediate incidence of 30%. In aging B6C3F1 mice, 30% of adenomas and 70% of carcinomas developing spontaneously contain activating codon 61 point mutations in the H-ras oncogene. This study evaluated oncogene activation in C3H/HeNcr spontaneous liver

tumors by NIH 3T3 transfection assay and characterized point mutations in the H-ras gene from PCR-amplified DNA fragments by dot blot analysis using mutation specific oligonucleotide probes, by XbaI or TaqI RFLPs, and by direct sequencing. If H-ras mutations are important in B6C3F1 spontaneous tumorigenesis, higher levels of mutations might be expected in the more susceptible C3H strain. However, only 8% of the adenomas (3/39) and 17% of the carcinomas (1/6) contained H-ras sequences mutated in codon 61. These data suggest that activation of H-ras is not obligatory for hepatocarcinogenesis in C3H/HeNcr mice.

We previously reported (Cancer Res. 50: 6139-6145, 1990) a significant frequency of activating point mutations in codon 12 of the K-ras oncogene in endometrial adenocarcinomas of the uterine corpus. We also observed in a limited number of samples that ovarian tumors in general do not have ras gene mutations with the possible exception of mucinous adenocarcinoma. To further investigate the role of ras activation in the development of endometrial adenocarcinoma, we surveyed cystic, adenomatous, and atypical hyperplasias of the uterine endometrium and additional cases of endometrial and cervical carcinoma for the presence of point mutations by dot blot analysis with mutation specific oligonucleotide probes and by direct sequencing after PCR amplification of the ras genes. Atypical hyperplasia is believed to be premalignant, whereas cystic and adenomatous hyperplasias are thought to be benign. Frequency of ras gene mutations was found to be significantly higher in endometrial adenocarcinoma of the uterine corpus (11/29 (38%), 10 K-ras, 1 N-ras) than in squamous cell carcinoma of the uterine cervix (1/23 (4%), 1 K-ras; $p=0.0041$). Ras gene mutations were lower, but not significantly, in atypical endometrial hyperplasias (2/16 (13%), 2 K-ras) than in grade 1 endometrial carcinomas (4/13, (30%), 3 K-ras, 1 N-ras). Neither 6 adenomatous hyperplasias nor 12 cystic hyperplasias contained any ras mutations. It is more likely that ras mutations occur as a later event than as an initiating event in the etiology of endometrial carcinoma. No significant correlation of clinical parameters with the presence of ras mutations was observed. Ras gene mutations were also detected in additional cases of ovarian tumors. K-ras mutations occurred significantly more frequently in mucinous adenocarcinomas (6/8, 75%) than in serous adenocarcinomas (2/10, 20%; $p=0.031$) or in all non-mucinous types of epithelial ovarian tumors combined (3/22, 14%; $p=0.0031$). These findings may imply the existence of an oncogenic pathway in mucinous adenocarcinomas that is also common to such morphologically similar tumors as pancreatic and colorectal carcinomas in which mucin secretion is common and frequent K-ras activation is also observed.

It has been demonstrated that point mutations in codon 12 of K-ras are frequently found in duct cell carcinomas of the pancreas (90%), adenocarcinomas of the lung (30%) and adenomas and adenocarcinomas of the colon (40%). On the other hand, adenocarcinoma of the stomach has been reported to have a very low incidence of ras mutation. We detected ras-gene mutations in stomach tumors by dot-blot hybridization with mutation-specific probes after amplification with PCR from sections of paraffin-embedded tissues. Point mutations were found specifically in 4 of 20 tumors that had the intestinal histological subtype: GGT (glycine) to GAT (aspartic acid) in 3 cases and to GGT (valine) in one case, all in codon 12 of K-ras. These mutations were also confirmed by direct sequencing. In contrast, none of 11 diffuse-type tumors showed K-ras point mutations. While K-ras point mutations may not be frequent events in gastric tumorigenesis, the similarity of the intestinal-type gastric tumors and colorectal tumors for K-ras point mutations as

well as the association of mutations in K-ras with a particular gastric tumor histology implicates K-ras activation in the development of these tumors.

Previously, we established that the K-ras oncogene is activated by point mutation at high frequency in chemically induced rat renal mesenchymal tumors (Sukumar, et al., Mol. Cell. Biol. 6:2716, 1986). These tumors are believed to originate in an embryonic component of the developing kidney and resemble the pediatric mesoblastic nephroma or the sarcomatous component of the Wilms' tumor. To determine if ras activation is similarly important to renal tumorigenesis in humans, the 4 major categories of pediatric renal neoplasms and, for comparison, adult renal cell carcinomas were evaluated for transforming mutations in K-ras sequences amplified by polymerase chain reaction (PCR) from histologic blocks from pathology archives. The pediatric tumors included mesoblastic nephromas, Wilms' tumors, clear cell sarcomas of the kidney, and malignant rhabdoid tumors of the kidney. Histologic sections from paraffin-embedded tumors were extracted with xylene and alcohol and PCR-amplified with primers surrounding codons 12 and 13 or codons 59-61 of K-ras. PCR-generated sequences were analyzed by selective oligonucleotide hybridization and dideoxy sequencing. By these techniques, 3/8 (38%) mesoblastic nephromas, 6/16 (38%) Wilms' tumors, 3/9 (33%) clear cell sarcomas, and 4/10 (40%) malignant rhabdoid tumors contained GGT-GAT transition mutations in codon 12 of the K-ras gene. No other mutations were detected in either region analyzed. Of the adult tumors, 0/24 renal cell carcinomas had mutations in codons 12 and 13 or 59-61. These results indicate that mutations in the K-ras oncogene occur specifically in pediatric and not adult renal tumors and suggest that ras activation may play a significant role in tumorigenesis during renal development.

Publication:

Enomoto T, Ward JM, Perantoni AO. H-ras activation and ras p21 expression in bladder tumors induced in F344/NCr rats by N-butyl-N-(4-hydroxybutyl)nitrosamine. Carcinogenesis 1990;11:2233-8.

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

PROJECT NUMBER

Z01CP05488-06 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Mechanisms of Inorganic Carcinogenesis

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

PI:	M. P. Waalkes	Research Pharmacologist	LCC	NCI
Others:	T. Coogan	Senior Staff Fellow	LCC	NCI
	A. O. Perantoni	Staff Fellow	LCC	NCI
	S. Rehm	Visiting Scientist	LCC	NCI
	R. A. Barter	Intramural Research Training Awardee	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology & Pathogen. Section	LCC	NCI

COOPERATING UNITS (if any) Program Resources, Inc./DynCorp., Frederick, MD (B. Diwan, H. Issaq); Data Management Services, Inc., Frederick, MD (C. Riggs); FDA, Rockville, MD (P. Goering); U. Western Ontario, Canada (B. Goyer, M. Cherian)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Inorganic Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21702-1201

TOTAL MAN-YEARS:

4

PROFESSIONAL:

3

OTHER:

1

CHECK APPROPRIATE BOX(ES)

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

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

The mechanisms by which cadmium and certain other metals induce cancer are currently under study at various levels. On the level of the whole animal, association of oral cadmium exposure and neoplasia of the rat prostate was confirmed, an important advance in support of cadmium as a factor in human prostatic cancer. Dietary zinc deficiency was found to either enhance or suppress cadmium carcinogenesis depending on the route of exposure to cadmium and/or the specific target tissue. The F344 rat was found to be particularly susceptible to sarcoma at the site of cadmium injection, indicating a genetic basis of susceptibility to tumor formation even at this target site. On the cellular and molecular level, it was found that cadmium is clearly capable of producing profound DNA damage, including frequent DNA single strand breakage. Pretreatments, such as zinc, which prevent cadmium carcinogenesis in certain tissues also prevent these genotoxic effects of cadmium. In testicular interstitial cells, several studies were directed at identifying factors involved in chemically-induced or genetically-based resistance. Testicular interstitial cells from strains of mice resistant to the effects of cadmium on the testes showed less cadmium uptake and more efflux than cells from susceptible mice. On the molecular level, it was determined that the testicular metallothionein gene does not play a major role in tolerance to cadmium carcinogenesis induced by pretreatment with zinc, as no differences in metallothionein mRNA levels were detected in tolerant cells. These findings support the concept that unresponsiveness to induction of the metallothionein gene is a consistent finding in target tissues of cadmium carcinogenesis.

PROJECT DESCRIPTION

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

M. P. Waalkes	Research Pharmacologist	LCC	NCI
T. Coogan	Senior Staff Fellow	LCC	NCI
A. O. Perantoni	Staff Fellow	LCC	NCI
S. Rehm	Visiting Scientist	LCC	NCI
R. A. Barter	Intramural Research Training Awardee	LCC	NCI
J. M. Ward	Chief, Tumor Pathology & Pathogen. Section	LCC	NCI

Objectives:

To determine the mechanisms by which cadmium and other metals produce tumors including target site specificity and susceptibility factors as well as the genetics of such tissue specificity.

Major Findings:

The mechanisms by which metals, especially cadmium and lead, induce the formation of tumors are currently under study at various levels of biological complexity. On the whole animal level, oral cadmium was confirmed to be an effective carcinogen in the rat, inducing tumors of the prostate, testes and hematopoietic system (leukemias). The association of oral cadmium exposure and neoplasia of the rat prostate is an important advance in support of a possible role of cadmium as an etiological factor in human cancer of the prostate. Dietary zinc deficiency was found to have highly complex effects on cadmium carcinogenesis, either enhancing or suppressing depending on the route of exposure to cadmium and/or the specific target site tissue. Dietary zinc deficiency in rats reduced cadmium-induced prostatic and testicular tumors from oral exposure but enhanced cadmium-induced subcutaneous injection site sarcomas. The F344 rat was found to be particularly susceptible to sarcoma formation at the site of cadmium injection, indicating a genetic basis of susceptibility to tumor formation even at this target site. Studies concerning the carcinogenic potential of lead in rodents, particularly transplacental carcinogenicity, are under way.

On the cellular and molecular level, the genotoxic effects of cadmium were assessed. Cadmium is clearly capable of producing profound DNA damage as evidenced by its ability to cause frequent DNA single strand breakage in cultured TRL1215 cells. Furthermore, it was found that pretreatments such as zinc, which we have previously shown to prevent cadmium carcinogenesis in certain tissues, prevented the genotoxic effects of cadmium in these cells. In specific target cells of cadmium carcinogenesis, namely, interstitial cells of the rodent testes, several studies were directed at determining factors involved in chemically-induced or genetically-based resistance. Interstitial cells from mouse strains resistant to the effects of cadmium on the testes showed less cadmium uptake and more efflux than cells from susceptible mice. Thus, differences in the toxicokinetics of cadmium may have some bearing on genetically based tolerance to cadmium at least in the testes. On the molecular level, studies were directed at determining the role of metallothionein, a cadmium binding protein that has been proposed as a key element in tolerance to cadmium toxicity. Using the p2A10 cDNA probe for the metallothionein gene, it was determined that the testicular metallothionein gene does not play a major role in

induced tolerance to cadmium toxicity and carcinogenesis generated by pretreatment with zinc or low dose cadmium, as no differences in metallothionein mRNA were detected in cells made tolerant to cadmium. These findings add further credence to the idea that unresponsiveness of the metallothionein gene consistently correlates with target tissue susceptibility to cadmium carcinogenesis.

In studies concerning non-metallic inorganic carcinogens, collaborative efforts are under way with the Food and Drug Administration to assess the carcinogenic potential of mammary augmentation device materials in rodents. Thus far, implantation techniques have been developed for such materials that duplicate human use. Subchronic and chronic studies are now being initiated.

Publications:

Koizumi T, Waalkes MP. Effects of zinc on the binding of cadmium to DNA: assessment with testicular interstitial cell and calf thymus DNAs. *Toxicol In Vitro* 1990;4:51-5.

Konishi N, Ward JM, Waalkes MP. Pancreatic hepatocytes in Fischer and Wistar rats induced by repeated injections of cadmium chloride. *Toxicol Appl Pharmacol* 1990;104:149-56.

Rehm S, Waalkes MP. Acute cadmium chloride-induced renal toxicity in the Syrian hamster. *Toxicol Appl Pharmacol* 1990;104:94-105.

Waalkes MP. Cadmium carcinogenesis in review. In: Collery MP, Maufait M, Etienne J-C, eds. *Proceedings of the first international symposium on metal ions in biology and medicine*. Montrouge, France: John Libbey Eurotex, Ltd., 1990;279-83.

Waalkes, MP, Diwan BA, Weghorst CM, Bare RM, Ward JM, Rice JM. Anticarcinogenic effects of cadmium in B6C3F1 mouse liver and lung. *Toxicol Appl Pharmacol* (In Press).

Waalkes MP, Goering PL. Metallothionein and other cadmium-binding proteins: recent developments. *Chem Res Toxicol* 1990;3:281-8.

Waalkes MP, Kovatch R, Rehm S. Effect of chronic dietary zinc deficiency on cadmium toxicity and carcinogenesis in the male Wistar [Hsd:(WI)BR] rat. *Toxicol Appl Pharmacol* (In Press).

Waalkes MP, Oberdoster G. Cadmium carcinogenesis. In: Foulkes ED, ed. *Biological effects of heavy metals*, vol. II. Mechanisms of metal carcinogenesis. Boca Raton: CRC Press, 1990;129-58.

Waalkes MP, Rehm S, Sass B, Konishi N, Bare RM, Ward JM. Carcinogenic and anticarcinogenic effects of single dose cadmium in the male Fischer rats. *Environ Res* (In Press).

Waalkes MP, Wahba ZZ, Koizumi T, Perantoni A, Rehm S. Cadmium-binding proteins in target tissues of cadmium carcinogenesis. In: Klaassen CD, Suzuki KT, eds. *Metallothionein in biology and medicine*. Caldwell, NJ: Telford Press (In Press).

Waalkes MP, Wahba ZZ, Rodriguez RE. Cadmium. In: Sullivan JB, Krieger KR, eds. Medical toxicology of hazardous materials. Baltimore: Williams & Wilkins Press (In Press).

Wahba ZZ, Hernandez L, Issaq H, Waalkes MP. Involvement of sulfhydryl metabolism in tolerance to cadmium in testicular interstitial cells. Toxicol Appl Pharmacol 1990;104:157-66.

Wahba ZZ, Waalkes MP. Cadmium-induced route specific alterations in essential trace element homeostasis. Toxicol Lett 1990;54:77-81.

Wahba ZZ, Waalkes MP. Effect of in vivo low-dose cadmium pretreatment on in vitro interactions of cadmium with isolated interstitial cells of the rat testes. Fund Appl Toxicol 1990;15:641-50.

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

PROJECT NUMBER
 Z01CP05673-01 LCC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Chemistry and Biology of Nitric Oxide

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:	D. Christodoulou	Intramural Research Training Awardee	LCC	NCI
	C. M. Maragos	Intramural Research Training Awardee	LCC	NCI
	J. E. Saavedra	Expert	LCC	NCI
	D. A. Wink	Staff Fellow	LCC	NCI

COOPERATING UNITS (if any) Temple U., Philadelphia, PA, (D. Morley); NHLBI, NIH (J. Diodati); PRI/DynCorp., Frederick, MD (J. Hrabie, A.W. Andrews); FDA, Rockville, MD (R. Elespuru); Glaxo, Inc., Research Triangle Park, NC (J. Allen)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, MD 27102-1201

TOTAL MAN-YEARS:

3

PROFESSIONAL:

3

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.)

Nitric oxide (NO) has been reacted with a variety of nucleophiles to form adducts of widely differing properties, extending our understanding of the electrophilic behavior of NO. Many of these compounds have shown both vasorelaxant activity in isolated rabbit aorta and *in vivo* hypotensive action on intravenous injection in rats. The *in vitro* potency was strongly correlated with the amount of nitric oxide these compounds were independently shown to release on spontaneous decomposition in physiological buffers, indicating that compounds in this series are of predictable potency based on physicochemical data. The most active of these agents proved comparable in potency to the clinical vasodilators, sodium nitroprusside and nitroglycerin. Duration of action in the *in vivo* experiments was most prolonged using a slow-release compound, the spermine/NO complex. The nucleophile/NO adducts have been derivatized by attaching alkyl groups or metal ion centers to either or both of the oxygen atoms; the resulting complexes have interesting properties as potential prodrugs. Other tests have shown the diethylamine/NO complex to be an inhibitor of platelet aggregation rivaling aspirin in potency. The results show that the nucleophile/NO complexes should be very useful tools for probing the involvement of nitric oxide in a variety of biomedical applications. Nitric oxide has also been shown to be capable of deaminating nucleic acids and their constituent units at pH 7.4 in the presence of air. NO-releasing compounds, including the nucleophile/NO complexes mentioned above and nitroglycerin, have been shown to induce point mutations in bacteria; nearly all the cases identified so far have been GC to AT transitions. The *in vivo* significance of these genotoxic deamination reactions is currently being evaluated.

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

L. K. Keefer	Chief, Chemistry Section	LCC	NCI
D. Christodoulou	Intramural Research Training Awardee	LCC	NCI
C. M. Maragos	Intramural Research Training Awardee	LCC	NCI
J. E. Saavedra	Expert	LCC	NCI
D. A. Wink	Staff Fellow	LCC	NCI

Objectives:

To examine the role of nitric oxide in bioregulatory phenomena from two unique vantage points: (1) the possibility that nitric oxide and its progenitors may be inherently genotoxic is being explored; and (2) the ability of nitric oxide to combine reversibly with various nucleophiles is under investigation as a tool for the controlled, spontaneous release of NO in a biologically useful form.

Major Findings:

The chemistry of the nucleophile/NO complexes has not previously been well investigated, despite having been known for almost two centuries. We have devoted considerable effort to this area, with special attention initially to synthesizing structurally diverse members of the series, including a number of adducts that were not previously described in the literature (collaboration with J. Hrabie). Rates of their spontaneous, unimolecular decomposition in aqueous buffers have been characterized by following the loss of their intense chromophore in the ultraviolet spectrum at 230-260 nm. The proportion of the material decomposed that is accounted for as NO was in turn measured by chemiluminescence techniques. An interesting dichotomy of mechanism was observed. Some of the compounds, such as the diethylamine and spermine adducts of NO, decompose to the parent amine and nitric oxide almost exclusively. Other compounds, such as the long-known sulfite adduct, decompose exclusively to nitrous oxide. To our surprise, analogs such as the isopropylamine derivative and $N_2O_3^{2-}$ ion suffer decomposition by both pathways, with the ratio between them being heavily dependent upon the concentration of the starting material.

These chemical results have several lines of significance, including offering insight into a three-decade-old controversy in the literature. Disagreement has been reported regarding the possible production of nitric oxide from Angeli's salt ($Na_2N_2O_3$), with essentially all recent commentators concluding that this did not happen. Our chemiluminescence and mass spectral data are incontrovertible in their demonstration that nitric oxide is produced; the fact that this occurs primarily at low (micromolar) concentrations only, with nitrous oxide being produced essentially exclusively at high concentrations, offers a means of rationalizing the previously published data.

The chemical results are also extremely useful in offering predictions of biological properties. Nitric oxide has only recently been identified with the action of endothelium-derived relaxing factor (EDRF), meaning that it is implicated as a central effector in the lowering of blood pressure. After confirming our hypothesis

that the nucleophile/NO complexes should be useful for lowering blood pressure via their ability to release nitric oxide *in vivo*, we quantified their *in vitro* vasorelaxant potencies and attempted to correlate them with the amount of nitric oxide released during the time frame of the experiment. The correlation coefficient was 0.996. We conclude that the ability of these complexes to relax the isolated rabbit aorta is quantitatively predictable based on their physicochemical properties (collaboration with Dr. Deborah Morley, Temple U.).

While in the *in vitro* experiments the diethylamine/nitric oxide complex gave the most interesting results (it was comparable in potency to the clinical nitrovasodilators, sodium nitroprusside and nitroglycerin), the spermine/NO complex expressed the advantage *in vivo* of long duration of action. Because it decomposes only one-twentieth as fast as the diethylamine adduct in pH 7.4 buffer, it showed little activity in the short time required to achieve maximum relaxation in aortic strip experiments. On the other hand, the spermine adduct was apparently able to titrate nitric oxide continuously into the blood stream over a prolonged period, keeping the blood pressure depressed for over an hour, while the diethylamine adduct and other similar derivatives had durations of action lasting only a few minutes. The *in vivo* studies were done in collaboration with Dr. Aaron Hoffman of the National Heart, Lung, and Blood Institute (NHLBI).

Another activity attributed to nitric oxide in the bioregulatory sphere is the inhibition of platelet aggregation. To test the hypothesis that our nucleophile/nitric oxide complexes should be active inhibitors of platelet aggregation, a collaboration with Dr. Jean Diodati of NHLBI was arranged. The drugs were added to whole blood or platelet-rich plasma (PRP) 1-20 minutes before mixing in collagen to initiate the coagulation process. The diethylamine complex proved to be as potent an inhibitor in whole blood as the agent used clinically for this purpose, aspirin. In PRP, the experimental drug was orders of magnitude more potent than aspirin. As predicted from the chemical data, the spermine adduct was also active, with the potency improving dramatically as the incubation period following exposure to the drug was increased from one to 20 minutes to allow more nitric oxide to be released from the slow acting agent.

As to the inherent genotoxicity potential of nitric oxide and its progenitors, exposure of deoxynucleosides, deoxynucleotides, and intact DNA to gaseous nitric oxide in the presence of air led to significant deamination of DNA bases bearing exocyclic primary amino groups at pH 7.4 and 37°C. Similar results were obtained when the nucleophile/NO complexes were used in place of the gaseous reagent. These results led to the hypothesis that if similar damage occurred in the living cell, point mutations might result. This was tested by administering the complexes to Ames *Salmonella* strain TA 1535 and searching for genotoxic consequences. The nitric oxide adducts with spermine, oxide, and diethylamine all proved mutagenic under the conditions tested (collaboration with A.W. Andrews). The numbers of revertants generated per plate by the spermine complex were sufficiently great relative to background that the identities of the point mutations induced could be determined with confidence (collaboration with Drs. Thomas Cebula, Rosalie Elespuru, and Walter Koch of the Food and Drug Administration). Using an oligonucleotide probing method to determine sequence changes at the *hisG46* locus, virtually all (113/114) of the induced mutants examined were the result of GC-AT transition mutations. Interestingly, nitroglycerin also proved mutagenic in this system. This clinically important cardiovascular drug is considered to be active via its metabolic

conversion to nitric oxide. In a collaboration with Dr. Jane Allen of Glaxo, Inc., dimethylsulfoxide solutions of 10% nitroglycerin on lactose powder, supplied as the article of commerce, generated a low but significant number of revertants at high concentrations of substrate. The results are consistent with the original hypothesis that exposure to NO and its progenitors in the presence of appropriate oxidizers can induce point mutations via nitrosative base deamination.

Additional types of biological activity in which nitric oxide has been inferred to play a bioeffector role are being investigated in an effort to characterize the overall biomedical potential of the nucleophile/NO complexes. To aid in the refinement of the biological properties, the nucleophile/NO adducts have been derivatized in two different ways: the oxygen atoms have been covalently attached to alkyl groups; they have been complexed with metal ion centers. Both types of derivative have prodrug character. The alkylated diethylamine/NO complex shows a prolonged hypotensive effect on intravenous injection in rats, consistent with the postulate that it requires metabolic activation (presumably by oxidative removal of the O-alkyl group) before NO release is possible and hypotensive activity can be expressed. Some of the metal complexes show excellent stability in aqueous solution, but appear to be activated on redox electron transfer from enzymatic or nonenzymatic sources. The chemistry and biology of these and related agents for controlled biological release of nitric oxide will be intensively investigated in the future in these laboratories.

Patents:

Keefer LK, Wink DA, Dunams TM, Hrabie JA. US Patent 4,954,526; Stabilized Nitric Oxide-Primary Amine Complexes Useful as Cardiovascular Agents, September 4, 1990.

ANNUAL REPORT OF

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

October 1, 1990 through September 30, 1991

The objectives 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, and to apply, whenever possible, the knowledge obtained from these studies towards effective prevention of cancer in man. The LEC seeks to accomplish this goal by bringing together and applying expertise in the diverse disciplines of cell biology, chemical and viral carcinogenesis, molecular biology, protein and nucleotide chemistry, and computer science towards characterization of the neoplastic process. Due to this integrated approach towards an understanding of mechanisms of the neoplastic process, considerable interaction occurs among the sections and the research groups in the LEC.

There are four research groups under the Office of the Chief (OC). The Image Processing and Computer Science and the Two-Dimensional Gel Electrophoresis Research groups continue to have considerable interaction both in developing the computer software needed for analysis of the 2-D gels and running, maintaining and developing the gel electrophoresis system. Although the main emphasis has been on 2-D analysis of the cellular proteins, recent development of two-dimensional DNA separation by using size fractionation of genomic DNA restriction fragments in combination with their sequence-dependent separation in denaturing gradient gels to measure mutational frequencies offers new and exciting possibilities. The 2-D Research Group is presently combining these two techniques in order to analyze the possible relationship between genomic instability and metabolic aberrations in neoplastic development.

The Food Derived Mutagen/Carcinogen Research Group was recently established under the OC. The main research focus of this Group is on the metabolic processing and genotoxicity of heterocyclic arylamines that have been isolated from cooked proteinaceous foods and as pyrolysis products of amino acids and proteins. These compounds are among the most mutagenic agents yet tested in the Salmonella mutagenicity assay, are carcinogenic in the rodent bioassay, and recently one of the heterocyclic amines (IQ) has been shown to be a potent carcinogen in nonhuman primates. Since the heterocyclic amines are formed under ordinary cooking conditions in foods which are staples of the American and Western European diet, it is important to evaluate the possible role that these compounds may have in the etiology of human cancer.

The tRNA Research Group joined the Laboratory of Experimental Carcinogenesis in 1986. The research is focused on characterizing the function, gene structure and regulatory elements which govern the expression of two minor opal suppressor tRNAs that are aminoacylated with serine and form phosphoseryl-tRNA in mammalian cells. The tRNA group is also involved in determining if the tRNAs utilized in ribosomal frameshifting in HIV and other vertebrate

retroviruses have any unique characteristics, and to identify the tRNAs involved in inframe suppression of the termination codons in mammalian type C retroviruses.

The research in the Chemical Carcinogenesis Section is predominantly focused on two areas: the mechanism(s) of neoplastic development in murine and human liver, and the molecular and cellular biology of the multidrug resistant gene family. The murine hepatocarcinogenesis model has been extensively used to study both the cellular and genetic events during chemically induced neoplasia in the liver. In particular, the research has been concentrated on characterizing the cellular biology of the hepatic stem cell compartment and to determine the possible role of these cells in hepatocarcinogenesis. We have also examined the possible role of TGF- β 1 as a major modulator of growth and differentiation in both normal liver and during hepatocarcinogenesis. In addition, considerable research efforts are focused on isolation and characterization of hepatic stem cells from rodents and primates, and on defining the genetic and epigenetic lineage determinants of the stem cells both *in vitro* and following autologous hepatic transplantation.

The major research endeavor associated with the multidrug resistant gene (*mdr*) family involves cloning and characterization of the rat *mdr* genes and determination of the molecular mechanism(s) responsible for the regulation of rat *mdr* genes by xenobiotics which will include characterization of the genetic element(s) responsible for the coinduction of *mdr* and cytochrome P450IA1 and P450IA2 genes. Our working hypothesis predicts that the induction of the *mdr* gene family by xenobiotics is mediated by a ligand-"receptor" complex(es) similar to and possibly related to the TCDD-"receptor" known to be involved in the induction of P450IA1 and IA2 genes.

The main area of research in the Hormone Action and Oncogenesis Section involves studies on chromatin structure and gene expression. In particular, the research is focused on the mechanism of transcriptional control by steroid receptors. The mouse mammary tumor virus has been used as the experimental model system to examine the regulation of transcription in mammalian cells both *in vivo* and *in vitro*. This has been achieved by generating a series of cell lines with the steroid-inducible MMTV LTR promoter mobilized on BPV vectors which are used to carry out an analysis of the hormone induced chromatin changes. The MMTV model has been used in extensive studies examining the impact of the chromatin structure of MMTV LTR on transcriptional regulation and for direct examination of proteins that interact with the MMTV promoter upon hormonal stimulation. Acquisition of a mouse mammary tumor cell line having an MMTV provirus integrated upstream from the *int-2* gene has made it possible to initiate studies aimed at identifying the regulatory elements responsible for MMTV tissue-specific expression as well as to explore the mechanism(s) involved in MMTV activation of the *int-2* oncogene. The mechanism of glucocorticoid repression of gene expression is also being investigated by using the proopiomelanocortin (POMC) gene. The POMC promoter can confer negative regulation on heterologous genes and the glucocorticoid receptor has been shown to footprint *in vitro* on several locations in the promoter, making this a useful model system for studying negative transcriptional control. Furthermore, studies analyzing the interaction of peptides corresponding to a putative zinc finger domain in the glucocorticoid receptor with the GRE

sequence as well as a project aimed at obtaining large quantities of functional glucocorticoid, progesterone, and vitamin D3 receptors by over-expressing these proteins in recombinant adenovirus have recently been initiated.

The research program in the Biopolymer Chemistry Section involves establishing and applying methodologies to study structure and function of proteins that are associated with neoplastic development. The present work is aimed at purifying and characterizing the plasma membrane polypeptides that are expressed at different stages in hepatocytes during chemically induced hepatocarcinogenesis. The use of high resolution 2D-PAGE allows the separation and identification of several hundred polypeptides from crude materials or partially purified extracts. The primary obstacle in effectively linking this technique to molecular and cellular biology is the difficulty in obtaining amino acid sequence data directly from the same polypeptides that are observed in specific gels. The ongoing research effort is focused on establishing highly sensitive protein sequencing methodologies capable of direct analysis on samples electroblotted from 2D-PAGE gels and thereby effectively breaking the barrier linking this powerful technique with that of protein chemistry, making it possible to obtain structural information on biologically important molecules.

Progress made in the past year in various phases of our research effort is summarized below:

Studies on Cellular and Molecular Biology of Hepatocarcinogenesis

The existence of a stem cell compartment in rat liver is now well established, and a considerable body of data indicate that the human liver also contains a stem cell compartment. The research is currently focused on defining the cellular and molecular biology of this stem cell compartment in normal and neoplastic liver (see also Project No. Z01CP05262). Rat liver epithelial (RLE) cells share many cellular markers with the "oval" cell population found in adult rat liver after a certain type of hepatic injury. We have hypothesized that these cell types are derived from a common stem cell compartment present in the adult liver. Investigation of patterns of cytokeratin expression in adult and fetal livers and during early stages of "oval" cell proliferation revealed that cytokeratin 14 was transiently expressed in early fetal hepatocytes and oval cells. The expression of cytokeratin 14 in adult rat liver was found in small epithelial cells in the periductal space. These results indicate that cytokeratin 14 may be a marker for early progeny derived from the hepatic stem cell compartment. Transplantation of spontaneous transformed RLE cells in vitro resulted in formation of highly differentiated hepato-cellular carcinomas. We have examined the stages of this in vitro transformation of RLE cells and discovered that coincident with the transformation process is an activation of a hepatocytic differentiation program that includes expression of albumin, alpha-fetoprotein, switching from connexin 43 to connexin 32 expression, and the expression of cytokeratins 8 and 18. These results formally demonstrate that progeny from the hepatic stem cell compartment such as the RLE cells and "oval" cells in vivo can be a target cell population in hepatocarcinogenesis and a precursor for hepato-cellular carcinomas. We have recently obtained evidence that epithelial cell lines obtained from adult pancreas have, similar to the RLE cells, an extended capacity for proliferation in vitro, and share close lineage relationship to

the RLE cells. These data indicate a common cell of origin for primitive cells isolated from rat liver and pancreas.

The expression of transforming growth factor α (TGF- α) was studied in hepatocytes following partial hepatectomy and also during hepatic differentiation in fetal, neonatal hepatocytes and in adult liver after acetylaminofluorene (AAF) administration by using both Northern blot and in situ hybridization methods. The level of TGF- α expression was increased six- to eightfold after partial hepatectomy and was also observed in the fetal and neonatal hepatocytes. Proliferating oval cells, perisinusoidal stellate cells as well as basophilic foci generated by the modification of Solt-Farber protocol were positive for TGF- α transcripts. The observation that TGF- α is expressed in both fetal and differentiating hepatic cells suggests that one of the TGF- α effects, in addition to the mitogenic stimulation, may involve the induction of hepatocytic differentiation in primitive liver cells. In contrast, heparin binding growth factor (HBGF-1) showed only a slight increase during fetal development but was observed to be significant in the liver treated with AAF.

Temporal and cellular distribution of transcripts for transforming growth factor (TGF)- β 1, procollagen I, III and IV, as well as for type IV collagenase (72 kDa gelatinase) were studied in order to elucidate (1) the pathogenesis of liver fibrosis (or cirrhosis) and (2) the possible role of TGF- β 1. Our study on liver fibrosis suggests that TGF- β 1 derived from inflammatory cells may have enhanced the expression of type I collagen as well as that of TGF- β 1 gene itself in desmin-positive perisinusoidal cells by paracrine mechanism. The simultaneous expression of TGF- β 1 and type I, III and IV collagen genes in mesenchymal cells during the fibrotic process also suggests the possibility that TGF- β 1 may have an important role in the production of liver fibrosis. We studied the regulatory mechanisms of synthesis and degradation of type IV collagen which is one of the main components of basement membrane and of accumulated fibrous tissue in liver fibrosis. The data suggest that the imbalance observed in 72 kDa gelatinase and type IV collagen expression in early stages of liver fibrosis may lead to the accumulation of type IV collagen. Furthermore, the data indicate that desmin-positive perisinusoidal cells play a central role in regulating type IV collagen deposition in CCl₄-induced rat liver fibrosis. In Solt-Farber's chemical hepatocarcinogenesis process, non-parenchymal cells of the liver, mainly desmin-positive perisinusoidal cells, are the principal source of TGF- β 1 production. Our data suggest that the close interaction between non-parenchymal cells and carcinoma cells may be necessary for the activation of latent TGF- β 1. It is hypothesized that regulatory effects of TGF- β 1 on growth of preneoplastic or carcinoma cells in the liver are exerted via the paracrine mechanism.

We have used the spontaneous transformation of rat liver derived epithelial (RLE) cells as a model to examine the change in the growth inhibitory effects of TGF- β 1 during the transformation process. The appearance of morphologically aberrant transformants correlated directly with an increased resistance of the population to the growth inhibitory effects of TGF- β 1. Clonal cell lines derived from the transformants were resistant to TGF- β 1-dependent inhibition of DNA synthesis. These cell lines were also highly tumorigenic, aneuploid with characteristic gross chromosomal abnormalities, and expressed a number of phenotypic markers common to rat liver epithelial

cells transformed by oncogenes or chemicals. In contrast, apparently normal-looking cell lines cloned from the same population were non-tumorigenic, near-diploid with few chromosomal gross abnormalities and were as sensitive to TGF- β 1 as early passage normal RLE cells. Morphologically normal late passage rat liver epithelial cells were sensitive to transformation by the DNA hypomethylating agent 5-aza-2-deoxycytidine, in contrast to earlier passage cells, and this transformation was accompanied by the development of resistance to the growth inhibitory effects of TGF- β 1. These findings suggest that acquisition of resistance to the effects of growth inhibitors such as TGF- β 1 is an important and possibly essential stage in the spontaneous transformation of RLE cells. In addition to the TGF- β 1 studies we are presently attempting to obtain partial protein sequence data on the highly purified fraction of LDGI.

Multidrug resistance is the phenomenon by which cells become cross-resistant to a range of unrelated compounds in response to exposure to a single agent; this resistance is the result of overexpression of a 170 Kd membrane protein, p-glycoprotein, which is encoded by the *mdr* gene(s). Previously, we have shown that exposure of rats to xenobiotic agents such as the carcinogens aflatoxin B1, isosafrole and 2-acetylaminofluorene (2-AAF) causes increased expression of *mdr* mRNA in the liver. To further study the mechanism by which xenobiotics regulate *mdr* gene expression, we have employed a primary hepatocyte culture system. Exposure of isolated hepatocytes to methycolanthrene (MC), 2-AAF but not 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) increased the expression of *mdr* mRNA expression; concomitant increases in cytochrome P4501A gene(s) expression were also observed with these agents. Inhibition of protein synthesis also increased the expression of *mdr* mRNA in these cells. The enhanced *mdr* expression caused by these compounds is a result of increased transcription. These data suggest that *mdr* expression is regulated by a protein that is distinct from the Ah receptor. To further investigate the mechanism of *mdr* regulation it was necessary to isolate the rat *mdr* genes. We have identified that the rat *mdr* gene family is comprised of three members; one of these genes has been isolated and characterized. Sequence analysis of a complete cDNA for a rat *mdr* cDNA indicated a high degree of identity to the mouse *mdr1b* gene (*mdr1*); thus, this rat cDNA was designated the *mdr1b* gene. Further studies on the 5' promoter and possible 3' mRNA stability regions are being performed. We observed in normal liver a slight but significant zonal difference of distribution of *mdr* transcripts (zone 1 > zone 3). Significant increase of *mdr* transcripts during regeneration was observed mainly in zone 1 hepatocytes. Our data further suggest that the increased *mdr* expression might represent a subpopulation of preneoplastic nodules which possibly undergo malignant transformation more specifically than GST-P positive nodules. Therefore, increased *mdr* expression could be a marker for hepatocytes which possibly undergo malignant transformation.

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

The two-dimensional gel electrophoresis (2DG) 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) the continued development of the computer system (dubbed ELSIE) to analyze gels and (2) the use of ELSIE to analyze experiments requiring computerized analysis of 2DGs. The ELSIE 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 a normal diploid karyotype and represent a homogeneous, clonal system for the study of transformation. Different retrovirus containing transforming oncogenes, such as v-H-ras, v-ras, v-raf, or a v-myc/v-raf chimera have been used to transform RLE cells or a human diploid fibroblast line (LG-1). The polypeptide patterns of the transformed cells differ significantly from their non-transformed progenitors. While qualitative differences are rare, over 30% of the spots may vary quantitatively. Very similar oncogenes, such as v-H-ras and v-N-ras, cause essentially indistinguishable 2DG patterns. These patterns are distinct from those caused by v-raf and v-myc/v-raf mediated transformation. The v-raf and v-myc/v-raf patterns are also readily distinguishable, but are more similar to each other than to non-transformed or to ras transformed cells.

Rat liver epithelial (RLE) cells provide a very valuable in vitro model to study both spontaneous and chemically induced hepatocarcinogenesis. Therefore, the main objectives of this project are to characterize the early cellular biochemical events as a result of genetic alterations and subsequent changes in protein expression during spontaneous and chemically mediated hepatocarcinogenesis. RLE cells can be transformed in vitro by various agents including chemical carcinogens and specific oncogenes as well as spontaneously when the cells are maintained under certain selective growth conditions. When injected into nude mice, the transformed cells produce a diverse variety of tumor types including those of a well-differentiated hepatocellular carcinoma-like tumor. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was utilized to construct comprehensive computerized topographical protein database maps of protein expression in each of the major subcellular fractions (whole cell, cytosol, nuclei, mitochondria, plasma membrane). Genetic analysis of aflatoxin B1 induced RLE transformants revealed a consistent point mutation consisting of a transition from G to A within codon 173 (CGC to GAG) in the tumor suppressor p53 gene. This mutation resulted in a change of the encoded amino acid from histidine to arginine. Mutations in the p53 gene are currently being analyzed in monkey genomic DNA extracted from paraffin embedded AFB induced tumors.

Two-dimensional gel electrophoresis (2DG) has been used to study the changes induced in dog plasma by the known urinary bladder carcinogens 4-aminobiphenyl (4-ABP) and 2-naphthylamine (2-NA). 3-Aminobiphenyl (3-ABP) and 1-naphthylamine (1-NA), both considered to be non-carcinogenic, were used as controls. The purpose of this study was: (1) to determine whether or not the apparent deletions on plasma proteins are specific to 4-ABP; (2) to measure the time course in the suppression of the major polypeptides during dosing and their re-synthesis during a recovery period; and (3) to determine, by micro-sequencing directly off the gels, the biochemical identity of the affected spots. The results indicate that the suppression is limited to 4-ABP, with 3-ABP, 2-NA and 1-NA causing no discernible change in the 2DG patterns over a 12 week dosing period. The 4-ABP caused dramatic suppression of two sets of spots. One of apparent molecular weight 32.5 kD, and pI 5.8-6.0, was identified to be the B chain of haptoglobin. This spot disappears after about two weeks of treatment and recovers slowly after dosing stops. Haptoglobin functions to bind with free hemoglobin and purge it from the blood stream. Since 4-ABP causes hemolysis, the disappearance of the haptoglobin is readily explained. Among the second set of spots, a MW 65 kD, pI 6.5-6.6 peptide,

disappears much faster than the haptoglobin, and recovers more quickly. The protein is about one-fifth the intensity of haptoglobin and appears to be blocked, as N-terminal microsequencing has been unsuccessful. Work is currently underway to obtain an internal sequence of this polypeptide in order to identify it.

Protein Structure and Function

Previously unidentified rat liver membrane glycoproteins, whose regulation is qualitatively and quantitatively altered during the course of chemically-induced hepatocarcinogenesis, have been observed by utilizing 2D-PAGE. The main goal of this project is to purify and characterize the specific glycoproteins which demonstrate such differences in expression in the plasma membranes of normal and neoplastic rat livers. This information will aid in understanding 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 sequenceable in this manner, presumably because of blocked N-termini. For this reason, research was continued to develop procedures for obtaining amino acid sequence information from all of these proteins, whether blocked or unblocked. This work focuses on methods development in areas that have become critical in order to obtain results from minute quantities of proteins isolated from 2D-gels. Various techniques involved in amino acid sequencing of transblotted samples have been notably improved. A new type of low volume reaction cartridge for the sequencer was used and the conditions modified for our specific purposes. This resulted in the attainment of sequencing data from transblotted samples that is equal to those obtained from the direct application of purified proteins to the sequencer on a polybrene-coated glass filter. In a test case we have identified 3 of 5 proteins analyzed that were purified from whole dog serum using a single 2D-polyacrylamide gel. We are currently making excellent progress in developing procedures to obtain internal sequence data from proteins purified in this manner.

Studies on Suppressor tRNA

We have sought to determine if host aminoacyl-tRNAs required for decoding the frameshift signal in human immunodeficient virus (HIV) and other retroviruses are altered (i.e., hypomodified) from the normal cellular aminoacyl-tRNAs. Preliminary evidence from this laboratory has suggested that these tRNAs in HIV and other retroviral infected cells are hypomodified. The mouse mammary tumor virus (MMTV) *gag-pro* frameshift signal (sequence A AAA AAC where C is the site of the frameshift) is being used as a model for these studies. We obtained the wild type frameshift signal and a series of mutations at the frameshift site in MMTV as a tool for determining if the tRNA required to alter the reading frame is hypomodified. The level of frameshifting is reduced dramatically with an AAU mutant even though AAC/AAU codons are decoded by the same asparagine isoacceptor with similar efficiencies at other mRNA sites. Hypomodified asparagine tRNA which lacks the highly modified Q base is known to preferentially read AAC over AAU codons. Thus, the observation that the AAC codon at the frameshift site promotes ribosomal frameshifting more efficiently than the AAU codon suggests that the tRNA lacking Q base is required for decoding the frameshift signal. We have not been successful in

utilizing in vitro assays thus far to demonstrate a requirement for hypomodified isoacceptors in ribosomal frameshifting due to the endogenous tRNA population in the assay system. However, we have developed an in vivo assay for ribosomal frameshifting utilizing microinjection techniques into *Xenopus* oocytes and are presently assaying the ability of purified hypomodified isoacceptors to alter the reading frame of the wild type and mutant MMTV gag-pro frameshift signals.

Two selenocysteine tRNA isoacceptors have been identified in mammalian cells in this laboratory. These tRNAs have two functions: 1) to read the termination codon, UGA, in protein synthesis and thus donate selenocysteine to the growing polypeptide chain in response to specific UGA codons; and 2) to serve as carrier molecules for the biosynthesis of selenocysteine. The pathway for the biosynthesis of selenocysteine is tRNA[Ser]Sec ---> seryl-tRNA[Ser]Sec ---> phospho-seryl-tRNA[Ser]Sec ---> selenocysteyl-tRNA[Ser]Sec. Both isoacceptors (designated NCA and CmCA on the basis of their anticodon sequences) arise from a single copy gene and differ from each other by five pyrimidine transitions which occur in the 5' half of the molecule. Therefore, one isoacceptor must be an edited version of the other. Both isoacceptors can be generated from the selenocysteine tRNA gene after its injection into *Xenopus* oocytes. Isolation of the primary transcript from *Xenopus* oocytes and its reinjection results in formation of both isoacceptors. We are presently investigating the pathway of synthesis of these isoacceptors as to whether one is a precursor of the other or both arise from a common precursor. The relative abundance of the selenocysteine tRNA population and the distributions of NCA and CmCA are influenced by the presence of selenium in mammalian cells grown in culture and in rat tissues taken from rats on diets with and without selenium, suggesting that tRNA editing occurs and the editing process responds to selenium. A selenocysteine tRNA that decodes UGA has been identified in animals, plants and protists in this laboratory (and in bacteria by other investigators) and thus the universal genetic code has been expanded to include selenocysteine as the 21st naturally occurring amino acid.

Studies on Chromatin Structure, Glucocorticoid Receptor and Gene Expression

Activation of the MMTV LTR promoter by steroid hormones proceeds through the assembly of a transcription initiation complex composed of factors NF1/CTF, octamer binding protein, and TFIID. We have found that loading of NF1 occurs constitutively on transiently transfected DNA, indicating that recruitment of some factors (NF1 requires a chromatin modification mechanism, whereas other factors (Oct-1) probably interact directly with receptor. To study the mechanism by which this process occurs, it is necessary to attempt reconstitution of this system in vitro. Steroid receptors are in low abundance in tissues and therefore difficult to purify. We have cloned steroid receptor cDNAs (glucocorticoid and vitamin D) into the adenovirus genome to form recombinant viruses which can infect cultured cells and express very high levels of functional receptors. These receptors have been partially purified, and shown to be highly active in specific DNA binding. We have further examined the mechanism of DNA binding by individually synthesizing the putative "zinc finger peptides" from the rat glucocorticoid receptor. Circular dichroism experiments demonstrated a significant alteration in secondary structure in the presence of zinc that is pH dependent and correlates directly with DNA binding. DNA binding experiments established that single fingers can successfully compete with the intact DNA binding domain in

a novel gel shift competition assay. Mutations in conserved cysteines fail to compete. Mutations in the CII finger establish that the final cysteine is required for DNA binding. The results suggest that a single synthetic "finger peptide" is able to bind to DNA in a sequence specific manner.

The sites to which steroid-receptors bind on the MMTV LTR are positioned on the surface of nucleosome B in a phased array of nucleosomes. Hormone activation of the MMTV promoter leads to modulation of nucleosome B structure in vivo which leads to a region of DNA hypersensitive to nucleolytic reagents. High-resolution analysis of the nucleosome position using PCR amplification indicates that the nucleosomes are positioned at base pair resolution, and that the hypersensitivity results not from nucleosome displacement, but from alteration in DNA structure across the nucleosome and into the A-B linker region. NF1, a component of the MMTV initiation complex is excluded from uninduced stable chromatin (see project ZOICP04986-14), but binds to transiently introduced DNA. We find that hormone induction also leads to H1 depletion from the A-B region in stable chromatin, whereas the core histone complement of this region is unchanged. We also find that a disomic structure composed of the A and B nucleosomes can be reconstituted in vitro, with the octamer cores accurately positioned, and that NF1 is excluded from this disomic structure. The glucocorticoid receptor, in contrast, can bind to the disome. Thus, two potential mechanisms exist for the exclusion of NF1, either (1) exclusion by nucleosome positioning, or (2) exclusion by a higher order, H1-dependent, chromatin structure. Modulation of the structure is necessary during transcription activation to permit binding of the initiation complex. These results indicate that a chromatin template containing specifically positioned nucleosomes is an active participant in transcriptional activation, and that modulation of this template structure is one feature of steroid hormone action.

The proopiomelanocortin (POMC) gene produces a complex precursor polypeptide that is posttranslationally cleaved into a series of peptide hormones. In response to stress, one of these hormones, ACTH, induces the production of glucocorticoids in the adrenal gland, which in turn act to suppress POMC transcription in the pituitary in a classical feedback loop regulatory mechanism. This locus manifests two regulatory features of interest in our laboratory, negative regulation of gene expression by steroids, and tissue-specific gene expression. The overall goals of the project are to identify and characterize tissue-specific and positive transacting factors that regulate POMC promoter activity, and to investigate mechanisms by which glucocorticoids mediate transcriptional repression. During characterization of potential regulatory regions in the POMC promoter by the classical mutagenesis and transfection approach, an unusual transcription factor (designated PO-B) was discovered. This protein activates POMC expression via a high-affinity DNA-binding site that is located between the TATA box and the cap site for initiation of RNA synthesis. The DNA recognition site for this factor has been characterized by the DNase I footprinting and DMS methylation interference techniques. In both in vitro transcription extracts and in vivo transfection studies, the TATA binding region is required, suggesting the factor acts cooperatively with TFIID. The factor has been extensively purified, and protein sequencing is underway.

The MMTV retrovirus is able to activate cellular oncogenes (so-called int loci) by insertion in the vicinity of these genes. This insertion is one of the key events leading to transformation of mammary epithelial cells. The mammary cell specificity of MMTV oncogenesis lies, in part, in tissue-specific expression of the MMTV promoter. Utilizing a recently developed cell line series (RAC cells) that retains many epithelial cell features in culture, we have discovered two regulatory elements in the viral regulatory sequences that are responsible for cell-specific viral transcription, and presumably also for tissue-specific oncogene activation. One of these elements, located at the 5' end of the LTR, acts as a cell-specific, positive enhancer region, and has been shown to contain binding sequences for at least two proteins, mp5 and mp4, both of which also appear to be limited in distribution to mammary cells. A second element, located internal to the LTR, acts as a negative transcription regulator, and also is restricted in its activity to a subset of mammary cells. Two factors that bind to this element and are tissue limited in distribution have been preliminarily characterized. Both the positive and negative factors correspond to similar activities present in humans cells, suggesting this regulatory system is active in man. These observations suggest that MMTV protooncogene activation is mediated by a combination of positive and negatively-acting tissue specific elements.

Studies on Food-Derived Mutagens/Carcinogens and Cytochrome P450

Heterocyclic arylamines (HAAs) are formed in meats such as beef, chicken and fish upon cooking at normal, household temperature. These compounds are potent promutagens in the Ames *Salmonella* mutagenicity assay and a number of these compounds have been shown to be carcinogenic in rodent bioassays. 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ) has been recently shown to be carcinogenic in cynomolgus monkeys. We have previously shown, using recombinant cytochromes P450-expressed in human cells, that cytochrome P450IA2 is the predominant cytochrome P450 responsible for the activation of these compounds via N-oxidation. Further studies have shown that N-hydroxy-IQ covalently binds to DNA directly and following further activation by O-acetyl- and sulfotransferases. Adducts of IQ have been examined in rodents and monkeys and found to be identical to those formed in vitro upon reaction of N-hydroxy-IQ with DNA and those found in *Salmonella*. In addition to in vitro studies of HAA metabolism we are studying the in vivo metabolism and distribution of HAAs in monkeys. 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ) is extensively metabolized in monkeys and excreted into urine and feces as metabolites. N-Sulfation, glucuronidation and N-demethylation play important roles in the detoxification and excretion of this compound. Metabolic processing also appears to be influenced by the dose of IQ. In light of this finding, we have begun to examine HAA-DNA adducts, and the distribution and excretion of HAA metabolites at food-equivalent doses by accelerator mass spectrometry. This is a highly sensitive method for counting ¹⁴C atoms following administration of low levels of radiolabeled compound. Studies concerning repair in specific genes using the UvrABC excinuclease and alkaline hydrolysis are also underway. Studies are being carried out in isolated genomic DNA, plasmids and cultured cells. With this methodology we are seeing heterogeneity in initial adduct formation in different gene regions and differences in adduct levels with N-hydroxy-IQ and N-hydroxy-MeIQx.

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 fields and are involved as consultants or advisers 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 research 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

Z01CP04986-14 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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: Trevor K. Archer Visiting Associate LEC NCI
 Ronald G. Wolford Microbiologist LEC NCI
 Diana S. Berard Biologist LEC NCI

COOPERATING UNITS (if any)

Metabolic Diseases Branch, NIDDKD (Catharine 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:

2.6

PROFESSIONAL:

2.1

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.)

Activation of the MMTV LTR promoter by steroid hormones proceeds through the assembly of a transcription initiation complex composed of factors NF1/CTF, octamer binding protein, and TFIID. We have found that loading of NF1 occurs constitutively on transiently transfected DNA, indicating that recruitment of some factors (NF1 requires a chromatin modification mechanism, whereas other factors (Oct-1) probably interact directly with receptor). To study the mechanism by which this process occurs, it is necessary to attempt reconstitution of this system in vitro. Steroid receptors are in low abundance in tissues and therefore difficult to purify. We have cloned steroid receptor cDNAs (glucocorticoid and vitamin D) into the adenovirus genome to form recombinant viruses which can infect cultured cells and express very high levels of functional receptors. These receptors have been partially purified, and shown to be highly active in specific DNA binding. We have further examined the mechanism of DNA binding by individually synthesizing the putative "zinc finger peptides" from the rat glucocorticoid receptor. Circular dichroism experiments demonstrated a significant alteration in secondary structure in the presence of zinc that is pH dependent and correlates directly with DNA binding. DNA binding experiments established that single fingers can successfully compete with the intact DNA binding domain in a novel gel shift competition assay. Mutations in conserved cysteines fail to compete. Mutations in the CII finger establish that the final cysteine is required for DNA binding. 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 L. Hager	Head, Hormone Action and Oncogenesis Section	LEC NCI
Trevor K. Archer	Visiting Associate	LEC NCI
Ronald G. Wolford	Microbiologist	LEC NCI
Diana S. Berard	Biologist	LEC NCI

Objectives:

(1) Determination of the molecular mechanism responsible for glucocorticoid receptor-mediated induction of transcription at hormonally regulated promoters.

(2) 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) Steroid receptor cDNAs are cloned into expression plasmids containing viral promoters.

(2) The expression plasmids are used to make recombinant adenovirus genomes by in vivo recombination with partial viral genomes.

(3) Recombinant viruses are purified and the receptors they produce upon infection of cultured cells are analyzed for size, interaction with specific antibodies, ligand binding and affinity, and DNA binding.

(4) Virally produced receptors are partially purified by well-defined techniques.

(5) Interactions of partially purified receptors with nucleosomes and other transcription factors are characterized by various footprinting techniques and gel mobility shift analysis.

(6) Partially purified receptors are used in transcription assays with promoters utilized as naked DNA templates, or reconstituted into chromatin.

(7) Zn finger peptide structure is analyzed by atomic absorption spectrophotometry and by circular dichroism spectroscopy.

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. 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 (-30 to +1 region), and Oct-1 (-45 to -30 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.

We have discovered that when DNA is introduced into the cell in a transient transfection protocol, NF1 loads on the DNA in a constitutive, hormone-independent manner. The hormone-dependent loading observed in stable chromatin must therefore result from some feature of chromatin organization. We propose that NF1 is excluded from the template by nucleoprotein structure, and that the receptor in some way perturbs this structure to permit NF1 binding. In contrast, other components of the transcription initiation complex, such as Oct-1, may interact directly with the receptor (or some bridging factor). Hormone activation therefore results from a bimodal mechanism, involving both modulation of chromatin structure and protein-protein contacts between transcription factors. Nucleoprotein structure and its maintenance, perhaps by specific negative modulatory factors, may be a critical determinant in regulation of gene expression.

In order to produce amounts of functional steroid receptors needed for reconstitution experiments, cDNAs for the human glucocorticoid and vitamin D receptors were cloned into expression vectors and inserted into the adenovirus genome. The receptors produced by these viruses have been characterized and found to be highly functional. Both receptors were found to bind DNA-cellulose and also manifest specific binding for the appropriate hormone response elements *in vitro*. These purified receptors can now be used to characterize their interaction with hormone response elements and other transcription factors in the context of naked DNA or reconstituted chromatin.

A fundamental understanding of the mechanism by which steroid receptors recognize and bind at their cis-regulatory sequences requires a detailed analysis of the DNA-protein interaction. We have synthesized peptides corresponding to the putative "zinc fingers" of the glucocorticoid receptor protein. The peptides were able to bind zinc and were 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. Thus, we have shown that several important aspects of the receptor-DNA interaction can be modelled with relatively small, chemically synthesized peptides.

Publications:

Archer TK, Hager GL, Omichinski JG. Sequence specific DNA binding by glucocorticoid receptor "finger peptides." Proc Natl Acad Sci USA 1990;87:7560-4.

Hager GL, Archer TK. The interaction of steroid receptors with chromatin. In: Parker MG, ed. Nuclear hormone receptors. London: Academic Press, 1991;217-34.

Smith CL, Hager GL, Pike JW, Marx S. Overexpression of the human vitamin D3 receptor in mammalian cells using recombinant adenovirus vectors. Mol Endocrinol (In Press).

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

PROJECT NUMBER

Z01CP05262-10 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Cellular Evolution of Chemically Induced Rat Hepatoma

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
	Zongyi-Hu	Visiting Fellow	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 main emphasis in this project has recently been on the role of transforming growth factor alpha (TGF- α) in the growth and differentiation of the hepatic stem cell compartment. The expression of (TGF- α) was studied in hepatocytes following partial hepatectomy and also during hepatic differentiation in fetal, neonatal hepatocytes and in adult liver after acetylaminofluorene (AAF) administration by using both Northern blot and in situ hybridization methods. The level of TGF- α expression was increased six- to eightfold after partial hepatectomy and was also observed in the fetal and neonatal hepatocytes. Proliferating oval cells, perisinusoidal stellate cells, as well as basophilic foci generated by the modification of the Solt-Farber protocol, were positive for TGF- α transcripts. The observation that TGF- α is expressed in both fetal and differentiating hepatic cells suggests that one of the TGF- α effects, in addition to the mitogenic stimulation, may involve the induction of hepatocytic differentiation in primitive liver cells. In contrast, heparin binding growth factor (HBGF-1) showed only a slight increase during fetal development but was observed to be significant in the liver treated with AAF.

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

Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Harushige Nakatsukasa	Expert	LEC NCI
Zongyi-Hu	Visiting Fellow	LEC NCI
Elizabeth Marsden	Biologist	LEC NCI

Objectives:

The objective of this project is to characterize the role of various growth factors in the proliferation and differentiation of primitive hepatic cells. The topics under investigation are: (1) the contribution of growth factors to the proliferation and differentiation of putative stem cells in the liver; (2) cell specific distribution of gene transcripts among epithelial and mesenchymal liver cells; (3) employment of the rat model to study human acute hepatic failure, in particular the effects of various growth factors on liver morphology and on the survival of the animals.

Methods Employed:

(1) Northern hybridization for observing changes in gene expression. (2) In situ hybridization for spatial localization of specific mRNA. (3) Quantitation of in situ hybridization data by using Magiscan Image Analysis system. (4) Isolation of plasmid DNA to be used for transcription of ³⁵S-labeled riboprobes for in situ hybridization.

Major Findings:

(1) Both extrahepatic and intrahepatic derived growth factors are thought to be involved in the mitogenic stimulation of hepatocytes after partial hepatectomy. Epithelial growth factor (EGF) and hepatocyte growth factor (HGF) are the principal extrahepatic growth factors, while heparin binding growth factor (HBGF-1) and transforming growth factor alpha (TGF- α) are the major intrahepatic growth factors. The role of mitogenic hepatic growth factors in the normal development and differentiation of the liver is still poorly defined. Of special interest are the intrahepatic growth factors, particularly TGF- α and HBGF-1. Previous studies from other laboratories have demonstrated an increase in TGF- α transcripts in hepatocytes after partial hepatectomy, in hepatocellular carcinomas and in various liver derived cell lines, especially after transformation. Therefore, an increased expression of TGF- α has usually been associated with neoplastic transformation. During the fetal development TGF- α is expressed as early as in the gastrulation stage of embryo, whereas during the embryonic development, its transcripts were only transiently present between day 8 and 10 of fetal life.

We have demonstrated both with Northern and in situ hybridization a significant expression of TGF- α transcripts in the embryonic and in neonatal rat livers. Quantitation of the number of silver grains by using image analysis revealed a six- to eightfold increase of TGF- α expression in fetal and neonatal livers as well as in livers after partial hepatectomy. In the differentiating cells of adult liver after acetylaminofluorene (AAF) administration, prominent expression of TGF- α was observed both in oval cells and in the differentiating small basophilic hepatocytes. The combination of in situ hybridization and immunohistochemistry using oval cell and Ito cell specific antibodies revealed the presence of TGF- α transcripts in both oval cells and in Ito cells.

Another intrahepatic growth factor, HBGF-1, is involved in the early proliferative process after partial hepatectomy by binding to high affinity receptors. Our preliminary data demonstrate a significant increase of this growth factor after AAF administration at the stage when a large number of primitive hepatic cells (oval cells) and mesenchymal perisinusoidal cells (Ito cells) occupy large areas of liver acini.

(2) There are important similarities between the lesions and functional disturbances observed in human acute hepatitis and those generated by the modification of Solt-Farber protocol utilizing the rat model. These include: (a) yellow staining of liver due to the failure of liver to conjugate bilirubin and excrete it in the bile, leading to the formation of bile thrombi; (b) failure to produce plasma proteins in normal amounts, particularly albumin; (c) focal necrosis; (d) disruption of liver cell plates; (e) and an increase in cellularity. Our efforts will be focused on the development of an appropriate administration route for various growth factors to rats undergoing acute hepatic failure and further, to analyze the effects of this treatment on the outcome of hepatic failure in the rats.

(3) Retinoic acid receptors alpha, beta and gamma (RAR- α , RAR- β , RAR- γ) belong to the steroid/thyroid hormone family of intranuclear receptors. Retinoids are known to play an important role in cellular differentiation and also in the pattern formation of the embryo. Retinoic acid receptors (RAR) function as transcriptional retinoic acid-inducible enhancer factors. This provides a basis for understanding how retinoic acid controls gene expression. Their spatial and temporal expression seem to indicate that each RAR controls the expression of a particular group of genes characteristic for that tissue. We have initiated studies concerning their role in hepatic differentiation and carcinogenesis, by using in situ hybridization for the spatial localization of retinoic acid receptors in liver during embryonic development and in chemical hepatocarcinogenesis.

Publications:

Thorgeirsson SS, Evarts RP. Experimental hepatocarcinogenesis: relationship between oval cells and hepatocytes in rat liver. In: Tabor E, Di Bisceglie AM, Purcell RH, Eds. Advances in applied biotechnology series, vol. 13. Etiology, pathology, and treatment of hepatocellular carcinoma in North America. Houston TX: Gulf Publishing, 1991;171-5.

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

PROJECT NUMBER

Z01CP05263-09 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Two-Dimensional Gel Analysis of Oncogene-Mediated Transformation

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
 Alfredo Romano Guest Researcher LEC NCI

COOPERATING UNITS (if any)

Michigan State University, East Lansing, MI (Dr. Justin McCormick)
 University Hospital, Geneva, Switzerland (Dr. Denis Hochstrasser)

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 unrounded 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) the continued development of the computer system (dubbed ELSIE) to analyze gels and (2) the use of ELSIE to analyze experiments requiring computerized analysis of 2DGs. The ELSIE 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 a normal diploid karyotype and represent a homogeneous, clonal system for the study of transformation. Different retrovirus containing transforming oncogenes, such as v-H-ras, v-N-ras, v-raf, or a v-myc/v-raf chimera have been used to transform RLE cells or a human diploid fibroblast line (LG-1). The polypeptide patterns of the transformed cells differ significantly from their non-transformed progenitors. While qualitative differences are rare, over 30% of the spots may vary quantitatively. Very similar oncogenes, such as v-H-ras and v-N-ras, cause essentially indistinguishable 2DG patterns. These patterns are distinct from those caused by v-raf and v-myc/v-raf mediated transformation. The v-raf and v-myc/v-raf patterns are also readily distinguishable, but are more similar to each other than to non-transformed or to ras transformed cells.

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

Mark J. Miller	Research Chemist	LEC NCI
Arthur David Olson	Computer Programmer	LEC NCI
Alfredo Romano	Guest Researcher	LEC NCI

Objectives:

The main objective of this project is to study the mechanism of carcinogenesis by employing the technique of quantitative two-dimensional gel electrophoresis of proteins. Since this technique allows for the simultaneous separation of 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 overall protein patterns as the cell undergoes neoplastic transformation. Our aim is to identify and characterize polypeptide patterns, as well as individual polypeptides, that are intimately associated with the transformed phenotype.

Methods Employed:

The principle methods employed are: (1) two-dimensional gel electrophoresis; (2) tissue culture techniques; (3) computer-based quantitation and analysis 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 (2DG) analysis system in order to facilitate the use of this important research technique in the analysis of the neoplastic process. The computer system developed here (dubbed ELSIE) is designed to be portable across a number of different computer systems and has been distributed to over 30 laboratories worldwide. In the LEC we are using ELSIE to compare the 2DG patterns of transformed and non-transformed cells in hopes of identifying polypeptides constantly associated with the transformed phenotype. We are also using the computer to compare the relative similarity of the 2DG patterns among different transformants. This may make it possible to use two-dimensional gel electrophoresis patterns to classify oncogenes by the biochemical phenotype they induce in transformed cells.

(1) Advances in the Computerized Analysis of Two-Dimensional Gels. Relatively little work has been done on further development on the ELSIE system in the past year. Some work has been done on the development of a new program for a more accurate matching of gel patterns across multiple gels. The ELSIE system builds its matched-spot database through the pair-wise matching of individual gels. Although the matching of individual pairs may be very accurate, errors

can accumulate leading to a large number of the spots in a set of gels having at least one mismatch somewhere in the set. Finding and eliminating these errors can be very difficult and labor intensive. This editing is currently the major bottleneck in the analysis of gels. However, it should be possible to use the information available from the accurate matching of most spots in a matched set to find and eliminate such errors automatically, while the sets of gels are being matched. Such a program, MATCHGROUPS, is under development in this laboratory. It considers an entire set of gels as it matches together spots, seeing if matchings are consistent across the set. At present, the program is about 90-95% accurate in the matching of spots, but takes a very long time to run (eight days for 12 gels, each containing about 1500 spots). Efforts to improve the speed and accuracy are underway.

(2) Analysis of Cellular Proteins of Human Fibroblasts Transformed In Vitro.

In collaboration with Dr. Justin McCormick at Michigan State University, quantitative two-dimensional gel electrophoresis was used to compare the cellular protein patterns of a normal foreskin-derived human fibroblast cell line (LG-1) and three infinite life span, tumorigenic derivatives of LG-1. One derivative, designated MSU-1.1 Vo, which was selected for its ability to grow in the absence of serum, has been shown to form low grade malignant tumors in athymic mice. The other two derivative cell strains, which were selected for focus-formation following transfection with the H-ras and N-ras oncogenes, form high grade malignant tumors. Both statistical and cluster-analysis techniques were used in the analysis. The gel patterns were readily separated into three distinct classes: LG-1, MSU-1.1 Vo, and the ras transformed. The differences between the classes were primarily quantitative in nature, with approximately 33% of the spots demonstrating significant differences in synthesis at some point in the analysis. Cluster analysis indicated the MSU-1.1 Vo cells were more closely related to the parental LG-1 than to the ras-transformed cells. The patterns from the two ras-transformed cell strains were almost indistinguishable from each other, i.e., less than the small differences observed in the patterns from duplicate samples.

(3) Analysis of Cellular Polypeptides in RLE Cells Transformed In Vitro.

The rat liver epithelial (RLE) cell lines were 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. We 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.

Retroviruses containing different transforming oncogenes have been used to infect and transform these cells. These viruses include pRNR-16 (v-H-ras), 3611-MSV (v-raf), and J-2 (v-myc/v-raf chimera). The transformed cell lines are designated "Rras", "F3611", and "FJ2" respectively. We are in the process of comparing the 2DG patterns of these and nontransforming cells.

The 2DG patterns of the transformed cells are readily distinguished from nontransformed cells and, usually, from each other. To gain a quantitative expression of these differences, we searched for spots which, on average, were

at least twofold different in intensity and whose distribution was such that there was only a 1% chance the differences were due to random events (T test). From 5 to 15% of the spots were classified as quantitatively different when any transformed cell line was compared to nontransformed RLE cells. Approximately the same fraction of spots were so classified when the various transformants were compared to each other. About 35% of the spots were capable of such quantitative variability in the course of some comparison. Principal component analysis readily clustered the samples. Separate Rras clones clustered together. F3611 and FJ2 clustered independently of each other but were more similar to each other than to Rras. All transformed cell line patterns were quite different from the nontransformed RLE cells. A preliminary search was made for spots which were associated with transformation regardless of the transforming oncogene. Twenty spots were flagged, of which five were confirmed visually and five others judged as possibles (the rest being caused by questionable matches or were judged statistical artifacts).

The above results must be considered to be preliminary because they have only been done with one experiment. The experiment is being repeated. Once polypeptides consistently associated with the process of transformation are found, we will attempt to identify them using microsequencing technology available in this laboratory.

Publications:

Miller MJ. Computer analysis of two-dimensional gel electrophoretograms. In: Dunn MJ, ed. Advances in electrophoresis. Weinheim: VCH Verlagsgesellschaft, 1989;181-217.

Miller MJ. Progress on development of a program for simultaneous matching of multiple gels. In: Dunn MJ, ed. Two-Dimensional gel electrophoresis, 1991. 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 1989;1:127-35.

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

PROJECT NUMBER

Z01CP05317-08 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Selenocysteine, the 21st Amino Acid in the Genetic Code

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

PI:	Dolph L. Hatfield	Research Chemist	LEC NCI
Others:	Byeong Jae Lee	Visiting Associate	LEC NCI
	In-Soon Choi	Visiting Fellow	LEC NCI

COOPERATING UNITS (if any)

Seoul National Univ. (Dr. Sang Dai Park and Mr. Jae-Hoon Joo); Univ. of Chicago Medical School, (Dr. Alan Diamond); National Heart, Lung, and Blood Institute (Dr. T. C. Stadtman); Vanderbilt University School of Medicine (Dr. Raymond Burk)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

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

Two selenocysteine tRNA isoacceptors have been identified in mammalian cells in this laboratory. These tRNAs have two functions: 1) to read the termination codon, UGA, in protein synthesis and thus donate selenocysteine to the growing polypeptide chain in response to specific UGA codons; and 2) to serve as carrier molecules for the biosynthesis of selenocysteine. The pathway for the biosynthesis of selenocysteine is tRNA[Ser]Sec ---> seryl-tRNA[Ser]Sec ---> phospho-seryl-tRNA[Ser]Sec ---> selenocysteyl-tRNA[Ser]Sec. Both isoacceptors (designated NCA and CmCA on the basis of their anticodon sequences) arise from a single copy gene and differ from each other by five pyrimidine transitions which occur in the 5' half of the molecule. Therefore, one isoacceptor must be an edited version of the other. Both isoacceptors can be generated from the selenocysteine tRNA gene after its injection into *Xenopus* oocytes. Isolation of the primary transcript from *Xenopus* oocytes and its reinjection results in formation of both isoacceptors. We are presently investigating the pathway of synthesis of these isoacceptors as to whether one is a precursor of the other or both arise from a common precursor. The relative abundance of the selenocysteine tRNA population and the distributions of NCA and CmCA are influenced by the presence of selenium in mammalian cells grown in culture and in rat tissues taken from rats on diets with and without selenium, suggesting that tRNA editing occurs and the editing process responds to selenium. A selenocysteine tRNA that decodes UGA has been identified in animals, plants and protists in this laboratory (and in bacteria by other investigators) and thus, the universal genetic code has been expanded to include selenocysteine as the 21st naturally occurring amino acid.

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 Associate	LEC NCI
In Soon Choi	Visiting Fellow	LEC NCI

Objectives:

The major goals of the project are to understand the structure, expression, function and evolutionary origin of the selenocysteine tRNA^{[Ser]^{Sec}} 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]^{Sec}} 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); (8) to investigate the distribution and evolutionary origin of this unique gene in nature; and (9) to determine the role of selenium in mammalian cells on the expression and function of these suppressors.

Methods Employed:

The selenocysteine tRNA^{[Ser]^{Sec}} gene was microinjected into *Xenopus* oocytes and the resulting primary transcript isolated and reinjected. The products generated from the reinjected primary transcript upon reinjection were isolated and are presently being characterized by sequencing.

Tissues (liver, brain, kidney, testis, muscle and heart) were obtained from two different sets of rats which were fed identical diets on selenium free yeast extract (for four months), except the diet of one set was supplemented with selenium. Transfer RNA was isolated from each tissue, aminoacylated with radioactive serine and the resulting seryl-tRNAs compared from tissues

with and without selenium by reverse phase chromatography. Yeast (*Saccharomyces cerevisiae*), *Thalassiosira pseudonana* and sugar beet cells were grown in culture on a chemically defined media. Se-75 was added to each cell culture, the resulting Se-75-selenocysteyl-tRNA^{[Ser]^{Sec}} was isolated and characterized by its coding properties and the selenocysteine moiety characterized by chromatographic techniques.

Internal control regions of the selenocysteine tRNA^{[Ser]^{Sec}} gene and the 5' and 3' flanking regions were changed by deletion substitution, insertion and site specific mutagenesis studies to analyze the effect of each of these areas on gene transcription. Transcription studies were carried out in vivo (by microinjection into *Xenopus* oocytes) and in vitro (in HeLa cell extracts).

A genomic library was constructed in *Cerebratulus* (a nonsegmented worm) and genomic libraries were obtained from two Echinoderms. Each library was screened by standard techniques with an appropriate probe for the purpose of isolating the selenocysteine tRNA^{[Ser]^{Sec}} gene.

Major Findings:

Injection of the selenocysteine tRNA gene into *Xenopus* oocytes resulted in synthesis of the primary processed transcript (Lee *et al.*, PNAS 84: 6384, 1987) and synthesis of either the isoacceptor designated NCA or a precursor to NCA. Reisolation of the latter isoacceptor from *Xenopus* oocytes and its reinjection resulted in synthesis of NCA and of the CmCA isoacceptors. Thus, both isoacceptors are generated from the same gene, demonstrating that the CmCA isoacceptor (which differs from the gene by three pyrimidine transitions including one in the anticodon) must arise by an editing process. We are presently confirming the structures of the different isoacceptors generated from the single gene by sequencing each in order to determine if the pathway of expression of NCA and CmCA is from a common precursor or whether NCA is the precursor to CmCA. This study represents only the second example of editing in mammalian cells and the first example of editing of a tRNA of any kind.

Editing of the selenocysteine tRNA isoacceptors is responsive to selenium as demonstrated in mammalian cells in culture and in tissues from animals fed diets with and without selenium. An analysis of the seryl-tRNA population by reverse phase chromatography in tRNA isolated from HL60 and rat mammary tumor cells grown on a chemically defined media in the presence and absence of selenium revealed that the selenocysteine tRNA population increased about 20% and a shift in the amount of tRNA from the NCA to CmCA isoacceptor was observed in response to selenium. Since an increase in the level of glutathione peroxidase is known to occur in these cells in response to selenium, it seems that the CmCA isoacceptor (which also increases in response to selenium) is utilized in the biosynthesis of glutathione peroxidase. In addition, the levels of the NCA and CmCA isoacceptors are altered in tissues in rats fed identical diets, except with and without selenium. The CmCA isoacceptor is enriched in liver and kidney, while the amount of NCA isoacceptor is slightly reduced. We are presently analyzing the selenocysteine isoacceptor levels in the other tissues.

Previous transcription studies in this laboratory on the selenocysteine tRNA gene from humans, rabbits, chickens and *Xenopus* demonstrated that the gene begins transcription, unlike that of any known tRNA, at the first nucleotide within the gene (Lee *et al.*, PNAS 84: 6384, 1987). Furthermore, transcription is terminated at a T cluster in the 3' flank and the trailer sequence is cleaved by a purified 3' processing enzyme. Three separate sites are present in the 5' flank for transcription: 1) a TATA box at position about -30 which is coupled with a second site; 2) an upstream GC rich region; and 3) an AT rich region which is present within nucleotides -62 to -76. More recent findings using deletion substitution, insertion and site specific mutagenesis studies show that the internal control regions which are involved in the level of expression of all other eukaryotic genes studied to date do not influence the amount of selenocysteine tRNA^{[Ser]Sec} expressed. These regions, designated as the A box and B box, respectively, play a role in processing of the trailer sequence. Site specific mutations generated in the upstream regulatory sites and these same mutations in combination with those prepared within the A and B boxes are currently being analyzed.

We had previously shown that expression of glutathione peroxidase (GPx) *in vitro* in wheat germ extracts or rabbit reticulocyte lysates results in very little GPx being synthesized in the absence of added suppressor tRNA. A peptide which corresponds to the region of the mRNA between the initiation codon (AUG) and the selenocysteine codon (UGA) is synthesized, demonstrating that the UGA codon serves as a termination codon. Exogenous tRNA^{[Ser]Sec} or addition of naturally occurring suppressor tRNA from yeast results in the expression of GPx showing that the UGA codon is suppressed. Addition of ⁷⁵Se-selenocysteyl-tRNA^{[Ser]Sec} results in a slight readthrough of the UGA codon and causes an overall inhibition of protein synthesis providing strong evidence that a factor (e.g., an elongation or antitermination factor) is required for the utilization of selenocysteyl-tRNA^{[Ser]Sec} in protein synthesis. We are presently attempting to demonstrate that the CmCA isoacceptor is utilized specifically for synthesis of GPx by injecting ⁷⁵selenocysteyl-tRNA_{CmCA} into *Xenopus* oocytes with GPx mRNA.

Previously, we administered ⁷⁵selenium to *T. pseudonana*, isolated the tRNA and demonstrated that the resulting ⁷⁵selenium containing isoacceptors were selenocysteyl-tRNA^{[Ser]Sec}. Yeast was found to have five ⁷⁵selenium containing isoacceptors, two of which were identified as selenomethionine isoacceptors (one was the initiator tRNA^{Met} and the other was the internal reading tRNA^{Met}) and two of which apparently have a selenonucleoside attached to the tRNA. The remaining ⁷⁵selenium containing tRNA in yeast may be the selenocysteine tRNA and our attention is focusing on this minor isoacceptor. Sugar beets contain two selenocysteine tRNAs, both of which recognize UGA in a ribosomal binding assay. Our studies have demonstrated that selenocysteyl-tRNA occurs in the animal, plant and protist kingdoms and evidence has been presented that this tRNA also occurs in fungi. The occurrence of selenocysteyl-tRNA in bacteria has been reported from another laboratory. Thus, it appears that selenocysteine tRNA is present in all five life kingdoms and we, therefore, have assigned selenocysteine to UGA within the universal genetic code.

A genomic library has been prepared from *Cerebratulus* for screening, isolating and sequencing the selenocysteine tRNA gene and genomic libraries from two Echinoderms have been screened and DNA regions subcloned which gave a positive signal with a human selenocysteine tRNA gene probe. Hybridization studies and studies with the gene product suggest that the *Cerebratulus* gene has undergone little evolutionary change from that of vertebrates, while the corresponding gene in Echinoderms is quite divergent. Interestingly, Echinoderms and vertebrates are much closer on the evolutionary scale than are vertebrates and *Cerebratulus*.

Publications:

Diamond A, Montero-Puerner Y, Lee BJ, Hatfield, D. Selenocysteine inserting tRNAs are likely generated by tRNA editing. *Nucleic Acids Res* 1990;18:6727.

Lee BJ, Choi I, Kang S, Kim Y, Rajagopalan M, You K, Hatfield D. Selenocysteine, the 21st naturally occurring amino acid within the universal genetic code. *Recent Prog Mol Biol Genet Eng in Korea* 1990;5:3-16.

Hatfield D, Lee BJ, Hampton L, Diamond A. Selenium induces changes in the selenocysteine tRNA^{[Ser]^{Sec}} population in mammalian cells. *Nucleic Acids Res* 1991; 19:939-43.

Hatfield D, Lee BJ, Price N, Stadtman T. Selenocysteyl-tRNA occurs in the diatom, *Thalassiosira*, and in the ciliate, *Tetrahymena*. *Mol Microbiol* (In Press).

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

PROJECT NUMBER

Z01CP05450-07 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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 L. Hager Head, Hormone Action and Oncogenesis Section LEC NCI

Others: Trevor K. Archer Visiting Associate LEC NCI
 Emery Bresnick Staff Fellow LEC NCI
 Ronald G. Wolford Microbiologist LEC NCI
 Diana S. Berard Biologist 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:

4.3

PROFESSIONAL:

3.6

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 sites to which steroid-receptors bind on the MMTV LTR are positioned on the surface of nucleosome B in a phased array of nucleosomes. Hormone activation of the MMTV promoter leads to modulation of nucleosome B structure in vivo which leads to a region of DNA hypersensitive to nucleolytic reagents. High-resolution analysis of the nucleosome position using PCR amplification indicates that the nucleosomes are positioned at base pair resolution, and that the hypersensitivity results not from nucleosome displacement, but from alteration in DNA structure across the nucleosome and into the A-B linker region. NF1, a component of the MMTV initiation complex is excluded from uninduced stable chromatin (see project Z01CP04986-14), but binds to transiently introduced DNA. We find that hormone induction also leads to H1 depletion from the A-B region in stable chromatin, whereas the core histone complement of this region is unchanged. We also find that a disomic structure composed of the A and B nucleosomes can be reconstituted in vitro, with the octamer cores accurately positioned, and that NF1 is excluded from this disomic structure. The glucocorticoid receptor, in contrast, can bind to the disome. Thus, two potential mechanisms exist for the exclusion of NF1, either (1) exclusion by nucleosome positioning; or (2) exclusion by a higher order, H1-dependent, chromatin structure. Modulation of the structure is necessary during transcription activation to permit binding of the initiation complex. These results indicate that a chromatin template containing specifically positioned nucleosomes is an active participant in transcriptional activation, and that modulation of this template structure is one feature of steroid hormone action.

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

Gordon L. Hager	Head, Hormone Action and Oncogenesis Section	LEC NCI
Trevor K. Archer	Visiting Associate	LEC NCI
Emery Bresnick	Staff Fellow	LEC NCI
Ronald G. Wolford	Microbiologist	LEC NCI
Diana S. Berard	Biologist	LEC NCI

Objectives:

The genetic information in mammalian cell exits 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 at 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, BPV vectors.
2. Chromatin of promoters and regulatory domains in the minichromosomes is examined for specific and reproducible nucleoprotein structures.
3. Nucleosome positioning is ascertained in vivo with high-resolution analysis of micrococcal-resistant core regions using PCR amplification of fragments.
4. Chromatin-bound proteins are detected in vivo by UV-induced DNA-protein cross-links, followed by selective adsorption of restriction enzyme digested chromatin fragments to a matrix with bound antibodies to the target protein.
5. Using purified DNA, histones, and specific DNA-binding proteins, chromatin is reconstituted in vitro.
6. 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 at the MMTV promoter with this system. Data with micrococcal nuclease and methidium propyl EDTA-FeII indicated that, at a resolution 10-20 base pairs, 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 high resolution by utilizing linear amplification of DNA fragments resulting from micrococcal digestion with thermal resistant Taq polymerase. This technology permits the analysis of nucleosome core positions at base pair resolution. We find that nucleosomes are reproducibly positioned over the MMTV LTR at base pair resolution. Thus, a nucleoprotein structure is generated when LTR DNA is replicated in stable chromatin that results in presentation of the DNA template to the soluble transcription apparatus in a highly reproducible form. 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 and the A-B linker region. The high resolution analysis now shows that the B region nucleosome is still present on the induced template, in a modified form. Thus, hypersensitivity results not from nucleosome displacement, but rather from a more subtle alteration in chromatin structure.

We have determined, using UV cross-linking technology and antisera specific to histone H1 and to the core histones, that the A-B region becomes depleted of H1 upon induction, but the octamer core histone complement remains unchanged. Thus, the region loses H1 in concert with the development of hypersensitivity and activation of the promoter.

We have also 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 have shown that NF1, one 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, whereas a DNA-binding fragment of the glucocorticoid receptor expressed in bacteria will bind to the A-B disome. We conclude that one transcription factor, the glucocorticoid receptor, can bind to its recognition site organized on a nucleosome. A second factor, NF1, is excluded from DNA in chromatin. When glucocorticoid receptor and NF1 are compared for binding to naked DNA, NF1 binds approximately 100 times more efficiently. Thus, the selective exclusion of NF1 from the reconstituted template is even more dramatic, in that NF1 has a much higher intrinsic affinity for MMTV promoter DNA.

Two mechanisms are currently under investigation to explain selective factor access. (1) Octamer core positioning itself may be responsible for differential access. Under this model, consistent with the in vitro reconstitution experiments, the NF1 site is sequestered in the core structure, whereas the receptor binding site is available. (2) Alternatively, some aspect of higher order structure, such as the formation of mature chromatosomes upon H1

deposition, may be involved in site sequestration. This model would be consistent with the observation that H1 is depleted from the nucleosome array upon hormone induction.

A second region of the LTR nucleosome array, corresponding to the octamer core designated F, has been examined at high resolution using the Taq polymerase technology. We also find for this core region that the boundaries mapped at high resolution are consistent with the previous low resolution positioning, and that this core adopts a unique position. These results support the general argument that nucleosomes across the MMTV LTR are highly positioned, and that transcription factors that bind to regulatory regions in this sequence interact with a highly organized nucleoprotein structure, and that this structure is active in modulating transcription factor access to DNA.

The MMTV LTR thus remains one of the most interesting models to study the interaction of transcription factors with specifically positioned nucleosomes, and to address the involvement of chromatin structure in gene regulation.

Publications:

Archer TK, Cordingley MG, Hager GL. Transcription factor access is mediated by accurately positioned nucleosomes on the MMTV promoter. *J Mol Cell Biol* 1991;11:688-98.

Bresnick EH, John S, Hager GL. Histone hyperacetylation does not alter the position or stability of phased nucleosomes on the MMTV LTR. *Biochemistry* 1991;30:3490-7.

Bresnick EH, Rories C, Marsaud V, Richard-Foy H, Hager GL. High resolution positioning of nucleosomes on the MMTV promoter: recruitment of transcription factors to a nucleosomal template. *J Mol Cell Biol* (In Press).

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

PROJECT NUMBER

Z01CP05453-07 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Cellular and Molecular Biology of the Hepatic Stem Cell Compartment

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

PI: Snorri S. Thorgeirsson Chief LEC NCI

Others: Hanne C. Bisgaard Visiting Fellow LEC NCI
 Hiroko Murakami Visiting Associate LEC NCI
 Minghuang Zhang Visiting Fellow LEC NCI
 Thomas Tan Clinical Associate LEC NCI
 Peter Nagy Visiting Scientist LEC NCI
 Ritva P. Everts Veterinary Medical Officer LEC NCI

COOPERATING UNITS (if any)

The Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, NC (Dr. Brian E. Huber); Northwestern University Medical School and Cancer Center, Chicago, IL (Dr. Philip M. Iannaccone)

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Laboratory of Experimental Carcinogenesis

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Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:

5.5

PROFESSIONAL:

3.5

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

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

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

The existence of a stem cell compartment in rat liver is now well established, and considerable body of data indicates that the human liver also contains a stem cell compartment. The research is currently focused on defining the cellular and molecular biology of this stem cell compartment in normal and neoplastic liver (see also Project No. Z01CP05262). Rat liver epithelial (RLE) cells share many cellular markers with the "oval" cell population found in adult rat liver after a certain type of hepatic injury. We have hypothesized that these cell types are derived from a common stem cell compartment present in the adult liver. Investigation of patterns of cytokeratin expression in adult and fetal livers and during early stages of "oval" cell proliferation revealed that cytokeratin 14 was transiently expressed in early fetal hepatocytes and oval cells. The expression of cytokeratin 14 in adult rat liver was found in small epithelial cells in the periductal space. These results indicate that cytokeratin 14 may be a marker for early progeny derived from the hepatic stem cell compartment. Transplantation of spontaneous transformed RLE cells in vitro resulted in formation of highly differentiated hepatocellular carcinomas. We have examined the stages of this in vitro transformation of RLE cells and discovered that coincident with the transformation process is an activation of a hepatocytic differentiation program that includes expression of albumin, alpha-fetoprotein, switching from connexin 43 to connexin 32 expression, and the expression of cytokeratin 8 and 18. These results formally demonstrate that progeny from the hepatic stem cell compartment such as the RLE cells and "oval" cells in vivo can be a target cell population in hepatocarcinogenesis and a precursor for hepatocellular carcinomas. We have recently obtained evidence that epithelial cell lines obtained from adult pancreas have, similar to the RLE cells, an extended capacity for proliferation in vitro, and share close lineage relationship to the RLE cells. These data indicate a common cell of origin for primitive cells isolated from rat liver and pancreas.

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

Snorri S. Thorgeirsson	Chief	LEC NCI
Hanne C. Bisgaard	Visiting Fellow	LEC NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Chu-chieh Hsia	Visiting Scientist	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; microinjection of cloned DNA fragments into the pronucleus of a fertilized mouse egg to generate transgenic mice, and polymerase chain reaction from DNA.

Major Findings:

- (1) The appearance of differentiated hepatocytes in the adult rat pancreas as well as pancreatic type-tissue in the adult rat liver can be experimentally induced. These observations suggest a lineage relationship between cell compartments present in rat liver and pancreas. The present data demonstrate that epithelial cell lines with almost identical phenotypes can be established from adult rat liver and pancreas. The established cell lines showed similar morphologies as established by light- and electron-microscopic studies. The

cell lines showed a unique expression pattern of intermediate filament proteins. Vimentin, actin and β -tubulin were present in all cell lines. In addition, simple epithelial type II cytokeratins 7 and 8 were found to be coexpressed with the type I cytokeratin 14 in several of the cell lines. Neither the type I cytokeratins 18 and 19, which are the normal partners for cytokeratins 8 and 7 in filament formation, or the type II cytokeratin 5 could be detected despite the fact that filaments were formed by both cytokeratin 8 and 14. This suggests that cytokeratin 14 acts as an indiscriminate type I cytokeratin in filament formation in the established cell lines. The cell lines expressed the same sets of LDH and aldolase isoenzymes and identical sets of glutathione transferase subunits. In addition, the epithelial cell lines from liver and pancreas were equally sensitive to the growth inhibitory effects of TGF- β 1. No expression of tissue or cell specific proteins such as α -fetoprotein, albumin, amylase, elastase or γ -glutamyl transpeptidase were detected. The almost identical phenotypes of the hepatic and pancreatic cell lines suggest that they may be derived from a common primitive epithelial cell type present in both rat liver and pancreas. In contrast to parenchymal cells, these cells have an extended capacity for proliferation in vitro and may represent a progeny from a "precursor" or "stem" cell compartment in vivo.

(2) Normal and transformed clonal cell lines were isolated from RLE cells during spontaneous transformation in vitro. We used this model to examine the possible involvement of growth regulation pathways and IC during the transformation process. The high passage normal cells showed no abnormal phenotypic characteristics, while the transformed clones were morphologically aberrant and produced hepatocellular carcinomas when transplanted onto nude mice. The transformed RLE cells were also less sensitive to the growth inhibitory effects of TGF β , had increased expression of TGF α , TGF β and the neoplastic marker GGT. In addition there was a dramatic shift in the expression of gap junction IC proteins from connexin 43 (more prominently expressed in rat heart and brain) to connexin 32 (expressed in hepatocytes and decreased in neoplastic liver nodules and carcinomas) with concurrent expression of α -fetoprotein and albumin. These data indicate the involvement of altered normal growth regulatory pathways and IC in neoplastic transformation of RLE cells. Furthermore, the data demonstrate that transformation of RLE cells can give rise to differentiated hepatocellular carcinoma.

(3) Cytokeratin 14 has long been considered a unique biochemical marker of basal cells in stratified epithelia and has not been reported expressed in any cell type of simple epithelia. Characterization of a number of apparent normal epithelial cell lines isolated from rat liver and pancreas revealed a unique expression of cytokeratin 14 together with the simple cytokeratins 7 and 8. Filaments were formed by both cytokeratin 14 and 8 despite the lack of expression of their normal partners cytokeratins 5 and 18, respectively. Rat liver epithelial cell lines share many common cellular markers with the "oval" cell population found proliferating in the adult rat liver after certain types of experimental injury. We have hypothesized that these cell types are derived from a common compartment of primitive progenitor cells present in the adult rat liver. This led us to investigate the pattern of cytokeratin expression in the developing and adult rat liver and during the early stages

of "oval" cell proliferation. Cytokeratin 14 was expressed in small epithelial cells in rat liver, including fetal hepatocytes, "oval" cells and cells in periductal space.

(4) The development of metastasis from transformed rat liver epithelial (RLE) cell lines in a syngeneic rat model were studied using the *E. coli lacZ* as a marker gene. RLE cells were transformed with either *v-raf* (T2) or *v-raf/v-myc* (J2). A retroviral vector carrying the *lacZ* gene was then used to stably express the bacterial form of β -galactosidase in the cell lines. Using a histochemical staining, primary tumors as well as micrometastasis could easily be visualized. Both J2 and T2 metastasized to the lungs after s.c. injection in the nude mouse. While J2 produced tumors with the histology of hepatoblastoma, T2 produced carcinoma-sarcoma. After injection into the systemic circulation of syngeneic rats, individual tumor cells were visualized in the lungs after 15 minutes, and micrometastasis within five days. Therefore, this rat model provides a powerful tool for quantitation and studies of early events of metastatic development following vascular dissemination of tumor cells.

(5) The histologic and ultrastructural features of oncogene transformed RLE cells in culture were studied and the tumors produced in nude mice by the transformed cells using *v-raf* and *v-myc* oncogenes, either independently or in combination, were also described. Spontaneously transformed RLE cell lines in culture and tumors induced in mice were also described. Two principal histologic types of tumors were encountered, namely, poorly or moderately well-differentiated carcinomas consistent with hepatoblastoma and sarcomatous tumors with varying degrees of differentiation and malignancy. In one of the five sarcomatous tumors, produced by the *v-raf* oncogene, focal areas of epithelial looking cells were encountered on transmission electron microscopy, while all other tumors were spindle shaped in morphology. In the spindle shaped tumor with epithelial cells, two morphologic features, exemplified by the presence of desmosomes and bile canalicular formation, were seen. In the epithelial tumors produced by a combination of *v-raf* and *v-myc* oncogenes, carcinomatous patterns, with hypervascularity, hypercellularity and abnormal forms of bile canalicular structures, were seen. In the sarcomatous tumors, produced by *v-raf* oncogene alone, the predominant cell type on electron microscopy resembled the spindle-shaped fibroblast with varying degrees of mitotic activity. The findings in this study suggest that a single oncogene, *v-raf*, can transform the RLE cell into a malignant spindle shaped tumor which is indistinguishable from a fibrosarcoma, while a combination of two oncogenes, *v-raf* and *v-myc*, produced an epithelial tumor which is typical and consistent with a carcinoma. The diverse effects of oncogenes on RLE cells show the potential of these cells to differentiate into two distinct morphologic cell types. The possible role of *v-myc* in switching the sarcomatous lineage (fibroblastic tumor) to an epithelial tumor lineage is considered to be interesting and worthy of further study. The mixed epithelial-mesenchymal tumors encountered in one of the *v-raf* transformed cells is consistent with carcino-sarcoma and the epithelial tumors produced by combined *v-raf* and *v-myc* oncogenes is consistent with an embryonal type of hepatoblastoma. The trabecular type of adult liver cell carcinoma encountered in the spontaneously

transformed RLE cell illustrates further the inherent potential of the RLE cell to undergo malignant change and confirms that these cells may be the precursor cells which precede the development of (rat) hepatocellular carcinoma.

(6) To obtain the liver specific expression of the *c-myc* gene, mouse *c-myc* cDNA was expressed under the control of the mouse albumin enhancer/promoter sequence. In order to stabilize mRNA from transgene, almost entire sequences of exon 1 and 3' non-coding region, which are supposed to be responsible for instability of *c-myc* mRNA, were deleted. Instead of *c-myc* poly A additional signal, the rabbit B-globin 3' non-coding sequence was inserted downstream of the termination codon of *c-myc* cDNA.

To assess the expression of transgene, the plasmid pLEC1 holding transgene construct was introduced into HepG2 cells by co-transfection with pSV2neo. HepG2 is a human hepatoma cell line known to be expressing albumin gene. After selection with G418, total RNA was extracted from transfected HepG2 cells and *c-myc* expression from the transgene was analyzed by Northern blot analysis. The transfected HepG2 cells produced considerable amounts of *c-myc* related transcript from the transgene, and the half life of it was about 2.5 hr which is more than five times longer than that of endogenous *c-myc* mRNA in HepG2 cells.

The 4.6 kb NaeI-KpnI fragment from pLEC1, containing transgene, was micro-injected into the one-cell embryos of (C57BL/6JxCBA) F2 mouse. The DNA samples extracted from tails were analyzed with Southern blotting. A total of 7 founder mice (5 males and 2 females) were found to be harboring the transgene. Each transgenic mouse was mated with a (C57BL/6JxCBA) F1 mouse to make F1 mice, and to date, transgenes from 5 males and 1 female were able to be transmitted to the next generation. Six F1 transgenic mice from each line were killed to be analyzed for the transgene expression. Total RNAs from liver, kidney, brain and heart were analyzed by Northern blotting. The 1.6 kb transcript from transgene was detected in the liver of 5 mice but not in other tissues. The steady state levels of mRNA from transgenes were considerably higher than that from the endogenous *c-myc* gene in the liver of three animals.

(7) Proliferation of a new population of epithelial cells with distinct morphology as well as cytokeratin and α -fetoprotein (AFP) expression was observed in noncancerous liver tissues of 14 cases of human HCC studied. These cells were characterized by oval nuclei, scanty pale cytoplasm, small cell size, and cross-reaction with a monoclonal antibody against rat oval cells (OV-6). These putative human oval cells displayed considerable heterogeneity of cytokeratin expression, particularly CK 19, and AFP expression. Proliferation of the oval cells was most prominent surrounding the preneoplastic cancer nodules. Cancer cells positive for CK 8, 18 and 19 were observed in half the HCC studied. Oval cells and transitional type of cells appear to be the principal producers of AFP in the hyperplastic and preneoplastic liver lesions. The data suggest that a new cell population morphologically similar to oval cells seen in early stages of chemical hepatocarcinogenesis in rats is consistently associated with hyperplastic or

preneoplastic liver lesions of HBV associated human HCC. Furthermore, it is possible that these oval type cells may account for the elevation of serum AFP frequently seen in preneoplastic stages of HBV associated human HCC.

Publications:

Bisgaard HC, Thorgeirsson SS. Evidence for a common cell of origin for primitive epithelial cells isolated from rat liver and pancreas. J Cell Physiol (In Press).

Huggett AC, Ellis PA, Ford CP, Hampton LL, Rimoldi D, Thorgeirsson SS. Development of resistance to the growth inhibitory effects of transforming growth factor beta-1 during the spontaneous transformation of rat liver epithelial cells. Cancer Res (In Press).

Nakatsukasa H, Evarts RP, Hsia CC, Marsden ER, Thorgeirsson SS. Expression of transforming growth factor- β 1 during chemical hepatocarcinogenesis in the rat. Lab Invest (In Press).

Thorgeirsson SS, Evarts RP. Hepatic stem cell compartment in the rat. In: Bock F, ed. Falk symposium no. 57 hepatic metabolism and disposition of endo- and xenobiotics. Lancaster, UK:Kluwer Academic Publishers (In Press).

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

PROJECT NUMBER

Z01CP05496-06 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Food-Derived Arylamine Carcinogens: Metabolic Processing and DNA Repair

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

PI: Elizabeth G. Snyderwine Senior Staff Fellow LEC NCI

Others: Snorri S. Thorgeirsson Chief LEC NCI
 Narayana Battula Expert LEC NCI
 Vilhelm Bohr Medical Officer LMP NCI
 Unnur Thorgeirsson Expert OD DCE NCI
 Richard H. Adamson Director DCE NCI
 Kazuhiro Nouse Guest Researcher LEC NCI

COOPERATING UNITS (if any)

Medical College of Ohio (H.A.J. Schut); Nestle, Lausanne, Switzerland (R. Turesky); National Cancer Center Research Institute, Tokyo, Japan (T. Sugimura); Lawrence Livermore National Laboratories (J. Felton and K. Turteltaub)

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.)

Heterocyclic arylamines (HAAs) are formed in meats such as beef, chicken and fish upon cooking at normal, household temperature. These compounds are potent promutagens in the Ames *Salmonella* mutagenicity assay and a number of these compounds have been shown to be carcinogenic in rodent bioassays. 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ) has been recently shown to be carcinogenic in cynomolgus monkeys. We have previously shown, using recombinant cytochromes P450 expressed in human cells, that cytochrome P450IA2 is the predominant cytochrome P450 responsible for the activation of these compounds via N-oxidation. Further studies have shown that N-hydroxy-IQ covalently binds to DNA directly and following further activation by O-acetyl- and sulfotransferases. Adducts of IQ have been examined in rodents and monkeys and found to be identical to those formed in vitro upon reaction of N-hydroxy-IQ with DNA, and those found in *Salmonella*. In addition to in vitro studies of HAA metabolism we are studying the in vivo metabolism and distribution of HAAs in monkeys. 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ) is extensively metabolized in monkeys and excreted into urine and feces as metabolites. N-Sulfation, glucuronidation and N-demethylation play important roles in the detoxification and excretion of this compound. Metabolic processing also appears to be influenced by the dose of IQ. In light of this finding, we have begun to examine HAA-DNA adducts, and the distribution and excretion of HAA metabolites at food-equivalent doses by accelerator mass spectrometry. This is a highly sensitive method for counting ¹⁴C atoms following administration of low levels of radiolabeled compound. Studies concerning repair in specific genes using the UvrABC excinuclease and alkaline hydrolysis are also underway. Studies are being carried out in isolated genomic DNA, plasmids and cultured cells. With this methodology we are seeing heterogeneity in initial adduct formation in different gene regions and differences in adduct levels with N-hydroxy-IQ and N-hydroxy-MeIQx.

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

Elizabeth G. Snyderwine	Senior Staff Fellow	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Narayana Battula	Expert	LEC NCI
Vilhelm Bohr	Medical Officer	LMP NCI
Unnur Thorgeirsson	Expert	OD DCE NCI
Richard H. Adamson	Director	DCE NCI
Kazuhiro Nouse	Guest Researcher	LEC NCI

Objectives:

The objectives of this study are to examine the metabolism and disposition of heterocyclic arylamines in nonhuman primates, identify reactive metabolites and DNA adducts, and examine the in vivo and in vitro repair of HAA-DNA adducts in the genome overall and in specific genes.

Methods Employed:

1. Chemical synthesis
2. HPLC
3. Mass spectrometry
4. Nuclear magnetic resonance
5. Ultraviolet absorption spectroscopy
6. Ames *Salmonella* mutagenicity assay
7. DNA isolation techniques
8. Agarose gel electrophoresis
9. Southern blotting
10. Southern hybridization
11. ³²P-postlabeling assay for DNA adduct detection
12. Cell culture
13. Recombinant cytochromes P450
14. Scintillation spectroscopy
15. Random prime labeling
16. Accelerator mass spectrometry

Major Findings:

2-Amino-3-methylimidazo[4,5-f]quinoline (IQ) was extensively metabolized in monkeys by ring hydroxylation and conjugation. Little of the parent compound was detected in urine or feces. In contrast, analysis of plasma by HPLC revealed the presence of IQ and N-demethyl-IQ. Urine was the predominant route of excretion accounting for approximately 60% of the administered dose 72 hr after IQ administration. Fecal excretion accounted for only 9% of the dose during this same time period. Twelve metabolites were found in the urine of monkeys. Metabolic processing occurs by cytochrome P450 mediate oxidation on the ring and at the exocyclic amino group followed by conjugation with

sulfate or glucuronide. The metastable metabolite N-hydroxy-N-glucuronyl-IQ was also identified in urine of IQ-fed monkeys, thus indicating that metabolic activation via N-hydroxylation occurs in vivo in monkeys. The role of this metabolite in extrahepatic DNA adduct formation and carcinogenicity is currently under investigation. Three metabolites were found in feces and the major metabolite was identified as IQ-N-sulfamate. Enteric bacterial metabolism also appears to play a significant role in the metabolism of IQ by oxidation of the ring. In contrast to studies in rodents, bacterial metabolism appears to play a larger role in the metabolism in monkeys. In addition, N-demethylation appears to be a predominant route of detoxification in the monkey unlike in the rat. No metabolism to glutathione conjugates or acetyl conjugates was observed. Preliminary studies in monkeys fed PhIP show that in contrast to the many metabolites found in IQ treated monkeys, two metabolites (4-hydroxy-PhIP and 4-sulfate-PhIP) were found in urine and bile of PhIP treated monkeys. Preliminary studies using accelerator mass spectrometry indicate that HAA-WBC adducts can be detected in monkeys fed 500 ng/kg of ¹⁴C-IQ. Peak adduct levels were observed between 6 and 24 hours.

Studies are being carried out on the DNA adduction and repair of adducts of HAAs and 4-nitroquinoline oxide, a structurally related compound, in specific genes. Studies have been conducted in carcinogen treated cells and in genomic and plasmid DNA reacted in vitro with N-hydroxylamines. The technique involves treatment of isolated DNA with the bacterial repair enzyme UvrABC excinuclease followed by quantitation of damage by Southern hybridization with probes for the gene of interest. We have found that IQ and MeIQx are excellent substrates for UvrABC which can easily detect as little as one IQ or MeIQx adduct per 14 kb gene fragment. Studies with 4-NQO in CHO-B11 cells (with amplified DHFR gene) show heterogeneity in the initial adduct levels in coding and downstream non-coding regions of the DHFR gene with initial adduct levels being higher in non-coding regions than coding. However, the extent of repair was 70% over 24 hrs in both regions. We have recently developed our gene-specific repair techniques to examine damage and repair in the coding and non-coding strands of the DHFR gene and also other non-amplified DNA sequences including *c-fos* and mitochondrial genes. Overall genomic damage and repair is being assayed by alkaline sucrose gradients, ³²P-postlabeling, and repair replication.

Publications:

Battula N, Schut HAJ, Thorgeirsson SS. Cytochrome P4501A2 constitutively expressed from transduced DNA mediates metabolic activation and DNA adduct formation of aromatic amine carcinogens in NIH 3T3 cells. *Mol Carcinog* (In Press).

Schut HAJ, Snyderwine EG, Zu H-X, Thorgeirsson SS. Similar patterns of DNA adduct formation of 2-amino-3-methylimidazo[4,5-*f*]quinoline in the Fischer-344 rat, CDF₁ mouse, cynomolgus monkey, and *Salmonella typhimurium*. *Carcinogenesis* 1991;12:931-4.

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

PROJECT NUMBER

Z01CP05555-04 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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 Jae Lee Visiting Associate LEC NCI
 Jae-Eon Jung Visiting Fellow LEC NCI

COOPERATING UNITS (if any)

University of California, San Francisco (Dr. Harold E. Varmus); Advanced BioScience Laboratories, Inc. (Dr. Stephen Oroszlan)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0.0

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.)

The major goal of this project is to determine if host aminoacyl-tRNAs required for decoding the frameshift signal in human immunodeficient virus (HIV) and other retroviruses are altered (i.e., hypomodified) from the normal cellular aminoacyl-tRNAs. Preliminary evidence from this laboratory has suggested that these tRNAs in HIV and other retroviral infected cells are hypomodified. The mouse mammary tumor virus (MMTV) *gag-pro* frameshift signal (sequence A AAA AAC, where C is the site of the frameshift) is being used as a model for these studies. We obtained the wild type frameshift signal and a series of mutations at the frameshift site in MMTV as a tool for determining if the tRNA required to alter the reading frame is hypomodified. The level of frameshifting is reduced dramatically with an AAU mutant even though AAC/AAU codons are decoded by the same asparagine isoacceptor with similar efficiencies at other mRNA sites. Hypomodified asparagine tRNA which lacks the highly modified Q base is known to preferentially read AAC over AAU codons. Thus, the observation that the AAC codon at the frameshift site promotes ribosomal frameshifting more efficiently than the AAU codon suggests that the tRNA lacking Q base is required for decoding the frameshift signal. We have not been successful in utilizing in vitro assays thus far to demonstrate a requirement for hypomodified isoacceptors in ribosomal frameshifting due to the endogenous tRNA population in the assay system. However, we have developed an in vivo assay for ribosomal frameshifting utilizing microinjection techniques into *Xenopus* oocytes and are presently assaying the ability of purified hypomodified isoacceptors to alter the reading frame of the wild type and mutant MMTV *gag-pro* frameshift signals.

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 Associate	LEC NCI
Jae-Eon Jung	Visiting Fellow	LEC NCI

Objectives:

The 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; 4) to purify altered tRNAs from infected cells and determine if they may have a role in the expression of the virus; and 5) to determine the efficiency of hypomodified and fully modified isoacceptors to decode wild type and mutant frameshift signals and thus establish their relative abilities to promote ribosomal frameshifting.

Methods Employed:

The wild type MMTV *gag-pro* overlap region encoding the A AAA AAC frameshift signal and the corresponding overlaps containing a series of mutations at the frameshift site (i.e., AAU, AAA and AAG), each subcloned into an expression vector, were obtained from H. E. Varmus, M.D. RNA was generated from each subclone, the resulting RNA capped and then used in protein synthesis either in vitro in rabbit reticulocyte lysates or in vivo in *Xenopus* oocytes. Asparagine tRNA with and without Q base and a hypomodified lysine tRNA have been purified previously from rabbit reticulocytes. Rabbit reticulocyte lysates were programmed with capped RNA (wild type and the corresponding mutants) and exogenous tRNA added to analyze ribosomal frameshifting in vitro and *Xenopus* oocytes were microinjected with these constituents to analyze ribosomal frameshifting in vivo.

Major Findings:

A correlation exists between the presence of a hypomodified tRNA in retroviral infected cells and utilization of the corresponding tRNA in ribosomal frameshifting or in viral replication (Hatfield et al., Virology 173: 736, 1989). To determine if the hypomodified tRNA utilized to decode the ribosomal frameshift site is a requirement to promote efficient frameshifting, we assayed the ability of a series of mutants within the MMTV *gag-pro* shift site to alter the reading frame in the -1 direction. The wild type signal which

contains the asparagine codon AAC promoted frameshifting most efficiently in rabbit reticulocyte lysates, while the AAU mutant was severalfold less efficient and the AAA and AAG mutants were most inhibitory. These results confirmed earlier observations from the Varmus laboratory. The important observation from these studies is that the same asparagine isoacceptor is required to decode AAC/AAU codons, suggesting (from studies in other laboratories) that the hypomodified asparagine tRNA may be required to decode the frameshift site. Addition of purified asparagine tRNA lacking Q base and/or a hypomodified lysine tRNA to the lysates, however, did not affect the levels of ribosomal frameshifting in any of the mutant or the wild type signals. This observation was not surprising, since it is known that rabbit reticulocytes contain substantial amounts of these hypomodified tRNAs (Hatfield *et al.*, *Biochim. Biophys. Acta* 564: 414, 1979). We, therefore, have subsequently focused our attention on an *in vivo* assay involving micro-injection of RNA and tRNAs into *Xenopus* oocytes. We had previously demonstrated that the HIV *gag* gene, but not the shift to the *pol* reading frame, occurred in *Xenopus* oocytes which provides us with an *in vivo* assay for frameshifting. The studies utilizing the MMTV wild type and mutant frameshift signals in *Xenopus* oocytes are presently in progress.

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

PROJECT NUMBER

Z01CP05558-04 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Negative Growth Regulators in Normal and Neoplastic Liver

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

PI: Snorri S. Thorgeirsson Chief LEC NCI

Others: Gil-Jong Kang Senior Staff Fellow LEC NCI

Rajalakshmi Padmanabhan Chemist LEC NCI

David C. Parmelee Expert LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.3

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 unreduced type. Do not exceed the space provided.)

The research in this project is focused on 1) defining the role of transforming growth factor- β 1 (TGF- β 1) in the early stages of hepatocarcinogenesis, and 2) isolation and characterization of a novel liver growth inhibitor (LDGI). We have used the spontaneous transformation of rat liver derived epithelial (RLE) cells as a model to examine the change in the growth inhibitory effects of TGF- β 1 during the transformation process. The appearance of morphologically aberrant transformants correlated directly with an increased resistance of the population to the growth inhibitory effects of TGF- β 1. Clonal cell lines derived from the transformants were resistant to TGF- β 1-dependent inhibition of DNA synthesis. These cell lines were also highly tumorigenic, aneuploid with characteristic gross chromosomal abnormalities, and expressed a number of phenotypic markers common to rat liver epithelial cells transformed by oncogenes or chemicals. In contrast, apparently normal-looking cell lines cloned from the same population were non-tumorigenic, near-diploid with few chromosomal gross abnormalities and were as sensitive to TGF- β 1 as early passage normal RLE cells. Morphologically normal late passage rat liver epithelial cells were sensitive to transformation by the DNA hypomethylating agent 5-aza-2-deoxycytidine, in contrast to earlier passage cells, and this transformation was accompanied by the development of resistance to the growth inhibitory effects of TGF- β 1. These findings suggest that acquisition of resistance to the effects of growth inhibitors such as TGF- β 1 is an important and possibly essential stage in the spontaneous transformation of RLE cells. In addition to the TGF- β 1 studies we are presently attempting to obtain partial protein sequence data on the highly purified fraction of LDGI.

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

Snorri S. Thorgeirsson	Chief	LEC NCI
Gil-Jong Kang	Senior Staff Fellow	LEC NCI
Rajalakshmi Padmanabhan	Chemist	LEC NCI
David C. Parmelee	Expert	LEC NCI

Objectives:

To define the role of growth inhibitors in cellular transformation and to delineate the mechanisms responsible for the altered growth control of transformed liver cells.

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:

(1) The temporary maintenance of a rat liver epithelial cell population at confluence before passaging followed by periods of rapid proliferation resulted in the generation of spontaneous transformants after about 108 population doublings. The appearance of morphologically aberrant transformants correlated directly with an increased resistance of the population to the growth inhibitory effects of transforming growth factor beta (TGF- β 1). Clonal cell lines derived from the transformants were resistant to TGF- β 1-dependent inhibition of DNA synthesis. These cell lines were also highly tumorigenic, aneuploid with characteristic gross chromosomal abnormalities, and expressed a number of phenotypic markers common to rat liver epithelial cells transformed by oncogenes or chemicals. In contrast, apparently normal looking cell lines cloned from the same population were non-tumorigenic, near diploid with few chromosomal gross abnormalities and were as sensitive to TGF- β 1 as early passage normal RLE cells. Morphologically normal late passage rat liver epithelial cells were sensitive to transformation by the DNA hypomethylating agent 5-aza-2-deoxycytidine, in contrast to earlier passage cells, and this transformation was accompanied by the development of resistance to the growth inhibitory effects of TGF- β 1. These findings suggest that acquisition of resistance to the effects of growth inhibitors such as TGF- β 1 is an important and possibly essential stage in the spontaneous transformation of rat liver epithelial (RLE) cells.

(2) The effects of transforming growth factor β (type I) (TGF- β 1) on DNA synthesis, cell proliferation and protein synthesis were examined in a series of *v-raf*-transformed rat liver epithelial (RLE) cells, which exhibit a range of transformed phenotypes. All of the transformed cells were relatively resistant to the growth-inhibitory effects of TGF- β 1, compared to normal RLE cells and control cells infected with a helper virus. The more tumorigenic cell lines had very few surface receptors for TGF- β 1 and showed no increase in the secretion of a number of specific proteins, including fibronectin, following TGF- β 1 treatment. In contrast, the more normal-looking, less tumorigenic *v-raf*-transformed cells bound similar amounts of TGF- β 1 as normal RLE and control cells and showed a similar pattern of TGF- β 1-stimulated protein secretion. These findings suggest that the effects of TGF- β 1 on cell proliferation and on the expression of certain secreted proteins are mediated through different mechanisms. Following transformation of RLE cells with *v-raf*, the signalling pathways controlling TGF- β 1 growth inhibition are perturbed, while those involved in regulating the synthesis of certain proteins may remain intact. Thus, the escape from the various distinct biological effects of TGF- β 1 may be an important stage in the progression of neoplastic transformation of RLE cells *in vitro*.

(3) Clonal cell lines were derived from RLE cells following their transformation with either *v-raf* or *v-raf/v-myc*. Cells transformed with *v-raf* alone showed reduced tumor incidence and tumor growth rates when implanted into nude mice, compared to cells also expressing the *v-myc* oncogene. A series of additional clones isolated from a tumor obtained following inoculation of an athymic nude mouse with the *v-raf*-transformed rat liver epithelial cells displayed an intermediate range of tumor aggressiveness. These findings indicate that unknown genotypic and/or phenotypic changes occur during tumor formation *in vivo*, which are required in addition to *raf* activation for complete expression of the malignant phenotype. This *in vitro* model of tumor progression was used to examine alterations in the expression of genes related to the growth control of liver epithelial cells, which may be involved in the malignant conversion of the preneoplastic cells. A close association was observed between the increased level of expression of the transforming growth factors α and β 1, the decreased expression of extracellular matrix proteins fibronectin collagen I, and the tumor aggressiveness (latency/growth rate), suggesting a causal role for these factors in the progression of *v-raf*-transformed RLE cells to the fully malignant phenotype.

Publications:

Hampton LL, Worland PJ, Yu B, Thorgeirsson SS, Huggett AC. Expression of growth-related genes during tumor progression in *v-raf*-transformed rat liver epithelial cells. *Cancer Res* 1990;50:7460-7.

Huggett AC, Hampton LL, Ford CP, Wirth PJ, Thorgeirsson SS. Altered responsiveness of rat liver epithelial cells to transforming growth factor beta-1 following their transformation with *v-raf*. *Cancer Res* 1990;50:7468-75.

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

PROJECT NUMBER

Z01CP05559-04 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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
	Salvatore Sechi	Visiting Fellow	LEC NCI
	Snorri S. Thorgeirsson	Chief	LEC NCI
	Kelli Beavers	Laboratory Worker	LEC NCI

COOPERATING UNITS (if any)

Division of Metabolism and Endocrine Drug Products, Food and Drug Administration
 (Dr. Chien-Hua Niu)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.0

PROFESSIONAL:

2.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

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

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

Previously unidentified rat liver membrane glycoproteins, whose regulation is qualitatively and quantitatively altered during the course of chemically-induced hepatocarcinogenesis, have been observed by utilizing 2D-PAGE. The main goal of this project is to purify and characterize the specific glycoproteins which demonstrate such differences in expression in the plasma membranes of normal and neoplastic rat livers. This information will aid in understanding 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 sequenceable in this manner, presumably because of blocked N-termini. For this reason, research was continued to develop procedures for obtaining amino acid sequence information from all of these proteins, whether blocked or unblocked. This work focuses on methods development in areas that have become critical in order to obtain results from minute quantities of proteins isolated from 2D-gels. Various techniques involved in amino acid sequencing of transblotted samples have been notably improved. A new type of low volume reaction cartridge for the sequencer was used and the conditions modified for our specific purposes. This resulted in the attainment of sequencing data from transblotted samples that is equal to those obtained from the direct application of purified proteins to the sequencer on a polybrene-coated glass filter. In a test case we have identified 3 of 5 proteins analyzed that were purified from whole dog serum using a single 2D-polyacrylamide gel. We are currently making excellent progress in developing procedures to obtain internal sequence data from proteins purified in this manner.

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
Salvatore Sechi	Visiting Fellow	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Kelli Beavers	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 and Tressyl-5PW, (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) A new 2D-PAGE system (2D-Investigatory-Millipore) was purchased, set up and tested that utilizes large format gels. There are several advantages to this system that will help to make the experimental results more reliable and reproducible. The latter point is crucial when isolating specific components from samples containing thousands of proteins.

(2) A general HPLC method was developed to remove more than 95% of albumin from serum and various other samples that must be applied to 2D gels. After the removal of this major protein contaminant, larger quantities of the unknown proteins can be utilized in each experiment. This will ultimately improve yields and save time by reducing the number of PAGE experiments necessary to obtain adequate amounts of specific samples for sequence analysis.

(3) We have continued to investigate the use of different stains that will not affect subsequent elution or that may block the N-terminal amino acid residues of proteins isolated by PAGE. Of several tried, stains containing cupric chloride seem to give the best result for SDS gels. The addition of iodoacetic acid eliminates an artifact band, observed in the region containing proteins of approximately 70,000 daltons, that is caused by dithiothreitol.

(4) The recovery of proteins continues to be a major problem when dealing with low amounts of protein (less than 200 pmoles). We have identified several problem areas by using standard whole proteins, CNBr fragments and tryptic digests. For example, more than 50% of any of these samples can be lost by simply placing them in tubes for 15 min. Yields can vary depending on the type of sample tubes utilized. Siliconizing and using various detergents has been found to greatly improve recovery. Drying the sample by Speed Vac or lyophilization can substantially reduce recovery if these steps are continued for a prolonged time. As an overall result, various experimental methods have been modified to reduce the number of steps that cause protein losses and to avoid certain procedures entirely.

(5) The conditions for CNBr cleavage of proteins (blocked or unblocked) have been improved. This reaction is straightforward with large amounts of protein, but is incomplete when dealing with small amounts of materials from PAGE experiments. We have modified conditions for obtaining better cleavages of these proteins, whether in the gel slices or on transblots. It is of major importance to insure that the methionine residues are not oxidized before the reaction. This problem can be reversed by treatment with dithiothreitol. However, there are still difficulties in recovering some fragments, even after the reaction proceeded properly. We have successfully obtained internal sequence information for a protein from dog plasma isolated by 2D-PAGE (a collaborative effort with Dr. Mark Miller). This led to its identification as apolipoprotein A-1. Another unknown protein was also identified as haptoglobin by N-terminal sequencing.

(6) Conditions and procedures have been improved for obtaining internal sequencing of proteins from 2D-gels. We have incorporated results from improving recovery of whole proteins and have investigated the elution rates of proteins from gel slices when equilibrated with different buffers. These improvements provided significantly better sequencing data for several internal peptides from different protein standards. We are currently applying these techniques, in collaborative experiments, to unknown proteins that are relatively easy to obtain. Following successes with these, we will attempt internal sequencing of the liver membrane glycoproteins mentioned above.

(7) The ABI gas phase sequencer was modified to increase repetitive and initial yields for proteins that are transblotted to membranes. Previously, this type of sample gave poor results. The modifications involved using a new type of reaction cartridge that has an extremely low volume. In addition, the sequencer programs had to be optimized with respect to reaction times, volumes and temperatures. The resulting current procedures give sequencing results which are equal in quality to samples that are added directly to the sequencer on polybrene coated glass fibers. This latter technique is the standard by which other techniques are judged.

(8) The ABI 430 Peptide Synthesizer has been upgraded to utilize the newly developed f-moc and NMP chemistry. This methodology simplifies cleavage of the peptide from the resin and improved coupling, respectively. Two peptides have been designed and synthesized that will be useful analytical tools to routinely check initial and repetitive yields of the ABI amino acid sequencer.

These peptides include all the amino acids that are normally observed, including several that are rarely seen. Some amino acids are difficult to detect during sequencing and having these peptides will confirm that the system will be able to identify all of them. These synthetic peptides will also be useful as controls for the many types of chemical modification reactions used on proteins (CNBr cleavage, reduction and alkylation by various methods, oxidations, DP-cleavage, NG-cleavage, etc.).

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

PROJECT NUMBER
 Z01CP05599-03 LEC

PERIOD COVERED
 October 1, 1990 to September 30, 1991

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
	Zongyi Hu	Visiting Fellow	LEC NCI
	Elizabeth Marsden	Biologist	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH
 Laboratory of Experimental Carcinogenesis

SECTION
 Chemical Carcinogenesis Section

INSTITUTE AND LOCATION
 NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.2	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 cellular distribution of transcripts for transforming growth factor (TGF)- β 1, procollagen I, III and IV, as well as for type IV collagenase (72 kDa gelatinase) were studied in order to elucidate (1) the pathogenesis of liver fibrosis (or cirrhosis) and (2) the possible role of TGF- β 1. Our study on liver fibrosis suggests that TGF- β 1 derived from inflammatory cells may have enhanced the expression of type I collagen as well as that of the TGF- β 1 gene itself in desmin-positive perisinusoidal cells by the paracrine mechanism. The simultaneous expression of TGF- β 1 and type I, III and IV collagen genes in mesenchymal cells during the fibrotic process also suggests the possibility that TGF- β 1 may have an important role in the production of liver fibrosis. We studied the regulatory mechanisms of synthesis and degradation of type IV collagen which is one of the main components of basement membrane and of accumulated fibrous tissue in liver fibrosis. The data suggest that the imbalance observed in 72 kDa gelatinase and type IV collagen expression in early stages of liver fibrosis may lead to the accumulation of type IV collagen. Furthermore, the data indicate that desmin-positive perisinusoidal cells play a central role in regulating type IV collagen deposition in CCl-4-induced rat liver fibrosis. In Solt-Farber's chemical hepatocarcinogenesis process, non-parenchymal cells of the liver, mainly desmin-positive perisinusoidal cells, are the principal source of TGF- β 1 production. Our data suggest that the close interaction between non-parenchymal cells and carcinoma cells may be necessary for the activation of latent TGF- β 1. It is hypothesized that regulatory effects of TGF- β 1 on growth of preneoplastic or carcinoma cells in the liver are exerted via paracrine mechanism.

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

Harushige Nakatsukasa	Expert	LEC NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC NCI
Snorri S. Thorgeirsson	Chief	LEC NCI
Zong-Yi Hu	Visiting Fellow	LEC NCI
Elizabeth Marsden	Biologist	LEC NCI

Objectives:

The objective of this project is to elucidate (1) the pathogenesis of liver fibrosis (or cirrhosis). This process is the most commonly observed terminal feature of chronic liver diseases and is also considered as a precancerous state; (2) the role of TGF- β 1 and (3) that of multidrug resistance (mdr) during the development of hepatocellular carcinomas by examining the expression of TGF- β 1 and mdr gene. TGF- β 1 has been reported to be involved in carcinogenesis through its multifunctional effects on both epithelial cells and mesenchymal cells. Increased expression of mdr in regenerating and neoplastic livers has been previously reported by our group.

Methods Employed:

(1) In situ hybridization for spacial localization of transcripts for type I, III and IV collagens, 72 kDa gelatinase (type IV collagenase), TGF- β 1 as well as mdr. (2) Immunohistochemistry for identification of desmin-positive perisinusoidal cells as well as for identification of TGF- β 1 (latent and mature forms) proteins and some of the collagens. (3) Techniques used in molecular biology including Northern blot analysis, amplification and isolation of plasmids. (4) Quantitation of in situ hybridization data by using Magiscan Image Analysis System. (5) 3 H-Thymidine uptake in rats to evaluate replicating cells after treatments.

Major Findings:

(1) Liver fibrosis was produced in rats by administering carbon tetrachloride by gavage once a week for 1 to 12 weeks. Increases in TGF- β 1 and type I collagen transcripts were observed primarily in periductal and periportal cells, as well as in endothelial cells of the portal and central veins after CCl₄ administration. In the centrilobular necrotic areas, TGF- β 1 and type I collagen transcripts were observed first in inflammatory cells and then in desmin-positive perisinusoidal cells and resulted in the accumulation of connective tissues. These data suggest that TGF- β 1 derived from inflammatory cells may have enhanced the expression of type I collagen as well as that of TGF- β 1 gene itself in desmin-positive perisinusoidal cells by the paracrine mechanism. This sequence of events may represent the initial stages of liver fibrogenesis.

During the advanced fibrotic stages, TGF- β 1 and collagen genes were similarly and predominantly expressed in desmin-positive perisinusoidal cells and fibroblasts, and their expression continued to be higher than that observed in control rats. The accumulation of extracellular matrix along small vessels and fibrous septa coincided with the expression of these genes. Desmin-positive perisinusoidal cells and fibroblasts appeared to play the principal role in synthesis of collagens in this model of hepatic fibrosis. Although collagens have been thought to be produced in hepatocytes, no transcripts for collagens were observed in hepatocytes throughout the process of liver fibrosis. The simultaneous expression of TGF- β 1 and collagen genes in mesenchymal cells during hepatic fibrosis suggests the possibility that TGF- β 1 may have an important role in the production of liver fibrosis.

Accumulation of extracellular matrices, particularly collagens, are the end results after synthesis and degradation of those molecules. In order to elucidate the regulatory mechanisms of synthesis and degradation of type IV collagen, we examined expression of type IV collagen and 72 kDa gelatinase/-type IV collagenase, which is thought to be a specific degrading enzyme for type IV collagen, during liver fibrogenesis. Type IV collagen is a main component of basement membrane and is one of the collagen types involved in extracellular matrix accumulation in liver fibrosis. After the initiation of fibrosis, the expression of type IV collagen increased (21-fold) and the transcripts were observed mainly in necrotic areas, where fibrogenesis occurred, and in portal areas. In contrast, no change in steady state level of mRNA for the 72 kDa gelatinase was observed by Northern blot analysis. However, in situ hybridization showed that transcripts of the 72 kDa gelatinase were present in the necrotic areas. Desmin-positive perisinusoidal cells were the principal source of both type IV collagen and 72 kDa gelatinase in fibrogenic areas, although fewer cells expressed 72 kDa gelatinase. The transcripts for type IV collagen and 72 kDa gelatinase were absent in hepatocytes before and after CCl₄ administration. These data suggest that the imbalance observed in 72 kDa gelatinase and type IV collagen expression in early stages of liver fibrosis may lead to the accumulation of type IV collagen. Furthermore, the data indicate that desmin-positive perisinusoidal cells play a central role in regulating type IV collagen deposition in CCl₄-induced rat liver fibrosis.

(2) In the hepatocarcinogenesis, TGF- β 1 transcripts were primarily observed in non-parenchymal cells, some of which were desmin-positive perisinusoidal cells, surrounding or within the preneoplastic nodules or carcinomas. Neither transcripts nor protein of TGF- β 1 were observed in preneoplastic or carcinoma cells. The distribution of latent TGF- β 1 protein was similar to the transcripts. However, mature TGF- β 1 was only detected in non-parenchymal cells and connective tissue associated with carcinomas, but was not observed in preneoplastic nodules. There was no difference in TGF- β 1 expression associated with tumor types or the differentiation status of primary hepatic carcinomas. The present study demonstrates that non-parenchymal cells are the principal source of TGF- β 1 production during hepatocarcinogenesis. Furthermore, the data suggest that the close interaction between non-parenchymal cells and carcinoma cells may be necessary for the activation of latent

TGF- β 1. It is hypothesized that the regulatory effects of TGF- β 1 on growth of preneoplastic or carcinoma cells in the liver are exerted via the paracrine mechanism.

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

PROJECT NUMBER

Z01CP05600-03 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Cloning of the Rat *mdr* Gene Family and Regulation in Normal and Neoplastic Liver

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 Senior Staff Fellow LEC NCI
 Timothy W. Gant Visiting Fellow LEC NCI
 Pamela A. Marino Senior Staff Fellow LEC NCI
 Harushige Nakatsukasa Expert LEC NCI
 Dieter Schrenk Guest Researcher 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:

3.4

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

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

Multidrug resistance is the phenomenon by which cells become cross-resistant to a range of unrelated compounds in response to exposure to a single agent; this resistance is the result of overexpression of a 170 Kd membrane protein, p-glycoprotein, which is encoded by the *mdr* gene(s). Previously, we have shown that exposure of rats to xenobiotic agents such as the carcinogens aflatoxin B₁, isosafrole and 2-acetylaminofluorene (2-AAF) causes increased expression of *mdr* mRNA in the liver. To further study the mechanism by which xenobiotics regulate *mdr* gene expression, we have employed a primary hepatocyte culture system. Exposure of isolated hepatocytes to methycolanthrene (MC), 2-AAF but not 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) increased the expression of *mdr* mRNA expression; concomitant increases in cytochrome P4501A gene(s) expression were also observed with these agents. Inhibition of protein synthesis also increased the expression of *mdr* mRNA in these cells. The enhanced *mdr* expression caused by these compounds is a result of increased transcription. These data suggest that *mdr* expression is regulated by a protein that is distinct from the Ah receptor. To further investigate the mechanism of *mdr* regulation it was necessary to isolate the rat *mdr* genes. We have identified that the rat *mdr* gene family is comprised of three members; one of these genes has been isolated and characterized. Sequence analysis of a complete cDNA for a rat *mdr* cDNA indicated a high degree of identity to the mouse *mdr1b* gene (*mdr1*); thus, this rat cDNA was designated the *mdr1b* gene. Further studies on the 5' promoter and possible 3' mRNA stability regions are being performed. We observed in normal liver a slight but significant zonal difference of distribution of *mdr* transcripts (zone 1 > zone 3). Significant increase of *mdr* transcripts during regeneration was observed mainly in zone 1 hepatocytes. Our data suggest that the increased *mdr* expression might represent a subpopulation of preneoplastic nodules which possibly undergoes malignant transformation more specifically than GST-P positive nodules.

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

Snorri S. Thorgeirsson	Chief	LEC NCI
Jeffrey A. Silverman	Senior Staff Fellow	LEC NCI
Timothy W. Gant	Visiting Fellow	LEC NCI
Pamela A. Marino	Senior Staff Fellow	LEC NCI
Harushige Nakatsukasa	Expert	LEC NCI
Dieter Schrenk	Guest Researcher	LEC NCI

Objectives:

The primary objectives of this project are to: (1) isolate and characterize members of the rat *mdr* gene family; (2) study the regulation of the rat *mdr* gene(s) by xenobiotics; and (3) define the mechanism(s) by which the expression of the rat *mdr* gene(s) is regulated. We are particularly interested in the normal physiologic role of the *mdr* gene(s) and the regulation of its expression in response to xenobiotic agents including carcinogens and inhibitors of protein synthesis. Possible co-regulatory pathways of the *mdr* gene and members of the cytochrome P450 gene superfamily are also being investigated.

Methods Employed:

Construction and screening of lambda phage vector genomic and cDNA libraries; subcloning and deletion mutation construction in plasmid vectors; Northern, Southern and Western blotting. RNase protection assays; transcription analysis, nuclear run-on and primer extension assays; polymerase chain reaction; DNA sequencing and computer analysis; primary hepatocyte isolation and tissue culture; DNA transfection into mammalian cells.

Major Findings:

(1) Cloning. Although the coding regions of the *mdr* genes are highly conserved between species, there are significant sequence differences, particularly in the flanking regulatory regions. Thus, we have been isolating and characterizing members of the rat *mdr* gene family. We have established that the rat gene family is comprised of three members; this is consistent with previous data on other rodent species. The complete sequence of a rat *mdr* cDNA was determined based on seven independent cDNA clones that correspond to the same gene. The longest of these clones contains a 4.3 kb insert which represents a full-length rat *mdr* cDNA. The longest open reading frame of this sequence is 3933 bp; the first ATG is at 103 bp, making the predicted protein 1277 amino acids long (141 kDa). This correlates well with previously identified P-glycoproteins. The sequence of this gene has a very high, >90%, degree of identity to the mouse *mdr1b* gene (also known as the *mdr 1* gene); therefore, we designate it the rat *mdr1b* gene. Transcription of this gene begins at a single start site 151 bp upstream from the start codon.

To further characterize the regulatory regions of this gene we are isolating and characterizing 5' flanking sequences from a rat liver genomic library. Several clones have been isolated which hybridize to probes which correspond to the 5' region of the human and rat *mdr* cDNAs. These clones are currently being subcloned and sequenced and will be used in promoter analyses.

(2) Regulation by Xenobiotics. We have used a primary hepatocyte culture model system to examine the regulation of the *mdr* gene(s) and possible relationships between induction of cytochrome P4501A1 and *mdr* expression. Both 3-methylcholanthrene (MC) and 2-acetylaminofluorene (2-AAF) were efficient inducers of *mdr* expression in a dose dependent relationship. Tetrachlorodibenzo-p-dioxin (TCDD) increased the expression of the P4501A genes but did not induce *mdr* expression at the doses used. These compounds also caused the expected responses in the levels of p4501A1 and IA2 mRNAs. These data suggest that *mdr* induction is not mediated via the Ah receptor. Western blot analyses further indicated that the increased expression of *mdr* mRNA was followed by an increase in the level of p-glycoprotein.

(3) Mechanism(s) of regulation. To begin to study the mechanism(s) by which the levels of *mdr* mRNA and, subsequently p-gp, are regulated, we used transcription rate analyses. Nuclear run-off assays indicated that the increases in *mdr* expression caused by both MC and 2-AAF were due to increases in transcription. Further, primer extension analyses indicated that the initiation of transcription in the drug induced cells was from the same site, 151 bases upstream from the ATG site, as in control cells. Identical results were obtained in vitro and in vivo.

To begin to analyze the mechanism of transcriptional regulation and the nature of potential regulatory proteins, we examined the regulation of *mdr* gene expression following administration of inhibitors of protein synthesis. Using cycloheximide, we inhibited protein synthesis by >90% in primary rat hepatocytes, rat H4II# cells, mouse HePal cells and human HepG2 cells. In each of these cell lines the level of *mdr* expression was strongly increased. Nuclear run-off analyses indicated that these increases were due to increases in transcription. Primer extension analysis confirmed the results of the Northern blot analyses and further indicated that the transcription initiation site was identical in the treated cells as in the control cells, 151 bases upstream from the initiation of translation. Similar analyses with the cytochrome P4501A genes have indicated the presence of both positive and negative regulatory elements in these genes.

In addition to the above investigations on the transcriptional regulation of the *mdr* gene, we have also initiated experiments to study the possible role of post transcriptional regulation of this gene. Previous investigations have indicated that the increase in *mdr* expression observed following partial hepatectomy is not a result of increased transcription. Thus, we have begun to investigate the possible contribution of post-transcriptional mechanisms, e.g., mRNA stability, to the observed increases in steady state levels of *mdr* mRNA. Segments of the 3' end of the human *mdr* gene are being subcloned into plasmid vectors that will permit the identification of sequences which may regulate mRNA stability.

(4) We observed in normal liver a slight but significant zonal difference of distribution of *mdr* transcripts (zone 1 > zone 3). This zonal difference was strongly enhanced during partial hepatectomy-induced liver regeneration. During CCl₄-induced liver regeneration, marked enhancement of *mdr* expression was observed at the early stages even before DNA synthesis occurs, and apparent shift of *mdr* mRNA species was observed. One of three species of mRNA for *mdr* may be directly induced by CCl₄ itself or its metabolites, and the other species were closely associated with hepatocyte replication. The enhancement of *mdr* transcripts was observed mainly in zone 1 hepatocytes regardless of the stimulants, such as hepatocyte replication or CCl₄ (xenobiotics). It is hypothesized that the species of *mdr* mRNA which are induced in the liver by CCl₄ and hepatocyte replication are different, and may be independently regulated.

During chemically induced hepatocarcinogenesis, we observed consistent elevation of *mdr* expression in enzyme-altered foci, preneoplastic nodules and hepatocellular carcinomas. This suggests that subpopulations of hepatocytes which are inside foci, preneoplastic nodules and carcinomas all have an increased constitutive expression of *mdr*. In situ hybridization study revealed that oval cells are negative for *mdr* expression. It has been previously hypothesized that during the early carcinogenesis process altered foci acquire the multidrug resistant phenotype, which may subsequently promote the initiated cells to expand and to transform to malignant phenotype. In contrast to the previous hypothesis, although the *mdr* expression is indeed significantly increased in the foci from the normal liver, the *mdr* expression in the foci-surrounding hepatocytes was much higher than that in the foci. This is due to the effect of 2-acetylaminofluorene, since this chemical compound is a well-known, potent inducer of *mdr*. In the late stages of hepatocarcinogenesis, the pattern of *mdr* distribution was opposite that observed in the early stages. The level of *mdr* transcripts in preneoplastic nodules was two to three times more than that found in foci. Although we found a similar distribution pattern of transcripts for *mdr* and GST-P, some GST-P positive nodules are negative for *mdr* overexpression. This suggests that the increased *mdr* expression might represent a subpopulation of preneoplastic nodules which possibly undergo malignant transformation more specifically than GST-P. Therefore, increased *mdr* expression could be another marker for special hepatocytes which possibly undergo malignant transformation.

Publications:

Gant TW, Silverman JA, Bisgaard HC, Burt RK, Marino PA, Thorgeirsson SS. Induction of multidrug resistance and cytochrome P450IA gene families in primary hepatocyte cultures by xenobiotics and protein synthesis inhibition. Evidence for both receptor and trans-acting mediated mechanisms of regulation. *Mol Carcinog* (In Press).

Silverman JA, Raunio H, Gant, TW, Thorgeirsson SS. Cloning and characterization of a member of the rat *mdr* gene family. *Gene* (In Press).

Thorgeirsson SS, Silverman JA, Gant TW, Marino PA. Multidrug resistance gene family and chemical carcinogens. In: Grunberger D, ed. Pharmacology and therapeutics. Great Britain: Pergamon Press 1991;283-92.

Thorgeirsson SS, Silverman JA, Gant TW, Marino PA. Regulation of mdr gene expression by xenobiotics. In: Bock F, ed. Falk symposium no. 57 hepatic metabolism and disposition of endo- and xenobiotics. Lancaster UK: Kluwer Academic Publishers (In Press).

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

PROJECT NUMBER

Z01CP05601-03 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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: Gordon L. Hager Head, Hormone Action and LEC NCI
 Oncogenesis Section

Others: Kenneth Carlson PRAT LEC NCI
 Ronald G. Wolford Microbiologist LEC NCI
 Diana S. Berard Biologist LEC NCI

COOPERATING UNITS (if any)

Department of Pharmacology, Georgetown University (Dr. Anna T. Riegel)

LAB/BRANCH

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TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

0.7

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 proopiomelanocortin (POMC) gene produces a complex precursor polypeptide that is posttranslationally cleaved into a series of peptide hormones. In response to stress, one of these hormones, ACTH, induces the production of glucocorticoids in the adrenal gland, which in turn act to suppress POMC transcription in the pituitary in a classical feedback loop regulatory mechanism. This locus manifests two regulatory features of interest in our laboratory, negative regulation of gene expression by steroids and tissue-specific gene expression. The overall goals of the project are to identify and characterize tissue-specific and positive trans-acting factors that regulate POMC promoter activity, and to investigate mechanisms by which glucocorticoids mediate transcriptional repression. During characterization of potential regulatory regions in the POMC promoter by the classical mutagenesis and transfection approach, an unusual transcription factor (designated PO-B) was discovered. This protein activates POMC expression via a high-affinity DNA-binding site that is located between the TATA box and the cap site for initiation of RNA synthesis. The DNA recognition site for this factor has been characterized by the DNase I footprinting and DMS methylation interference techniques. In both in vitro transcription extracts and in vivo transfection studies, the TATA binding region is required, suggesting the factor acts cooperatively with TFIID. The factor has been extensively purified, and protein sequencing is underway.

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

Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Kenneth Carlson	PRAT Fellow	LEC NCI
Ronald G. Wolford	Microbiologist	LEC NCI
Diana S. Berard	Biologist	LEC NCI

Objectives:

- (1) To characterize the mechanism by which the PO-B factor regulates the transcription of POMC and, perhaps, other genes.
- (2) To identify and characterize other positive transacting factors and tissue-specific factors that regulate POMC promoter activity.
- (3) To understand the mechanism of negative regulation of transcription by the glucocorticoid receptor using the POMC gene as a model.
- (4) 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.

Methods Employed:

- (1) To identify regions of the POMC promoter important for negative regulation: oligonucleotide-directed mutagenesis, deletion mutational analysis, liposome-mediated transient transfection, gel retardation analysis.
- (2) To detect specific interactions of transcription factors with DNA: gel retardation assay, exonuclease III footprinting, DNase I footprinting, and methylation interference assay.
- (3) In vitro transcription assay. Primer extension analysis of transcription products.
- (4) Protein isolation and purification techniques are utilized to prepare sufficient quantities of the PO-B factor to determine its partial amino acid sequence in preparation for molecular cloning of the protein.

Major Findings:

We have previously demonstrated that a factor (PO-B), detected in a number of mammalian cell lines, binds specifically between the TATA box and the transcription initiation site of the POMC gene. The binding site of this protein, -3 to -15, does not overlap the POMC TATA box or cap site, and does not

resemble the binding site of any previously characterized transcription factor. Mutation of the PO-B binding site significantly decreased the transcriptional activity of the POMC promoter after transient transfection into the ATt-20 mouse pituitary tumor cell line and also in in vitro transcription assays. Because of the unusual location of its binding site and of its presence in a number of cell lines, PO-B may represent a new class of transcription factor that may be able to facilitate gene expression by interacting with components of the transcription initiation complex.

Two lines of experimentation have been followed in order to obtain a molecular clone of this interesting protein. One approach involves the generation of high-titer cDNA libraries using mRNA from cells with significant PO-B levels, to introduce these libraries in vectors capable of producing potentially functional fusion proteins including the PO-B reading frame, and to identify such clones by the detection of a DNA binding activity that recognizes the PO-B recognition site. This approach has not yet produced recombinant clones that can be unambiguously shown to express the PO-B DNA binding activity.

A second approach is to extensively purify the protein, and obtain a partial amino acid sequence, either N-terminal or internal, that would permit the preparation of PO-B specific oligonucleotide probes based on the protein sequence. Accordingly, extensive purification of the protein has been achieved, and initial efforts at partial protein sequencing are underway. Given the failure of the expression cloning approach, future efforts in characterization of the protein and molecular cloning will follow the second approach.

Publications:

Riegel AT, Remenick J, Wolford RG, Berard DS, Hager GL. A novel transcriptional activator (PO-B) binds between the TATA box and cap site of the pro-opiomelanocortin gene. *Nucleic Acids Res* 1990;18:4513-21.

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

PROJECT NUMBER

Z01CP05659-02 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Detection of Polypeptide and Genetic Alterations during Hepatocarcinogenesis

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

PI:	Peter J. Wirth	Supervisory Research Chemist	LEC NCI
Others:	Lin-di Luo	Visiting Associate	LEC NCI
	Yoshinori Fujimoto	Guest Researcher	LEC NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

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SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

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

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

Rat liver epithelial (RLE) cells provide a very valuable in vitro model to study both spontaneous and chemically induced hepatocarcinogenesis. Therefore, the main objectives of this project are to characterize the early cellular biochemical events as a result of genetic alterations and subsequent changes in protein expression during spontaneous and chemically mediated hepatocarcinogenesis. RLE cells can be transformed in vitro by various agents including chemical carcinogens and specific oncogenes as well as spontaneously when the cells are maintained under certain selective growth conditions. When injected into nude mice the transformed cells produce a diverse variety of tumor types including those of a well-differentiated hepatocellular carcinoma-like tumor. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was utilized to construct comprehensive computerized topographical protein database maps of protein expression in each of the major subcellular fractions (whole cell, cytosol, nuclei, mitochondria, plasma membrane). Genetic analysis of aflatoxin B1 induced RLE transformants revealed a consistent point mutation consisting of a transition from G to A within codon 173 (CGC to GAG) in the tumor suppressor p53 gene. This mutation resulted in a change of the encoded amino acid from histidine to arginine. Mutations in the p53 gene are currently being analyzed in monkey genomic DNA extracted from paraffin embedded AFB induced tumors.

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

Peter J. Wirth	Chemist	LEC NCI
Lin-di Luo	Visiting Associate	LEC NCI
Yoshinori Fujimoto	Guest Researcher	LEC NCI

Objectives:

Working on the hypothesis that the acquisition of the neoplastic phenotype would bring about both qualitative and quantitative changes in cellular function quite different from those observed under normal and/or preneoplastic conditions and that these changes should be reflected both on the DNA and cellular protein level, we have undertaken a detailed analysis of genetic and cellular polypeptide alterations during chemically and spontaneously induced neoplastic transformation of rat liver epithelial (RLE) cells. Initial work was directed toward the construction of computer accessible databases of cellular polypeptides from RLE cells utilizing two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in combination with the ELSIE 5 gel analysis system. Databases were constructed utilizing both [³⁵S]-methionine- and [³²P]-orthophosphate-labeled as well as silver stained polypeptides from normal RLE cells. In addition, particular emphasis was placed on the sub-cellular fractionation and assignment of individual polypeptides to specific subcellular compartments. We felt that such an analysis would be quite valuable not only as a result of increasing the "viewing window" of our RLE polypeptide database but would also provide meaningful information regarding possible biochemical functions of each protein and allow us to determine if certain polypeptides are differentially modulated during cellular growth, differentiation, and transformation.

In parallel with our protein studies, work was directed toward the analysis of possible genetic alterations, in particular, the activation of specific oncogenes (e.g. *ras*, *myc*, *raf*, etc) or the inactivation of certain tumor suppressor genes (e.g., p53) during either chemically- or spontaneously-induced transformation of RLE cells.

Methods Employed:

Methods used in these studies include: (1) tissue culture techniques, DNA, RNA, and protein purification; (2) 2D-PAGE; (3) Southern capillary blotting/semi-dry DNA electroblotting; (4) computer assisted image analysis; and (5) polymerase chain reaction (PCR), DNA cloning and sequencing.

Major Findings:

2D-PAGE analysis of [³⁵S]-methionine labelled whole cell lysates from RLE cells yielded high resolution maps of 2100-2500 individual polypeptides. Multiple exposures of individual autoradiograms allowed for the detection and

quantitation of polypeptide spots expressed as low as 0.003-0.005% of the total amount of protein loaded on the gels. Since estimates have been made that the typical cell may contain on the order of 10,000-20,000 individual polypeptides, we, therefore, sought to increase the "viewing window" of our analysis by performing subcellular fractionation of the intact RLE cells and constructing 2D-PAGE polypeptide maps of the individual cellular compartments. Fractionation of cells into cytosolic (C) and crude particulate (P) fractions provided a significant increase in resolution of the individual polypeptides, although the total number of polypeptides separated was not significantly increased (total 2400). Although certain polypeptides were detected in both fractions, a significant number of polypeptides were localized to either the P or C fraction. Since the P fraction contains such major cellular components such as plasma membrane, mitochondria, nuclei, lysosomes, endoplasmic reticulum, whole cells were fractionated into intact nuclei, membrane-associated, mitochondrial, and cytoskeletal components. 2D-maps were constructed from each fraction and databases established consisting of 1500 nuclear, 1700 membrane-associated, and 415 mitochondrial polypeptides. Cytoskeletal components consisted of α , β -tubulins, α , β , γ -actins, vimentin, and minor amounts of vimentin degradation polypeptides. Cell-free *in vitro* translation of poly-A selected RLE mRNA was performed and the 2D-PAGE patterns of the resultant 770 polypeptide products were mapped back to original whole cell lysate maps. Greater than 70% of the *in vitro* translation products could be mapped to the original whole cell lysate electrophoretograms. RLE cells were labelled with either [³⁵S]-methionine and [³²P]-orthophosphate, samples mixed and blended samples subjected to 2D-PAGE analysis. Electrophoretograms were silver-stained and the dried gels exposed simultaneously to two X-ray films placed back to back. The first film which was appressed directly to the electrophoretogram visualized both [³⁵S] and [³²P], while the second film recorded only exposure to [³²P] due to the differential energy levels of the two isotopes (β -particle from [³⁵S] unable to pass through film emulsion and mylar film). This method allowed for the unambiguous mapping of post-translationally modified phosphoproteins into the database of unfractionated [³⁵S]-labelled polypeptides. In addition, the rates of synthesis of individual polypeptides were accessed following comparison of silver stained images with the autoradiograms of the metabolically labelled proteins. Approximately 200 phosphate-labelled and over 1500 silver stained polypeptides were accurately cataloged in this manner. Comparison of whole cell lysate, nuclear, and *in vitro* translated polypeptides among normal RLE cells and AFB transformed and spontaneously transformed cell lines revealed significant qualitative and quantitative polypeptide differences. In all AFB transformants the α -isoform was markedly increased as compared to either normal RLE or spontaneously transformed RLE cells. In addition, two major groups of higher molecular weight membrane associated polypeptides (205 kD and pI ranges of 6.6-7.4 and 120 kD and pI ranges of 6.5-7.4, respectively) were either not expressed or expressed at greatly reduced levels in both AFB and spontaneously induced transformants.

Analysis of RLE cells previously transformed with acutely transforming retroviral oncogenes, namely *v-Ha-ras* (F22 and F22T), *v-raf* (F3611), the *v-raf/v-myc* (FJ-2) chimera with normal RLE cells revealed characteristic differences among the various transformants.

Epithelial cell lines were established from adult rat pancreas and compared to RLE cells. Two clonal rat liver lines (NA7 and NF12) and two pancreatic epithelial lines (RPE3 and RPR4) were labelled with [³⁵S]-methionine and whole cell lysates were analyzed using 2D-PAGE. Essentially no qualitative and only minor quantitative differences were observed among the 1500 polypeptides compared, suggesting that these cells may be derived from a common primitive epithelial cell type present in both rat liver and pancreas.

RLE cells transformed either with aflatoxin B1 (AFB) or spontaneously were studied for alterations of the *ras* oncogene and the p53 tumor suppressor gene. Although Kirsten *ras*(codon 12) was neither mutated nor amplified in any of the spontaneously or AFB transformed RLE cell lines, the p53 gene was mutated in all the AFB lines. Sequencing of amplified cDNA by reverse transcriptase-polymerase chain reaction of p53 mRNA indicated that all six AFB transformed cell lines showed the same point mutation consisting of a transition from G to A within codon 173 (CGC to GAG) resulting in a change of the encoded amino acid from arginine to histidine. However, no mutation in this region was observed in either the parental RLE cell line or in a series of highly tumorigenic, spontaneously transformed RLE cell lines. The p53 mRNAs were highly expressed in all of the AFB transformed lines as compared to the parental cell line. Genomic DNA analysis indicated that no wild type alleles of the p53 gene were present in any of the AFB transformed cell lines. Subcutaneous implantation of the AFB cell lines into nude mice showed a variety of tumor types including hepatocellular carcinoma, cholangiocarcinoma, mixed type carcinoma, and hepatoblastoma. 2D-PAGE analysis of cellular polypeptides yielded similar but characteristic patterns for each cell line as described above, suggesting that although all six AFB cell lines showed the same mutation (codon 173) they were clonally independent.

In order to check the potential role of this mutation in the transformation of RLE cells, appropriate plasmid constructs (PCMVneo) were made containing the mutated p53 gene sequence, wild type, p53 as well as wild type antisense p53 sequences. The SV40neo selectable marker gene was ligated into the PCMV β plasmid containing the cytomegalovirus promoter. The respective p53 gene inserts were then ligated into the NotI site of PCMVneo plasmid. The respective constructs will be introduced into the RLE cells using lipofection and after selection on G418 the growth rates and tumorigenicities of the resultant cell lines will be determined.

Epidemiological studies have suggested a link between dietary AFB and cancer developemnt and recent data have shown that the p53 gene is mutated in certain human hepatocellular carcinomas. In monkeys AFB induces a variety of tumors, including hepatocellular carcinoma, cholangiosarcoma, osteosarcoma, hemangiosarcoma, and pancreatic duct adenocarcinomas. In order to examine the possible involvement of the p53 gene in AFB induced tumorigenesis, DNA was extracted from a series of paraffin embedded monkey tumors and exons 4-8 of the p53 gene were amplified using PCR. Routinely, 900 bp of genomic DNA could be amplified from paraffin extracted DNA, while PCR amplification of DNA extracted from fresh tissue yielded 2.2 kb fragments scanning the entire genomic sequence. Using appropriately designed primers the conserved region

II (exons 4-5) of the monkey p53 gene was amplified and cloned into pGEM for sequencing. Similarly, specific primers were synthesized for the amplification and sequencing of the conserved regions III (exon 7) and IV (exon 8), respectively.

Publications:

Huber BE, Wirth PJ, Newbold JE. Effects of WELLFERON® (human lymphoblastoid interferon) on proliferation, gene expression and tumorigenicity of human hepatoma cell lines. *Interferon Res* (In Press).

Wirth PJ, Fujimoto Y, Mori M, Nagao M, Sugimura T. Two-dimensional electrophoretic analysis of hepatitis associated polypeptides in livers of LEC rats developing spontaneous hepatitis. In: Mori M, Yoshida MC, Takeichi N, Taniguchi N, eds. *The LEC rat: a new model for hepatitis and liver cancer*. Tokyo: Springer-Verlag (In Press).

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

PROJECT NUMBER

Z01CP05660-02 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Tissue-Specific Expression of MMTV and Mechanism of Protooncogene Activation

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

PI: Gordon L. Hager Head, Hormone Action and LEC NCI
 Oncogenesis Section

Others: Philippe Lefebvre Visiting Fellow LEC NCI
 Sam John Graduate Student LEC NCI
 Kenneth Carlson PRAT Fellow LEC NCI
 Diana S. Berard Biologist 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:

2.9

PROFESSIONAL:

2.6

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

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

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

The MMTV retrovirus is able to activate cellular oncogenes (so-called int loci) by insertion in the vicinity of these genes. This insertion is one of the key events leading to transformation of mammary epithelial cells. The mammary cell specificity of MMTV oncogenesis lies, in part, in tissue-specific expression of the MMTV promoter. Utilizing a recently developed cell line series (RAC cells) that retains many epithelial cell features in culture, we have discovered two regulatory elements in the viral regulatory sequences that are responsible for cell-specific viral transcription, and presumably also for tissue-specific oncogene activation. One of these elements, located at the 5' end of the LTR, acts as a cell-specific, positive enhancer region, and has been shown to contain binding sequences for at least two proteins, mp5 and mp4, both of which also appear to be limited in distribution to mammary cells. A second element, located internal to the LTR, acts as a negative transcription regulator, and also is restricted in its activity to a subset of mammary cells. Two factors that bind to this element and are tissue limited in distribution have been preliminarily characterized. Both the positive and negative factors correspond to similar activities present in human cells, suggesting this regulatory system is active in man. These observations suggest that MMTV protooncogene activation is mediated by a combination of positive and negatively-acting tissue-specific elements.

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

Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI
Philippe Lefebvre	Visiting Fellow	LEC NCI
Sam John	Graduate Student	LEC NCI
Kenneth Carlson	PRAT Fellow	LEC NCI
Diana S. Berard	Biologist	LEC NCI

Objectives:

- (1) Determination of the molecular mechanism responsible for activation of proto-oncogenes by MMTV, and the interaction of steroid receptor-mediated and tissue-specific induction pathways.
- (2) Characterization of nuclear DNA-binding factors specific for MMTV regulatory sequences.
- (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 DNA's.
- (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) The impact of putative transcription factors is monitored in vitro by the reconstitution of specific transcription initiation in cell-free extracts.
- (5) The nucleoprotein structure of regions found to harbor binding sites for transcriptional regulatory proteins is characterized to determine if alterations to chromatin structure are involved in controlling access to the DNA template.

Major Findings:

We have examined the mechanism of activation of the proto-oncogene int-2 by MMTV during viral-induced mammary carcinogenesis in a series of cell lines that are able to grow under two differentiation states. One of these cells, RAC 11P, retains many epithelial cell features in culture, but is unstable and spontaneously dedifferentiates into a series of less differentiated cells, illustrated by the RAC 5E cell line. These cell lines have a proviral MMTV DNA integrated upstream of the int-2 gene, whose activation was responsible for the original tumor event. The rate of expression of proviruses is strikingly different between the two cell types, and the rate of expression of the int-2 gene is directly correlated with the MMTV expression level. This cell line thus provides a highly relevant system to study both the mechanism leading to a cell-specific expression of the MMTV and also its possible relationship with protooncogene activation.

A series of deletion mutants over the MMTV LTR were constructed. These mutants were tested both for efficiency of expression and response to hormone in RAC cells and other cell lines. An upstream region was found to contain a positive-acting enhancer activity functional not only in RAC cells, but also in other mouse mammary cell lines known for their high rate of MMTV transcription (C127 cells, 34i cells) and not in mouse fibroblasts (NIH 3T3 cells). Potential transcription factors active at this element have been characterized by gel shift and footprinting techniques. Two separate proteins, designated mp5 and mp4, have been characterized thus far. Both have recognition sites in the region necessary for enhancer function, and both are detected only in cells of mammary origin. Proteins corresponding to these two factors have also been detected in human mammary cell lines, indicating the regulatory mechanisms represented by these activities are operative in human mammary tissues as well.

In addition to the enhancer domain, a repressor element was detected that is located central to the MMTV LTR. This element is active in RAC 11P cells, but not in 5E cells, or cells of non-mammary origin, and is thus even more limited in its cellular activity profile than the enhancer region. This element is probably distinct from another region, already described by several authors, that manifests repressor activity in many cell types. At least two proteins that bind to the negative element have been characterized thus far. The region is quite complex, however, and it is anticipated that other factors will be involved.

Both the positive and negative cell-specific transcriptional elements apparently cooperate with glucocorticoid receptors in activating MMTV transcription. They serve, therefore, as interesting models to investigate mechanisms of cooperation between multiple transcription elements. Potential cooperativity with other steroid receptors has yet to be investigated.

The activity of the activated int-2 locus in RAC cells is refractory to hormone modulation. We therefore propose that the cell-specific activating regions of MMTV can interact directly with int-2 promoter elements to give hormone independent activation, but can also act synergistically with the HRE

to given cooperative activation of MMTV itself. In agreement with this model, preliminary experiments suggest that introduction of DNA sequences containing the mp4-mp5 domain into RAC cells inhibits int-2 transcription. Competition for these factors apparently blocks their action at the int-2 locus. This experimental approach should eventually permit a direct identification of the factors binding to MMTV that are required for int-2 activation.

Publications:

Lefebvre P, Berard DS, Hager GL. Two regions of the mouse mammary tumor virus LTR regulate the activity of its promoter in mammary cell lines. J Mol Cell Biol (In Press).

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

PROJECT NUMBER

Z01CP05675-01 LEC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Plasma Proteins as Early Biomarkers of Exposure to Carcinogenic Aromatic Amines

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: Snorri S. Thorgeirsson Chief LEC NCI
 David C. Parmelee Expert LEC NCI

COOPERATING UNITS (if any)

National Center for Toxicological Research (NCTR), Jefferson, AK (Dr. Fred Kadlubar)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.4

PROFESSIONAL:

0.4

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

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

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

The objective of this project is to evaluate changes in plasma proteins as an early biomarker of exposure to carcinogenic aromatic amines. Two-dimensional gel electrophoresis (2DG) has been used to study the changes induced in dog plasma by the known urinary bladder carcinogens 4-aminobiphenyl (4-ABP) and 2-naphthylamine (2-NA). 3-Aminobiphenyl (3-ABP) and 1-naphthylamine (1-NA), both considered to be non-carcinogenic, were used as controls. The purpose of this study was: (1) to determine whether or not the apparent deletions on plasma proteins are specific to 4-ABP; (2) to measure the time course in the suppression of the major polypeptides during dosing and their re-synthesis during a recovery period; and (3) to determine, by microsequencing directly off the gels, the biochemical identity of the affected spots. The results indicate that the suppression is limited to 4-ABP, with 3-ABP, 2-NA and 1-NA causing no discernible change in the 2DG patterns over a 12 week dosing period. The 4-ABP caused dramatic suppression of two sets of spots. One of apparent molecular weight 32.5 kD, and pI 5.8-6.0, was identified to be the B chain of haptoglobin. This spot disappears after about two weeks of treatment and recovers slowly after dosing stops. Haptoglobin functions to bind with free hemoglobin and purge it from the blood stream. Since 4-ABP causes hemolysis, the disappearance of the haptoglobin is readily explained. Among the second set of spots, a MW 65 kD, pI 6.5-6.6 peptide, disappears much faster than the haptoglobin, and recovers more quickly. The protein is about one-fifth the intensity of haptoglobin and appears to be blocked, as N-terminal microsequencing has been unsuccessful. Work is currently underway to obtain an internal sequence of this polypeptide in order to identify it.

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
David C. Parmelee	Expert	LEC NCI

Objectives:

The main objective of this project is to evaluate the changes in plasma proteins that occur as a result of exposure to carcinogenic aromatic amines. Two-dimensional gel electrophoresis, which is capable of resolving many hundreds of proteins on a single gel matrix, was used to observe these changes. It is hoped that these efforts will lead to the identification of early biochemical markers of neoplasia, markers that may precede both histopathologic changes and alterations in the expression of cellular genes controlling growth and differentiation.

Methods Employed:

The principal methods are: (1) two-dimensional gel electrophoresis; (2) silver-staining techniques; and (3) microsequencing techniques.

Major Findings:

4-Aminobiphenyl (4-ABP), a known component of tobacco smoke, is a potent urinary bladder carcinogen in both dogs and humans. 2-Napthylamine (2-NA) also induces bladder tumors in dogs and humans, but is appreciably less potent than 4-ABP. On the other hand, 3-aminobiphenyl (3-ABP) and 1-napthylamine (1-NA) are considered to be non-carcinogenic. In this experiment, 4-ABP and 2-NA, as well as their non-carcinogenic isomers, have been evaluated for their ability to induce early changes in blood plasma proteins during a chronic dosing regimen that has been used previously in carcinogenicity studies. Two-dimensional gel electrophoresis (2DG), coupled with high-sensitivity silver staining, has been used to monitor these changes.

In this study, female beagle dogs were orally administered one of the aromatic amines, or a placebo (two dogs each for a total of 10 dogs) for five days a week, over a 12 week period. The animals were allowed to recover six additional weeks before being euthanized. Blood samples were drawn three times a week (MWF) for the first two weeks of dosing and at weekly intervals thereafter. Plasma was prepared and stored at -70°C. There were a total of 23 samples per dog, 230 samples total.

Visual inspection of the gels showed there were no significant changes in the 2DG patterns of control or of the 3-ABP, 2-NA, or 1-NA treated dogs. The 4-ABP treated dog plasmas were characterized by dramatic loss of several polypeptides, most notably two groups of three proteins each. One had an apparent

molecular weight of 32.5 kD and apparent pIs of 5.8, 5.9 and 6.0. The central spot was one of the most abundant in the 2DG map and was easily extracted and the N-terminal sequence determined in the LEC's microsequencing laboratory. Nineteen amino acid residues were sequenced and there was a 100% homology with the dog haptoglobin β chain sequence (IMGGSVDAKGSFPWQAKMV) stored in the Protein Identification Resource database. Although haptoglobin has sometimes been associated with neoplasia, its major function seems to be to bind and clear hemoglobin from the blood stream. If sufficient hemoglobin is present, haptoglobin can completely disappear from the blood stream. 4-ABP has been shown to cause hemolysis (F.C. Kadlubar, in press), so the loss of haptoglobin is readily explained and unlikely to be a marker for early neoplasia.

The second set of three polypeptides have an apparent molecular weight of 65 kD and an apparent pI range of from 6.5 to 6.6. These will be referred to collectively here as "spot B". These disappear much more quickly than does the haptoglobin spot and recover more quickly and more completely. Spot B is fully suppressed within one week of dosing, whereas haptoglobin took 3-4 weeks in one dog and 1-2 weeks in the other. Spot B is fully recovered 1-2 weeks after cessation of 4-ABP dosing, while it is 3-4 weeks before haptoglobin begins to recover and recovery is not complete even after six weeks. Spot B is not repressed to the same extent as haptoglobin and can often be detected during the dosing period. Spot B is thus more closely associated with the 4-ABP dosing than is haptoglobin. We have been unable to obtain an N-terminal sequence for Spot B, which is apparently blocked. Work is currently underway to obtain an internal sequence for spot B in hopes of determining its biochemical identity.

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

October 1, 1990 through September 30, 1991

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

The Laboratory of Experimental Pathology (LEP), continuing its studies of experimental respiratory carcinogenesis, has focused its current research on the mechanisms responsible for the cytotoxic and carcinogenic activities of crystalline silica and other inorganic particulate materials. 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 microns) it is known to induce a progressive obstructive pulmonary disease, silicosis, characterized by progressive granulomatous and fibrogenic reactions. Since 1984, strong evidence has been obtained that silica induces high incidences of lung cancer in rats, and epidemiologic studies have shown that human silicotic subjects are at increased risk for lung cancer.

Results reported by LEP in the last few years, following single intratracheal instillations of silica in different species and strains of rodents, showed markedly different host responses. Progressive pulmonary fibrosis (silicosis) was induced in rats and in mice, but not in hamsters. Alveolar type II epithelial cell proliferation and high incidences of pulmonary carcinomas were induced only in rats, but not in mice nor in hamsters. Silica-induced lung tumors in rats reached incidences of 85-100%, and were mostly adenocarcinomas, of which about 30% developed around fibrotic nodules, reproducing the pathology of human pulmonary scar cancer. The rat, therefore, appears as a singularly sensitive animal model of silica-induced carcinogenesis and offers a unique model for the study of the pathogenesis of scar cancer, an important form of human lung cancer. Host factors in vivo appear as important determinants of the response. Thus, the mechanisms of action of silica need to be investigated not only to clarify cytotoxic, granulomatogenic and fibrogenic mechanisms resulting in silicosis, but also to elucidate those mechanisms that lead to progressive epithelial proliferation and lung cancer.

The current LEP approach to these problems includes four coordinated projects. (A) Cell biology studies in the BALB/3T3/A31-1-1 cell line, on the mechanisms of silica uptake, phagocytosis, cytotoxicity, and neoplastic transformation, with molecular characterization of the silica-transformed, tumorigenic cells lines. (B) Studies of the reactions to silica *in vivo*, including silica-induced lung tumors in rats and silicosis-associated lung cancers in human subjects, extended by studies for the molecular characterization of silica-induced lung carcinomas, using paraffin sections for DNA analysis by polymerase chain reaction with specific probes for oncogenes and tumor suppressor genes. (C) Studies of the effects of silica on epithelial cells in culture. (D) Studies of molecular mechanisms of DNA damage and mutation related to physico-chemical properties and biological activity of silica crystalline surfaces.

These studies make use of several crystalline silica samples with different surface properties and characteristics, and other particulates for comparison. Physico-chemical properties of the silica surface are investigated in order to evaluate their role in determining biological effects.

Results Obtained in the Current Year

Several samples of crystalline silica of respirable size (mostly 1-5 μm in diameter) were studied for different surface reactive properties and their biological effects. The following samples were used: (A) quartz samples: Min-U-Sil 5 (MQZ, a standard U.S. preparation), hydrofluoric-acid-etched MQZ (HFMQZ, prepared for this laboratory in order to remove impurities from the silica surface by HF-etching), DQ12 and F600 (two standard preparations from Germany) and CSQZ (a standard preparation from China); (B) other crystalline silica forms: cristobalite and tridymite (prepared synthetically for this laboratory); and (C) non-fibrogenic dusts for comparison, usually ferric oxide.

Neoplastic transformation was obtained in the BALB/3T3/A31-1-1 mouse embryo cell line by exposure of the cells to different samples of crystalline silica. Optimal conditions for the assays of insoluble particulates in this cell line were established. Transformation was obtained, so far, with three samples: MQZ showed the highest activity, followed by HFMQZ and F600. The incidence of induced neoplastic transformed foci was found to be dose-dependent at dust concentrations on the dish surface up to 25-50 $\mu\text{g}/\text{cm}^2$ and near plateau level at higher concentrations. The induced transformed foci were subcultured, cryopreserved for molecular analysis and also inoculated in nude mice, where they all rapidly gave positive results for tumorigenicity. The cells of the transformed foci and of the resulting tumors are being analyzed for karyotypic alterations. The untreated control cells gave consistently negative results in the transformation and tumorigenicity tests. The silica-transformed cells are currently analyzed for the identification of activated oncogenes.

The cytotoxicity of silica particles was studied in the same BALB/3T3/A31-1-1 cell line. The cells, grown in the current optimized culture conditions, were confirmed to have high phagocytic activity. Cell growth and phagocytosis occurred when the dust was added over previously attached cells (the method of choice) and also when the dust was previously settled on the dish and the cells seeded on top of it. Cell survival, as measured by colony forming efficiency (CFE), was decreased by exposure to various silica dusts with a range of toxicity levels, the lowest cytotoxicity being shown by the F600 quartz. After confluence, cells remained quiescent even

when loaded with dust particles, with no further signs of toxicity. The intracellular storage of particles, mostly in the perinuclear cytoplasm, appeared analogous for dusts of markedly different initial toxicity, suggesting that after completion of the phagocytic process the dust particles are no longer toxic, possibly as a consequence of being segregated in membrane-bound phagosomes. Current results suggest that the mechanisms responsible for cytotoxicity, and possibly transformation, take place in the time interval between the encounter of the particles with the cell membrane and the completion of phagocytosis. The hypothesis that toxic effects occur only during this initial phase is being tested by treating the cells for selected periods of time with various inhibitors of the mechanisms of toxicity.

Decreasing the serum concentrations in the medium progressively decreased the CFE of control cells and of quartz-treated cells, the latter always showing a proportionally lower CFE than control cells. Cells exposed to media conditioned by incubation with high doses of silica showed no toxicity, suggesting that the cytotoxic effect is not due to soluble factors released from silica into the medium.

Since the mechanisms of silica toxicity on DNA *in vitro* were found to be dependent on the production of reactive oxygen radicals catalyzed by iron cations, as reported below, studies were undertaken to determine the role of oxygen radicals and of divalent and trivalent iron cations on cell toxicity, using selective factors, such as inhibitors of reactive oxygen species, iron chelators and specific iron cations.

Studies with epithelial cells in culture were continued, using the previously established mouse skin keratinocyte (MK) cell lines in serum-free medium, confirming the finding that silica inhibits colony formation and cell growth rate. The MK cell line 2057C, previously selected to grow in the presence of high levels (1 ng/ml) of TGF- β , was cultured for further mechanisms studies. Methods were explored for the culture of rat pulmonary alveolar type II cells, which are, *in vivo*, the target cells for silica carcinogenesis in the lung.

The mechanisms of silica toxicity and carcinogenicity were investigated by several methods, aimed at characterizing the reactivity of crystalline surfaces and their effects on DNA damage. The underlying hypothesis is that the highly toxic hydroxyl radical is produced by a cascade of reactions triggered by silica surfaces, probably through their iron impurities. Hydroxyl radical production occurs through the Haber-Weiss reaction (hydrogen peroxide reacting with superoxide radical anion to yield hydroxyl radical). This reaction is greatly accelerated by action of iron cations: trivalent iron reacts with superoxide radical to yield divalent iron and oxygen; divalent iron reacts with hydrogen peroxide to yield hydroxyl radical while regenerating trivalent iron (Fenton reaction).

The surface of crystalline silica (silicon dioxide, SiO₂) becomes partly hydrated in aqueous medium to form silanol groups (-SiOH) and partially ionized silanol groups (-SiO⁻), which impart a negative charge to the silica surface. This charge is decreased when divalent or trivalent iron cations (or other metals) are bound to the available negative charge sites (iron-coordination sites). A new spectrophotometric assay was developed, using the cationic dye Janus Green B, to measure the adsorption of the dye on the silica surface, as an indicator of the negative charge on given silica samples. Dye adsorption was undetectable on ferric oxide, low on F600 quartz and cristobalite, intermediate on MQZ, HFMQZ and tridymite, and high on CSQZ and on DQ12 quartz. These results indicated that dye adsorption on the tested samples was

roughly inversely proportional to the extent of in vitro DNA damage obtained in the presence of hydrogen peroxide (see following paragraph).

The induction of DNA double strand breaks was measured by in vitro exposure of lambda phage DNA *Hind*III digest to silica suspensions, in the presence or absence of hydrogen peroxide and/or inhibitors. No DNA damage was induced by silica alone, or by hydrogen peroxide alone, but the exposure to silica in the presence of hydrogen peroxide induced extensive DNA double strand breakage, revealed after agarose gel electrophoresis and ethidium bromide staining by the degradation of the discretely sized bands. The severity of this DNA damage increased with time of exposure and varied for the different dust samples. Ferric oxide had the highest activity. Among the silica dusts, the highest DNA damage was induced by cristobalite, followed by F600 and by MQZ; lower levels of activity were found with DQ12, HFMQZ and tridymite and nearly no activity was detected with CSQZ. Since these effects are likely to be due to reactive oxygen radicals generated by an iron-dependent reaction, selective inhibitors were tested. The DNA damage induced by MQZ quartz in the presence of hydrogen peroxide was significantly inhibited by the iron chelator deferoxamine, but, in contrast, it was accelerated by another iron chelator, diethyl-triamino-pentaacetic acid (DETAPAC). This apparent discrepancy is explained by the different mechanisms of these two chelating agents. Deferoxamine blocks the availability of chelated iron for oxidation/reduction reactions, whereas DETAPAC does not. Thus, iron chelation per se is insufficient to reverse this mechanism of induction of DNA damage, which occurs only when the chelated iron remains chemically reactive. DNA damage in this system was also partially inhibited by the hydroxyl radical scavenger dimethylsulfoxide (DMSO). Superoxide dismutase (SOD, copper-zinc type), which can generate hydrogen peroxide by dismutation if superoxide is present, allowed DNA damage to take place, without added hydrogen peroxide, in the presence of MQZ quartz, although requiring a long incubation time. This effect was not induced when ferric oxide was similarly tested. Possible molecular mechanisms that would explain these findings are currently under study.

A shuttle vector, plasmid pSI89, containing the *supF* reporter gene, was used to study DNA damage and mutation occurring inside mammalian cells exposed to silica. The plasmid was transfected into the 293 cell line (adenovirus 5 transformed human embryo kidney cells). The cultured cells were subsequently exposed to silica for 72 hours and the plasmid was then extracted by an alkaline lysis method. Following digestion with *Dpn*I (to destroy all the original unreplicated bacterially derived plasmid), the mammalian-cell replicated plasmid DNA was used to transform an appropriate bacterial indicator strain (*E. coli* MBM 7070). The mutation frequency induced in the bacteria was calculated by counting the colonies formed in agar plates containing X-galactose: in a lawn of blue colonies, the mutant colonies appear white. Preliminary results showed a marked mutagenic effect for F600 quartz, and much lower effects for MQZ and CSQZ. The white colonies were restreaked on plates and their DNA is being characterized by sequencing with the dideoxy chain termination method of Sanger to identify changes in the *supF* gene.

With the advice and collaboration of specialized experts, an effort is being made to define critical physico-chemical properties of crystalline silica surfaces, relevant to their biological activities.

The in vitro mechanisms of silica activity need to be interpreted in light of its in vivo effects, both in experimental animals and in human subjects. The initial interaction of silica with pulmonary macrophages in vivo bears a close resemblance to the phagocytic and cytotoxic reaction observed in cultured cells, but animal

studies showed a marked species variation. While rats and mice showed phagocytosis and killing of macrophages, granulomatous reactions and fibrosis, hamsters were found singularly resistant to silica toxicity and their pulmonary reaction was limited to phagocytosis and storage. Cells from these different species should represent useful models for mechanism studies. In the susceptible species, including the human, the complex granulomatous reactions are supported by a continuous recruitment of new cells, and it is known that macrophages and granulocytes are active producers of reactive oxygen radicals, as well as of several cytokines that can influence the pathogenesis of silicosis. Endogenous iron sources may play a critical role in modifying the surface reactions of silica. Little is known about the mechanisms of stimulation of the alveolar epithelia that eventually give rise to proliferative lesions and to carcinomas.

The histological material, obtained in several previous serial sacrifice and long term studies in rats, mice and hamsters, was further analyzed and compared with the corresponding morphology of cells in culture. The marked differences in the reaction to silica, that were previously reported in the three tested species, were further studied: rats developed typical silicosis and high incidences of adjacent carcinomas of the lung (arising from hyperplastic areas of alveolar type II cells); mice developed discrete silicotic nodules, but no persistent epithelial reactions or tumors; and hamsters developed only phagocytic storage reactions with no progressive fibrosis or epithelial proliferation. These three distinct types of host responses are being investigated for their pathogenetic significance and are correlated with the cellular and molecular reactions observed in the cell biology and molecular biology studies.

Human pathology of lung cancer in silicotic subjects was studied by histopathological examination of additional cases. These observations confirmed the presence of areas of epithelial hyperplasia adjacent to silicotic granulomas, analogous to some of the proliferative epithelial lesions observed in the experimental animals. These observations were made possible through the courtesy of several academic departments of pathology. The observations, however, remain sporadic, due to the limited extent of lung tissue present in the available slides.

A new investigation has been undertaken to identify molecular lesions underlying the induction of lung cancer by silica, applicable to both human and animal tumors. The first phase of this study, currently under way, investigates molecular lesions in silica-induced lung tumors in rats. These studies could then be extended to detect corresponding molecular lesions in human lung cancers associated with silicosis and other types of pulmonary fibrosis. The carcinomas, arising near fibrotic silicotic nodules in rats, were identified histologically, and the corresponding areas from adjacent paraffin sections were isolated and their DNA extracted and processed by polymerase chain reaction for the amplification of selected genes. Analysis with a panel of gene probes has been initiated. The selected probes include the p53 gene and the *ras* genes. The tumor suppressor gene p53 is currently under analysis for the presence of mutations, using a pair of p53 rat primers specific for exons 4-8. This gene has been implicated in the control of the cell cycle and is known to act as a tumor suppressor in the pathogenesis of adenocarcinomas from other tissues (i.e., colon). It has also been shown to cooperate with the *ras* genes in cellular transformation. Although the role of *ras* oncogenes has been described in lung carcinomas, their interaction with the p53 protooncogene expression needs to be investigated. The experimental production of high incidences of lung carcinomas in the rat by silica represents a unique model for human lung cancer arising from a fibrotic lesion (scar cancer) and provides an appropriate source of normal,

hyperplastic, preneoplastic and frankly neoplastic epithelium for the study of mutations in the p53 gene and their possible interaction with *ras* and other oncogenes. Similar molecular investigations in the silica-transformed cells in culture, described above, will determine whether common specific pathways of gene activation exist in different target cells affected by silica.

In conclusion, the current studies on silica-induced carcinogenesis have shown that methods of physical chemistry, molecular biology, cell biology, experimental pathology and human pathology can be effectively combined in order to interpret the mechanisms involved in the neoplastic transformation induced by crystalline particulate materials that give rise to fibrogenesis and carcinogenesis in the lungs of susceptible hosts. It is hoped that these studies will lead to the detection of susceptibility mechanisms, applicable to the identification of human subjects at high risk for lung cancer induced by silica and/or other fibrogenic factors.

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

PROJECT NUMBER
 Z01CP04491-15 LEP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Quantitative Studies on Concurrent Factors in Neoplastic Transformation

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

PI:	U. Saffiotti	Chief	LEP	NCI
Others:	L.N. Daniel	Senior Staff Fellow	LEP	NCI
	Y. Mao	Visiting Fellow	LEP	NCI
	N. Ahmed	Microbiologist	LEP	NCI
	A. Knapton	Biologist	LEP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.9

PROFESSIONAL:

1.1

OTHER:

1.8

CHECK APPROPRIATE BOX(ES)

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

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

Crystalline silica samples in the respirable size (1-5 microns in diameter) were studied for their effects in the BALB/3T3/A31-1-1 mouse embryo cell line. The following samples were used: (A) quartz samples: Min-U-Sil 5 (MQZ, a standard U.S. preparation), hydrofluoric-acid-etched MQZ (HFMQZ, prepared for this laboratory in order to remove impurities from the silica surface by HF-etching), DQ12 and F600 (two standard preparations from Germany) and CSQZ (a standard preparation from China); (B) other crystalline silica forms: cristobalite and tridymite (prepared synthetically for this laboratory); and (C) non-fibrogenic dusts for comparison, usually ferric oxide. Neoplastic transformation was induced by three samples of quartz in the following order of activity: MQZ, HFMQZ and F600. Cells from the transformed foci, all found to be positive for tumorigenicity in nude mice, were cryopreserved and used for karyotyping and for molecular analysis of activated oncogene expression. Cytotoxicity, induced by initial exposure to silica and expressed by decreased colony forming efficiency (CFE), was followed by storage of phagocytosed particles with no further signs of toxicity in the surviving cells. Current results suggest that the mechanisms responsible for cytotoxicity, and possibly for transformation, take place in the time interval between the encounter of the particles with the cell membrane and the completion of phagocytosis. The hypothesis that toxic effects occur only during this initial phase is being tested by treating the cells for selected periods of time with various inhibitors of the mechanisms of toxicity. Decreasing the serum concentrations in the medium progressively decreased the CFE of control cells and of quartz-treated cells, the latter always showing a proportionally lower CFE than control cells. Exposure of cells to media conditioned by incubation with large doses of silica showed no toxicity, suggesting that the effect is not due to soluble factors.

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

U. Saffiotti	Chief	LEP	NCI
L.N. Daniel	Senior Staff Fellow	LEP	NCI
Y. Mao	Visiting Fellow	LEP	NCI
N. Ahmed	Microbiologist	LEP	NCI
A. Knapton	Biologist	LEP	NCI

Objectives:

The general objective of this project is to study mammalian cell culture systems quantitatively for induction kinetics and mechanisms of neoplastic transformation, related cytotoxicity, mutagenicity and DNA damage. Current studies are designed to investigate the mechanisms of cellular toxicity, genetic damage and neoplastic transformation induced by inorganic particulates.

Methods Employed:

The BALB/3T3/A31-1-1 mouse embryo cell line was used under test conditions that were further optimized for use in the assays for colony forming efficiency (CFE), toxicity and transformation, induced by particulate materials. Computer programs for storage, processing and analysis of cell culture data and results were further extended. The following particulate materials of respirable size (mostly 1-5 μm in diameter) were used: (A) quartz samples: Min-U-Sil 5 (MQZ, a standard U.S. preparation), hydrofluoric-acid-etched MQZ (HFMQZ, prepared for this laboratory in order to remove impurities from the silica surface by HF-etching), DQ12 and F600 (two standard preparations from Germany) and CSQZ (a standard preparation from China); (B) other crystalline silica forms: cristobalite and tridymite (prepared synthetically for this laboratory); and (C) non-fibrogenic dusts for comparison, usually ferric oxide.

Major Findings:

Optimal culture conditions for the BALB/3T3/A31-1-1 cell line were re-established after screening several cell sublines and new batches of fetal bovine serum (FBS). Formation of smooth monolayers of cells was obtained with inoculum sizes ranging from 200 to 10,000 cells/50 mm dish, with no significant spontaneous transformation during the assay (5 weeks). The choice method of exposure to particulates consisted of plating the cells and allowing them to attach, and then changing the medium to one containing the dust that settles on the cells and dish surface. Silica-treated cells, after the initial toxic effect, went on to form good continuous monolayers of cells, without further expression of toxicity. The silica-treated cells showed persistent phagocytosis of particles, which remained present in nearly all monolayer cells (usually many particles per cell in a perinuclear location). Such intracellular storage of particles appeared analogous for dusts of markedly different initial toxicity, suggesting that after completion of the phagocytic process the dust particles are no longer toxic, possibly as a consequence of being segregated in membrane-bound phagosomes.

Only at relatively high doses of silica exposure ($100 \mu\text{g}/\text{cm}^2$ or higher) were many dust-laden dead cells observed above the monolayer, which still retained its continuity. Silica-laden cells appeared sensitive to variable culture conditions, such as temperature, CO_2 level and serum batch; considerable "fine tuning" was required in order to obtain the current optimal results for CFE and transformation assays. Current results suggest that the mechanisms responsible for cytotoxicity, and possibly transformation, take place in the time interval between the encounter of the particles with the cell membrane and the completion of phagocytosis. The hypothesis that toxic effects occur only during this initial phase is being tested by treating the cells for selected periods of time with various inhibitors of the mechanisms of toxicity.

Neoplastic transformation was induced by 3 samples of quartz (MQZ, HFMQZ and F600). MQZ-induced transformation was obtained either by (i) exposing the culture to dust and then washing out the excess dust after 2-3 days, or by (ii) changing only the supernatant medium and allowing all the dust to remain in the dish. The latter method is more accurate and was used in subsequent studies. Dose-response studies showed a marked increase of induced neoplastic transformation frequencies in cells treated with MQZ at concentrations from 6.25 to $25 \mu\text{g}/\text{cm}^2$, reaching a plateau of about 300 transformants per 10^5 surviving cells (vs. none in controls) for doses between 25 and $100 \mu\text{g}/\text{cm}^2$. The transforming activity of HFMQZ and of F600 was lower, reaching a level of 30-40 transformants per 10^5 surviving cells at $50 \mu\text{g}/\text{cm}^2$. F600 had lower activity than the other samples at the lower doses.

Tumorigenicity assays by inoculation of quartz-transformed cells in nude mice were all positive. The transformed cells were subcultured and cryopreserved for karyotypic analysis and molecular biology studies. The silica-transformed cells are currently being investigated for the identification of activated oncogenes.

The inhibition of CFE of BALB/3T3/A31-1-1 cells by silica was used as a measure of toxicity. Dose-dependent toxicity was found with all types of crystalline silica. The quartz sample F600, with significant transforming activity, was found to have very low cytotoxicity, an interesting finding in view of its high level of activity in tests for DNA damage in vitro in the presence of H_2O_2 , and for mutagenesis in the plasmid pS189 shuttle vector (see Project Z01CP05674-01). Minimal toxicity was found for amorphous silica and ferric oxide. Decreasing the serum concentrations in the medium progressively decreased the CFE of control cells and of quartz-treated cells, the latter always showing a proportionally lower CFE than control cells, at all serum concentrations. Exposure of cells to media conditioned by incubation with silica (up to $3200 \mu\text{g}/\text{cm}^2$ MQZ) showed no toxicity, suggesting that the effect is not due to soluble factors.

Because crystalline silica was found to be dependent on iron-mediated reactive oxygen mechanisms for its production of DNA double strand breaks in vitro (see Project Z01CP05674-01), studies were undertaken to evaluate the role of oxygen radicals and of divalent or trivalent iron cations on the cell response to silica, through the use of selective inhibitors such as iron chelators and oxygen radical scavengers.

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

PROJECT NUMBER
 Z01CP05274-10 LEP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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:	A.O. Williams	Visiting Scientist	LEP	NCI
	L.N. Daniel	Senior Staff Fellow	LEP	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.4

PROFESSIONAL:

1.0

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.)

A) Histopathology. Further studies were made of the cellular reactions induced by a single intratracheal instillation of Min-U-Sil 5 quartz (MQZ) or of hydrofluoric-acid-etched MQZ in F344 rats, using tissues from previously conducted lifetime and serial sacrifice experiments. These studies resulted in the induction of high incidences of peripheral lung carcinomas, derived from hyperplastic areas of alveolar type II epithelium, adjacent to silicotic granulomas. The cellular reaction to silica had previously been found to be markedly different in three tested species. Rats develop typical silicotic granulomas and adjacent epithelial alveolar hyperplasia and, eventually, high incidences of lung carcinomas (mostly adenocarcinomas); mice develop silicosis but no persistent epithelial hyperplasia and no tumors; hamsters develop macrophagic storage lesions but no progressive fibrosis and no epithelial proliferation. Further histological observations of human lungs with silicosis and lung cancer showed similarities with two of the lesions observed in the experimental silica-treated rats, namely, (a) adenocarcinoma growing among fibrotic tissue and around it ("scar cancer"); and (b) epithelial hyperplasia and proliferative lesions adjacent to silicotic granulomas, in areas distant from the carcinoma. (B) Molecular biology. Formalin-fixed, paraffin-embedded rat lungs containing silica-induced carcinomas were sectioned and the areas with carcinoma were carefully excised, subjected to DNA extraction, and processed by the polymerase chain reaction method for amplification of selected genes. The p53 tumor suppressor gene is currently being investigated, using a pair of p53 rat primers specific for exons 4-8. The amplified products are subjected to electrophoresis in 0.8% agarose gel and stained with ethidium bromide; the p53 gene expressions are sequenced and their mutation frequencies calculated.

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

U. Saffiotti	Chief	LEP	NCI
A. O. Williams	Visiting Scientist	LEP	NCI
L. N. Daniel	Senior Staff Fellow	LEP	NCI

Objectives:

The general objective of this project is the study of models and pathogenetic mechanisms of respiratory tract cancers induced by chemical and physical factors, alone or in combination. Animal models for respiratory carcinogenesis are developed, characterized and compared to their human counterpart. The induced tumors are analyzed by molecular biology methods to identify the critical molecular lesions.

The objectives of current studies are: (A) the pathogenesis of lung cancers induced by crystalline silica particles and their relationship with the pathology of lung cancer in human silicotic subjects, including the relationship between chronic pulmonary granulomatosis/fibrosis and the development of adjacent epithelial proliferation and lung cancer; and (B) the molecular characterization of gene alterations in silica-induced lung cancers.

Methods Employed:

(A) Histopathology.

(1) Experimental animal studies. Tissues were obtained from recently completed lifetime and serial sacrifice studies of rats, hamsters, and mice of the following inbred strains: Fischer 344 rats; 15.16/EHS:CR hamsters; and A/JCr, BALB/cAnNcr and Ncr:NU (athymic nude) mice. All test animals received a single intratracheal instillation of particulate suspensions of one of the following materials: crystalline silica as Min-U-Sil 5 quartz (MQZ), hydrofluoric acid-etched MQZ (HFMQZ), cristobalite and tridymite and the non-toxic ferric oxide. The cellular reactions in the respiratory tissues were studied by histologic, histochemical, and immunochemical methods.

(2) Human pulmonary pathology material was examined histologically from cases of silicosis with lung cancer, made available through the courtesy of several university departments of pathology.

(B) Molecular biology. Formalin-fixed, paraffin-embedded rat lungs containing silica-induced carcinomas were sectioned and the areas with carcinoma were carefully excised, subjected to DNA extraction, and processed by the polymerase chain reaction method for amplification of selected gene expressions. The p53 gene is currently being investigated, using a pair of p53 rat primers specific for exons 4-8. The amplified products are subjected to electrophoresis in 0.8% agarose gel and stained with ethidium bromide; the p53 gene expressions are sequenced and their mutation frequencies calculated.

Major Findings:

(A) Histopathology.

(1) In the current year, further histopatologic studies were conducted on previous animal experiments to investigate the cellular reactions to crystalline silica particles in the lung, the induction of epithelial hyperplasia and epithelial cancers adjacent to granulomatous/fibrogenic reaction areas, and their pathogenetic correlations. The marked differences in the reaction to silica, that were previously reported in the three tested species, were further studied: rats developed typical silicosis and high incidences of adjacent carcinomas of the lung (arising from hyperplastic areas of alveolar type II cells); mice developed discrete silicotic nodules, but no persistent epithelial reactions or tumors; and hamsters developed only phagocytic storage reactions with no progressive fibrosis or epithelial proliferation. These three distinct types of host responses are being investigated for their pathogenetic significance and are correlated with the cellular and molecular reactions observed in the studies reported in the other LEP projects.

(2) Further histopathological examination of human cases of lung cancer in silicotic lungs confirmed the presence of areas of epithelial hyperplasia adjacent to silicotic granulomas, analogous to some of the proliferative epithelial lesions observed experimentally. These observations, however, remain sporadic, because the limited extent of lung tissue samples present in the available slides is inadequate to estimate the extent and frequency of proliferative epithelial lesions in whole silicotic human lungs.

(B) Molecular biology.

A new investigation has been undertaken to identify molecular lesions underlying the induction of lung cancer by silica, applicable to both human and animal tumors. The first phase of this study, currently under way, investigates molecular lesions in silica-induced lung tumors in rats. These studies could then be extended to detect corresponding molecular lesions in human lung cancers associated with silicosis and other types of pulmonary fibrosis. The slides from rat lungs treated with a single instillation of silica were screened. Areas with the following lesions were selected: carcinomas arising near fibrotic silicotic nodules, areas of preneoplastic reaction, and areas of non-neoplastic lung from the same animals. The corresponding areas from adjacent paraffin sections were cut and excised and their DNA was extracted and processed by polymerase chain reaction for the amplification of selected gene expressions. Analysis with a panel of gene probes has been initiated. The selected probes include the p53 gene and the *ras* genes. The tumor suppressor gene p53 is currently under analysis for the presence of mutations. This gene has been implicated in the control of the cell cycle and is known to act as a tumor suppressor in the pathogenesis of adenocarcinomas from other tissues (i.e., colon). It has also been shown to cooperate with the *ras* genes in cellular transformation. Although the role of *ras* oncogenes has been described in lung carcinomas, their interaction with the p53 protooncogene expression needs to be investigated. The experimental production of high incidences of lung carcinomas in the rat by silica represents a unique model for human lung cancer arising from fibrotic lesions (scar cancer) and provides an appropriate source of normal, hyperplastic, preneoplastic and frankly neoplastic epithelium for the study of

mutations in the p53 gene and their possible interaction with ras and other oncogenes. Similar molecular investigations in the silica-transformed cells in culture, described above, will determine whether common specific pathways of gene activation exist in different target cells affected by silica.

Publications:

Saffiotti U. Lung cancer induction by crystalline silica. In: D'Amato R, Slaga TJ, Farland W, Henry C, eds. Relevance of animal studies to evaluate human cancer risk. New York: John Wiley & Sons (In Press).

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

PROJECT NUMBER
 Z01CP05276-10 LEP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Growth Control in Epithelial Cells and its Alteration in Carcinogenesis

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

PI:	U. Saffiotti	Chief	LEP	NCI
Others:	A. Knapton	Biologist	LEP	NCI

COOPERATING UNITS (if any)

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.1

OTHER:

0.4

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 mouse epidermal keratinocyte cell line MKDC4, previously established in this project, was further studied for growth in two specially formulated media: (a) LEP/MK2, containing 0.1 mg/ml bovine serum albumin (BSA) and 0.5% bovine pituitary extract (BPE, equivalent to 0.09 mg protein/ml), and (b) LEP/MK4, containing no BPE and 1.5 mg/ml BSA. Growth in the LEP/MK4 medium was found to support colony formation but failed to support extended growth or subculture. The MKDC4 cell line in LEP/MK2 medium was further studied in the presence of inorganic particulates. Ferric oxide not only did not decrease colony forming efficiency, but at certain doses induced a stimulation of cell growth and colony formation. Crystalline silica particles of various types, in contrast, induced a marked dose-dependent inhibition of these parameters. The MKDC4-derived cell line, 2057C, selected for its ability to grow continuously in the presence of high levels of transforming growth factor beta (1 ng/ml), was reestablished for further mechanism studies. Methods were explored for the establishment of cultures of pulmonary alveolar type II epithelial cells from F344 rats, the target cell for silica-induced carcinogenesis in vivo.

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

U. Saffiotti	Chief	LEP	NCI
A. Knapton	Biologist	LEP	NCI

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) and rat respiratory epithelia have been used so far in this project. Specific current objectives include: (a) establishment and characterization, in serum-free media, of cell lines with selected states of differentiation; (b) study of the penetration of particulate materials (including crystalline silica) in these cells and analysis of their toxic and/or transforming effects; (c) analysis of mechanisms and development of selective conditions for the transformation of these cells in combination with oncogenes and/or growth factors and inhibitors.

Methods Employed:

Previously established cell lines of BALB/c newborn mouse epidermal cells were cultured in serum-free media developed in this laboratory. The growth medium LEP/MK2, previously reported, contains bovine pituitary extract (0.5%) and bovine serum albumin (0.1 mg/ml). Tests of a new medium, LEP/MK4, were continued: in this medium, 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 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:

The new chemically defined, serum-free growth medium, LEP/MK4, initially reported last year, was derived from the previously established 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). Both media were found to support colony growth of MKDC4 cells (a mouse keratinocyte cell line previously isolated in this laboratory), but LEP/MK4 was found to be unable to sustain continuous cell growth and subculture. Addition of selected, chemically defined growth factors is planned in order to develop a chemically defined medium with improved nutrient properties.

Further tests were conducted in the cell line MKDC4 in LEP/MK2 medium for growth and colony formation in the presence of inorganic particulates (silica and ferric oxide). Colony forming efficiency and growth rate (population doublings/day) were not inhibited by the non-toxic ferric oxide particles, which appeared to

stimulate excess growth at certain dose levels. In contrast, crystalline silica particles induced a marked dose-dependent inhibition of colony formation.

The cell line 2057C, derived from the MKDC4 line of mouse keratinocytes, was previously established by selective growth in the presence of high levels of transforming growth factor beta (TGF- β , 1 ng/ml) that were highly toxic to the parent cell line. Cell line 2057C was reestablished in culture for further mechanism studies.

Pilot studies were undertaken to establish cultures of pulmonary alveolar type II epithelial cells from F344 rats, representing the selective susceptible target cell type for silica lung carcinogenesis in vivo.

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

PROJECT NUMBER
 Z01CP05674-01 LEP

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Gene Damage Induced by Crystalline Silica

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

PI:	L. N. Daniel	Senior Staff Fellow	LEP	NCI
Others:	U. Saffiotti	Chief	LEP	NCI
	Y. Mao	Visiting Fellow	LEP	NCI

COOPERATING UNITS (if any)

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.8

PROFESSIONAL:

1.4

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

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

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

Double stranded DNA (phage lambda Hind III digest) was exposed in vitro to test particulate materials (see Project Z01CP04491-15 LEP), either directly or through a dialysis membrane. No DNA damage was induced by dusts alone, or by hydrogen peroxide alone. In the presence of hydrogen peroxide, however, exposure to several dusts induced marked DNA damage. The effect was highest for ferric oxide, followed by cristobalite and various samples of quartz, low for tridymite and nearly absent for Chinese standard quartz. The mechanism of DNA damage was mediated by reactive oxygen species catalyzed by iron cations, as shown by its inhibition by the iron chelator deferoxamine and by the hydroxyl radical scavenger dimethylsulfoxide. Other modifying agents were studied. Spectrophotometric analysis of the adsorbance of the cationic dye Janus Green B on silica particles was found to be appropriate for the measurement of free negative charges (or iron coordination sites) on silica surfaces. The role of divalent and trivalent iron cations was studied to characterize the reactivity of silica surfaces. In the tested samples, Janus Green B adsorption was found to be roughly inversely proportional to the induction of DNA double strand breaks. Plasmid DNA from the shuttle vector pS189 (which contains the supF reporter gene) was transfected into the human epithelial cell line 293. The cells were later exposed to test silica samples and pS189 DNA reextracted and digested with DpnI (to destroy all bacterially derived plasmid DNA). E. coli MBM 7070 strain, transformed with the mammalian-cell replicated plasmid DNA, was tested for mutant colony frequency in selective medium. The altered colonies were restreaked and their DNA sequenced to identify changes in the supF gene. Preliminary results showed a marked increase over background mutation levels following exposure to F600 quartz. Two other quartz samples, MQZ and CSQZ, showed a much lower effect. Characterization of the induced mutations by sequence analysis is currently under way.

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

L. N. Daniel	Senior Staff Fellow	LEP	NCI
U. Saffiotti	Chief	LEP	NCI
Y. Mao	Visiting Fellow	LEP	NCI

Objectives:

The main objectives of this project are: (A) to establish methods for analysis of silica-induced DNA damage and mutations, (B) to correlate physico-chemical properties among different silica preparations with their biological effects, and (C) to assess the contribution of free radical mechanisms to the biological effects of silica.

Methods Employed:

(A) Analysis of in vitro DNA double strand breakage. Double stranded DNA (phage λ *Hind* III digest) was exposed in vitro to crystalline silica, either directly or through a dialysis membrane, in an aqueous medium in the presence or absence of various free radical generating and/or modifying agents. These agents included: hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), catalase, dimethylsulphoxide (DMSO), and the iron chelators deferoxamine and diethyl-triamino-pentaacetic acid (DETAPAC). DNA double strand breakage was assessed after agarose gel electrophoresis and ethidium bromide staining by evidence of degradation of the discretely sized bands.

(B) Surface adsorption of the cationic dye Janus Green B on different silica samples was measured by spectrophotometric analysis of the absorbance after exposure of silica suspensions to known concentrations of the dye. Total dye adsorption was calculated from standard curves of absorbance vs. concentration.

(C) Plasmid DNA from the shuttle vector pS189 (which contains the *supF* reporter gene) was transfected into cells of the 293 line (adenovirus 5 transformed human embryonic kidney cells). The transfected cells were later exposed to silica for 72 hrs and pS189 DNA reextracted by an alkaline lysis method. Following digestion with *DpnI* (to destroy all of the original unreplicated bacterially derived plasmid DNA) the mammalian-cell replicated plasmid DNA was used for transformation of an appropriate bacterial indicator strain (*E. coli* MBM 7070). The mutation frequency induced in the bacteria was calculated by counting the colonies formed in X-gal containing agar plates: on a lawn of blue colonies, the mutant colonies appear white (colorless). The white colonies were restreaked on plates and their DNA was sequenced by the dideoxy chain termination method of Sanger to identify changes in the *supF* gene.

The test dusts, all in the respirable size, included samples of quartz (MQZ, HFMQZ, DQ12, F600 and CSQZ), cristobalite, tridymite and ferric oxide (See Project Z01CP05274-10).

Major Findings:

(A) DNA double strand breaks in vitro were not induced by crystalline silica alone, or by H_2O_2 alone, but only when dusts acted in the presence of H_2O_2 . Studies with Min-U-Sil 5 quartz (MQZ) showed that this DNA damage was easily detectable and its severity increased with time of exposure. Since the effect is likely to be due to reactive oxygen radicals generated by an iron-dependent reaction, selective inhibitors were tested. In the presence of H_2O_2 , the iron chelator deferoxamine significantly inhibited the DNA damage induced by MQZ, but another iron chelator, diethyl-triamino-pentaacetic acid (DETAPAC) accelerated DNA damage by MQZ. This apparent discrepancy may be explained by the different mechanisms of the two chelating agents. Deferoxamine blocks the availability of chelated iron for oxidation/reduction reactions, whereas DETAPAC does not. Thus, iron chelation per se is insufficient to reverse this mechanism of induction of DNA damage, which occurs only when the chelated iron remains chemically reactive. DNA damage in this system was also partially inhibited by the hydroxyl radical scavenger dimethylsulfoxide (DMSO). Superoxide dismutase (SOD, copper-zinc type), which can generate hydrogen peroxide by dismutation if superoxide is present, allowed DNA damage to take place, without added hydrogen peroxide, in the presence of MQZ quartz, although requiring a long incubation time. This effect was not induced when ferric oxide was similarly tested. Possible molecular mechanisms that would explain these findings are currently under study.

The dust samples tested in this system showed significant differences in their ability to induce DNA damage in the presence of H_2O_2 . Ferric oxide had the highest activity, but it was not activated by SOD alone. Among the crystalline silica samples tested, the highest DNA damage was induced by cristobalite, followed by F600, MQZ, DQ12, HFMQZ, tridymite and CSQZ.

(B) The tested dust samples were found to differ significantly in their ability to adsorb the cationic dye Janus Green B. Higher dye adsorption was found generally to correlate with a lower activity in the H_2O_2 -dependent DNA damage assay. A partial exception to this inverse correlation was observed for silica sample DQ12. These findings generally support the hypothesis that the reactivity of silica in vitro is mediated by an iron-dependent reactive oxygen pathway, but also suggest that other parameters should be taken into account, such as surface properties and the ratio of divalent to trivalent iron. The advice and collaboration of specialized experts is being sought in order to investigate critical physico-chemical properties of crystalline silica surfaces, relevant to their biological activities.

(C) Preliminary results obtained with the shuttle vector pS189 plasmid mutation assay showed a marked increase over background mutation levels following exposure of the plasmid-transfected human cell line to F600 quartz. Two other quartz samples, MQZ and CSQZ, showed a much lower effect. Characterization of the induced mutations by sequence analysis is currently under way.

ANNUAL REPORT OF

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

October 1, 1990 through September 30, 1991

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: the In Vitro Carcinogenesis Section (IVCS), the Molecular Genetics and Carcinogenesis Section (MGCS), and the 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; and 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, as well as 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 and Biostatistics 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 exchange 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.

SUMMARIES OF RESEARCH PROJECTS

A. Neoplastic Transformation of Human Epithelial Cells by Transfected Activated Protooncogenes In Vitro

Five families of activated protooncogenes, ras, raf, jun, erbB-2 (neu) and myc have so far been associated with human bronchogenic carcinoma. 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. Overexpression of erbB-2 leads to neoplastic transformation of human bronchial epithelial cells. The molecular mechanisms of ras and raf/myc induction of neoplastic transformation is the major focus of current and future studies. We investigated the role of ras and raf in the transduction of signals from ligand activated cellular membrane receptors to the nucleus and subsequent altered expression of growth and terminal differentiation genes. We hypothesize that receptor-mediated phosphorylation of the raf kinase causes both an increase in its activity and a translocation to the nucleus. Possible substrates of the activated raf kinase include PI kinase and nuclear regulatory proteins, myc and p53. The effects of the mutant p53 gene on cell growth and terminal squamous differentiation is being assessed. The cooperativity of mutant p53 and ras genes in the "immortalization" and neoplastic transformation of normal human bronchial epithelial cells will also be determined. Since the Ha-ras or myc/raf transformed bronchial epithelial cells are highly invasive and metastatic in athymic nude mice, we plan to investigate the regulation and expression of genes considered to be involved in tumor metastasis including Nm23, collagenase IV and TIMP-2.

B. Tumor Suppressor Genes in Human Carcinogenesis

Mutations in the evolutionarily conserved codons of the p53 tumor suppressor gene are common in diverse types of human cancer. The p53 mutational spectrum differs among cancers of the colon, lung, esophagus, breast, liver, brain, lymphomas and leukemias. Transitions predominate in cancers of the colon, brain and lymphoid malignancies, whereas G:C to T:A transversions are the most frequent substitutions observed in cancers of the lung and liver. Mutations at

A:T base pairs are seen more frequently in esophageal carcinomas than in other solid tumors. The distributions of mutations in the p53 sequence are also distinct. Most transitions in colorectal carcinomas, brain tumors, leukemias and lymphomas are at CpG dinucleotide mutational hotspots (codons 175, 248, 273 and 282). G to T transversions in lung, breast and esophageal carcinomas are dispersed among numerous codons, whereas in liver tumors from geographic areas in which both aflatoxin B₁ and hepatitis B virus are cancer risk factors, most mutations are at one nucleotide pair of codon 249. These differences may reflect the etiological contributions of both exogenous and endogenous factors in human carcinogenesis.

C. Development of specific and sensitive methods for molecular dosimetry of carcinogen exposure in humans

Studies in laboratory animals and tissues and cells *in vitro* have shown that the formation of carcinogen-DNA adducts is necessary but not sufficient for chemical carcinogenesis. Following environmental exposure, it seems reasonable that these types of adducts are formed in humans. Since the biologically effective dose of a chemical carcinogen is governed by the amount of carcinogen that becomes internalized and activated to a chemical species capable of damaging DNA, it seems reasonable to develop methods to detect these adducts. Human carcinogen dosimetry at the molecular level requires sensitive and specific methods for carcinogen-macromolecular adduct quantitation. A number of different types of methods have been developed for carcinogen-DNA dosimetry in humans. These include the 32P-nucleotide postlabeling assay, immunoassays, fluorescence spectroscopy, electrochemical conductance and gas chromatography/mass spectroscopy (GC/MS). Each technique has advantages and limitations, and within the framework of epidemiological surveys multiple corroborative end-point analyses often provide the most useful information. Our studies are focused on the use of separatory techniques to achieve chemical specificity prior to sensitive detection by different corroborative methods.

D. Molecular Epidemiology of Human Lung Cancer

Generation of hypotheses that can be tested in experimental systems is a goal of molecular epidemiology. Identification of specific genetic lesions that are important in carcinogenesis is a major effort. In order to adequately address these projects it is logical to seek consistent genetic mutations in primary tumors and examine their effects in normal or malignant cells in tissue culture. A primary tumor panel that can be used to determine which gene mutations are worthy of further study or to determine particular spectra of mutations that may relate to specific environmental exposures (e.g., tobacco, industrial effluent, coal smoke or cooking vapors) is a valuable resource. Expression of specific oncogenes and tumor suppressor genes (erbB-2, p53, p21, transforming growth factor-alpha and epidermal growth factor-receptor) in lung cancer are also being studied as potential markers of either early detection or prognosis (survival). Genetic polymorphisms that have been suggested to have an association with human cancer risk are the DNA-restriction fragment length polymorphisms (DNA-RFLPs) at the human HRAS1, L-*myc*, p53, CYP1A1, CYP2E1 and CYP2D6 gene loci. In the case of HRAS, L-*myc*, CYP1A1, CYP2E1 and p53, rare or minor restriction fragments (alleles) may predispose to certain cancers or may be associated with poor prognosis. There are no previously existing reports that describe examination of these markers within an epidemiological study.

E. In Vitro Studies of Human Mesothelial Cell Carcinogenesis

Recent studies indicate that loss or mutation of tumor suppressor genes is frequently associated with many types of human cancer. The etiology of these cancers is not completely clear. In the case of mesothelioma, the carcinogenic agent(s) are generally considered to be asbestos fibers. It was, therefore, of interest to examine a relatively large (19) collection of cell lines for loss of expression of the retinoblastoma (Rb) gene or mutation in the p53 gene. Our studies to date suggest that loss of Rb expression is not common in mesothelioma. Protein studies will be performed to confirm this conclusion. The status of the p53 gene in mesothelioma is being studied by immunocytochemistry and direct sequencing. To date only 10% (2/20) of tumors show mutations, while 3/20 show protein staining higher than normal mesothelial cells. None of the tumors positive for p53 mutations have mutations in Ki-ras codon 61. Examination of expression of platelet-derived growth factor (PDGF)-A chain in tumors retrieved after inoculation of athymic nude mice with derivatives of the immortalized human mesothelial cell MET-5A showed that tumors from PDGF-A chain transfectants but not EJ-ras transfectants overexpressed PDGF-A chain from the endogenous gene. Interestingly, rare tumors from control plasmid transfected cells also overexpressed the endogenous PDGF-A chain gene, suggesting that this may be a common alteration correlating with tumorigenic progression.

F. In Vitro Model for Human Liver Carcinogenesis Studies

Techniques have been developed and media have been formulated that will support replicative cultures of monkey and human liver epithelial cells that exhibit some hepatocellular characteristics for 20 and 12 culture population doublings, respectively. However, monkey and transformed human liver epithelial cells established in these media will cease replicating after a few passages unless the media are supplemented with arginine. A similar observation was made using the human hepatoblastoma cell line Hep-G2. Thus, we speculate that hepatocytes may commonly lose portions of the urea cycle metabolic pathway as they undergo "retrograde differentiation" and/or transformation. In addition, we have found by measuring albumin and keratin species 18 and 19 that, initially, the transformed human liver epithelial (THLE) cells probably originate from hepatocytes or from cells that are of the hepatocyte lineage. However, with continued growth the THLE cells also co-exhibited liver ductal epithelial cell characteristics. Preliminary investigations have demonstrated that intersplenic injection protocols and "tissue equivalent" matrices may be efficacious for inducing replicative cultures of normal and transformed liver epithelial cells to re-express hepatocellular phenotypic expressions. THLE cells should be a useful model for investigating the interactive effects of chemical carcinogens, e.g., aflatoxin B₁, and hepatitis viruses, e.g., HBV and HCV, in liver carcinogenesis. In addition, the molecular mechanisms of p53 mutations in liver carcinogenesis can be studied.

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

PROJECT NUMBER

Z01CP05435-07 LHC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Development of Methods for Human Molecular Dosimetry

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

P. I.:	Ainsley Weston	Visiting Scientist	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Shunji Kato	Visiting Fellow	LHC	NCI
	Stefano Petruzelli	EORTC Fellow	LHC	NCI
	Miriam C. Poirier	Research Chemist	CCTP	NCI
	Peter G. Shields	Sr Clin Investigator	LHC	NCI
	Bonita G. Taffe	IRTA Fellow	LHC	NCI
	Glennwood E. Trivers	Research Biologist	LHC	NCI

COOPERATING UNITS (if any)

Cambridge Bioscience, Worcester, MA (M. Newman); M.R.C., Carshalton, England (P. Farmer); MIT, Boston, MA (S. Tannenbaum, J. Essigmann); Am. Health Foundation, Valhalla, NY (S. Hecht, P. Foiles); IARC, Lyon, France (C. Wild, R. Montesano).

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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.)

Studies in laboratory animals and tissues and cells *in vitro* have shown that the formation of carcinogen-DNA adducts is necessary but not sufficient for chemical carcinogenesis. Following environmental exposure, it seems reasonable that these types of adducts are formed in humans. Since the biologically effective dose of a chemical carcinogen is governed by the amount of carcinogen that becomes internalized and activated to a chemical species capable of damaging DNA, it seems reasonable to develop methods to detect these adducts. Human carcinogen dosimetry at the molecular level requires sensitive and specific methods for carcinogen-macromolecular adduct quantitation. A number of different types of methods have been developed for carcinogen-DNA dosimetry in humans. These include the ³²P-nucleotide postlabeling assay, immunoassays, fluorescence spectroscopy, electrochemical conductance and gas chromatography/mass spectroscopy (GC/MS). Each technique has advantages and limitations, and within the framework of epidemiological surveys multiple corroborative end-point analyses often provide the most useful information. Our studies are focused on the use of separatory techniques to achieve chemical specificity prior to sensitive detection by different corroborative methods.

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

Ainsley Weston	Visiting Scientist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Shunji Kato	Visiting Fellow	LHC	NCI
Stefano Petruzelli	EORTC Fellow	LHC	NCI
Miriam C. Poirier	Research Chemist	CCTP	NCI
Peter G. Shields	Sr Clin Investigator	LHC	NCI
Bonita G. Taffe	IRTA Fellow	LHC	NCI
Glennwood E. Trivers	Research Biologist	LHC	NCI

Objectives:

Development and validation of methods for the detection and quantitation of "bulky" carcinogen-macromolecular adducts in humans, for example, polycyclic aromatic hydrocarbon (PAH) or aromatic amine adducts, formed with either DNA or protein.

Development and validation of methods for the detection and quantitation of "small" carcinogen macromolecular adducts in humans, for example, alkylation and oxidation products.

Methods Employed:

Immunoaffinity Chromatography: Immunoaffinity columns were prepared from anti-benzo[α]pyrene-diol epoxide (BPDE)-DNA monoclonal antibody 8E11. One column was used for calibration with radiolabeled BPDE-DNA and the others were reserved for use with human DNA exclusively to avoid potential contamination with standard materials. Monoclonal antibodies have also been used for O^6 -methyldeoxyguanosine and 8-OH-deoxyguanosine purification.

High Performance Liquid Chromatography (HPLC): Reverse-phase HPLC was conducted at room temperature with Vydac C18 columns (25 cm x 4.6 mm). Samples were eluted with linear methanol/water gradients (30-60% over 10 min, followed by 60-100% over 5 min) at a flow rate of 1 ml/min. Eluates were collected in 0.5 ml fractions. Labeled benzo[α]pyrene-tetrahydrotetrol that was extracted from hydrolyzed tritium labeled BPDE modified DNA eluted in a major peak at 14.5-15.0 min with a final recovery of 60% of the radioactivity.

Second Derivative Synchronous Fluorescence Spectroscopy (SFS): Spectral data acquired by driving the excitation emission monochromators of a fluorescence spectrophotometer (Perkin Elmer Corp., Rockville, MD 20850) simultaneously with a fixed-wavelength difference of 34 nm were converted to second derivatives using the Savitsky-Golay algorithm.

Preparation of N7-Methyldeoxyguanosine and N7-Ethyldeoxyguanosine Monophosphate and Bisphosphate. Synthetic standard was synthesized by treatment of 2'-deoxyguanosine mono- or bisphosphate (17 mg) with methyl iodide or ethyl iodide (20 μ l) in dimethylsulfoxide (4 ml). The reaction mixture was stirred at 20 - 24°C overnight and then purified by anion exchange HPLC. The structure was confirmed by both ultraviolet (UV) spectroscopy, excitation and emission fluorescence and thin layer chromatography analysis following treatment with acid and alkali.

HPLC Separation of Normal and Methylated Nucleotides: HPLC was performed using an ion-pair column (50 μ m 4.6 mm X 25 cm). Triethylamine acetate (0.1 mM; pH 7.0) (Applied Biosystems, Foster City, CA) and 1% acetonitrile were mixed isocratically for 20 min. The acetonitrile was increased to 5% for 10 min, held isocratically for 10 min and then increased to 10% over the next 5 min. Alternatively, weak anion exchange HPLC was used with 0.1-0.5 triethylamine acetate over 35 minutes. Flow rates were 1 ml/min and UV absorbance was monitored at 254 nm. Fractions (1 ml) were collected, pooled and lyophilized for ³²P-postlabeling.

HPLC/³²P-postlabeling of DNA: DNA (10 - 100 μ g) was digested to nucleoside 3'-monophosphates. The entire digestion mixture was fractionated by HPLC, fractions containing normal or adducted nucleotides were pooled and lyophilized. One-thousandth of the fractions containing deoxyguanosine monophosphate (dGp) were added back to the fraction containing the adducts. The mixture was dissolved in H₂O (5 μ l), dithiotretol (1 μ l; 0.1 M) and buffer (bicine-0.1 M, spermidine-1.0 nM, MgCl₂-0.1 M; pH 7.0). ATP (0.8 μ l; 0.5 mM) and [γ -³²P]-ATP (2 μ l) were added in the presence of T4-poly nucleotide kinase (10 units) resulting in a transfer of the gamma-phosphate group from ATP to the 5' position of the nucleotide. The mixture was incubated at 37°C for 1 hr. An additional aliquot of T4 poly nucleotide kinase (2 μ l) was added. After 1 hr, a portion (5-10 μ l) of the mixture was spotted on 20 X 20 cm polyethylenimine cellulose plates (Merck, Germany). The N7-ethyldeoxyguanosine was also treated with nuclease P1 and identified as a 5'-monophosphate. The plates were developed in 2 dimensions and x-ray plates were exposed to them. Adduct containing areas were measured by liquid scintillation counting.

Major Findings:

An improved method for the ³²P-postlabeling assay that permits standardized quantitation of polycyclic aromatic hydrocarbon-DNA adducts has been developed. This method relies upon concomitant labeling of 2'-deoxyguanosine as an internal standard and thin layer chromatography that identifies unmodified nucleotides along with polycyclic aromatic hydrocarbon-DNA adducts on the same thin layer chromatography plate. This approach confirms labeling efficiency, detects the presence of unknown inhibitors, assesses the adequacy of digestion, and allows for the development of calibration curves for directly determined molar ratios (adduct:internal standard). Chemically synthesized adduct standards and quantitative ³²P-postlabeling data have been corroborated by UV spectroscopy, fluorescence spectroscopy and liquid scintillation counting where radiolabeled

materials are available. Labeling efficiencies of the polycyclic aromatic hydrocarbon-DNA adducts were found to be 2- to 100-fold less than expected and depended upon both the adduct and adduct levels (lower levels being less efficiently detected). The presence of unmodified nucleotides resulted in a 2-fold lower labeling efficiency which has implications for interpretation of results in previous reports. Mixtures of polycyclic aromatic hydrocarbon-DNA adducts did not affect the labeling efficiencies of each other. Treatment with nuclease-P1 also did not affect detection. This method can be adopted for use with butanol extraction procedures but not nuclease-P1 enrichments due to insufficient inactivation of the enzyme and loss of the internal standard. The data suggest that previous ³²P-postlabeling assay studies for polycyclic aromatic hydrocarbon-DNA adducts may have underestimated adduct levels. Greater emphasis needs to be placed on chemically identifiable adducts before meaningful molecular dosimetry can be performed.

Improved techniques have been developed for the specific identification of BPDE-DNA adducts in human tissues. Immunoaffinity chromatography, synchronous fluorescence spectroscopy and second derivative synchronous fluorescence spectroscopy have previously been used to detect BPDE-DNA adducts in human placenta. These methods, together with high performance liquid chromatography and the generation of complete fluorescence excitation-emission matrices, have been used to unequivocally identify BPDE-DNA adducts in samples of human lung. Benzo[α]pyrene-diol epoxide nucleotide adducts were isolated with immunoaffinity chromatography columns bearing antibodies raised against the \pm anti-7,8-diol-9,10-epoxide-deoxyguanosine adduct of benzo[α]pyrene. These adducts were hydrolyzed to tetrahydrotetrols and the hydrolysis products subjected to high performance liquid chromatography. The major product isolated by high performance liquid chromatography, benzo[α]pyrene-7,10/8,9-tetrahydrotetrol, was detected by fluorescence spectroscopy. Using this method, levels of BPDE-DNA adducts in the range of 1-40 in 10^8 nucleotides were measured in 6 out of 25 samples, with a lower detection limit of 1 adduct in 10^8 nucleotides. The data may also indicate that adduct levels show regional variation in different parts of the same lung.

Aryl hydrocarbon hydroxylase activity was determined (an indicator of benzo[α]pyrene metabolism) for 16 placenta samples. BPDE-adduct levels were determined by SFS from DNA extracted from syncytiotrophoblasts from the same placentas. For these samples, there was no correlation between smoking history and BPDE-DNA adduct levels as detected by SFS. There was, however, a significant association of aryl hydrocarbon hydroxylase activity with the presence of BPDE-DNA adducts in human placenta.

The use of micropreparative techniques is important for accurate assessment of carcinogen-DNA adduct levels. We have combined ion-pair reverse phase and weak anion exchange HPLC for alkyl deoxyguanosine DNA adducts to improve chemical specificity. Single step HPLC has not been adequate for complete isolation of N7-methyldeoxyguanosine monophosphate as previously reported. Elution profiles with both HPLC methods were determined for synthesized N7-methyldeoxyguanosine monophosphate and N7-ethyldeoxyguanosine monophosphate (and their corresponding

depurinated and ring-opened forms), N7-methyldeoxyguanosine monophosphate and O⁶-methyldeoxyguanosine monophosphate. Authenticity was confirmed by UV, spectrophotofluoroscopy and HPLC elution compared with commercially available standards. These micropreparative techniques were then applied to the ³²P-postlabeling of N7-methyldeoxyguanosine monophosphate and N7-ethyldeoxyguanosine monophosphate in a two-dimensional thin layer chromatography system. The detection limit was 1 adduct in 10⁷ to 10⁸ dGp residues, with a labeling efficiency of 100%. Improvement of labeling efficiency will be investigated.

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CONTRACT IN SUPPORT OF THIS PROJECTUNIVERSITY OF MARYLAND (N01-CP-15623, Replaces N01-CP-71012)Title: Resource for Human Esophageal Tissues and Cells from Donors with Epidemiological ProfilesCurrent Annual Level: \$87,575Person 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 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

In this first year of the new contract, 81 donors were acquired for ninety-three specimen collections and histopathological characterization. Sixty-four intermediate autopsies and 3 immediate autopsies provided single specimen collections of normal esophageal epithelium; 14 surgeries provided matching esophageal carcinoma specimens and organ site normal epithelial tissues. Complete epidemiological profiles were obtained for each surgical donor and from next-of-kin of the deceased whenever allowed by the Medical Examiner.

AIDS patients, known drug overdose victims, intravenous drug users, and HIV-profiled individuals were routinely excluded from the qualified donors.

Also in this period, the contractor has provided an additional 13 Chinese esophageal tissues from the People's Republic of China. The collections are obtained via Dr. Zhu of the Sun Yat Sen Medical School and Dr. He of the Cancer Research Institute of China Medical University of Shenyang. Some specimens have matching serum.

2. Viability and Characterization

Similar to the procedures described for the multi-organ collections, tissue and cellular characterizations for each collection included cytochemical, morphological, and immunocytochemical examinations, and are delivered to the LHC upon request. Each case was sampled for histological evidence of cell injury, viability, and general condition. As above, tests are conducted for specific biochemical markers (HCG_β, AFP, CEA, etc.) occurring in normal human esophageal epithelium. Tissues from each case are preserved in major fixatives (Bouin's, ethanol, and/or aldehydes) for additional characterization when required.

3. Cell Bank

The contractor maintains a cell bank of normal and malignant esophageal cells and tissue explants in a Queue -135° freezer in his facility (centralized tissue culture lab, MSTF 7-60). The inventory of the old cultures from normal frozen stock are essentially unchanged. However, SV40 virus transfection is being employed to produce immortalize cells for carcinogenesis studies.

4. Epidemiological Profile Construction and Storage

Epidemiologic data are important for the study of relationships between esophageal tumors, selected risk factors and exposure to carcinogens. Donor histories, medical records, and computer storage of the compiled histories of environmental exposure for each tissue are vital components of this resource.

In this period, donor profiles were obtained using the OMB-approved, LHC questionnaire, completed in interviews by the contractor's trained epidemiology technician. Profiles were completed for 11 esophagus surgery patients; 41 medical records were abstracted for surgery and autopsy tissue donors. In data processing, a total of 153 records were coded for computer storage and analysis.

The total number of cases with these data, collected from the beginning of the contract (from seven participating hospitals), are listed below:

<u>Period</u>	<u>Un. Hosp.</u>	<u>LRVA</u>	<u>Un. Mem.</u>	<u>MD Gen.</u>	<u>WVAMC</u>	<u>Sinai Center</u>	<u>Med. Exam.</u>	<u>China</u>	<u>Total</u>
Previous	180	18	1	2	8	2	695	33	955
Current	17	6	0	0	2	1	7	13	46
Total	197	24	1	2	10	3	702	46	1001

To date, the categories of information that have been completed for esophagus donors in this period are as follows:

Refused Interview

<u>Period</u>	<u>Med. Rec.</u>	<u>Interviewed</u>	<u>Coded</u>	<u>Patient/Doctor/UMH</u>	<u>Med. Ex.</u> ¹		
Previous	675	76	663	12	1	0	670
Current	41	11	153	0	0	0	46
Total	716	87	816	12	1	0	716

¹ Profiles limited to medical record extracts only--personal contacts routinely prohibited by ME policy.

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

PROJECT NUMBER

201CP05480-06 LHC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Molecular Epidemiology of Human Lung Cancer

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

P.I.: Ainsley Weston Visiting Scientist LHC NCI

Others: Curtis C. Harris Chief LHC NCI
 William P. Bennett Medical Staff Fellow LHC NCI
 Neil Caporaso Medical Staff Fellow EEB NCI
 Robert Hoover Chief EEB NCI
 Greenwood E. Trivers Biologist LHC NCI
 Patricia Steeg Research Biologist LP NCI

COOPERATING UNITS (if any)

New England Medical Center, Boston, MA (T. Krontiris); University of Maryland, Baltimore, MD (B. F. Trump)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

2.0

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

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

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

Generation of hypotheses that can be tested in experimental systems is a goal of molecular epidemiology. Identification of specific genetic lesions that are important in carcinogenesis is a major effort. In order to adequately address these projects it is logical to seek consistent genetic mutations in primary tumors and examine their effects in normal or malignant cells in tissue culture. A primary tumor panel that can be used to determine which gene mutations are worthy of further study or to determine particular spectra of mutations that may relate to specific environmental exposures (e.g., tobacco, industrial effluent, coal smoke or cooking vapors) is a valuable resource. Expression of specific oncogenes and tumor suppressor genes (erb-B2, p53, p21, transforming growth factor-alpha and epidermal growth factor-receptor) in lung cancer are also being studied as potential markers of either early detection or prognosis (survival). Genetic polymorphisms that have been suggested to have an association with human cancer risk are the DNA-restriction fragment length polymorphisms (DNA-RFLPs) at the human HRAS1, L-myc, p53, CYP1A1, CYP2E1 and CYP2D6 gene loci. In the case of HRAS, L-myc, CYP1A1, CYP2E1 and p53, rare or minor restriction fragments (alleles) may predispose to certain cancers or may be associated with poor prognosis. There are no previously existing reports that describe examination of these markers within an epidemiological study.

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

Ainsley Weston	Visiting Scientist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
William P. Bennett	Medical Staff Fellow	LHC	NCI
Neil Caporaso	Medical Staff Fellow	EEB	NCI
Robert Hoover	Chief	EEB	NCI
Glennwood E. Trivers	Biologist	LHC	NCI
Patricia Steeg	Research Biologist	LP	NCI

Objectives:

To examine the DNA restriction patterns of normal and lung tumor tissues with human genomic and cDNA probes. Studies are focused on genetic loci implicated in lung cancer. Polymorphic loci throughout the human genome have 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. In addition, an epidemiological format has been used to study other polymorphisms that may be potential indicators of cancer risk and prognosis.

Methods Employed:

High molecular weight DNA was extracted from fresh or frozen tissues by standard procedures. Enzymes were used to degrade protein and RNA. Hydrolysates were extracted with phenol, phenol:chloroform and chloroform; high molecular weight DNA was spooled after alcohol precipitation.

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 immobilized on nylon or nitrocellulose filters. The filters were hybridized to cloned human genomic or cDNA fragments of known genetic loci, which were previously radiolabeled to high specific activity with $\alpha^{32}\text{P}$ -dCTP using the method of random priming. X-ray films were exposed to the filters in light-proof cassettes at -70°C for periods of between 4 hr and 6 days.

After dewaxing, inactivation of endogenous peroxidase activity, and blocking cross-reactions with normal goat serum, tissue sections are incubated overnight at 4°C with a saturating dilution of a polyclonal rabbit antiserum, CM-1, raised against wild type human p53 protein which was produced in a bacterial expression system. Localization of the primary antibody is detected by subsequent application of a biotinylated goat antirabbit antibody, an avidin-biotin complex conjugated to horseradish peroxidase (Vectastain Elite Kit, Vector Laboratories,

Burlingame, CA), and diaminobenzidine osmicated with nickel sulfate. The intensity and pattern of p53 immunostaining indicate p53 overexpression.

DNA is amplified using appropriate primers and Taq polymerase by the thermocycling technique known as the polymerase chain reaction (PCR). This technique has been used prior to analysis of the PCR products by dideoxy chain termination DNA sequence analysis and restriction enzyme analysis.

A modification of an ultrasensitive enzyme radioimmunoassay has been developed to detect the presence of anti-p53 antibodies in human serum. This assay might be useful as a screen for early cancer detection. The p53 protein is immobilized on a microtitre plate and human serum is introduced into the wells. Human antibodies that recognize p53 are then detected with an alkaline phosphatase conjugated goat anti-human IgG.

Major Findings:

Loss of heterozygosity studies have been conducted in primary lung cancer materials obtained at surgery. The potential candidate genes for involvement in lung cancer that have been studied include protein-tyrosine-phosphatase gamma (PTP γ), nm23 and p53. The PTP γ gene mapped to a region of human chromosome 3, 3p21, which is frequently deleted in lung cancer. One of the functions of PTP γ is to reverse the effect of protein-tyrosine kinases, many of which are oncogenes, suggesting that some PTP γ genes may act as tumor suppressor genes. An inclination of tumor suppressor genes is that they are deleted in tumors in which their inactivation contributes to the malignant phenotype. Allelic loss of PTP γ was found in 5 of 10 lung carcinoma tumor samples tested. Importantly, one PTP γ allele was lost in three lung tumors which had not lost flanking loci. Thus, the PTP γ gene has characteristics which suggest it is a candidate tumor suppressor gene at 3p21.

Tumor progression to the metastatic phenotype is accompanied in certain cell types by reduced expression of the nm23 gene. Human nm23-H1 has been localized to chromosome 17, p11-q11 by somatic cell hybrid analysis and in situ hybridization. Somatic allelic deletion of nm23-H1 was observed in human lung carcinoma DNA samples when compared to DNA from matched normal tissues. In many cases, allelic deletions at nm23-H1 and other chromosome 17p and q loci did not occur concurrently. The data identify nm23-H1 as a novel, independent locus for allelic deletion in human cancer, a characteristic shared with previously described suppressor genes.

A series of primary human non-small cell lung tumors have been analyzed for p53 mutations (by immunohistochemistry and dideoxy-mediated chain-termination DNA sequencing) and chromosome 17p sequence deletions (by Southern hybridization). In general, gene mutations are generally consistent with positive immunohistochemical staining, although false positive and false negative results are being investigated. These data suggest that inactivation of p53 (and its role of p53 as a tumor suppressor gene) is an important step in human lung carcinogenesis. Thus far evidence for the presence of anti-p53 antibodies has

been found in 3 of 15 lung cancer patients. This assay is now suitable, however, for a larger screening effort in an epidemiological framework.

Further analyses of the variable tandem repeat restriction fragment length polymorphism (RFLP) located at the 3' end of the HRAS-1 protooncogene are in progress in populations of whites and blacks residing in the Baltimore-Washington metropolitan area. These studies address the question of whether specific alleles (rare alleles) of the HRAS-1 protooncogene locus vary in their distribution among different racial groups. Using the 6.6kb BamHI human HRAS-1 recombinant fragment from the plasmid pEC, a total of 30 allelomorphs of different sizes have been detected in a total of 342 study subjects. An association was observed between race and specific alleles. Rare alleles were more frequent in black subjects compared to whites (both cancer patients and autopsy donors). Within each racial category, lung cancer patients had an excess of rare alleles. Differences in allelic distribution were not found to be associated with either age or gender of the study subjects. These studies are continuing in conjunction with studies that propose to assess the relevance of a 5' located RFLP that has been reported to be in linkage disequilibrium with the 5' polymorphism.

DNA-RFLPs of cytochrome P450 enzymes (CYP1A1 and CYP2D6) have been claimed to indicate lung cancer risk, presumably through their influence on drug and xenobiotic disposition. In a case control analysis of lung cancer, the CYP1A1 RFLP, which detects the presence or absence of an MspI restriction site that is located approximately 1.5 kb to the 3' end of the structural gene, no difference was found between lung cancer cases and controls in a US population. However, a difference in the distribution of the two allelomorphs was found between blacks and whites. At the CYP2D6 gene locus an XbaI polymorphism, analyzed by Southern hybridization to the hdb-1 probe, failed to find an association, whereas phenotyping by urinalysis did. This is probably because the Southern analysis fails to find a common mutant gene encoded by a 29 kb XbaI restriction fragment. A PCR based method to further analyze samples containing the 29 kb fragment has been validated in a small group of volunteers (12). In each case the volunteers were dosed with 10 mg of debrisoquine and gave blood (50 ml) and urine samples (8 hr). The PCR method correctly identified individuals who were found to be poor metabolizers of the drug. The extent to which this method predicts metabolic heterozygotes remains to be determined.

Publications:

Caporaso NE, Tucker MA, Hoover R, Hayes RB, Pickle LW, Issaq H, Muschik G, Green-Gallo L, Buivys D, Aisner S, Resau J, Trump BF, Tollerud D, Weston A, Harris CC. Lung cancer and the debrisoquine metabolic phenotype. *JNCI* 1990;85:1264-72.

Harris CC. Molecular epidemiology of human cancer in the 1990's. In: Gledhill BL, Mauro F, eds. *New horizons in biological dosimetry*. New York: Wiley-Liss (In Press).

- Harris CC. Molecular epidemiology: overview of biochemical and molecular basis. In: Groopman J, Skipper P, eds. Molecular dosimetry and human cancer. Boca Raton: CRC Press (In Press).
- Sinopoli N, Ficorells C, Trivers GE, D'Andrea V, Cagnazzo P, Tomao S, Harris CC, Frati L. Determination of PAH-DNA adducts in lung tissue of cancer patients. In: Columbano A, ed. Chemical carcinogenesis: 5th Sardinian meeting on modulating factors in multistage carcinogenesis. New York: Plenum Press (In Press).
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- Sugimura H, Caporaso NE, Shaw GL, Modali RV, Gonzalez FJ, Hoover RN, Resau JH, Trump BF, Weston A, Harris CC. Human debrisoquine hydroxylase gene polymorphisms in cancer patients and controls. Carcinogenesis 1990;11:1527-30.
- Sugimura H, Weston A, Caporaso NE, Shields PG, Bowman ED, Metcalf RA, Harris CC. Biochemical and molecular epidemiology of cancer. In: Chang LW, Hart R, eds. Recent advances in biomarker research. New York: Academic Press (In Press).
- Tamai S, Sugimura H, Caporaso NE, Resau JH, Trump BF, Weston A, Harris CC. Restriction fragment length polymorphism analysis of the L-myc gene locus in a case-control study of lung cancer. Int J Cancer 1990;46:411-5.
- Weston A, Caporaso NE, Hoover RN, Tannenbaum SR, Skipper PL, Resau JH, Trump BF, Harris CC. Measurement of 4-aminobiphenyl-hemoglobin adducts in lung cancer cases and controls. Cancer Res (In Press).
- Weston A, Harris CC. Chemical carcinogenesis. In: Holland JF, Frei E III, Bast RC Jr, Kufe DW, Morton DL, Weichselbaum RR, eds. Cancer Medicine. 3rd ed. Philadelphia: Lea and Fibiger (In Press).
- Weston A, Vineis P, Caporaso NE, Krontiris TG, Lonergan JA, Sugimura H. Racial variation of HRAS-1 in lung cancer patients and a disease free population. Mol Carcino (In Press).

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

PROJECT NUMBER

Z01CP05505-07 LHC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Neoplastic Transformation of Human Epithelial Cells by 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:	Brenda I. Gerwin	Research Chemist	LHC	NCI
	John F. Lechner	Chief, IVCS	LHC	NCI
	William Bennett	Medical Staff Fellow	LHC	NCI
	Kathleen Forrester	Staff Fellow	LHC	NCI
	Masakazu Murakami	Special Volunteer	LHC	NCI
	Masayuki Noguchi	Special Volunteer	LHC	NCI
	Bonita G. Taffe	IRTA Fellow	LHC	NCI

COOPERATING UNITS (if any)

Fox Chase Cancer Center, Philadelphia, PA (A. Klein-Szanto); Cetus Corporation, Emeryville, CA (F. McCormick); Johns Hopkins, Baltimore, MD (B. Vogelstein); Georgetown University, DC (M. Lippman)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

5.0

PROFESSIONAL:

2.0

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.)

Five families of activated protooncogenes, ras, raf, jun, erbB-2 (neu) and myc have so far been associated with human bronchogenic carcinoma. 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. Overexpression of erbB-2 causes neoplastic transformation of human bronchial epithelial cells. The molecular mechanisms of ras and raf/myc induction of neoplastic transformation is the major focus of current and future studies. We are investigating the role of ras and raf in the transduction of signals from ligand activated cellular membrane receptors to the nucleus and subsequent altered expression of growth and terminal differentiation genes. We hypothesize that receptor-mediated phosphorylation of the raf kinase causes both an increase in its activity and a translocation to the nucleus. Possible substrates of the activated raf kinase include PI kinase and nuclear regulatory proteins, myc and p53. The effects of mutant p53 gene on cell growth and terminal squamous differentiation will be assessed. The cooperativity of mutant p53 and ras genes in the "immortalization" and neoplastic transformation of normal human bronchial epithelial cells is being studied. Human papillomaviruses 16 and 18 have been shown to "immortalize" normal bronchial epithelial cells. Since the Ha-ras or myc/raf transformed bronchial epithelial cells are highly invasive and metastatic in athymic nude mice, we plan to investigate the regulation and expression of genes considered to be involved in tumor metastasis including Nm23, collagenase IV and TIMP-2.

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

Curtis C. Harris	Chief	LHC	NCI
Brenda I. Gerwin	Research Chemist	LHC	NCI
John F. Lechner	Chief, IVCS	LHC	NCI
William Bennett	Medical Staff Fellow	LHC	NCI
Kathleen Forrester	Staff Fellow	LHC	NCI
Masakazu Murakami	Special Volunteer	LHC	NCI
Masayuki Noguchi	Special Volunteer	LHC	NCI
Bonita G. Taffe	IRTA Fellow	LHC	NCI
James C. Willey	Expert	LHC	NCI
Lance A. Liotta	Chief	LP	NCI
Patricia Steeg	Senior Staff Fellow	LP	NCI

Objectives:

Determination of the functional role of oncogenes associated with human lung cancers in the neoplastic transformation of human bronchial epithelial cells in vitro.

Investigation of the dysregulation by transfected oncogenes of the growth and differentiation pathways in human bronchial epithelial cells.

Investigation of the role of oncogenes in metastasis of neoplastic human bronchial epithelial cells.

Methods Employed:

1. In vitro culture of human bronchial epithelial cells.
2. Gene transfer by transfection or infection.
3. Nucleic acid hybridization.
4. Immunoprecipitation and PAGE analysis.
5. Immunocytochemistry of "tumor markers."
6. Tumorigenicity assay in athymic nude mice.
7. Collagenase type IV assays.
8. In vitro matrigel invasion assay.

Major Findings:

Human bronchial epithelial cells transformed by the c-raf-1 and c-myc protooncogenes induce multidifferentiated carcinomas in nude mice: a model for lung carcinogenesis. We have previously described the neoplastic transformation of immortalized human bronchial epithelial cells (BEAS-2B) by the combination of the c-raf-1 and c-myc protooncogenes and the concomitant induction of neuron-specific enolase mRNA expression. In this paper we describe the morphological,

biochemical and immunohistochemical characteristics of the primary c-raf-1/c-myc tumors, xenografts of these tumors and tumors that originated from cell lines of the primary neoplasm. The tumors were morphologically characterized by the appearance of desmosomes and tonofilaments, microvilli and dense core granules representing markers of squamous, glandular and neuroendocrine differentiation, respectively. Eleven out of 13 tumors were positive by immunohistochemical techniques for neuron-specific enolase, serotonin (9/13), and calcitonin (6/13). Keratins were expressed in 11/13 tumors, and while specific keratins (K5, K7, K16/K17) decreased, there was an increase of vimentin in the tumor cells. Gastrin releasing peptide (GRP) immunoreactivity was detectable in a small number of tumors (5/13). BEAS-2B cells transfected with the c-raf-1 and c-myc protooncogenes and cell lines established from the primary tumors expressed major histocompatibility class II antigen which has been found on small cell lung carcinoma cells. The tumors induced by the c-raf-1 and c-myc protooncogenes resemble the multi-differentiated phenotype of small cell lung cancer frequently detected in vivo and present a defined model to study the relation between molecular markers, phenotypical appearance and response to chemotherapeutic agents and radiation.

Biological consequences of overexpression of a transfected c-erbB-2 gene in "immortalized" human bronchial epithelial cells. After introduction of a human c-erbB-2 expression vector into the large T antigen immortalized human bronchial epithelial cell line, BEAS-2B, six subclones (B2BE1, 2, 3, 4, 5 and 6) were isolated. Three subclones (B2BE2, 5, and 6) expressed levels of c-erbB-2 detectable by immunocytochemistry. More than 80% of B2BE2 and -6 cells expressed c-erbB-2 but less than 10% of B2BE3 and -5 cells expressed it. These results correlated with the amount of 9.0 kb mRNA expression detected by northern blot analysis. The tumorigenic cell line (B2BE6) constitutively expressed TGF- α mRNA in addition to a high level of c-erbB-2 protein, while B2BE2, which expressed an equally high level of c-erbB-2, was not tumorigenic. These data indicate that overexpression of c-erbB-2 can contribute to a tumorigenic phenotype in human bronchial epithelial cells in combination with expression of TGF- α and/or other growth regulators. Further analysis of these two cell lines may provide insight into the mechanisms of transmembrane signalling by c-erbB-2. Colony forming efficiency assays and immunocytochemical analysis of Ki67 revealed no significant differences in proliferative capacity correlated with c-erbB-2 expression. One of the clones (B2BE6) induced slow growing, cystic tumors in 4 out of 10 athymic nude mice. A cell line (B2BE6T) derived from a tumor explant was immunocytochemically positive for c-erbB-2 expression. The tumorigenic subclone (B2BE6) constitutively expressed TGF- α mRNA, whereas the non-tumorigenic subclones did not.

Immortalization of normal human bronchial epithelial cells by human papillomaviruses 16 or 18. Human papillomaviruses (HPV) are associated with papillomatosis of the larynx, trachea and bronchi in decreasing order of frequency and these papillomatosis lesions may become malignant. When the patients are not selected for a history of papillomatosis, the frequency of HPV in bronchogenic carcinoma tissue is 1-5%. In order to develop a model for investigating the role of HPV in human bronchogenic carcinogenesis, normal human

bronchial epithelial (NHBE) cells were transfected with cloned full-length HPV-16 or HPV-18. Two HPV-18 transformed cell lines (BEP1 and 2) and one HPV-16 transformed cell line (BEP3) were established. These non-tumorigenic epithelial cell lines have: (a) attained over 100 population doublings *in vitro*; (b) mutually exclusive human marker chromosomes; (c) HPV DNA in forms that are consistent with chromosomal integration by Southern analysis; (d) HPV E6, E7 and E6* mRNA transcripts by Northern and reverse transcriptase-polymerase chain reaction analysis; and (e) diminished confluence-induced squamous differentiation. These cell lines should be useful for studying mechanisms involved in proliferation, differentiation and neoplastic transformation of human bronchial epithelial cells.

Development of tumorigenicity in SV40 T antigen-positive human bronchial epithelial cell lines. Of five simian virus 40-transformed clonal human bronchial epithelial cell lines previously shown to be non-tumorigenic at early passages, two lines (BES-1A1 and BEAS-2B) from different donors have become weakly tumorigenic with further passaging. BES-1A1 passage 26 cells formed tumors in 3/10 athymic nude mice injected subcutaneously, whereas BEAS-2B passage \geq 32 cells formed highly cystic tumors at 8 of 58 injection sites after long latency periods. Tumor cell lines were established from several BEAS-2B tumors, and these are resistant to the growth inhibitory effects of serum, an inducer of squamous differentiation in BEAS-2B and normal bronchial epithelial cells. This finding supports the hypothesis that development of resistance to inducers of terminal differentiation may be a step in the process of bronchial carcinogenesis. One of these tumor cell lines, B39-TL, is significantly more tumorigenic than the others and has a deletion from the short arm of chromosome 3 as has previously been described for some naturally occurring human bronchial carcinomas. Thus, from the clonally-derived BEAS-2B cell line, cell populations with various degrees of tumorigenicity have developed. Analysis of the changes in these cells may yield insights into the multiple events involved in acquisition of the tumorigenic phenotype.

Publications:

Iman DS, Harris CC. Oncogenes and tumor suppressor genes in human lung carcinogenesis. *CRC Crit Rev Oncogenesis* 1991;2:161-71.

McDonald JW, Brash DE, Oseroff AR, Harris CC. Photosensitizing dyes for the selection of nontumorigenic revertants from human lung cancer cell lines. *Cancer Res* 1990;50:5369-73.

Pfeifer AMA, Jones RT, Bowden PE, Mann D, Klein-Szanto AJP, Trump BF, Harris CC. Human bronchial epithelial cells transformed by the c-raf-1 and c-myc protooncogenes induce multidifferentiated carcinomas in nude mice: a model for lung carcinogenesis. *Cancer Res* (In Press).

Reddel RR, Hsu IC, Mass MJ, Hukku B, Gerwin BI, Salghetti SE, Somers ANA, Galati AJ, Gunning WT III, Harris CC, Stoner GD. A human bronchial epithelial cell strain with unusual in vitro growth potential which undergoes neoplastic transformation after SV40 T antigen gene transfection. *Int J Cancer* (In Press).

Stoner GD, Kaighn ME, Reddel RR, Resau JH, Bowman D, Naito Z, Matsukura N, You M, Galati AJ, Harris CC. Establishment and characterization of SV40 T-antigen immortalized human esophageal epithelial cells. *Cancer Res* 1991;51:365-71.

Trump BF, Berezsky IK, Smith MW, Jones RT, Phelps PC, Miyashita M, Harris CC. Studies of human bronchial epithelium in vitro: changes of $[Ca^{2+}]_i$ in relation to injury, growth, and differentiation. *Toxicol In Vitro* 1990;4:646-53.

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

PROJECT NUMBER

Z01CP05541-04 LHC

PERIOD COVERED

October 1, 1990 to September 1991

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

In Vitro Model for Human Liver Carcinogenesis Studies

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
	Takayoshi Tokiwa	Guest Researcher	LHC	NCI
	Duane Smoot	Special Volunteer	LHC	NCI
	Tsung-tang Sun	Visiting Scientist	LHC	NCI

COOPERATING UNITS (if any)

Laboratory of Hepatitis Research, CBER/FDA (S.M. Feinstone)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

6.0

PROFESSIONAL:

3.0

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.)

Techniques have been developed and media have been formulated that will support replicative cultures of monkey and human liver epithelial cells that exhibit some hepatocellular characteristics for 20 and 12 culture population doublings, respectively. However, monkey and transformed human liver epithelial cells established in these media will cease replicating after a few passages unless the media are supplemented with arginine. A similar observation was made using the human hepatoblastoma cell line Hep-G2. Thus, we speculate that hepatocytes may commonly lose portions of the urea cycle metabolic pathway as they undergo "retrograde differentiation" and/or transformation. In addition, we have found by measuring albumin and keratin species 18 and 19 that, initially, the transformed human liver epithelial (THLE) cells probably originate from hepatocytes or from cells that are of the hepatocyte lineage. However, with continued growth the THLE cells also co-exhibited liver ductal epithelial cell characteristics. Finally, preliminary investigations have demonstrated that inter-splenic injection protocols and "tissue equivalent" matrices may be efficacious for inducing replicative cultures of normal and transformed liver epithelial cells to re-express hepatocellular phenotypic expressions.

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
Takayoshi Tokiwa	Special Volunteer	LHC	NCI
Duane Smoot	Special Volunteer	LHC	NCI
Tsung-tang Sun	Expert	LHC	NCI

Objectives:

1. Develop techniques for replicative cultures of normal human liver epithelial cells.
2. Investigate normal human liver epithelial cell multiplication and differentiation control processes.
3. Investigate the molecular mechanisms of human liver carcinogenesis by chemical carcinogens and/or hepatitis viruses.

Methods Employed:

1. In vitro culture of normal and transformed human epithelial cells.
2. Determination of cytotoxicity by measuring colony forming efficiency of treated cells.
3. Analysis of growth factor mitogenic activity using [³H]thymidine incorporation.
4. Gene transfer by DNA mediated transfection.
5. Analysis of tumorigenicity by subcutaneous inoculation into athymic nude mice.
6. Immunocytochemistry.

Major Findings:Experiments with human liver epithelial cells: replicative cultures.

Hepatocytes are obtained by collagenase/dispase perfusion of the lower lobe of livers of non-cancerous "immediate autopsy" donors, using methods previously described. The cultures are initiated by inoculating the cells at a density of 6,700 cells/cm² into flasks that had been precoated with Vitrogen® collagen.

The cultures are incubated overnight in Waymouth's medium containing 10% serum. The next day the cultures are rinsed and the medium is replaced with the serum-free liver cell medium (LCM).

To date the liver cells from five donors have been cultured; all underwent four successive (1:4 split ratio) subculturings or an estimated 12 population doublings, with a cell doubling time of 3 days, before division ceased. However, in contrast to the Rhesus monkey liver epithelial cell situation, the cultures did not originate from colonies. Instead, initial cell attachment was more than 90% and sporadic mitotic cells were seen throughout the culture. Immunostaining of 3rd passage cells showed them to be uniformly positive for keratin 18 but negative for keratin 19, a keratin species not found in hepatocytes but present in ductal cells. In addition, 50% of the same passage cells exhibited human albumin. Thus, our medium and culture conditions are at least minimally adequate for replicative cultures of liver epithelial cells that have hepatocyte features. On the other hand, additional investigations are necessary to establish whether the nutrient formula, surface requirements and medium supplements are optimal.

Cells from the second donor were transfected with a DNA construct containing the SV40 T antigen gene linked to the Rous sarcoma virus LTR. Transformed colonies with morphologies varying from epithelial-like to fibroblastic-like were discernible 6-8 weeks later, at which time we attempted to isolate and subculture individual clones. However, these efforts were unsuccessful; the cells were exceptionally sensitive to trypsin and they consistently sloughed from the culture dish surface within two days after subculturing. Therefore, the remaining colonies were pooled as a mass culture. With each successive subculturing the THLE cells became progressively resistant to trypsin. THLE cells would not form colonies; thus, their clonal growth rate could not be measured. However, 5th passage moderately dense cultures exhibited a mean generation time of 36 hrs. The cells continued replicating for 11 (1:4 split ratio) subculturings or approximately 40 population doublings before growth ceased.

When THLE cells were examined at the 3rd passage, roughly 30% of the cells stained positively for the SV40 T antigen gene. However, when re-examined 2 passages later, virtually 100% of the cells expressed the SV40 T antigen in their nuclei. Passage 3 cells were uniformly positive and negative for keratins 18 and 19 respectively, suggesting that the cells arose from hepatocytes or from cells of the hepatocyte lineage. However, even though 100% of the cells exhibited keratin 18 at the 10th passage, 30% of the cells also displayed keratin 19. Transferrin, fibrinogen and albumin expression was measured in 10th passage cells. None of the cells showed evidence of transferrin or fibrinogen. On the other hand, 20% of the cells were positive for albumin. These albumin expressing cells were always found in clusters of 8-12 cells. Analogous to what has been reported for rat liver epithelial cells, the number of albumin positive cells increased to 30-40% if the THLE cells were incubated for 48 hrs in medium supplemented with 10% serum.

Experiments with human liver epithelial cells: differentiation studies. The second assumption of our hepatocyte culture strategy is that techniques and media can be devised that will induce the replicating liver epithelial cells to stop dividing and concomitantly, differentiate into cells expressing many hepatocyte characteristics. We recently inaugurated this phase of the research using Hep-G2 cells. Surprisingly, these human hepatoblastoma cells will not multiply when incubated in LCM medium unless it is supplemented with 1 mM arginine. (Once aware of this arginine requirement, we speculated that THLE cells might also have progressed from arginine independent to dependent at the time growth ceased, i.e., passage 11; this possibility is currently being tested).

Both in vivo and in vitro techniques are being tested for their ability to induce Hep-G2 cells to express more of the traits exhibited by hepatocytes in vivo. The in vivo approach has been to inject the cells into the spleen of athymic nude mice. Hematoxylin and eosin stained sections of spleens removed 3-5 weeks after injection showed colonies of liver epithelial cells. Experiments to quantitate the hepatocyte-specific characteristics of the Hep-G2 cells within the spleen are still in progress.

High concentrations of amino acids, hyperosmolality, transforming growth factor- β , interleukin-1, interleukin-6, tumor necrosis factor- α , dimethylsulfoxide, sodium butyrate, collagen matrices, liver basement membranes and co-culturing with rat liver oval cells are known culture conditions that promote the expression of liver-specific proteins. Thus, prior to initiating our in vitro differentiation experiments, there was considerable information in the literature to guide our efforts. Our approach is an adaptation of the collagen/fibroblast mesenchyme "tissue equivalent" matrices technique, described by Bell and associates, to induce organotypic differentiation of thyroid cells. However, in contrast to their observations, we have found that hepatoblastoma cells, when inoculated onto "tissue equivalents," do not have to be xenotransplanted to athymic nude mice in order for (some of) the cells to aggregate/grow into tissue-like structures. Determinations of the presence of albumin and other hepato-specific proteins by both immunostaining and in situ hybridization methodologies are in progress.

Publications:

Harris CC. Hepatocellular carcinogenesis: recent advances and speculations. *Cancer Cells* 1990;2:146-8.

Lechner JF, Smoot DT, Pfeifer AMA, Cole KH, Weston A, Groopman JD, Shields PG, Tokiwa T, Harris CC. A non-tumorigenic human liver epithelial cell culture model for chemical and biological carcinogenesis investigations. In: Rhim JS, Dritschilo A, eds. *Neoplastic transformation in human cell systems: mechanisms of carcinogenesis*. Clifton, New Jersey: Humana Press (In Press).

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

PROJECT NUMBER

Z01CP05543-04 LHC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Tumor Suppression Genes in Human Carcinogenesis

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

PI:	Curtis C. Harris	Chief	LHC	NCI
Others:	Brenda I. Gerwin	Research Chemist	LHC	NCI
	John F. Lechner	Chief, IVCS	LHC	NCI
	William Bennett	Medical Staff Fellow	LHC	NCI
	Chad Giri	Guest Researcher	LHC	NCI
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	Robert A. Metcalf	Medical Staff Fellow	LHC	NCI
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COOPERATING UNITS (if any)

Key Med. Ctr., Baltimore, MD (E. Gabrielson); Univ. of CA, Irvine, CA (E. Stanbridge); Johns Hopkins Univ., Baltimore, MD (B. Vogelstein); Qidong Liver Cancer Inst. (N. Wang); Temple Univ. (C. Croce, K. Huebner); NYU (J. Schlessinger)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Molecular Genetics and Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

7.5

PROFESSIONAL:

4.0

OTHER:

3.5

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.)

Mutations in the evolutionarily conserved codons of the p53 tumor suppressor gene are common in diverse types of human cancer. The p53 mutational spectrum differs among cancers of the colon, lung, esophagus, breast, liver, brain, lymphomas and leukemias. Transitions predominate in cancers of the colon, brain and lymphoid malignancies, whereas G:C to T:A transversions are the most frequent substitutions observed in cancers of the lung and liver. Mutations at A:T base pairs are seen more frequently in esophageal carcinomas than in other solid tumors. The distributions of mutations in the p53 sequence are also distinct. Most transitions in colorectal carcinomas, brain tumors, leukemias and lymphomas are at CpG dinucleotide mutational hotspots (codons 175, 248, 273 and 282). G to T transversions in lung, breast and esophageal carcinomas are dispersed among numerous codons, whereas in liver tumors from geographic areas in which both aflatoxin B₁ and hepatitis B virus are cancer risk factors, most mutations are at one nucleotide pair of codon 249. These differences may reflect the etiological contributions of both exogenous and endogenous factors in human carcinogenesis. A receptor protein tyrosine phosphatase, PTP gamma, is a candidate tumor suppressor gene on human chromosome 3p21 that may be involved in human lung and renal carcinogenesis. An antimetastasis gene, nm23, is frequently altered in human lung, breast and renal cancers.

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

Curtis C. Harris	Chief	LHC	NCI
Brenda I. Gerwin	Research Chemist	LHC	NCI
John F. Lechner	Chief, IVCS	LHC	NCI
William Bennett	Medical Staff Fellow	LHC	NCI
Kathleen Forrester	Staff Fellow	LHC	NCI
Chad Giri	Microbiologist	LHC	NCI
Teresa A. Lehman	Senior Staff Fellow	LHC	NCI
Robert A. Metcalf	Medical Staff Fellow	LHC	NCI
Haruhiko Sugimura	Visiting Fellow	LHC	NCI
Anne Van Der Meerem	Visiting Fellow	LHC	NCI
Ainsley Weston	Visiting Scientist	LHC	NCI
Tsung-Tang Sun	Visiting Scientist	LHC	NCI
Kirsi Vahakangas	Special Volunteer	LHC	NCI
Lance A. Liotta	Chief	LP	NCI
Patricia Steeg	Senior Staff Fellow	LP	NCI

Objectives:

Determine the functional role and molecular mechanisms of putative tumor suppressor genes, e.g., p53 and protein tyrosine phosphatase γ , that are frequently inactivated during lung carcinogenesis.

Isolate and investigate the molecular mechanisms of novel tumor suppressor genes.

Determine the functional role and molecular mechanisms of putative anti-metastasis genes, e.g., nm23, in human lung carcinogenesis.

Methods Employed:

1. In vitro culture of normal and neoplastic human bronchial epithelial cells.
2. Gene transfer by transfection or infection.
3. Nucleic acid hybridization.
4. Immunoprecipitation and Western analysis of oncogene and tumor suppressor gene products.
5. Somatic cell fusion.
6. Microcell transfer of human chromosomes.
7. Preparation of cDNA subtraction libraries in expression vectors.
8. Polymerase chain amplification and DNA sequencing.

Major Findings:

Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Human hepatocellular carcinomas (HCC) from patients in Qidong, a high incidence area of China, in which both hepatitis B virus and aflatoxin B₁ are risk factors, were analyzed for mutations in the p53 gene. Eight of the 16 HCC had a point mutation at the third base position of codon 249. The G→T transversion in 7 HCC DNA samples and the G→C transversion in the other HCC are consistent with the mutational spectrum caused by aflatoxin B₁ in experimental mutagenesis studies. No mutations were found in exons 5, 6, 8 and the remainder of exon 7. These data contrast sharply with the previously reported p53 mutational spectra in human lung, colon, esophagus, and breast carcinomas and sarcomas where the mutations are found primarily scattered over four of the five evolutionary conserved domains, which includes codon 249. We hypothesize that the resultant mutant p53 protein may lead to a selective clonal expansion of hepatocytes during carcinogenesis.

Archival analysis of p53 genetic and protein alterations in Chinese esophageal cancer. A strategy and methods for archival analysis of genetic and protein alterations in the p53 tumor suppressor gene are presented. The tumor series includes 43 paraffin-embedded esophageal carcinomas from two high-incidence regions in the People's Republic of China. More than half contained elevated p53 protein levels which were detected by a high titer polyclonal antiserum and a sensitive immunohistochemical method. To estimate the frequency of underlying mutations, DNA was isolated from conventional paraffin sections, amplified by the polymerase chain reaction, and examined by dideoxy termination sequencing. Analysis of exons 5-8 in a subset of 10 tumors revealed missense point mutations in 4 out of 5 immunostain positive tumors and a mutation encoding a stop codon in 1 of 5 immunostain negative tumors. In this report of archival material, we conclude that detectable levels of p53 protein correlate closely with the occurrence of missense mutations. Furthermore, these methods render large repositories of paraffin-embedded tumor and non-tumor tissues accessible to analysis. Immunohistochemical screening for elevated protein levels followed by sequence analysis represents an efficient strategy for the evaluation of the mutational spectrum within the p53 coding region.

p53 mutations, ras mutations and p53-heat shock 70 protein complexes in human lung cell lines. The p53 tumor suppressor gene is frequently mutated and the K-ras oncogene is occasionally mutated in primary specimens of human lung carcinomas. These mutated genes also cooperate in the immortalization and neoplastic transformation of rodent cells. To determine whether these mutations are necessary for maintenance of the immortalized and/or neoplastically transformed states of human bronchial epithelial cells, the p53 gene and regions of the ras (K-, H- and N-) genes were sequenced in nine human lung carcinoma cell lines. Detection of p53 mutations by polymerase chain amplification and direct DNA sequencing was corroborated by p53 immunocytochemistry and coimmunoprecipitation of p53 with heat shock protein 70. p53 and ras genes were frequently, but not always, mutated in the carcinoma cell lines. These data are

consistent with the hypothesis that multiple genetic changes involving both protooncogenes and tumor suppressor genes occur during lung carcinogenesis.

Frequent mutation of the p53 gene in human esophageal cancer. Sequence alterations in the p53 gene have been detected in human tumors of the brain, breast, lung and colon, and it has been proposed that p53 mutations spanning a major portion of the coding region inactivate the tumor suppressor function of this gene. Neither transforming mutations in oncogenes nor mutations in tumor suppressor genes have been reported previously in human esophageal tumors. We examined four human esophageal carcinoma cell lines and fourteen human esophageal squamous cell carcinomas by polymerase chain reaction amplification and direct sequencing for the presence of p53 mutations in exons 5, 6, 7, 8 and 9. Two cell lines and five of the tumor specimens contained a mutated allele (one frameshift and six missense mutations). All missense mutations detected occurred at G:C base pairs in codons at or adjacent to mutations previously reported in other cancers. The identification of aberrant p53 gene alleles in one-third of the tumors we tested suggests that mutations at this locus are common genetic events in the pathogenesis of squamous cell carcinomas of the esophagus.

Receptor protein-tyrosine-phosphatase, PTP γ , is a candidate tumor suppressor at human chromosome region 3p21. The protein-tyrosine-phosphatase gene, PTP γ , maps to a region of human chromosome 3, 3p21, which is frequently deleted in renal cell carcinoma and lung carcinoma. One of the functions of protein-tyrosine-phosphatases is to reverse the effect of protein-tyrosine kinases, many of which are oncogenes, suggesting that some protein-tyrosine-phosphatase genes may act as tumor suppressor genes. A hallmark of tumor suppressor genes is that they are deleted in tumors in which their inactivation contributes to the malignant phenotype. In this study, one PTP γ allele was lost in 3 of 5 renal carcinoma cell lines and 5 of 10 lung carcinoma tumor samples tested. Importantly, one PTP γ allele was lost in three lung tumors which had not lost flanking loci. PTP γ mRNA was expressed in kidney cell lines and lung cell lines but not expressed in several hematopoietic cell lines tested. Thus, the PTP γ gene has characteristics which suggest it as a candidate tumor suppressor gene at 3p21.

Somatic allelic deletion of nm23 in human cancer. Tumor progression to the metastatic phenotype is accompanied in certain cell types by reduced expression of the nm23 gene. We have localized human nm23-H1 to chromosome 17 by somatic cell hybrid analysis. Regional localization in the CEPH database and *in situ* hybridization is reported. Somatic allelic deletion of nm23-H1 was observed in human breast, renal, colorectal and lung carcinoma DNAs, as compared to DNA from matched normal tissues. A homozygous deletion of nm23-H1 was observed in a lymph node metastasis of a colorectal carcinoma, indicating that nm23-H1 can be recessively inactivated. The data identify nm23-H1 as a novel, independent locus for allelic deletion in human cancer, a characteristic shared with previously described suppressor genes.

Publications:

Bennett WP, Hollstein MC, He A, Zhu SM, Resau J, Trump BF, Metcalf RA, Welsh JA, Gannon JV, Lane DP, Harris CC. Archival analysis of p53 genetic and protein alterations in Chinese esophageal cancer. *Oncogene* (In Press).

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Hollstein M, Metcalf RA, Welsh J, Montesano R, Harris CC. Frequent mutation of the p53 gene in human esophageal cancer. *Proc Natl Acad Sci USA* 1990;87:9958-61.

Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science* (In Press).

Hsu IC, Metcalf RA, Welsh J, Sun T, Wang NJ, Harris CC. p53 gene mutational hotspot in human hepatocellular carcinomas from Qidong, China. *Nature* 1991;350:427-8.

LaForgia S, Morse B, Levy J, Barnea G, Cannizzaro LA, Li F, Nowell PC, Boghosian-Sell L, Glick J, Weston A, Harris CC, Drabkin H, Patterson D, Croce CM, Schlessinger J, Huebner K. Receptor-linked protein-tyrosine-phosphatase, PTPy, is a candidate tumor suppressor at human chromosome region 3p21. *Proc Natl Acad Sci USA* (In Press).

Lehman TA, Bennett WP, Metcalf RA, Reddel R, Welsh JA, Ecker J, Modali RV, Ullrich S, Romano JW, Appella E, Testa JR, Gerwin BI, Harris CC. p53 mutations, ras mutations and p53-heat shock 70 protein complexes in human lung cell lines. *Cancer Res* (In Press).

Lehman TA, Harris CC. Oncogene and tumor suppressor gene involvement in human lung carcinogenesis. In: Milo GE, Casto B, Schuler C, eds. Transformation of human epithelial cells: molecular and oncogenetic mechanisms. Boca Raton: CRC Press (In Press).

Lehman TA, Reddel RR, Pfeifer AMA, Spillare E, Kaighn ME, Weston A, Gerwin BI, Harris CC. Oncogenes and tumor suppressor genes. *Environ Health Perspect* (In Press).

Leone A, McBride OW, Weston A, Wang M, Anglard P, Cropp CS, Gorpe J, Lidereau R, Callahan R, Linehan WM, Rees RC, Harris CC, Liotta LA, Steeg PS. Somatic allelic deletion of nm23 in human cancer. *Cancer Res* (In Press).

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

PROJECT NUMBER

Z01CP05611-03 LHC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

In Vitro Studies of Human Mesothelial Cell Carcinogenesis

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
	Teresa Lehman	Senior Staff Fellow	LHC	NCI
	Robert Metcalf	Medical Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

Institute of Occupational Health, Helsinki, Finland (K. Linnainmaa); University of Uppsala, Uppsala, Sweden (C. Betsholtz); 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.)

Recent studies indicate that loss or mutation of tumor suppressor genes is frequently associated with many types of human cancer. The etiology of these cancers is not completely clear. In the case of mesothelioma, the carcinogenic agent(s) is generally considered to be asbestos fibers. It was, therefore, of interest to examine a relatively large (19) collection of cell lines for loss of expression of the retinoblastoma (Rb) gene or mutation in the p53 gene. Our studies to date suggest that loss of Rb expression is not common in mesothelioma. Protein studies will be performed to confirm this conclusion. The status of the p53 gene in mesothelioma is being studied by immunocytochemistry and direct sequencing. To date only 10% (2/20) of tumors show mutations, while 3/20 show protein staining higher than normal mesothelial cells. None of the tumors positive for p53 mutations have mutations in Ki-ras codon 61. Examination of expression of platelet-derived growth factor (PDGF)-A chain in tumors retrieved after inoculation of athymic nude mice with derivatives of the immortalized human mesothelial cell MET-5A showed that tumors from PDGF-A chain transfectants but not EJ-ras transfectants overexpressed PDGF-A chain from the endogenous gene. Interestingly, rare tumors from control plasmid transfectant cells also overexpressed the endogenous PDGF-A chain gene, suggesting that this may be a common alteration correlating with tumorigenic progression.

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
Teresa Lehman	Senior Staff Fellow	LHC	NCI
Robert Metcalf	Medical Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

- 1) To determine whether the tumor suppressor genes Rb (retinoblastoma) and/or p53 may be involved in the pathogenesis of human mesothelioma.
- 2) Analysis of possible association of ras gene mutations with human mesothelioma.
- 3) To test the hypothesis that PDGF might be an autocrine factor involved in the pathogenesis of human mesothelioma.
- 4) To test whether growth factor overexpression is common in mesothelioma.

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, polyacrylamide gel electrophoresis analysis and Western blotting. Mutations in genes such as c-ras or p53 are analyzed by polymerase chain reaction (PCR) amplification of DNA from cell lines or tumors followed by direct sequence analysis. Structural changes in specific chromosomes are studied by RFLP analysis. Tumorigenicity of cell lines is determined by subcutaneous inoculation of viable cells into athymic nude mice.

Major Findings:

Tumor Suppressor Studies: Studies of mRNA expression of the RB gene in mesothelioma and normal mesothelial cells have shown that neither the level of expression nor the size of the normal retinoblastoma gene messenger RNA is altered in mesothelioma. Current studies are addressing the production of retinoblastoma protein by mesothelioma cells. Two instances of mutation in the p53 gene have been found using the PCR method for gene amplification followed by direct sequencing. These point mutational events are of interest since this

type of mutation might not have been predicted as an initiating event in fiber-induced carcinogenesis. The p53 status of 19 mesothelioma cell lines is being examined by immunostaining and mRNA expression as well as sequencing. Three cell lines show a heightened level of p53 staining, while one expresses no p53 mRNA. These data suggest the presence of mutant protein or of gene rearrangements. Thus, it is possible that mutation or loss of p53 may occur during the progression of this tumor type.

ras Gene Mutation Studies: A line of non-tumorigenic but immortalized human mesothelial cells (MET-5A) has been developed in this laboratory. It has been shown that introduction of the mutated EJ-ras oncogene into this cell line results in the development of cells which induce rapidly growing tumors in athymic nude mice. These tumors pathologically resemble the epithelial sub-type of human mesotheliomas. Thus, it was thought that ras mutations might play a role in the pathogenesis of the human disease. Accordingly, human mesothelioma cell lines were examined by PCR and direct sequencing for mutations in the H-ras or K-ras genes. Results to date indicate that ras mutations are found infrequently in human mesothelioma cell lines. Thirteen mesothelioma lines tested are wild type at K-ras codon 61 and seven lines are wild type for K-ras codon 12.

Autocrine Growth Factors in Mesothelioma: Several lines of evidence suggest that overproduction of PDGF may be important in the pathogenesis of mesothelioma. First, we have previously observed that human mesothelioma cell lines overproduce PDGF-A chain and, less frequently, -B chain message in comparison to NHM cells. Furthermore, we have shown that NHM cells respond to mitogenic signals from PDGF. We transfected the non-tumorigenic, immortalized MET-5A cells with PDGF-A chain, PDGF-B chain and a vector control generating three cell lines. The PDGF-A chain transfected cells have proved to induce slow but progressively growing tumors in athymic nude mice. It has been found that MeT-5A EJ-ras tumors do not show overexpression of PDGF-A, while tumors from PDGF-A transfectants and rare tumors from vector control transfectants do overexpress the endogenous A chain mRNAs. These tumors can be transplanted and resemble the spindleoid sub-type of human mesothelioma. These studies are being pursued to analyze the growth factor production and responses of these cells in comparison to the other transfectants and the original MET-5A cell line. Of particular interest are expression levels of epidermal growth factor, transforming growth factor- α , fibroblast growth factor and other pertinent growth factors in the tumorigenic EJ-ras transfected mesothelial cells.

Asbestos Resistance: In studies of asbestos cytotoxicity, we have found that mesothelioma cell lines are much less sensitive than normal mesothelial cells to this cytotoxic effect. The immortalized non-tumorigenic MeT-5A cells are almost as sensitive as normal mesothelial cells. We asked whether populations which were resistant to asbestos cytotoxicity were selected after asbestos exposure or whether this resistance was a feature of tumorigenic mesothelial cells unrelated to asbestos exposure. We compared four cell lines derived from MeT-5A. These lines were the tumorigenic PDGF-A chain expressing cells, the tumorigenic EJ-ras producing cells, a non-tumorigenic population 30 passages after treatment with

amosite and a similar population after erionite treatment. The fiber treated populations were more resistant to fiber cytotoxicity than untreated MeT-5A cells, while the tumorigenic MeT-5A transfectants were as sensitive as the parental line.

Publications:

Gabrielson EW, Lechner JF, Gerwin BI, Harris CC. Cultured human mesothelial cells are selectively sensitive to cell killing by asbestos and related fibers: a potential in vitro assay for carcinogenicity. In: Brown RC, Hoskins J, Johnson N, eds. Proceedings of NATO advanced research workshop on mechanisms in fiber carcinogenesis. (In Press).

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Lechner JF, Gerwin BI, Reddel RR, Gabrielson EW, Van Der Meeren A, Linnainmaa K, Somers ANA, Harris CC. Studies on human mesothelial cells: effects of growth factors and asbestiform fibers. In: Harris CC, Lechner JF, Brinkley BR, eds. Cellular and molecular aspects of fiber carcinogenesis. New York: Cold Spring Harbor Laboratory Press, 1991;115-29.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05435-07 LHC, Z01CP05480-06 LHC, Z01CP05505-07 LHC, Z01CP05541-04 LHC, Z01CP05543-04 LHC, and Z01CP05611-03 LHC

UNIVERSITY OF MARYLAND (N01-CP-95624)

Title: Collection and Evaluation of Human Tissues and Cells from Patients with an Epidemiological Profile

Current Annual Level: \$528,066

Person Years: 5.54

Objectives: Collection, characterization, and delivery (to the NIH in aseptic and viable condition) 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 with an epidemiological profile, obtained and available upon request.

Major Contributions:

1. Collection of Tissues

Four hundred seventeen tissue collections were made from a total of 188 donors. Specimens were collected and transported to the NIH within 2 hours of release by the pathologist. These included the following collection categories:

A. Surgical Specimens:

Tumor and noninvolved, "normal", organ-site tissue specimens were collected from tumor cases whenever possible. One hundred forty-nine collections were obtained from 83 patients, with or without cancer, following cardiothoracic (44), colonic (37) and hepatic (7) surgeries. We received 78 tumor specimens (36 lung, 5 bronchus with trachea, 36 colon, and 6 liver) and 71 specimens of uninvolved, "normal" organ site tissues (37 lung, 30 colon, and 4 liver) from tumor patients when available;

B. Intermediate Autopsy (between 2 and 12 hours after death):

One specimen each of bronchus, with lung attached, and liver are usually obtained from each case. There were 83 cases (including 21 SIDS cases, 3 wks to 1 year) autopsied under the auspices of the Medical Examiner. From these we received 170 specimens (72 lung, 22 liver, 5 specimens of bronchus with trachea and 71 sera). [Note: Sera were collected for 49 lung specimen cases, 17 lung and liver cases and the 5 cases with bronchus and trachea collections.]

C. Immediate Autopsy (IA) (within 60 minutes after death):

One specimen each of colon, bronchus with lung attached, and liver are usually obtained from each case. 12 collections (4 lung, 5 liver, 2 sera, 1 colon) were obtained from 5 non-cancer cases during immediate autopsy.

D. Case Control Study:

Twenty-two preoperative workups for a lung cancer case-control study provided a total of 88 specimens: 22 specimens each of two timed-urines, red blood cells, and buffy coats.

Except for the case control specimens, tissues collected were throw-away residuals from materials taken for corrective or diagnostic purposes and not for research per se. Routinely excluded from the collection procedure were AIDS patients, known drug abusers or overdose victims, intravenous drug users, and HIV-profiled individuals. All tissues, tumors and normal, were defined and classified as described below.

The contractor retained amounts of tissue for histopathological characterization and assessment of viability. However, the major portion of all specimens collected were sent to the NIH within 2 hours of their availability from the pathologist.

2. Viability Evaluations

Bronchus: Surgical and immediate autopsy cases have been routinely shown viable and fail to culture only in cases with septicemia.

Colon: These tissues are currently being held for use in studies of the putative tumor suppressor/oncogene, p53.

Liver: The contractor uses a two-step profusion method (Hank's solution and L-15 medium with collagenase) for the isolation of viable liver cells from IA cases, with good viability results.

3. Definition and Classification of Non-neoplastic and Neoplastic Tissue

Bronchus: Characterization of human primary lung carcinomas routinely includes morphological and histochemical examination for p53, erb-B2, calcitonin, adrenocorticotrophic hormone (ACTH), alpha-fetoprotein (AFP), keratin, somatostatin, (beta human) chorionic gonadotropin (HCG-beta), serotonin, neuron specific enolase, calmodulin, and tubulin.

Normal and abnormal bronchial epithelial cells contain keratin, calmodulin, and tubulin; lung tumors contain HCG and keratin in, respectively, 80% and 75% of non-small cell tumors; in contrast, lung tumors rarely contain somatostatin, ACTH, HCG, AFP, or calcitonin.

Liver: Tissues and cultured cells are examined by electron microscopy and light microscopy for histological and pathological evaluation. Primary liver cells are cultured and the ultrastructural appearance of liver at autopsy is applied as a parameter for the viability in culture. This approach has given good results in cultures started from IA cases.

4. Epidemiological Profile Construction and Storage

Epidemiologic data (see below) are required for the study of relationships between tumor type, selected risk factors and exposure to known carcinogens of lung and liver (e.g., benzo[a]pyrene, N-nitrosamines, and aflatoxin B,) that bind to DNA in normal human epithelium, and potentially influences tissue responses in in vitro experimentation or genetic analysis. Donor histories, medical records, and computer storage of the compiled history of environmental exposure for each collected tissue are important components of this resource.

In this period, donor profiles were obtained using the OMB-approved, LHC questionnaire, completed in interviews conducted by the contractor's trained personnel. Profiles were completed for 53 patients (20 bronchus and 33 colon); 141 medical records were abstracted for surgery and autopsy patients (93 thoracic and 48 colonic); in data processing, a total of 206 records (13 colonic and 193 bronchus) 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 nine participating hospitals, and the medical examiner), are listed below:

	<u>Univ. Hosp.</u>	<u>LRVA</u>	<u>Un. Mem.</u>	<u>BCG</u>	<u>St. Ag.</u>	<u>S. Baltimore Gen. Hospital</u>
Bronchus	405(25)	206(14)	57(0)	15(8)	90(4)	10(0)
Colon	473(34)	155(5)	33(0)	8(2)	2 (0)	0(0)
	<u>Washington Veterans Admin. Hospital</u>	<u>Sinai Center</u>	<u>Mercy</u>	<u>Medical Examiner</u>	<u>Total</u>	
Bronchus	7(0)	14(0)	8(2)	758(82)	1573(138)	
Colon	51(0)	27(0)	2(0)	0(0)	751(41)	

The total number of epidemiological profiles completed to date and in this period are as follows:

Refused Interview

	<u>Med. Rec.</u>	<u>Interviewed</u>	<u>Coded</u>	<u>Patient/Doctor/UMH</u>			<u>Med. Ex.</u> ¹
Bronchus	1457(93)	582(20)	1348(193)	38(0)	7(0)	55(0)	758(82)
Colon	646(48)	501(33)	556(13)	31(0)	6(0)	33(0)	0
Total ²	2103(141)	1083(53)	1904(206)	69(0)	13(0)	88(0)	758(82)

{ } = increased number accomplished in this report period.

¹ Profiles limited to medical record extracts only--personal contacts routinely prohibited by ME policy.

² Grand total for the period = 482.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05480-06 LHC, Z01CP05541-04 LHC, and Z01CP05611-03 LHC

GEORGETOWN UNIVERSITY (NO1-CP-85606)

Title: Collection and Evaluation of Human Tissues and Cells from Donors with an Epidemiological Profile

Current Annual Level: \$80,138

Person Years: 0.96

Objectives: To provide the LHC with (1) a source of human lung and bronchial tissues collected at the time of surgery; (2) human peripheral blood lymphocytes (PBLs), human bronchial alveolar cells (BALCs) and supernatants (BALC's) from bronchial lavage of normal, smoking and nonsmoking volunteers with three (3) lung function tests and chest x-rays, and (4) completed epidemiological questionnaires for medical and environmental histories.

Major Contributions: This is the final year of the current procurement. In this period, the contractor provided a total of 145 specimens. These included 16 specimens of bronchi from 11 surgeries; 35 specimens of colon from 23 surgeries; pleural fluid (3) from 1 heart failure, 1 pneumonia, and 1 postcardiotomy syndrome cases; 7 matching lung tumor and uninvolved tissue sets from 6 cases of adenocarcinoma and 1 case of squamous cell carcinoma; 6 uninvolved normal tissues from 2 adenocarcinomas, and 2 broncho-alveola carcinoma, and 2 aspergilloma; 12 matching colon tumor and uninvolved normal tissues from a total of 13 cases of adenocarcinoma of the colon, one a tumor only collection; and 12 normal specimens of colon from 12 cases with normal polyps, inflammation or diverticulitis.

In addition, we received samples from 20 sets of matching PBMs (95% lymphocytes), BALCs (85% macrophages), and sera from normal volunteers. From 12 different BALC donors (6 smoker, 14 nonsmokers), in addition to the sera and the PBLs, we received the clarified lavage fluids and red blood cells. In total, we received 91 samples of blood and lung fluid components from these normal donors.

Medical and environmental histories were completed for all participating patients and normal volunteers. These records were filed in the contractor's facility for future use by LHC.

The resources provide specimens for the In Vitro Carcinogenesis and Molecular Epidemiology Sections of the laboratory. Lung and colonic tissues are used to develop assays for carcinogen DNA adducts, genetic restriction fragment length polymorphisms, and DNA repair in humans with exposure to known or suspected environmental carcinogens.

This effort constitutes the laboratory's only source of immunocompetent cells (i.e., alveolar macrophages and PBMs) from normal, non-hospitalized, smoking and nonsmoking adult human volunteers. Experiments with these cells serve to provide a macromolecular data base of normal levels, frequencies, and distribution of humans positive for certain carcinogen-induced damage to genes and gene products, and chemical markers considered potential indices for

persons at risk for chemical carcinogenesis. The detection of 8-hydroxydeoxyguanosine in normal human donors is ongoing using electrochemical methodology. Detection of carcinogen-DNA and carcinogen-hemoglobin adducts with fluorescence, postlabelling, and immunoassay is continuing.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05435-07 LHC and Z01CP05480-06 LHC

WALTER REED ARMY MEDICAL CENTER (Y01-CP-70500)

Title: Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: \$26,078

Person Years: 1.0

Objectives: To collect, and deliver to the NIH, pathologically characterized and deliver to the NIH specimens of malignant and noninvolved human bronchus and lung epithelium (obtained at time of surgery for cancer or for benign lesions); to obtain epidemiological profiles (medical and environmental histories) for each donor.

Major Contributions: Materials received through this interagency agreement are used in in vitro/in vivo studies of the mechanisms of carcinogenesis similar to those described above. In this first year of the latest renewal of the agreement, the agency has provided a total of 138 specimens from 18 cardiothoracic surgeries. These include 12 sets of matching tumor and uninvolved normal tissue specimens plus sets of PBLs, RBCs, and plasma from the following patients: one case each of carcinoma (ca), infiltrating ca, nonsmall cell ca, large cell ca, and a fish adenocarcinoma; 2 small cell ca, 2 squamous cell ca, and 7 adenocarcinomas. There were 4 cases of matching tumor and uninvolved normal tissue without the blood components, and two collections of normal tissue only, one from an E wing sarcoid case and one from a fish adenocarcinoma.

Personal profiles of medical and environmental histories were obtained and stored for all cooperating patients. These records were filed in the contractor's facility and are delivered to NIH upon request.

As with the projects described previously, these resources are also used in in vitro carcinogenesis and molecular epidemiology studies in the laboratory. These lung tissues are similarly used to develop assays for chemical and genetic markers denoting human exposure to known or suspected environmental carcinogens.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05505-07 LHC, Z01CP05541-04 LHC, Z01CP05543-04 LHC, and Z01CP05611-03 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: \$355,796

Person Years: 3.40

Objectives: 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: One thousand one hundred twenty eight Swiss litters (-1%) were delivered during this period. The total of 8,466 pups (-1%) (7.5/litter, +10%) included 3,831 (45%, -3%) nu/nu pups; 55% (+4%) of the nu/nu newborns survived, giving a total of 2,119 nudes or 1.9 nudes/litter.

During the year, the contractor maintained a monthly average colony population of 1,233 (+16) mice: 179(+0) breeders, 243 (-50) newborn weanlings for new experiments, and 793 (-72) mice in experimental protocols. An average of 9 (-4) experimental animals died and 202 (+108) were sacrificed monthly. As expected, changes in the research demands effect a fluctuating rate of use of colony resources. Experiments in this period employed approximately 2,749 irradiated nude mice, with 830 ongoing at the end. The objectives of the experimental designs include growth, differentiation, tumorigenicity and/or transplantability of cells as in the following examples:

1) Esophageal primary and tissue culture cells with and without *in vitro* modifications, comprising varying engineered or natural gene constructions of interest (7 ongoing and new experiments, 228 mice used, 97 surviving).

2) Transformed human liver epithelial cells implanted subcutaneously into nude mice after attachment to collagen gel matrices (4 ongoing and 6 new experiments, 78 mice used, 37 surviving).

3) Mesothelioma cells and mesothelial cell lines implanted by different routes with and without irradiation, and exposure to Amosite asbestos (20 ongoing and 6 new experiments, 404 mice used, 253 surviving).

4) A human lung carcinoma cell line, HuT-292, as host for transfected putative suppressor chromosomes (11 and 3) implanted to determine the influences of chromosomes on the tumorigenicity (20 ongoing and 7 new experiments, 394 mice used, 128 surviving).

5) Bronchial epithelial (BE) cells, SV40 immortalized and transfected with genes (e.g., BEAS-2B): a) BEAS-2B zip-v-ha-ras control BE cells, b) BES-1A-1

cells, BEAS-2B cells, c) B39-TL tumor cells from BEAS-2B cells grown in nude mice, d) BZR-T33 cells for tumor suppression, e) different BEAS-2B/ras-transfection constructs in age related susceptibility (43 ongoing and 28 new experiments, 1,643 mice used, 646 surviving).

These efforts resulted in 132 experiments ongoing during this period; 50 newly initiated; 2,749 mice on experiment during this period; 1038 mice surviving at the end of the period.

The level of colony performance relates to experimental designs optimized to accomplish in vivo characterization of in vitro modifications in sufficient but economically effective time. Experiments are allowed a predetermined shelf time (6 weeks usually, to 52 weeks as the maximum) and are routinely terminated with prior notification to the investigators. To keep space for new experiments, tumor transfers are held to a minimum. Therefore, turnover is rapid and new experiments have priority.

Xenotransplantation of H-ras-transfected human bronchial epithelial cells and zip-ras adeno 12 SV40 (hybrid virus)-transfected cell lines routinely produce tumors in nude mice. Similarly, combinations of the immortalizing SV40 T antigen, ras, and raf, have produced tumors in nude mice.

Histological and pathological diagnosis, by high resolution and/or electron microscopy, is performed by an assigned veterinarian pathologist from the contractor's staff.

A Dbase IV computer program, written by a professional programmer for this system, is used for storage and analysis of colony data. The result is completely modernized ability to collect and organize this increasingly complicated and evolving mass of records from this very important research method. These programs are proving to be invaluable in this rapidly expanding field of study.

Xenografts of human tissue (bronchus, pancreatic duct, colon, breast, prostate, and esophagus) are maintained for extended periods (more than 16 months), with viable-appearing epithelium and normal histology, detection of radiolabeled precursors in their epithelial cells, and positive test for human isozymes.

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

October 1, 1990 to September 30, 1991

The Laboratory of Molecular Carcinogenesis (LMC) plans, develops, and conducts research designed to (1) understand the molecular mechanisms 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 the genetic, environmental, and endogenous factors that contribute to 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, identify susceptible populations and prevent human cancer. The 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 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 highly advanced techniques of molecular and cell biology, immunology, enzymology and protein chemistry. The staff are highly experienced in DNA recombinant and related molecular biology techniques, cell biology, protein chemistry, enzyme kinetics and hybridoma technology. The power and precision of these technologies have driven considerable success in the development of 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 Cockayne's syndrome (CS), and with Bloom's syndrome (BS) are being studied. New assays using plasmids as tools to measure DNA repair, DNA end joining, and mutagenesis at the molecular level in cultured human cells were developed. The DNA repair and replication systems in both lymphoblastoid cells and fibroblasts from the same XP patient induced more mutations into the UV treated shuttle vector plasmid, pZ189, than normal controls. There was an additional mutagenic hotspot in the plasmid passed through the XP lymphoblasts not seen with the XP fibroblasts. This demonstrates variability of mutagenic

hotspots with different cell types. CS cells were found to have reduced repair of cyclobutane dimer type photoproducts in the UV treated plasmid normal repair of non-dimer photoproducts. Bloom's syndrome cells were shown to have normal levels of topoisomerase II activity despite increased spontaneous chromosome breakage. The mutagenic properties and specificity of cytochrome P450 activated carcinogens in repair deficient human cells is being studied. Immune studies revealed reduced natural killer (NK) cell activation and impaired interferon production in XP patients. A registry of XP patients has been established. We found high dose (2 mg/kg/da) oral 13-cis retinoic acid to be effective in preventing skin cancers in XP patients but very toxic. A variable response to low dose (0.5 mg/kg/da) oral 13-cis retinoic acid was found in different patients ranging from almost complete tumor prevention to no beneficial effect.

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 P450 with the use of monoclonal antibodies for the detection, purification, and identification of forms of cytochrome P450 responsible for different carcinogen and drug metabolism; (3) the protein structure and membrane topology of cytochrome P450 with the goal of understanding structure-function relationships; and (4) use of expression vectors to produce pure P450 for determination of functional specificity.

Analysis of cytochrome P450 function was determined with the two complementary approaches of inhibitory monoclonal antibodies and cDNA expression. The MAb techniques were used to analyze stereospecific metabolism of benzo(a)pyrene, nitropyrene toluene, methyl-N-amylnitrosamine as well as their DNA binding. Other studies concerned 8-methoxy psoralen metabolism and DNA binding. The MABs also were used to analyze the mechanism of P450 inhibitors of heme destruction. The cDNA expressed P450s were characterized for their enzyme activity and mutagen activation activity for hydrocarbons, heterocyclic amine food pyrolysate products, aflatoxin and nitroasamines. The cDNA expression systems utilized a lytic vaccinia virus vector yielding a large number of animal and human P450s that could be expressed in various host cells including human cells. The cDNAs were also expressed with a herpes-like vector in a stable transfection in a human lymphoblastoid cell line. The complementary methods define the role and contribution of individual P450s in drug and carcinogen detoxification and activation.

UDP-glucuronosyltransferase (UDPGTase) has an important role in the conjugation and excretion of products related to detoxification processes. Monoclonal antibodies (MABs) to the human UDPGTase are being prepared with chemically synthesized peptides of 20 amino acid residues (UDPGTase 394 - 413) essential for UDPGTase activity. One of six hybridomas produced IgG1 type of MABs and five hybridomas produced IgM types. The MABs of each type bound to the immunogen by RIA. The library of MABs to the peptides may be useful for the determination of amino acid sequences essential for UDPGTase activity, and for the identification, reaction phenotyping and quantification of UDPGTase in different organs and tissues of humans.

In order to study the functional role of several P450s and cytochrome b_5 , we have prepared monoclonal antibodies (MABs) to several P450s and b_5 . Thirteen hybridomas were generated to rabbit cytochrome b_5 . Six of the hybridomas produced

IgG1 type of MAbs and seven hybridomas produced IgM types. Six hybridomas were generated to an eight amino acid peptide of P4503A1. The MAbs to b₅ and the P4503A1 peptide may be useful in distinguishing subgroups of the P450 3 family. These MAbs also will be useful for the identification, quantification, and purification of the protein antigens in animal and human tissues.

Progress has been made on the characterization of structure-function relationships for the cytochromes P450. A cross-linking study of rat liver microsomes revealed that P450c is specifically associated with both P450 2a and P450 reductase. Benzo[a]pyrene (BP) metabolism data indicate that P450 2a further metabolizes one of the phenol metabolites generated by P450c. These results support the membrane cluster model in which P450s and P450 reductase exist as stable complexes rather than as monomers. The binding of BP to purified P450c was examined by fluorescence energy transfer and polarization techniques. The results showed that the role of reductase extends beyond that of an electron donor since it also enhances binding of BP to P450c. Monoclonal antibody (MAB) 1-7-1 inhibited reductase-mediated changes, which suggests binding near the reductase receptor region of P450c. A thermodynamic study of the interaction of benzphetamine with microsomal P450b revealed that MAB 4-7-1 alters the P450 spin equilibrium of substrate-bound but not substrate-free P450b, and that a membrane phase transition near 20°C alters the binding of benzphetamine to P450b. A three dimensional model of mammalian P450 is being developed using both theoretical and experimental approaches. The latter includes identification of exposed surface regions on P450s by protease digestion experiments, cross-linking experiments to identify spatially proximate regions within the P450 primary sequence, and the use of antibodies to synthetic P450 peptides to identify functionally significant sequences. A membrane topography study involving microsomal P450c, P450b, and P450j revealed a proteolytically sensitive region common to all three P450s. This region has been identified and corresponds to a predicted turn in a relatively exposed region of the P450 surface. This finding is consistent with the concept of a common tertiary structure for different classes of mammalian P450s. Experiments are underway to evaluate whether the structural integrity of this region is essential for substrate binding and activity.

The ubiquitous superfamily of enzymes, the cytochrome P450s, derive from their ability to catalyze the oxidation of a wide variety of lipophilic endogenous substrates such as steroids, prostaglandins and fatty acids, and exogenous drugs and environmental chemicals. Molecular modeling techniques were developed to predict a cytochrome P450 hydrogen abstraction reaction. The p-nitrosophenoxy radical was used as a model for the P450 active oxygenating species and a linear correlation was observed between ΔH^\ddagger and a combination of ΔH_r and either the modified Swain-Lupton resonance parameter or the ionization potential of the radical formed. The latter relationship gave an estimated standard deviation of the predicted ΔH^\ddagger of 0.8 kcal/mol, suggesting that it may be possible to obtain an estimate of the relative ability for any carbon-hydrogen bond to undergo P450 mediated hydrogen atom abstraction by calculating the relative stability and ionization potential of the resulting radical. This model is now being tested as well as expanded to include aromatic and olefinic oxidations. In addition, reactants and products for 54 hydroxylation and desaturation reactions were modeled and used to predict the relative tendency for each reaction to occur. Finally, a model for the aromatase catalyzed formation of estrogen has been developed.

The cytochromes P450 are a family of isozymes capable of oxidizing a wide variety of both endogenous and exogenous compounds. Two characteristics of these enzymes make it possible for a limited number of isozymes to metabolize a vast and varied array of chemical compounds. The first is due to the generally broad substrate and regio-specificity presumably due to relatively nonspecific substrate binding characteristics and multiple binding orientations. The second is a versatile active oxygenating species that is capable of oxidizing a variety of functional groups. The goal of this research is to explore the mechanisms of oxygen activation, substrate oxidation and the topology of the P450 active sites. Methods used in the project include recombinant DNA techniques, determination of enzyme and isotope effect kinetics, and kinetic analysis of both wild type and mutant enzymes. In the past, we have derived several equations for comprehensive kinetic models to describe the observed kinetic isotope effects on cytochrome P450 catalyzed oxidations. These models suggest that the observed isotope effects can provide information on both binding conformations and the amount of uncoupled electron flux that results in water formation. Previous studies on the metabolism of testosterone by several of the expressed P450 isozymes and their chimeric and mutant forms (developed in the Nucleic Acids Section) revealed that modification of a few amino acid residues in critical positions can markedly affect the pattern of metabolites. Full kinetic analyses is now in the process including isotope effect and stoichiometry experiments to characterize wild-type enzymes and the effect of the mutations on the enzyme mechanisms.

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.

Four general areas are being investigated: 1) Regulation of cytochrome P450s and their genes by inducing agents and during development. Tissue specific gene regulation is also being studied, in particular expression of pulmonary P450s. We uncovered the involvement of the transcription factor HNF-1 in regulation of the constitutively-expressed CYP2E1 gene by use of *in vitro* transcription and DNA-binding studies and by analysis of mutant mice that lack expression of both HNF-1 and CYP2E1. The human CYP4B1 gene promoter is also being studied. This promoter is only expressed in lung cells thus accounting for the tissue specific expression of CYP4B1 in lung Clara cells. 2) The structure-function relationships of P450 are under examination to determine those amino acid residues in the P450 that are required for substrate binding and catalytic activities. cDNA expression and site-directed mutagenesis are being used for these studies. An amino acid residue Phe₃₈₁ determines the substrate selectivity of the CYP2D1 P450 for the drugs bufuralol and debrisoquine. 3) Catalytic activities of human P450s are being studied. Several P450 cDNAs have been isolated and expressed into active enzymes. These are the most abundantly expressed P450s in liver and lung. Catalytic activities towards common drugs and different classes of procarcinogens have been found. For example, five P450s are capable of metabolically activating aflatoxin B₁, whereas only single P450, CYP1A2, exhibits significant activity toward food pyrolysate promutagens. 4) The mechanisms and diagnosis of human P450 polymorphisms are being studied. Normal and mutant P450 genes are isolated and sequenced and this information is used to

develop PCR assays to detect defective P450 genes in the human population. The most common mutant CYP2D6 allele was cloned and completely sequenced. This gene is inactive by virtue of a single G to A base change at the junction of intron 3 and exon 4 which changes the consensus 3 prime splice acceptor site.

Another area under investigation by the Nucleic Acids Section is the regulation of the human thyroid peroxidase gene. The complete gene has been cloned and its promoter dissected and inserted into heterologous expression vectors to determine cis- and trans-acting factors required for thyroid-specific gene expression. A thyroid specific enhancer element was uncovered about 6 kilobase pairs upstream of the transcription start site of the peroxidase gene. A thyroid-specific transcription factor was also cloned and shown to interact with the enhancer element to activate the thyroid peroxidase gene.

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 in normal and neoplastic cells is being investigated. Present efforts are concentrated on determining the cellular function of two non-histone chromosomal proteins, HMG-14 and HMG-17, which may be involved in modulating the structure of transcriptionally active chromatin. We have cloned the human and chicken genes coding for HMG-14 and HMG-17. Analysis of the expression of these genes during myogenesis and erythropoiesis indicates that during differentiation, the HMG-14/-17 mRNA levels are down-regulated. This result is consistent with a role for HMG-14/-17 protein in chromatin patterning. The down-regulation is associated with distinct changes in the chromatin structure of the genes. The expression of chromosomal protein HMG-14, whose gene is located on chromosome 21, is elevated in cells and tissues of patients with Down's syndrome and in trisomy 16 mice. Aberrant expression of protein HMG-14 may have a pleiotropic effect on the expression of several genes and, therefore, may be a contributing factor to the etiology of Down's syndrome. Immunochemical analysis of the organization of the proteins in chromatin indicates that the negatively charged, C-terminal regions are exposed and available to bind to other proteins. In contrast, the central domain is tightly bound to DNA. A peptide corresponding to the DNA binding domain can act as an independent functional entity. These studies are aimed at dissecting the molecules into functional domains and at understanding the molecular mechanisms involved in the generation of transcriptionally active chromatin.

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

PROJECT NUMBER
 Z01CP04496-14 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Chromosomal Proteins and Chromosomal Functions

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

PI:	Michael Bustin	Section Chief	LMC	NCI
Others:	James Pash	Staff Fellow	LMC	NCI
	Massimo Crippa	Visiting Associate	LMC	NCI
	Donald Lehn	Guest Researcher	LMC	NCI
	Pedro J. Alfonso	IRTA Fellow	LMC	NCI

COOPERATING UNITS (if any)

Dept. of Physiology, Johns Hopkins University (R. Reeves); Natl. Center for Biotechnology Info., NLM (David Landsman); NIDDK (A. Wolffe, J. Nickol).

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 5.0

PROFESSIONAL: 5.0

OTHER: 0

CHECK APPROPRIATE BOX(ES)

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

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

The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes in normal and neoplastic cells is being investigated. Present efforts are concentrated on determining the cellular function of two non-histone chromosomal proteins, HMG-14 and HMG-17, which may be involved in modulating the structure of transcriptionally active chromatin. We have cloned the human and chicken genes coding for HMG-14 and HMG-17. Analysis of the expression of these genes during myogenesis and erythropoiesis indicates that during differentiation, the HMG-14/-17 mRNA levels are down-regulated. This result is consistent with a role for HMG-14/-17 protein in chromatin patterning. The down-regulation is associated with distinct changes in the chromatin structure of the genes. The expression of chromosomal protein HMG-14, whose gene is located on chromosome 21, is elevated in cells and tissues of patients with Down's syndrome and in trisomy 16 mice. Aberrant expression of protein HMG-14 may have a pleiotropic effect on the expression of several genes and, therefore, may be a contributing factor to the etiology of Down's syndrome. Immunochemical analysis of the organization of the proteins in chromatin indicates that the negatively charged, C-terminal regions are exposed and available to bind to other proteins. In contrast, the central domain is tightly bound to DNA. A peptide corresponding to the DNA binding domain can act as an independent functional entity. These studies are aimed at dissecting the molecules into functional domains and at understanding the molecular mechanisms involved in the generation of transcriptionally active chromatin.

Project Description

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

Michael Bustin	Section Chief	LMC	NCI
James Pash	Staff Fellow	LMC	NCI
Massimo Crippa	Visiting Associate	LMC	NCI
Donald Lehn	Guest Researcher	LMC	NCI
Pedro J. Alfonso	IRTA Fellow	LMC	NCI

Objectives:

To understand the mechanism of gene regulation and its relation to neoplasia by studying the role of defined chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes in normal and transformed cells. Studies are designed to give insights into the chemical nature of chromosomal proteins, their immunological specificities, the manner in which they interact with DNA, and the regulation of the expression of genes coding for these proteins.

Methods Employed:

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 plasmids, and the DNA sequence determined. The cDNA clones are used to isolate the genes coding for the proteins and to study gene expression in various tissues by analyzing the RNA. Cells are constructed by inserting the cDNA fragment at defined restriction sites in the vector. Cells are transformed by the diethylaminoethyl (DEAE) cellulose method. Transcription is determined by Slot blot, Northern analysis and RNase protection. 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:

We have previously cloned and fully analyzed the cDNAs and genes for HMG-14/-17 from several species. In addition, we prepared antibodies to the proteins and developed techniques for protein purification and analysis. We have used these reagents and methods to study the expression of chromosomal high mobility group proteins HMG-14 and HMG-17 during cellular differentiation in cultured mouse myoblasts and in chicken erythroid cells. During myogenesis the expression of these proteins is down-regulated. In fully differentiated myotubes the mRNA levels decreased by approximately 80%, compared to myoblasts. Likewise, during chicken erythropoiesis the levels of mRNA for these proteins changes significantly. Erythroid cells from 5 day chicken embryos contain 2.5 to 10 times more HMG mRNAs than cells from 14 day

embryos, whereas circulating cells from adult animals are devoid of HMG and most other mRNAs. Nuclear run-off experiments and Northern analysis of RNA from various developmental stages and from Percoll-fractionated cells indicate that the genes are transcribed in early cells of either the primitive or definitive erythroid lineage. The rate of synthesis of the various HMGs changes during erythropoiesis: in erythroid cells from 7 day embryos the ratio of HMG-14b or HMG-17 to HMG-14a is, respectively, 8 and 10 times lower than in 9 day erythroids. HMG-14a, the major chicken HMG-14 species, is synthesized mainly in primitive cells, while HMG-14b is preferentially synthesized in definitive cells. Thus, the change from primitive to definitive erythroid lineage during embryogenesis is accompanied by a change in the expression of HMG chromosomal proteins. Conceivably, these changes in HMG expression in these two experimental systems reflect changes in the structure of certain regions in chromatin; however, it is not presently clear whether the switch in HMG protein gene expression is a consequence or a prerequisite for proper differentiation.

The transcriptional down regulation of the HMG-17 gene, during chicken erythrocyte maturation, is associated with major alterations in the chromatin structure of the gene. The 5' region of the gene contains both constitutive and developmental stage-specific deoxyribonuclease I (DNase I) hypersensitive sites. The constitutive sites bracket the "CpG island" present in the gene, which remains hypomethylated throughout the various developmental stages. During erythropoiesis the gene acquires a distinct structure which, upon digestion with micrococcal nuclease (MNase), yields an unusual repeat. Two nucleosomes, with a 200 bp repeat, are positioned immediately downstream from the start of transcription. Immediately down- and upstream from these nucleosomes the boundaries between MNase sites change to a 75 bp repeat which indicates an unusual chromatin structure. The differentiation related changes in the DNase I and MNase digestion pattern in the 5' region of the gene suggest that sequences present in the first intron may be involved in gene regulation. The results may be relevant to the regulation of the entire HMG-14/-17 gene family.

To study whether the levels of HMG proteins have a direct influence on the differentiation process, myoblast cells were transfected with plasmids expressing HMG protein under the control of the dexamethasone-sensitive MMTV promoter. Stable transformants were selected by resistance to neomycin and cell lines were established. Induction of the MMTV promoter by treatment of the cells with Dexamethasone prevented myotube formation in cells transformed with plasmids expressing the HMG protein but not in colonies transformed with various control plasmids. The results are consistent with the possibility that the differentiation process requires proper expression of the HMG proteins.

The structure of the HMG-14/-17 protein family has features which are similar to those of certain transcription factors. The distribution of charged amino acid residues along the polypeptide chains is asymmetric with positive charges clustered toward the N-terminal region, while the C-terminal regions have the potential to form α -helices with negatively charged surfaces. The ability of HMG proteins to function as transcriptional activators is studied in yeast cells expressing lexA-HMG fusion proteins which bind to reporter molecules containing the β -galactosidase gene downstream from a lexA operator. Fusion constructs expressing deletion mutants of the HMG proteins are also tested. The ability of the fusion construct to bind to their cognate sequences was tested by mobility-shift assays. None of the lexA-HMG fusion proteins were capable of significantly elevating the level of β -galactosidase

activity in transfected yeast cells. Thus, although the structure of the HMG proteins is similar to that of acidic transcriptional activators, these chromosomal proteins do not function as activators in this test system.

In a separate study the organization of the proteins in chromatin was tested. One aim of the study was to determine whether the acidic regions, which potentially may serve some function in transcriptional activation, are available to interact with other macromolecules. Antisera were elicited against synthetic peptides corresponding either to regions common to all HMG-14/-17 proteins or to distinct domains of the HMG-14 or HMG-17 subgroup. The antisera were used to probe the accessibility of various HMG domains in chromatin. Competitive ELISA assays indicate that the central region of the proteins, which contains their DNA binding domain and is positively charged, is exposed to a smaller degree than the C-terminal region of the proteins which has a net negative charge. The C-terminal regions of the HMG-14 and HMG-17 proteins are exposed and available to interact with other proteins.

Recently we found that the HMG-14/-17 proteins specifically bind to nucleosomal cores, as opposed to free DNA. Furthermore, a 22 amino acid long peptide whose sequence corresponds to that of the HMG-14/-17 nucleosome-core binding domain, acts as an independent functional entity. The peptide competes for core particles with the intact protein, the binding of the peptide to the cores stabilizes the core structure, as determined by Tm analysis and, the peptide shifts the mobility of the nucleosome core in a fashion similar to that of the intact protein. These studies are aimed at dissecting the molecule into distinct functional domains.

We found that the gene for human chromosomal protein HMG-14 is located in region 21q22.3, a region associated with the pathogenesis of Down's syndrome, one of the most prevalent human birth defects. Examination of the RNA and protein levels for HMG-14 in both cultured cells and brain tissue samples, obtained from Down's syndrome patients, revealed a gene dosage effect on the expression of this gene. The expression of this gene was also analyzed in mouse embryos that are trisomic in chromosome 16 and are considered to be an animal model for Down's syndrome. Northern analysis and detailed analysis of HMG-14 protein levels indicate that mouse trisomy 16 embryos have approximately 1.5 times more HMG-14 mRNA and protein than their normal litter-mates, suggesting a direct gene dosage effect. The HMG-14 gene may be an additional marker for Down's syndrome. Chromosomal protein HMG-14 is a nucleosomal binding protein which may confer distinct properties to the chromatin structure of transcriptionally active genes and therefore may be a contributing factor in the etiology of the syndrome.

Recently we initiated a study on the effect of HMG-17 on the replication, chromatin assembly and transcription of the satellite I gene of Xenopus laevis. The effects are studied with low-speed extracts of Xenopus laevis eggs. The chromatin assembled in the presence of a large molar excess of HMG-17, has a different linking number than that assembled in the absence of the protein. The presence of the HMG results in a 5-fold elevation in the amount of RNA transcribed from the template. The results are consistent with the possibility that the chromatin assembled in the presence of HMG has an altered conformation and that HMG-17-containing chromatin is more readily transcribed than chromatin lacking this protein.

These studies are pertinent to the elucidation of the molecular mechanisms involved in the generation of transcriptionally active chromatin.

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

PROJECT NUMBER
 Z01CP04517-15 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

DNA Repair in Human Cancer-Prone Genetic Diseases

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

PI: Kenneth H. Kraemer Research Scientist LMC NCI
 Others:
 C. Parris Visiting Fellow LMC NCI S. Adelberg Biologist LMC NCI
 D. Levy IRTA Fellow LMC NCI M. Seidman Spec Volun LMC NCI
 S. Seetharam Spec Volun LMB NCI R. Tarone Biometrician BB NCI
 K. Sanford Section Chief LCMB NCI J. Robbins Sr. Invest. DB NCI
 J. DiGiovanna Expert DB NCI

COOPERATING UNITS (if any)

T. Fleisher Chief, Immuno Serv CC, Dept of Pathology, NJ School of Medicine (W.C. Lambert); New York Blood Center (J. German); Dept of Dermatology, Univ of Rochester (A. Gaspari); Tel Aviv Univ (H. Slor); Kyoto Univ (T. Yagi)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Office of the Chief

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 unreduced type. Do not exceed the space provided.)

Molecular, cellular and clinical abnormalities in patients with xeroderma pigmentosum (XP), with Cockayne syndrome (CS), and with Bloom's syndrome (BS) are being studied. We developed new assays using plasmids as tools to measure DNA repair, DNA end joining, and mutagenesis at the molecular level in cultured human cells. The DNA repair and replication systems in both lymphoblastoid cells and fibroblasts from the same XP patient induced more mutations into the UV treated shuttle vector plasmid, pZ189, than normal controls. There was an additional mutagenic hotspot in the plasmid passed through the XP lymphoblasts not seen with the XP fibroblasts. This demonstrates variability of mutagenic hotspots with different cell types. CS cells had reduced repair of cyclobutane dimer type photoproducts in the UV treated plasmid but normal repair of non-dimer photoproducts. Bloom's syndrome cells were shown to have normal levels of topoisomerase II activity despite increased spontaneous chromosome breakage. The mutagenic properties of cytochrome P450 activated carcinogens in repair deficient human cells is being studied. Immune studies revealed reduced natural killer (NK) cell activation and impaired interferon production in XP patients. A registry of XP patients has been established. We found high dose (2 mg/kg/da) oral 13-cis retinoic acid to be effective in preventing skin cancers in XP patients but very toxic. A variable response to low dose (0.5 mg/kg/da) oral 13-cis retinoic acid was found in different patients ranging from almost complete tumor prevention to no beneficial effect.

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

K. Kraemer	Research Scientist	LMC NCI
C. Parris	Visiting Fellow	LMC NCI
D. Levy	IRTA Fellow	LMC NCI
S. Adelberg	Biologist	LMC NCI
M. Seidman	Special Volunteer	LMC NCI
S. Seetharam	Special Volunteer	LMB NCI
K. Sanford	Section Chief	LCMB NCI
R. Tarone	Biometrician	BB NCI
J. DiGiovanna	Expert	DB NCI
J. Robbins	Sr. Investigator	DB NCI
T. Fleisher	Chief, Immunology Service	CC

Objectives:

Human cancer prone genetic diseases are being studied to identify groups of people with an increased susceptibility to environmental carcinogenesis. We are attempting to 1) understand the molecular basis of the 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 DNA damage-modifying enzymes or with restriction endonucleases to create linear plasmids. The extent and sites of damage are assessed by endonuclease-sensitive site assay. DNA transfection with calcium-phosphate co-precipitation, DEAE dextran or electroporation 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). 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) are examined with particular emphasis on cutaneous abnormalities. Cultures of skin fibroblasts or peripheral blood lymphocytes are established for laboratory analysis. Blood is obtained for plasmid mutagenesis and for immune studies. 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 including patients with both XP and Cockayne's syndrome, a sun sensitive disorder without tumor formation. 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 functioning of cellular repair enzymes. Plasmid expression was up to 100-fold higher in normal than in XP group A fibroblasts and lymphoblasts. In XP-A 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 fibroblasts and lymphoblasts also cannot repair non-dimer photoproducts. In contrast, CS cells are defective in repairing dimer photoproducts but have normal repair of non-dimer photoproducts.

The shuttle vector plasmid, pZ189, was used to measure replication and mutagenesis after UV treatment and transfection into XP-A fibroblasts and lymphoblasts. Plasmid survival was markedly reduced in the XP cells reflecting their repair deficiency and cellular hypersensitivity to the cytotoxic effects of UV. The frequency of mutations introduced into UV damaged pZ189 by DNA repair and replication enzymes of the cells was greater than normal with the XP-A, reflecting the cellular UV-hypermutable of these disorders. The spectrum of mutations in the plasmid passed through the XP-A fibroblasts was similar to that in the XP lymphoblasts. With all lines, more than 85% of the mutations were G:C to A:T transitions. However, different mutagenic hotspots were present with each cell line. In particular, there was an additional mutagenic hotspot in the UV treated plasmid passed through the XP-A lymphoblast line in comparison to the plasmid passed through the fibroblast line from the same patient. This hotspot variability indicates a cell type contribution to UV mutagenesis.

Similar results were obtained with UV treated pZ189 passed through Japanese XP-A and XP-F cells. There was reduced plasmid survival, increased plasmid mutability and a predominance of G:C → A:T base substitution mutations. The spectrum of plasmid mutations with both Japanese cell lines was similar to that of the US XP-A patient despite markedly different clinical features.

Cells from patients with Bloom's syndrome (BS) were reported by others to have reduced *in vitro* DNA ligase I activity, with levels 30 - 50% of normal and increased spontaneous chromosome breakage. We found normal levels of topoisomerase II activity in these BS cells.

In a 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. Patients receiving oral retinoid (13-cis

retinoic acid) showed less chromosome breakage following in vitro X-ray exposure of their peripheral blood lymphocytes, indicating a radioprotective effect of retinoids.

The XP Registry (a joint effort of 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.

We provide clinical consultation to physicians who contact NIH concerning diagnosis or treatment of XP patients. We wrote and published the first information booklet for layman describing XP. A review of 20 years of study of XP at NIH was compiled.

A collaborative long term clinical trial of skin cancer prevention utilizing oral 13-cis retinoic acid (Accutane) in patients with XP is in progress. XP patients were selected who had a documented high rate of skin tumor formation. All pre-existing tumors were surgically removed and high dose (2 mg/kg/da) oral 13-cis retinoic acid was given for 2 years. The drug was then stopped for an additional 1 year observation period. In the 5 XP patients who completed the study there was a greater than 60% reduction in frequency of skin cancer formation during the time of drug treatment and an increase to pre-treatment levels when the drug was withdrawn. All patients experienced the side effect of mucocutaneous toxicity, and, in addition, some patients developed elevated liver function tests, elevated triglycerides and calcification of the tendons and ligaments. Patients who completed the high dose protocol were treated with lower doses to determine if the effectiveness will remain with less toxicity. There was variable response to the low dose treatment ranging from complete tumor suppression to no difference from control interval. Patients had a dose related toxicity. Calcification of ligaments and tendons was observed on x-ray in most treated patients.

Possible immune defects were studied to understand better their role in tumor formation in XP. Natural killer (NK) cell activity was reduced in 5 of 8 XP patients with cancer but normal in 3 others. Thus, reduced NK activity was not linked to cancer. All XP patients studied showed impaired NK cell activation and impaired interferon production in vitro.

Publications:

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

PROJECT NUMBER
 Z010P05086-13 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Monoclonal Antibodies to Human EDP-glucuronosyltransferase

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

PI:	Sang Shin Park	Expert/Guest Scientist	LMC	NCI
Others:	Harry V. Gelboin	Chief, LMC	LMC	NCI
	Waydell Walker	Microbiologist	LMC	NCI

COOPERATING UNITS (if any)

National Institute of Child Health and Development, NIH, (I.S. Owens); Ehwa Womans University, Seoul, Korea (Y.Y. Sheen).

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.)

UDP-glucuronosyltransferase (UDPGTase) is one of the enzymes which plays an important role in the conjugation and excretion of biotransformation products in detoxification processes. In order to study the functional role of UDPGTase in these processes, we are preparing a library of monoclonal antibodies (MAbs) to the human UDPGTase. Female Balb/c mice were immunized with chemically synthesized peptides of 20 amino acid residues (UDPGTase 394 - 413) which are essential for UDPGTase activity. The peptides were conjugated with KLH prior to immunization. Six independent hybridomas were generated by the fusion of myeloma cells with spleen cells from mice immunized with the chemically synthesized peptides. One of the hybridomas produced IgG1 type of MAbs and five hybridomas produced IgM types. All MAbs of each type in culture fluids bound to the immunogen in RIA but not more than 3 times that for nonspecific MAbs. No immunoprecipitin occurred between the MAbs and the peptides in Ouchterlony double immunodiffusion analysis. The library of MAbs to the peptides would be useful for the determination of amino acid sequences which are essential for UDPGTase activity, and also for identification, reaction phenotyping and quantification of UDPGTase in different organs and tissues of humans.

Project Description

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

Sang Shin Park	Expert/Guest Scientist	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Waydell Walker	Microbiologist	LMC	NCI

Objectives:

Uridine diphosphate glucuronosyltransferase (UDPGTase) plays important roles in the metabolism of benzo(a)pyrene, bilirubin, hydrodeoxycholic acid, 3,4-cathecholestrogen and 17-epiestriol. Following the hydroxylation of the substrates by cytochrome P-450, the hydroxylation products become conjugated to UDPG by UDPGTase for secretion. Through chimeric studies of two highly homologous cDNA clones, one of which showed 100 times greater UDPGTase activity, it was found that amino acid residues from 385 to 469 were necessary for full UDPGTase activity. Monoclonal antibodies (MAbs) to the peptides of amino acid sequences required for UDPGTase activity would be useful tools for the determination of the essential amino acid sequences and reaction phenotyping of UDPGTase for different organs and tissues of humans.

Methods Employed:

The peptides of 20 amino acids which correspond to amino acid residues 394 to 413 of human UDPGTase were chemically synthesized and conjugated with KLH. The conjugated peptides were emulsified in Freund's complete adjuvant (10 ug/0.2 ml) and female Balb/c mice were inoculated with 0.2 ml of the mixture. The primed spleen cells were fused with myeloma cells (SP 2/0), hybrid cells were selected in HAT medium, MAb-producing cells were determined by RIA, and mouse Ig subtypings were carried out by Ouchterlony double immunodiffusion. The organ and tissue specific expression of UDPGTase were accomplished by Western blotting with human liver microsomes and each type of MAbs in ascites.

Major Findings:

Six independent hybridomas were obtained by the fusion of myeloma cells with the primed spleen cells. One hybridoma produced mouse IgG1 type of MAbs and five hybridomas produced IgM types. All the MAbs in culture fluids bound significantly to the peptides in RIA but not more than three times that for nonspecific MAbs. Although the MAbs bound to the peptides, no immunoprecipitin reaction was observed between the MAbs and the peptides in Ouchterlony double immunodiffusion analysis. MAb inhibition studies on catalytic activity and reaction phenotyping will be carried out with microsomal preparations from various organs and tissues of humans.

Publications:

Alston K, Robinson RC, Park SS, Gelboin HV, Friedman D. Interaction among cytochromes P-450 in the endoplasmic reticulum: monoclonal antibody-based detection of chemically cross-linked complexes. J Biol Chem 1991;266:735-9.

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

PROJECT NUMBER
 Z01CP05125-10 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Preparation of monoclonal antibodies to rabbit b₅ and rat P-450 3A1

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	LMC	NCI
	Fred K. Friedman	Research Scientist	LMC	NCI
	Richard C. Robinson	Biologist	LMC	NCI
	Waydell Walker	Microbiologist	LMC	NCI
	Tsuyoshi Kakefuda	Oncologist	OIA	NCI

COOPERATING UNITS (if any)

Shinshu University, Shin-Shu, Japan (T. Aoyama)

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 mainly consist of three components: cytochrome P-450 (P-450), NADPH-P-450 reductase and phospholipid. With several P-450s, including 2C6, 2C11, 2B4 and 2E1, cytochrome b₅ (b₅) may participate in the monooxidase pathway by serving in electron transfer from NADPH cytochrome P-450 reductase to P-450. In order to study the functional role of each P-450s and b₅, we are preparing a library of monoclonal antibodies (MAbs) to P-450 and b₅. Female Balb/c mice were immunized with chemically purified rabbit b₅ and the chemically synthesized peptide of N-terminal eight amino acid residues (Lys-Asp-Lys-Glu-Ser-His-Thr-Ala) of rat liver microsomal P-450 3A1 as immunogens. Thirteen hybridomas were generated by the fusion of myelomas with spleen cells from mice immunized with rabbit b₅. Six of the hybridomas produced IgG1 types of MAbs and seven hybridomas produced IgM types. Six hybridomas were generated by the fusion between the myeloma cells and spleen cells from the mice immunized with the peptides. All of the MAbs in culture fluids bound to the immunogens 10 - 20 times greater than control MAbs. The subtypes of MAbs were IgG1, IgG2a and IgM. The MAbs to b₅ would be useful for the study of b₅ function in MFO systems and the MAbs to P-450 3A1 peptide would be more specific in distinguishing subgroups of the P-450 3 family. These MAbs also will be useful for the identification, quantification, and purification of their immunogens 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
Fred K. Friedman	Research Chemist	LMC	NCI
Richard C. Robinson	Biologist	LMC	NCI
Waydell Walker	Microbiologist	LMC	NCI
Tsuyoshi Kakefuda	Oncologist	OIA	NCI

Objectives:

Mixed function oxidase systems (MFO) metabolize many drugs, chemical carcinogens, fatty acids, prostaglandins and some steroids. Cytochromes P-450 (P-450) play a major role in the substrate specificity for the metabolism of their substrates, but the other components of MFO, cytochrome b_5 (b_5) and NADPH-P-450-reductase are also important in determining the balance of the metabolism which leads to carcinogenic or detoxifying processes. The P-450 3 family has many subfamilies of P-450. MABs to specific N-terminal sequences will be very helpful in identifying the subfamily isozymes.

Methods Employed:

Cytochrome b_5 was purified from rabbit liver microsomes, and the peptide of the N-terminal eight amino acid residues (Lys-Asp-Lys-Glu-Ser-His-Thr-Ala) of rat liver microsomal P-450 3A1 was chemically synthesized and conjugated with KLH. The b_5 and peptides were emulsified in Freund's complete adjuvant (10 μ g/0.2 ml) and used to inoculate female Balb/c mice. The primed spleen cells were fused with myeloma cells (SP 2/0), hybrid cells were selected in HAT medium, MAB-producing cells were determined by RIA, and mouse Ig subtypes were carried out by Ochterloney double immunodiffusion.

Major Findings:

Thirteen independent hybridomas were obtained by the fusion of myelomas cells with spleen cells from mice immunized with rabbit b_5 . Six of the hybridomas produced IgG1 types of MABs and seven hybridomas produced IgM types. Six independent hybridomas were obtained by the fusion between the myeloma cells and spleen cells from the mice immunized with the peptides. All the MABs in culture fluids bound to the immunogens 10 - 20 times greater than non specific MABs. The subtypes of MABs were IgG1, IgG2a and IgM. The MABs to b_5 will be useful for the study of b_5 function in MFO systems and the MABs to the P-450 3A1 peptide would be more specific in distinguishing subgroups of the P-450 3 family. These MABs also will be useful for the identification, quantification, and purification of their immunogens in animal and human tissues.

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

PROJECT NUMBER
 Z01CP05318-09 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Structure-Function of Cytochrome P450

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
 Harry V. Gelboin Chief LMC NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control 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.)

The focus of this project is the characterization of structure-function relationships for the cytochromes P450. A cross-linking study of rat liver microsomes revealed that P450c is specifically associated with both P450 2a and P450 reductase. Benzo[a]pyrene (BP) metabolism data indicate that P450 2a further metabolizes one of the phenol metabolites generated by P450c. These results support the membrane cluster model in which P450s and P450 reductase exist as stable complexes rather than as monomers. The binding of BP to purified P450c was examined by fluorescence energy transfer and polarization techniques. The results showed that the role of reductase extends beyond that of an electron donor since it also enhances binding of BP to P450c. Monoclonal antibody (MAb) 1-7-1 inhibited reductase-mediated changes, which suggests binding near the reductase receptor region of P450c. A thermodynamic study of the interaction of benzphetamine with microsomal P450b revealed that MAb 4-7-1 alters the P450 spin equilibrium of substrate-bound but not substrate-free P450b, and that a membrane phase transition near 20°C alters the binding of benzphetamine to P450b. A three dimensional model of mammalian P450 is being developed using both theoretical and experimental approaches. The latter includes identification of exposed surface regions on P450s by protease digestion experiments, cross-linking experiments to identify spatially proximate regions within the P450 primary sequence, and the use of antibodies to synthetic P450 peptides to identify functionally significant sequences. A membrane topography study involving microsomal P450c, P450b, and P450j revealed a proteolytically sensitive region common to all three P450s. This region has been identified and corresponds to a predicted turn in a relatively exposed region of the P450 surface. This finding is consistent with the concept of a common tertiary structure for different classes of mammalian P450s. Experiments are underway to evaluate whether the structural integrity of this region is essential for substrate binding and activity.

Project Description

Names, 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
Harry V. Gelboin	Chief	LMC	NCI

Objectives:

To characterize the structure-function relationships of the multiple forms of cytochrome P450 in both the purified and microsomal state.

Methods Employed:

Cross-linking of microsomal membrane proteins was carried out with the cross-linker sulfosuccinimidyl(4-azidophenyl)dithio)propionate. Cross-linked P450s were identified after disulfide cleavage and identified using monoclonal antibodies to various P450s. Analytical methods employed include gel electrophoresis, Western blots, spectral analyses, and enzyme assays for P450 catalyzed activities. A laser flash photolysis apparatus was designed and assembled from various components and used to monitor the kinetics of CO binding to P450.

Major Findings:

The organization of P450s in the microsomal membrane was studied by cross-linking of microsomal membrane proteins and subsequent immunopurification with monoclonal antibodies to different P450s. P450c was found to be specifically associated with P450 2a, but not P450d or P450j. The data also demonstrated a complex between P450c and P450 reductase, an essential electron carrier during the catalytic cycle. There are two models for P450 membrane organization: 1) P450s are monomeric and freely diffusing; 2) P450s are polymeric and exist in clusters. Our data support the cluster model for P450c, and also show that P450 reductase is part of the cluster. Since P450 substrates are often acted upon by multiple P450s, specific complex formation among P450s may influence secondary metabolism of P450 substrates. We found that the primary phenol products of BP hydroxylation are indeed further metabolized by P450 2a.

The active site structure of purified and microsomal P450c was examined in binding studies using the substrate BP. BP fluorescence was quenched upon binding to P450c and was utilized as a probe for binding of BP to P450c. Addition of P450 reductase increased the affinity of P450c for BP. This demonstrates that in addition to its well-known conventional role as an electron carrier, binding of P450 reductase to P450 also transmits a conformational change to the active site that modulates substrate binding. An inhibitory monoclonal antibody to P450c (1-7-1) inhibited the P450 reductase-induced binding changes, and may thus bind near the reductase receptor region of the P450 surface. Flash photolysis experiments of CO recombination with the P450 heme yielded parallel kinetic data for the effect of BP on active site dynamics.

MAb 4-7-1 to P450b was used to examine the thermodynamics of the P450b-benzphetamine interaction within the microsomal membrane. This MAb alters the P450 spin equilibrium of substrate-bound but not substrate-free P450b. The data also show that a microsomal membrane phase transition occurs near 20°C, which alters the binding parameters of benzphetamine to microsomal P450b. This MAb-based approach has thus successfully characterized the fundamental interactions of a P450 in its native, membrane-bound state. This approach contrasts with conventional methods that involve P450s in artificially reconstituted membrane systems.

We are constructing a three dimensional model of mammalian P450 using both theoretical and experimental approaches. Sequence similarity and structure prediction algorithms along with molecular modeling and the known structure of P450cam have been used to predict structural features of P450d and P450c. Experimental evaluation of the model is in progress. Regions of P450c that are distant in primary structure but spatially proximate are being identified by internal cross-linking of proximate residues of P450c followed by peptide mapping analysis. A membrane topography study involving microsomal P450c, P450b, and P450j revealed a proteolytically sensitive region common to all three P450s. This region has been identified and corresponds to a predicted turn in a relatively exposed region of the P450 surface. This finding is consistent with the concept of a common tertiary structure for different classes of mammalian P450s. Experiments are underway to evaluate whether the structural integrity of this region is essential for substrate binding and activity.

In addition, we are evaluating the use of antibodies to various synthetic P450 peptides for defining the structure-function role of various regions of the primary sequence. P450d peptides have been used to generate polyclonal and monoclonal antibodies to P450d, as detailed in Project Report number Z01CP05436-07. We have also prepared peptides from the rat P450p and human hPCN P450 forms.

Publications:

Alston K, Robinson RC, Park SS, Gelboin HV, Friedman FK. Interactions among cytochromes P450 in the endoplasmic reticulum: monoclonal antibody-based detection of chemically crosslinked complexes. *J Biol Chem* 1991;266:735-9.

Carubelli R, Graham SA, McCay PB, Friedman FK. Prevention of 2-acetylaminofluorene-induced loss of nuclear envelope cytochrome P450 by the simultaneous administration of 3-methylcholanthrene. *Biochem Pharmacol* 1991;41:1331-4.

Myers M, Liu G, Miller H, Gelboin HV, Robinson RC, Friedman FK. Synthetic peptide antigens elicit monoclonal and polyclonal antibodies to cytochrome P450IA2. *Biochem Biophys Res Commun* 1990;169:171-6.

Omata Y, Friedman FK. A fluorescence study of the interactions of benzo[a]pyrene, cytochrome P450c and NADPH-cytochrome P450 reductase. *Biochem Pharmacol* (In Press).

Wilson JD, Miller H, Gelboin HV, Friedman FK. Variation in inducibility of cytochrome P450c and aryl hydrocarbon hydroxylase in rat liver, lung, kidney, pancreas and nasopharynx. *Pharmacology* 1990;41:256-62.

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

PROJECT NUMBER
 Z01CP05436-07 LMC

PERIOD COVERED

October 1, 1990 to September 1991

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

Reaction Phenotyping with Monoclonal Antibodies and cDNA Expressed P-450s

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	Section Chief	LMC NCI
	Kenneth Korzekwa	Senior Staff Fellow	LMC NCI
	Fred Friedman	Research Chemist	LMC NCI
Others:	Sang Shin Park	Guest Researcher	LMC NCI
	Gao Liu	Visiting Fellow	LMC NCI
	Hala Awney	Guest Researcher	LMC NCI

COOPERATING UNITS (if any)

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Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section and Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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 unreduced type. Do not exceed the space provided.)

Analysis of cytochrome P-450 function was determined with the two complementary approaches of inhibitory monoclonal antibodies and cDNA expression. The MAB techniques were used to analyze stereospecific metabolism of benzo(a)pyrene, nitropyrene toluene, methyl-N-amyl-nitrosamine as well as their DNA binding. Other studies concerned 8-methoxy psoralen metabolism and DNA binding. The MABs also were used to analyze the mechanism of P-450 inhibitors of heme destruction. The cDNA expressed P-450s were characterized for their enzyme activity and mutagen activation activity for hydrocarbons, heterocyclic amine food pyrolysate products, aflatoxin and nitroasamines. The cDNA expression systems utilized a lytic vaccinia virus vector yielding a large number of animal and human P-450s that could be expressed in various host cells including human cells. The cDNAs were also expressed with a herpes like vector in a stable transfection in a human lymphoblastoid cell line. The complementary methods define the role and contribution of individual P-450s in drug and carcinogen detoxification and 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
Kenneth Korzekwa	Senior Staff Fellow	LMC NCI
Fred Friedman	Research Chemist	LMC NCI
Sang Shin Park	Guest Researcher	LMC NCI
Gao Liu	Visiting Fellow	LMC NCI
Hala Awney	Guest Researcher	LMC NCI

Objectives:

The goal of this project is to (a) use inhibitory monoclonal antibodies to different P-450s to determine the quantitative contribution of each P-450 to mutagen activation and the metabolism of environmental agents and drugs; (b) express enzymatically active individual human and animal cytochrome P-450s from recombinant vaccinia virus vectors; (c) to analyze the expressed proteins for enzymatic activities; and (d) to test for mutagen activation and cell transformation activity of the individual P-450s.

Methods Employed:

Monoclonal antibodies are produced by the hybridoma technology, and mutagen activation assays are carried out using the Ames test. A variety of drug metabolism assays were used as described in major findings. 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, and 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.

Major Findings:

Four monoclonal antibodies (MAbs) to phenobarbital-induced cytochrome P-450 (PB-P-450) show different patterns of inhibition of PB-P-450 catalyzed aryl hydrocarbon hydroxylase (AHH), 7-ethoxycoumarin deethylase, benzphetamine demethylase and ethylmorphine demethylase. The inhibition constants vary depending on the individual monoclonal antibody and the individual substrate. Two of the four monoclonal antibodies completely inhibit the reduction of cytochrome P-450 by NADPH cytochrome c (P-450) reductase. The same cytochrome P-450 bound to carbon monoxide, however, can be reduced chemically by sodium dithionite in the presence of the monoclonal antibody. These data indicate that the two MAbs examined completely prevent electron transfer by NADPH cytochrome c (P-450) reductase. Substrate binding is partially inhibited by the monoclonal antibody. The type I substrate-binding spectrum of benzphetamine is inhibited more than the type II binding spectrum of

aniline. The degree of inhibition of the substrate binding as indicated by the spectrum is less than that observed for the inhibition of catalytic enzyme activity by the monoclonal antibodies. The data indicate that each of the MABs are directed toward epitopes on the cytochromes P-450 with different relationships to the active catalytic site.

Monoclonal antibodies (MABs) were used to study the contribution of cytochromes P450IA1/IA2, P450IIB1/IIB2, P450IIC11/IIC6 and P450IIE1 to toluene side-chain (benzyl alcohol, BA formation) and ring (o- and p-cresol formation) oxidation in liver microsomes from fed, one-day fasted, and phenobarbital (PB)-, 3-methylcholanthrene (MC)- and ethanol-treated rats. MAB 1-7-1 against P450IA1/IA2 inhibited markedly o-cresol formation and slightly p-cresol formation but not BA formation only in microsomes from MC-treated rats. MABs 2-66-3, 4-7-1 and 4-29-5 against P450IIB1/IIB2 strongly inhibited BA, o-cresol and p-cresol formation only in PB-induced microsomes. MAB 1-68-11 against P450IIC11/IIC6 inhibited BA formation at high toluene concentration in the following order: fed>fasted>ethanol>MC>PB, and ethanol>fed>fasted>MC>PB on the basis of the percentage and net amount of inhibition, respectively. MAB 1-91-3 against P450IIE1 inhibited BA formation at low toluene concentration, but not at high concentration, in the following order: ethanol>fasted>fed>MC, and ethanol>fasted>fed>MC on the basis of percentage and net inhibition, respectively. MABs 1-68-11 and 1-91-3 also inhibited p-cresol formation at high and low toluene concentrations, respectively. These results indicate that (i) both P450IIE1 and P450IIC11/IIC6 are constitutive isozymes mainly responsible for the formation of BA and p-cresol from toluene as low- and high- K_m isozymes, respectively; (ii) P450IIE1, but not P450IIC11/IIC6, is induced by one-day fasting and ethanol treatment; (iii) both P450IIE1 and P450IIC11/IIC6 are decreased by PB and MC treatments; (iv) P450IIE1 is inhibited by a high concentration of toluene; (v) P450IIB1/IIB2 can contribute to the formation of BA, o- and p-cresol from toluene, while P450IA1/IA2 preferentially contributes to the formation of o-cresol.

The proportion and amount of benzo(a)pyrene that binds to DNA through the carcinogenic (+)-anti-benzo(a)pyrene-7,8-diol-9,10-epoxide[(+)-anti-BaPDE] increases with length of time of exposure to BaP in cell cultures derived from a number of species. Primary rat hepatocyte cell cultures were used to establish the role of induction of specific P450 isozymes in this increase in (+)-BaPDE formation. Pretreatment of primary rat hepatocyte cultures for 12 hours with benzo(a)pyrene increased the metabolism of benzo(a)pyrene 47% and the binding of benzo(a)pyrene to DNA by 53% compared with acetone-pretreated hepatocytes during a subsequent show exposure to [3 H]BaP. The amount of (+)-anti-BaPDE bound to DNA increased 275% in DNA from hepatocytes pretreated with benzo(a)pyrene compared to acetone-pretreated cells. Benzo(a)pyrene pretreatment also increased the binding to DNA of 7,8-dihydroxy-7,8-diol-benzo(a)pyrene twofold while the binding of (±)-anti-benzo(a)pyrene-7,8-diol-9,10-epoxide to DNA was the same or slightly decreased compared to acetone-pretreated hepatocytes. Western blotting showed that cytochrome P450IA1 was selectively increased by benzo(a)pyrene treatment. The percentage of benzo(a)pyrene converted to water-soluble metabolites by microsomes from benzo(a)pyrene-pretreated hepatocytes was decreased from 20% to 4% in the presence of a monoclonal antibody which inhibits cytochrome P450IA1 activity. The presence of this monoclonal antibody decreased the binding of benzo(a)pyrene to DNA by almost tenfold. These results indicate that the time-dependant increase in the formation of (+)-anti-BaPDE-DNA adducts results from induction of P450IA1 by the BaP itself. The importance of P450IA1 induction by the BaP for its activation to an ultimate

carcinogenic metabolite suggests that BaP exposure of a cell or organism previously exposed to inducers of P450IA1 could result in activation of a higher proportion of BaP to the carcinogenic (+)-anti-BaPDE.

A monoclonal antibody, MAB 1-68-11, prepared to constitutive cytochrome P-450 IIC11 (2c/RLM5) from male Sprague-Dawley rat liver, was used to study the contribution of the class of cytochrome P-450s epitopically related to P-450 IIC11 to the regiospecific metabolism of benzo[a]pyrene (BP) and its binding to DNA. The effect of MAB 1-68-11 was determined on the conversion of BP to 9,10-dihydrodiol, 7,8-dihydrodiol, 4,5-dihydrodiol, phenols and quinones and the P-450 dependent DNA binding catalyzed by P-450 in microsomes from uninduced male and female Wistar and Sprague-Dawley, as well as 3-methylcholanthrene and phenobarbital (PB) induced male Wistar rat liver. In liver microsomes from untreated male rats, MAB 1-68-11 inhibited BP 9,10-dihydrodiol formation by 80%; with liver microsomes from untreated female rats, the inhibition was 100%. BP 7,8-dihydrodiol formation was inhibited from 38 to 77% in microsomes from males and 50% in those from females. In microsomes from PB-induced rats, inhibition of the 9,10-dihydrodiol and 7,8-dihydrodiol was 90% and 73%, respectively, whereas BP 4,5-dihydrodiol formation was enhanced 80%. In microsomes from 3-methylcholanthrene-treated rats, no inhibitory effect of MAB 1-68-11 was observed on either the metabolism of BP or its binding to DNA. In contrast, the binding of BP to DNA was completely inhibited by MAB 1-68-11 in microsomes from uninduced male Wistar rats and 70% in PB-induced microsomes. ³²P-postlabeling analysis showed that formation of the major stable adduct, BP diol epoxide bound at C-10 to the 2-amino of deoxyguanosine, was strongly inhibited with uninduced and PB-induced microsomes. Formation of the major labile BP-DNA adduct, 7-(BP-6-yl)Gua, was inhibited about 60% with microsomes from untreated male Wistar rats. These results show that MAB 1-68-11 regiospecifically inhibits cytochrome P-450 IIC11 and epitopically related P-450s that metabolize BP at the 7,8,9 and 10 positions. MAB 1-68-11 also inhibits enzyme-catalyzed binding of BP to DNA in the specific formation of 7-(BP-6-yl)Gua and adducts detected by the ³²P-postlabeling technique.

Inhibitory monoclonal antibodies (MAbs) were used to determine the contribution of epitope-specific cytochrome P-450 isozymes in rat liver microsomes to hydroxylation of the esophageal carcinogen methyl-n-amylnitrosamine. These P-450-catalyzed reactions form 2-, 3-, 4-, and 5-hydroxymethyl-n-amylnitrosamine, formaldehyde (demethylation), and pentaldehyde (depentylation). With uninduced microsomes from male rats, MAB 1-68-11 inhibited 4-hydroxylation by 73% and demethylation by 46%. This indicated the major contribution of constitutive male-specific P-450 IIC11 to the metabolism. Inhibition studies with MAbs 2-66-3 and 1-91-3 indicated that P-450 IIB1 contributed 19% and IIE1 35% to demethylation. With uninduced microsomes from females, MAB 1-68-11 produced similar inhibitions to those in male rats, indicating that female-specific P-450 IIC12 (which is closely related to IIC11) also catalyzed 4-hydroxylation and demethylation. With microsomes from 3-methylcholanthrene-induced male rats, P-450 IA1 and/or IA2 were responsible for 60% of 3-hydroxylation and 40% of depentylation. With microsomes from phenobarbital-treated rats, P-450 IIB1 and IIB2 catalyzed all 6 reactions but especially 4-hydroxylation and depentylation, which were 50-75% inhibited by MAB 2-66-3. Microsomes from Aroclor-induced males behaved as if they were induced by both 3-methylcholanthrene and phenobarbital. After treatment with isoniazid (a P-450 IIE1 inducer), inhibition by MAB 1-91-3 indicated a 45% contribution of P-450 IIE1 to demethylation, and both P-450 IIE1 and IIB1 (or IIB2) appear to have been induced. A major finding with

uninduced microsomes was the high specificity of MAb 1-68-11 for inhibiting 4-hydroxylation, indicating that P-450 IIC11 and IIC12 catalyzed most of this ω -1-hydroxylation. In microsomes from induced rats, the MAB inhibitions showed the role of the induced P-450 IA1 (or IA2), IIB1 (or IIB2), and IIE1 in methyl-n-aminonitrosamine hydroxylation at different positions, as well as the presence of P-450 IIC11. This study illustrates the usefulness of inhibitory MABs for defining the contribution of individual P-450s to position-specific metabolism.

The mutagenicity of N-nitroso-N-benzyl-methylamine (NBzMA), N-benzyl-N-nitrosourea (BzNU) and N-methyl-N-nitrosourea (MNU) in Salmonella typhimurium strains was investigated. BzNU selectively mutated TA100 strain as compared to TA1535, whereas MNU showed an inverse strain response, an effect probably related to the fact that benzylation of DNA is a stronger inducer of SOS DNA repair than methylation, as indicated by the higher activity of BzNU in the SOS chromotest. Benzylation of bacterial DNA by NBzMA, as deduced from the differential strain responsiveness, contributed predominantly to its mutagenicity in the presence of liver preparations from untreated, Aroclor, or ethanol-treated rats. Since benzyl alcohol, a metabolite of NBzMA, was not mutagenic in S. typhimurium, it appears that benzyl carbonium cations responsible for the mutagenicity of NBzMA in TA100 are formed via cytochrome P450-mediated hydroxylation of the methyl group. Neither ferric-EDTA nor desferrioxamine altered the mutagenicity of NBzMA, suggesting that activation occurs mainly within the catalytic site of P450. Experiments with isozyme-specific monoclonal antibodies showed that P450IIE1 did not contribute to N-demethylation of NBzMA at either low or high substrate concentrations and that P450IA contributed only weakly. Debenzylation was catalyzed predominantly by P450IA at high NBzMA concentration. Antibodies against rat liver P450IIB enhanced NBzMA mutagenicity in S. typhimurium TA1535 strain up to 17-fold at low substrate concentration, but were without effect at high concentration. In liquid incubation assays, a 100% GSH-dependent reduction of NBzMA mutagenicity was found with liver S9 from untreated Wistar rats. The reducing effect of GSH was less pronounced in the presence of liver S9 from BDVI or Fischer 344 rats.

The in vitro bioactivation of 8-MOP was studied in liver microsomes of male CD-1 mice. In 10-min incubations with 40 μ M [14 C]8-MOP, covalent binding (mean \pm S.D.) was 1.8 \pm 0.4, 3.1 \pm 0.6 and 5.4 \pm 0.4 nmol/mg protein, respectively, in microsomes from mice pretreated for 3 days with vehicle, phenobarbital (PB) or β -naphthoflavone (BNF). A monoclonal antibody (MAB 1-7-1), which recognizes isozymes of cytochrome P-450 induced by 3-methylcholanthrene (P₁-450 and P₂-450), selectively inhibited the metabolism of 8-MOP (-57%) and covalent binding of its metabolites (-40%) in microsomes from mice pretreated with BNF, but had no effect in microsomes of mice pretreated with PB or vehicle. MAB 2-66-3, which recognizes the major isozymes of rat cytochrome P-450 induced by phenobarbital and unknown isozymes in the mouse, enhanced the covalent binding of 8-MOP metabolites in microsomes of mice pretreated with vehicle (+74%), PB (+44%) or BNF (+31%) without affecting the disappearance of 8-MOP. Preincubation of liver microsomes from BNF-pretreated mice with 40 μ M 8-MOP decreased the activity of 7-ethoxycoumarin deethylase (ECD) in a time-dependent manner. Preincubation with 40 μ M 8-MOP for 10 min, decreased the V_{max} from 3.4 to 1.2 nmol/min/mg protein and increased the K_m from 46 to 90 μ M, thus demonstrating mixed competitive and noncompetitive inhibition of ECD. Cysteine trapped three-fourths of the reactive intermediates of 8-MOP but was ineffective in preventing the irreversible inhibition of ECD activity or the 45% spectral loss of cytochrome P-450. Cysteine was ineffective probably because it did not prevent the irreversible

binding of metabolites of 8-MOP to cytochrome P-450. There was no spectral evidence that 8-MOP formed cytochrome P-420 or metabolite-intermediate complexes with cytochrome P-450. These findings support the hypothesis that irreversible inactivation of cytochrome P-450 by 8-MOP is caused by modification of the apoprotein by reactive metabolites.

Various 4-alkyl analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) cause mechanism-based inactivation of cytochrome P-450 (P-450) via heme destruction. We have examined the time course of effects of DDC analogues on the catalytic activities and apoproteins of the major β -naphthoflavone-, dexamethasone-, and phenobarbital-inducible isozymes of rat liver P-450 following *in vivo* administration. In β -naphthoflavone-treated rats, all DDC analogues examined caused loss of the P-450 chromophore and dramatic loss of 7-ethoxyresorufin-O-deethylase activity, a catalytic marker for P-450c. The isopropyl, hexyl, and isobutyl analogues caused the most pronounced loss/alteration of P-450c apoprotein levels, as revealed by two monoclonal antibodies (MAbs), 1-31-2 and 1-7-1. The apoprotein of P-450d was not altered. In dexamethasone-treated rats, all analogues except 4-hexyl-DDC caused loss of the P-450 chromophore and erythromycin N-demethylase activity, a catalytic marker for P-450p-related isozymes. Only 4-isopropyl-DDC caused significant loss/alteration of the apoprotein of P-450p-related forms, as revealed by MAb 2-13-1. In phenobarbital-treated rats, all analogues reduced the level of the P-450 chromophore, whereas only 4-hexyl-DDC and 4-isopropyl-DDC lowered 7-pentoxoresorufin O-dealkylase activity, a catalytic marker for P-450b. MAbs 2-66-3 and 2-8-1 revealed no change in the level of phenobarbital-inducible apoproteins recognized by these probes. In agreement with our previous *in vitro* studies [Mol. Pharmacol. 35:626-634 (1989)], P-450 c and p are targets for mechanism-based inactivation by DDC analogues. However, unlike the situation *in vitro*, loss of enzyme activity *in vivo* is, at least in some instances, accompanied by loss/alteration of the corresponding P-450 apoprotein.

Two peptide sequences from cytochrome P450 IA2 were synthesized, coupled to ovalbumin and used as antigens to generate anti-peptide monoclonal and polyclonal antibodies. Antisera to both peptides reacted with rat IA2 but not the structurally similar IA1 form as determined by enzyme-linked immunosorbent assay. However, antisera to both peptides detected both rat IA2 and IA1 on immunoblots. In addition immunoblots of human liver microsomes revealed that both antisera recognized human IA2, but not IA1. Monoclonal antibodies generated against one of the peptides recognized rat IA2 and IA1 but did not detect human IA2. These results demonstrate the utility of anti-peptide antisera as a practical approach for the generation of P450 specific antibodies.

The role of P450 IA2 in the hydroxylation of acetanilide was examined using an inhibitory monoclonal antibody (MAb) 1-7-1 and vaccinia cDNA expression producing murine P450 IA1 (mIA1), murine P450 IA2 (mIA2), or human P450 IA2 (hIA2). Acetanilide hydroxylase (AcOH) activity was measured using an HPLC method with more than 500-fold greater sensitivity than previously described procedures. This method, which does not require the use of radioactive acetanilide, was achieved by optimizing both the gradient system and the amount of enzyme needed to achieve detection by uv light. MAb 1-7-1 inhibits up to 80% of the AcOH activity in both rat liver microsomes and cDNA expressed mouse and human P450 IA2. MAb 1-7-1, which recognizes both P450 IA1 and P450 IA2, completely inhibits the aryl hydrocarbon hydroxylase (AHH) activity of cDNA expressed in IA1. The inhibition of only 80% of

the AHH activity present in MC liver microsomes by MAb 1-7-1 suggests that additional P450 forms are contributing to the overall AHH activity present in methylcholanthrene (MC)-liver microsomes as MAb 1-7-1 almost completely inhibits the AHH activity of expressed mIA1. Maximal inhibition of IA2 by 1-7-1 results in an 80% decrease in acetanilide hydroxylase activity in both liver microsomes and expressed mouse and human IA2. The capacity of MAb 1-7-1 to produce identical levels of inhibition of acetanilide hydroxylase activity in rat MC microsomes (80%) and in expressed mouse (81%) and human P450 IA2 (80%) strongly suggests that P450 IA2 is the major enzyme responsible for the metabolism of acetanilide. These results demonstrate the complementary utility of monoclonal antibodies and cDNA expression for defining the contribution of specific P450 enzymes to the metabolism of a given substrate.

The metabolism of [³H]-nitropyrene by specific forms of human cytochrome P450 was investigated in vitro using Vaccinia virus expression of P450 cDNAs in HepG2 cells. Cell lysates were infected individually with recombinant Vaccinia virus containing human P450 cDNA (P450 IA2, IIA3, IIB7, IIC8, IIC9, IID6, IIE1, IIF1, IIIA3, IIIA4, IIIA5 and IVB1). Only IIIA3 and IIIA4 demonstrated significant activity in the C-oxidation of 1-nitropyrene. The principal metabolite from both P450 forms was 1-nitropyren-3-ol, produced in at least 4-fold greater abundance than the mixture of 1-nitropyren-6-ol and 1-nitropyren-8-ol, or the K-region dihydrodiols. This is in contrast to the metabolism in many species where 6-ol and 8-ol formation predominate over 3-ol formation. In fact, in rats and rabbits, P450 forms quite distinct from the IIIA P450s catalyze the majority of the metabolism of this pollutant. This is the first demonstration of the role of individual human P450 forms in the metabolism of a representative chemical in this important class of environmental pollutants. The importance of these observations in the overall carcinogenic risk of humans to these chemicals remains to be established. These studies furthermore establish a marked species difference in the metabolism of nitrated polycyclic aromatic hydrocarbons.

Twelve forms of human hepatic cytochrome P450 were expressed in hepatoma cells by means of recombinant vaccinia viruses. The expressed P450s were analyzed for their abilities to activate the potent hepatocarcinogen aflatoxin B₁ to metabolites having mutagenic or DNA-binding properties. Five forms, P450s IA2, IIA3, IIB7, IIIA3, and IIIA4, activated aflatoxin B₁ to mutagenic metabolites as assessed by the production of His revertants of Salmonella typhimurium in the Ames test. The same P450s catalyzed conversion of aflatoxin B₁ to DNA-bound derivatives as judged by an in situ assay in which the radiolabeled carcinogen was incubated with cells expressing the individual P450 forms. Seven other human P450s, IIC8, IIC9, IID6, IIE1, IIF1, IIIA5, and IVB1, did not significantly activate aflatoxin B₁ as measured by both the Ames test and the DNA-binding assay. Moreover, polyclonal anti-rat liver P450 antibodies that cross-react with individual human P450s IA2, IIA3, IIIA3, and IIIA4 each inhibited aflatoxin B₁ activation catalyzed by human liver S-9 extracts. Inhibition ranged from as low as 10% with antibody against IIA3 to as high as 65% with antibody against IIIA3 and IIIA4. These results establish that metabolic activation of aflatoxin B₁ in human liver involves the contribution of multiple forms of P450.

Eight forms of human liver microsomal P-450 were individually expressed in human hepatoma Hep G2 cells with a vaccinia virus cDNA expression system. Using the Ames test, each expressed P-450 was examined for its ability to activate to mutagenic

products the compounds, 2-amino-3-methylimidazo[4,5-f]quinoline, 2-amino-3,4-dimethylimidazo-[4,5-f]quinoline, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline, and 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline, respectively. Three forms of human P-450 significantly activated 2-amino-3-methylimidazo[4,5-f]quinoline when the latter was at high substrate concentrations, but only a single form, P-450IA2, showed very high activation of all promutagens at lower substrate concentrations. Human IA2 had extraordinarily high affinity towards four promutagens tested and is likely the predominant P-450 enzyme responsible for their mutagenic activation in human liver.

A human lymphoblastoid cell line stably expressing a human cytochrome P450IA2 cDNA was developed. This recombinant cell line displayed P450IA2 protein and estradiol 2-hydroxylase activity, neither of which was detected in the parental cell line. The recombinant cell line was also approximately 1000-fold more sensitive to the cytotoxicity and mutagenicity of the carcinogenic mycotoxin aflatoxin B₁ than was the parent cell line. The increase in mutagenicity was supported by a corresponding increase in the level of aflatoxin B₁ binding to DNA in cells expressing P450IA2 relative to control cells.

Five cDNA clones representing coding sequences for human CYP1A2, CYP2A3, CYP2E1, CYP3A4 and microsomal epoxide hydrolase (mEH) were inserted into two independent vectors with independent means of selection in human cells. These vectors were introduced sequentially into a human B-lymphoblastoid cell line which contains native CYP1A1 activity. The resulting cell line, designated MCL-5, stably expressed all 5 cDNAs and the native CYP1A1 as measured at the enzyme level. The mutagenicity of 7 model procarcinogens was examined at the hypoxanthine guanine phosphoribosyl transferase (hgrprt) and thymidine kinase (tk) loci. Direct exposure to benzo(a)pyrene (BP), 3-methylcholanthrene (3MC), N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), aflatoxin B₁, (AFB), 2-acetylaminofluorene (AAF) and benzidine (BZD) resulted in a statistically significant increase in mutant frequency. The lowest exposure-concentrations to yield significant increases in mutant fraction were 3 ng/ml BP, 30 ng/ml 3MC, 20 ng/ml NDMA, 100 ng/ml NDEA, 5 ng/ml AFB, 20 ug/ml AAF and 100 ug/ml BZD. For most of these procarcinogens, the response observed at the lowest concentration tested was substantially above the limit of detection. Calculation (via linear interpolation) of the concentration of procarcinogen necessary to result in a doubling of mutant fraction in MCL-5 cells and the parent AHH-1 cell line revealed that, for all chemicals except BZD, MCL-5 cells were significantly more sensitive than AHH-1 cells. The increase in sensitivity ranged from 3 fold for AAF to greater than 40,000 fold for NDMA. The MCL-5 cell system has the potential to be a useful screening tool for the examination of human procarcinogen activation.

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

PROJECT NUMBER
 Z01CP05521-04 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Polymorphic Drug Oxidation: The Human CYP2D6 Gene

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

PI: Frank J. Gonzalez Section Chief LMC NCI
 Others: Harry V. Gelboin Chief, LMC LMC NCI
 Shioko Kimura Visiting Scientist LMC NCI

COOPERATING UNITS (if any)

Biocenter University of Basel, Switzerland (Urs A. Meyer); Gentest Corporation, Woburn, Massachusetts (Charles Crespi)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH Bethesda, MD 20892

TOTAL MAN-YEARS: .75	PROFESSIONAL: .75	OTHER: 0
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(a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

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

The debrisoquine/sparteine genetic polymorphism is due to mutant alleles of CYP2D6. Individuals lacking production of this cytochrome P450 are unable to metabolize over 30 drugs. In several instances, lack of metabolism of a drug results in toxicity or exaggerated responses. About 7.5% of Caucasians possess two mutant alleles of CYP2D6. In order to characterize these mutant genes and in an effort to develop simple polymerase chain reaction-based diagnostic tests, a mutant CYP2D6 allele was directly cloned from leukocyte DNA taken from an individual that was unable to metabolize debrisoquine. The gene was completely sequenced and found to contain a G to A transition that alters the consensus 3 prime splice site at the junction of intron 3 and exon 4 of CYP2D6. A simple non-radioactive PCR test was developed, based on this single mutation, that can be used to screen individuals by analyzing leukocyte DNA for presence of mutant P450 genes. This mutant allele, designated CYP2D6(B), represents about 75% of all mutant CYP2D6 genes.

A second mutant CYP2D6 gene, designated CYP2D6(C), was identified that produces a protein lacking a single amino acid. This enzyme is catalytically active but is not stably expressed in human liver. A polymerase chain reaction-based test was also developed for CYP2D6(C). This is a rare allele representing less than 2% of all mutant genes.

Complementary DNA expression systems have been developed to evaluate the ability of CYP2D6 to metabolize drugs and activate chemical carcinogens. A lymphoblastoid cell containing the CYP2D6 cDNA was constructed and used to demonstrate metabolic activation of a carcinogen found in tobacco smoke.

Project Description

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

Frank J. Gonzalez	Section Chief	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Shioko Kimura	Visiting Scientist	LMC	NCI

Objectives:

1. To determine the sequences of mutant CYP2D6 alleles.
2. To develop polymerase chain reaction-based diagnostic tests for mutant CYP2D6 alleles.

Methods Employed:

Human leukocyte genomic libraries were constructed using the vector lambda EMBL 3. DNAs were sequenced by shotgun cloning into M13 and dideoxy chain termination reactions. Sequence data were analyzed by use of the Beckman Microgenie program.

Major Findings:

The debrisoquine polymorphism is a common genetic defect that results in deficient oxidation of debrisoquine and numerous other drugs. These compounds are metabolized by a form of cytochrome P450, designated CYP2D6. Some 5-10% of Caucasians are unable to metabolize debrisoquine, because of mutant alleles of CYP2D6. A CYP2D6 allele was isolated from leukocyte DNA of an individual who was deficient in debrisoquine metabolism. The gene was completely sequenced, including 725 bp of upstream and 400 bp of downstream DNA. Several base changes were uncovered within the exons, resulting in four amino acid differences between the mutant and wild-type allele. Most important, a single base change, G₁₉₃₄ - A, at the junction of the third intron and fourth exon would result in an incorrectly spliced primary transcript and an mRNA having a single base deletion. This deletion presumably disrupts the mRNA reading frame, resulting in a truncated protein. These data establish unequivocally that the debrisoquine polymorphism is the result of mutant CYP2D6 alleles and provide a framework to design a genetic test for this drug oxidation deficiency. A defective CYP2D7 allele was also isolated and completely sequenced, providing evidence that gene conversions have occurred between CYP2D6 and CYP2D7.

A variant CYP2D6 P450 protein was found in livers characterized by deficient microsomal metabolism of bufuralol and sparteine, prototypical substrates for the debrisoquine/sparteine drug oxidation polymorphism. This protein was present at decreased levels in liver and had a slightly lower relative mobility on SDS-polyacrylamide gels. cDNA cloning and sequencing of the variant, designated CYP2D6(C), revealed that its mRNA lacked a single codon resulting in deletion of Lys₂₈₁. This was the result of a 3-base-pair deletion at the 3' end of CYP2D6 exon 5. The CYP2D6(C) P450, produced in Hep G2 cells using vaccinia-virus mediated cDNA expression, displayed Kms toward bufuralol, debrisoquine and sparteine that were not significantly different from wild type CYP2D6. These data indicate that the poor

metabolizer phenotype in livers expressing CYP2D6(C) is due to a quantitative decrease of the P450 protein in microsomal membranes. A polymerase chain reaction-based procedure, developed to detect CYP2D6(C) alleles, indicates that this variant probably represents less than 2 % of all CYP2D6 alleles.

Epidemiological studies have suggested that individuals who are deficient in CYP2D6 expression and who smoke are protected against lung cancer. To explore the biochemical basis of this association we examined the ability of cDNA-expressed CYP2D6 to metabolically activate carcinogens found in tobacco smoke. We have developed a human B-lymphoblastoid cell line, designated 2D6/Hol, which stably expresses the human cytochrome P450 CYP2D6 cDNA. This cell line exhibits bufuralol 1'-hydroxylase activity and an immunologically detectable CYP2D6 protein. The specific activity of (+)-bufuralol 1'-hydroxylase in microsomes from 2D6/Hol cells was comparable to that observed in human liver microsomes. This cell line was used to examine the mutagen activation of three tobacco smoke-derived nitrosamines, N-Nitrosornicotine (NNN), 1-(N-Methyl-N-nitrosamine)-1-(3-pyridyl)-4-butanol (NNA) and 4-(Methylnitrosamino)-1-butanone (NNK), by CYP2D6. Exposure of 2D6/Hol cells to NNK concentrations of 30 to 90 µg/ml induced a concentration-dependent decrease in relative survival and an increase in mutant fraction at the hypoxanthine guanine phosphoribosyl transferase (hprt) locus. In contrast, NNK was non-mutagenic and non-cytotoxic to control cells at exposure concentrations up to 150 µg/ml. NNK mutagenicity in 2D6/Hol cells was compared to the responses observed in isogenic cell lines expressing human CYP1A2 (1A2/Hol), human CYP2A3 (2A3/Hol) and human CYP2E1 (2E1/Hol). These three additional human cytochrome P450-expressing cell lines were also found to be sensitive to NNK-induced mutagenicity and cytotoxicity. We found no evidence of CYP2D6-mediated activation of NNN or NNA. NNN was non-cytotoxic and non-mutagenic to both control and 2D6/Hol cells. NNA was equally cytotoxic and mutagenic to control cells and 2D6/Hol cells. The activation of NNA to a mutagen may have been carried out by a P450 native to the AHH-1 TK⁻ cell line. The 2D6/Hol cell line, in conjunction with the control cell line and other isogenic cell lines expressing other human cytochrome P450 cDNAs, provides a useful system for the examination of the role of the polymorphic CYP2D6 in human procarcinogen activation and drug metabolism.

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Sugimura H, Caporaso NE, Shaw GL, Modali RV, Gonzalez FJ, Hoover RN, Resau JH, Trump BF, Weston, A, Harris CC. Human debrisoquine hydroxylase gene polymorphisms in cancer patients and controls. Carcinogenesis 1990; 11:1527-30.

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

PROJECT NUMBER
Z01CP05522-04 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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: Shiko Kimura Visiting Scientist LMC NCI

Others: Kimio Mizuno Visiting Associate LMC NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 1.5 PROFESSIONAL: 1.5 OTHER: 0

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

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

Thyroid peroxidase is only expressed in the thyroid gland and plays a major role in synthesizing the thyroid hormones, T3 and T4. Patients with autoimmune thyroid disorders, such as Graves' disease and Hashimoto's thyroiditis, recognize thyroid peroxidase as an autoantigen and produce autoantibodies against it resulting in thyroid hypertrophy and destruction of normal thyroid function.

The cDNA and the gene for human thyroid peroxidase have been used as probes to understand the molecular mechanism of thyroid hormone synthesis and of thyroid autoimmunity. We have recently identified a thyroid-specific enhancer element of 230 bp that is located approximately 5.5 kbp upstream of the gene's transcription start site. By using a double-stranded oligonucleotide within this 230-bp sequence, which showed the strongest affinity towards a DNA binding protein(s), we were able to clone a cDNA encoding a thyroid-specific enhancer-binding protein designated T/EBP.

Expression of the T/EBP conferred thyroid-specific enhancer activity to nonpermissive human hepatoma Hep G2 cells when a T/EBP cDNA-expression vector was co-transfected with a luciferase reporter gene connected to the human thyroid peroxidase gene upstream sequence containing the critical enhancer element. Vaccinia virus-expressed T/EBP was further shown by gel mobility shift assays, to specifically bind to the enhancer-derived double-stranded oligonucleotide. These results clearly indicated that the binding of T/EBP to the specific *cis*-acting enhancer element is largely responsible for thyroid-specific enhancer activity.

Project Description

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

Shioko Kimura	Visiting Scientist	LMC	NCI
Kimio Mizuno	Visiting Associate	LMC	NCI

Objectives:

1. Clone and characterize a cDNA encoding a thyroid-specific enhancer-binding protein (T/EBP).
2. Characterize T/EBP in terms of binding to the specific DNA sequence and its role for enhancer activity.

Methods Employed:

A lambda gt11 library constructed from rat thyroid FRTL-5 cells poly(A) RNA, was screened without amplification by using a ³²P-labeled double-stranded oligonucleotide. Sequence of the oligonucleotide corresponded to one of the three DNA-protein binding regions within a 230-bp enhancer element that exhibited the highest affinity towards the thyroid-specific DNA-binding proteins. A cDNA clone thus obtained which encodes a T/EBP was subcloned into pUC9 and sequenced by using the shotgun cloning and dideoxy chain terminator sequencing methods. Expression of the T/EBP mRNA was determined by Northern blot analyses using total RNAs obtained from various rat tissues. A cDNA encoding T/EBP was subcloned into an expression vector pCMV4 which expresses T/EBP under control of human cytomegalovirus major immediate early gene promoter. This vector DNA was transfected into human hepatoma Hep G2 cells by using calcium phosphate precipitate method together with a plasmid containing the luciferase reporter gene preceded by either 6.3 or 3.1 kbp of upstream DNA derived from the human thyroid peroxidase gene. T/EBP was further expressed by using the vaccinia-virus expression system and the expressed protein was analyzed by gel mobility shift and DNase I footprinting assays.

Major Findings:

- We have previously identified a thyroid-specific enhancer element of 230 bp that lies approximately 5.5 kbp upstream of the human thyroid peroxidase transcription start site. We have further determined three cis-acting DNA elements that are encompassed within the 230-bp enhancer element, all of which appear to be thyroid-specific as judged by DNase I footprinting analyses. An attempt to isolate a cDNA(s) coding for an enhancer-binding protein(s) was begun by using the in situ detection system of sequence-specific DNA binding activity specified by a recombinant bacteriophage. A double-stranded oligonucleotide whose sequence corresponds to one of three thyroid-specific DNA-protein binding regions that exhibited the strongest affinity towards DNA-protein bindings, was used as probe to screen a lambda gt11 FRTL-5 rat thyroid library. A single cDNA clone was isolated from approximately 5x10⁵ recombinant phage. This cDNA insert was used to rescreen the library and three more cDNA clones were obtained.

Nucleotide and deduced amino acid sequences of the clones revealed that all four cDNA sequences are basically identical to those of thyroid-specific transcription factor 1 (TTF-1) that has been reported by Guazzi et al. This factor has been characterized as a new type of mammalian homeodomain-containing DNA-binding protein which confers thyroid specificity to thyroglobulin gene expression. Northern blot analyses of total RNAs obtained from various rat tissues using the homeodomain sequence as a probe revealed that T/EBP mRNA is expressed in not only thyroid but also lung. This is very interesting since neither thyroid peroxidase nor thyroglobulin is expressed in lung. In order to determine if T/EBP by itself reconstitutes enhancer activity upon binding to an enhancer element, human thyroid peroxidase genes upstream DNA of about 6.3 and 3.1 kbp were connected to the promoter-less luciferase vector. Based on our previous results on identification of a thyroid-specific enhancer sequence, the 6.3-kbp luciferase construct contains an enhancer element, whereas the 3.1-kbp construct does not.

These construct DNAs were co-transfected with DNA of the recombinant vector that expresses T/EBP under control of human cytomegalovirus major immediate early gene promoter, into human hepatoma Hep G2 cells and luciferase activity was measured. Hep G2 cells were previously shown to exhibit no enhancer activity when used as host cells in transfection experiments of human thyroid peroxidase upstream sequence deletion mutants. Further, Northern blot analyses of RNAs from rat liver and Hep G2 cells, using homeodomain sequence as a probe, did not detect any mRNA corresponding to T/EBP. DNA co-transfection experiments of the construct expressing T/EBP with only the 6.3- kbp luciferase construct markedly enhanced luciferase activity, but not with the 3.1-kbp construct. This enhancing activity was detected as high as 26-fold.

T/EBP was further expressed in Hep G2 cells using the vaccinia-virus expression system and the expressed protein was shown to specifically bind to the enhancer-derived specific oligonucleotide by gel mobility shift analyses. We believe that the binding of T/EBP to its specific *cis*-acting enhancer element is the principle event responsible for enhancer activity. Specific binding of the expressed protein to its specific enhancer element is also being studied by using DNase I footprinting assays.

Publications:

Booth KS, Caughey WS, Kimura S, Ikeda-Saito M. Calcium binding sites in myeloperoxidase and lactoperoxidase. In: Heizmann C, ed. Novel calcium-binding proteins. Heidelberg: Springer Press (In Press)

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Isozaki O, Tsushima T, Emoto N, Saji M, Tsuchiya Y, Demura H, Sato Y, Shizume K, Kimura S, Kohn LD. Methimazole regulation of thyroglobulin biosynthesis and gene transcription in rat FRTL-5 thyroid cells. Endocrinology (In Press)

Kikkawa F, Gonzalez FJ, Kimura S. Characterization of a thyroid-specific enhancer located 5.5 kilobase pairs upstream of the human thyroid peroxidase gene. *Mol Cell Biol* 1990;10:6216-24.

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

PROJECT NUMBER
 Z01CP05561-04 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Transcription Regulation of Cytochrome P450 Genes

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

PI:	Frank J. Gonzalez	Section Chief	LMC	NCI
Others:	Harry V. Gelboin	Chief, LMC	LMC	NCI
	Su-yan Liu	Visiting Fellow	LMC	NCI
	Talia Sher	Visiting Associate	LMC	NCI
	Masahiko Yano	Visiting Associate	LMC	NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 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 unreduced type. Do not exceed the space provided.)

P450 genes are either individually or are constitutively expressed. The inducible genes are usually expressed at low levels in the absence of an inducing agent. Constitutively expressed P450 genes are also developmentally regulated. Typically P450s are absent in livers of animals prior to birth. Immediately after parturition, several P450 genes are activated and become fully expressed within a few days after birth. To understand the mechanism of this developmentally programmed gene activation, the CYP2E1 gene is being studied. The upstream DNA of this gene becomes demethylated immediately after birth coincident with its transcription activation. The nuclear transcription factor HNF-1 was found to bind to a DNA element immediately adjacent to the CYP2E1 promoter and this binding is thought to mediate transcription. Nuclease hypersensitivity analysis indicated that the chromatin of the CYP2E1 gene becomes sensitive within one day after birth suggesting that a factor is bound to the DNA. It is still unknown whether demethylation or HNF-1 binding is the signal of developmental gene activation.

A strain of radiation deletion mice were found that have a deletion of a 1.2 centiMorgan segment of DNA on chromosome 7. These mice die within a few days after birth due to liver failure. Analysis of CYP2E1 gene expression revealed that newborn mice homozygous for the deletion do not express CYP2E1. Heterozygotes and homozygous normal mice do produce CYP2E1 mRNA. Since the Hnf-1 gene is on chromosome 5, and CYP2E1 is on chromosome 7, but proximal to the deleted region in these mice, the lack of CYP2E1 gene expression might be due to the binding of a trans-acting transcription factor that is epistatic in the regulatory cascade that includes HNF-1 expression.

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

Frank J. Gonzalez	Section Chief	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Su-yan Liu	Visiting Fellow	LMC	NCI
Shioko Kimura	Visiting Scientist	LMC	NCI
Masahiko Yano	Visiting Fellow	LMC	NCI

Objectives:

1. To characterize the cis-acting DNA elements required for control of P450 genes.
2. To identify DNA factors that bind to the cis-acting elements and initiate the events required for transcriptional activation of P450 genes.

Methods Employed:

In vitro nuclear extracts were prepared and used to study factor binding to CYP2E1 gene DNA and transcription using a template derived from the CYP2E1 gene. Mice heterozygous for a deletion on chromosome 7 were mated and offspring were phenotyped using the eye pigment color test. RNA was prepared and analyzed for CYP2E1 and beta-actin mRNA using Northern blotting. Nuclei were prepared from newborn and adult rats and digested with DNase I. DNase-I-hypersensitive sites in the CYP2E1 gene were assayed by Southern blotting.

Major Findings:

In vitro transcription of the cloned CYP2E1 gene and various derivatives of the gene in which upstream DNA was deleted were used to establish the presence of a cis-acting DNA element. Gel mobility shift assays using double-stranded DNA of known liver-specific transcription factors were used to determine whether this domain could bind to the hepatocyte nuclear factor-1 (HNF-1). A variant form of HNF-1 was found in newborn and prenatal rat liver at a time in development in which the CYP2E1 gene was not transcribed.

Nuclease sensitivity studies were carried out on liver nuclei of prenatal newborn and adult rats. A hypersensitive site in the DNA upstream of the CYP2E1 gene was found only in nuclei of rats expressing the gene. This site was not precisely coincident with the HNF-1 binding cis-acting element. The CYP2E1 gene in liver nuclei of rats not expressing the gene was resistant to nuclease digestion indicating a tight DNA protecting chromatin structure. A model was developed in which the chromatin of the CYP2E1 gene opens up after birth allowing binding of HNF-1 and perhaps other factors resulting in transcriptional activation.

Thus, in vitro evidence suggests that the rat CYP2E1 gene is positively regulated by the transcription factor HNF-1 or protein displaying DNA-binding properties similar to HNF-1. To investigate the relationship between HNF-1 and CYP2E1 an in vivo model was used. In contrast to newborn mice, homozygous mice for this deletion do not show significant expression of the CYP2E1 and Hnf-1 genes. However, the CYP2E1 and Hnf-1 structural genes are not in the chromosome 7 segment deleted in these mice.

Although CYP2E1 maps to chromosome 7, it is distal to this deletion; Hnf-1 maps to chromosome 5. These data suggest that the deleted region of chromosome 7 contains a gene encoding a trans-acting factor which is upstream (epistatic) in the regulatory cascade that includes Hnf-1 gene expression.

Publications:

Gonzalez FJ, Liu S-Y, Kozac CA, Nebert DW. Decreased Hnf-1 gene expression in mice homozygous for a 1.2-centiMorgan deletion chromosome 7. *DNA Cell Biol.* 1990; 9: 771-776.

Ueno T, Gonzalez FJ. Transcriptional control of the rat hepatic CYP2E1 gene. *Mol Cell Biol* 1990;10:4495-505.

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

PROJECT NUMBER
 Z01CP05562-04 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Identification and Characterization of New Human P450s

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

PI:	Frank J. Gonzalez	Section Chief	LMC	NCI
Others:	Harry V. Gelboin	Laboratory Chief	LMC	NCI
	Hirokazu Furuya	Visiting Fellow	LMC	NCI
	Maceij Czerwinski	Pre IRTA	LMC	NCI
	Kenneth R. Korzekwa	Senior Staff Fellow	LMC	NCI

COOPERATING UNITS (if any)

Genest Corporation, Woburn, MA (C. Crespi); Department of Medicinal Chemistry, University of Washington, Seattle, WA (W. Trager, A.E. Rettie); Department of Environmental Health, Case Western Reserve University, Cleveland, OH (Paul Howard)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Nucleic Acids Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 3.5

PROFESSIONAL: 3.5

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.)

Cytochrome P450s, the principal enzymes of foreign compound metabolisms, oxidize and inactivate most of the clinically used drugs so that they can be more easily eliminated from the body. P450s also activate chemical carcinogens to highly reactive electrophiles capable of binding to DNA and mutating genes. A large degree of species differences exist for P450 genes, particularly between rodents and humans. These differences are at the regulatory and catalytic level and are most prevalent between genes in the various CYP2 subfamilies. The CYP2A, CYP2B and CYP2C subfamilies are particularly complex with many members. cDNAs encoding two different human CYP2A and CYP2B P450s and four CYP2C P450s have been identified in humans. Since numerous rodent P450s within each of these subfamilies have been identified and the presence of multiple human CYP2A, CYP2B and CYP2C genes are suggested by Southern blotting analysis, we believe that other human P450s within these subfamilies have yet to be identified. We therefore developed strategies using polymerase chain reactions (PCR) to find and isolate new human P450s. CYP2C18 was identified by PCR and its cDNA was cloned and completely sequenced. The cDNA was expressed into a protein with a relative molecular weight of 49,000. We have not, however, identified a favorable substrate for this enzyme. A PCR-based assay was developed to quantify levels of CYP2C8, CYP2C9 and CYP2C18 mRNA.

Studies are in progress to determine the catalytic specificities of human P450s using cDNA expression. Metabolism of the anticoagulant warfarin was studied using vaccinia virus-expressed P450s. Metabolism of carcinogens to mutagenic metabolites was analyzed by use of the lymphoblastoid cDNA expression system.

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

Frank J. Gonzalez	Section Chief	LMC	NCI
Harry V. Gelboin	Laboratory Chief	LMC	NCI
Hirokazu Furuya	Visiting Fellow	LMC	NCI
Shioko Kimura	Visiting Scientist	LMC	NCI
Kenneth R. Korzekwa	Senior Staff Fellow	LMC	NCI

Objectives:

- 1) To identify and clone cDNAs for new human P450s.
- 2) To produce catalytically active P450s by cDNA-directed expression.

Methods Employed:

Total RNA was isolated from human liver specimens using guanidine isothiocyanate. DNA was prepared from human liver and leukocytes. Polymerase chain reactions (PCR) were carried out using standard protocols supplied by Cetus-Perkin Elmer. PCR products were subcloned into pUC9 and sequenced by the dideoxynucleotide chain termination strategy. cDNA-directed expression of human P450s was carried out using vaccinia virus. Enzyme assays were performed using standard procedures and HPLC for metabolite separations.

Major Findings:

Sequencing of genomic polymerase chain reaction (PCR) products synthesized using primers generated from the CYP2C8 and CYP2C9 cDNAs revealed the presence of a new CYP2C gene in the human genome. Primers specific to exons of this new gene were used to further perform PCR on human liver cDNA libraries and cDNA synthesized from human liver mRNA to generate a cDNA containing the complete cytochrome P450 amino acid reading frame. This P450 cDNA, designated CYP2C18, displayed 87% and 85% nucleotide and 81% and 77% amino acid sequence similarities, respectively, with cDNAs and proteins of CYP2C8 and CYP2C9. cDNA-directed synthesis of CYP2C18 revealed a protein with a relative Mr 49,000 which is considerably less than that calculated from the deduced amino acid composition Mr 55,747. A preferred substrate for this enzyme has not been uncovered. Expression of CYP2C8, CYP2C9 and CYP2C18 mRNAs were examined in seventeen human liver specimens using a PCR-based assay and normalization to tubulin mRNA. CYP2C18 mRNA was found in all livers examined albeit at mean levels of seven- to eightfold lower than mRNAs encoding CYP2C8 and CYP2C9. Marked interindividual differences in levels of expression of all three CYP2C mRNAs were also found.

The anticoagulant effect of racemic warfarin is mediated largely by the (S)-enantiomer, which is approximately six times more potent than its antipode. Inhibition of the formation of (S)-7-hydroxywarfarin, the inactive, major metabolite

of racemic warfarin in humans, is known to be the cause of several of the drug interactions experienced clinically upon co-administration of warfarin with other therapeutic agents. In order to investigate approaches that would lead to the establishment of meaningful *in vitro-in vivo* correlations for these interactions, we have determined the catalytic activity of eleven human liver cytochrome P-450 forms expressed in Hep G2 cells, towards phenol formation from both (R)- and (S)-warfarin. P450s 2A3, 2D6, 2E1 and 4B1 displayed no detectable catalytic activity towards either enantiomer of the drug. P450 1A2 was predominantly an (R)-6-hydroxylase. P-450s 2B7 and 2F1 were (R)-4'-hydroxylases, whereas P450 2C8 was principally an (S)-4'-hydroxylase. The major phenolic metabolite obtained from P450 3A4 was also (S)-4'-hydroxywarfarin. However, this form was principally an (R)-10-hydroxylase, as was 3A5. P450 2C9 was the only form examined which was predominantly an (S)-7-hydroxylase. In addition, this form was highly stereoselective, and did not form detectable quantities of phenolic metabolites from (R)-warfarin. Although both 1A2 and 3A4 did form appreciable quantities of (S)-7-hydroxywarfarin, consideration of their overall metabolite profiles indicated that they must contribute to a negligible extent to the clearance of (S)-warfarin *in vivo*. Both P450 2C9 and human liver microsomal preparation from three separate donors exhibited a K_m for (S)-warfarin of 4 μ M. Sulfaphenazole selectively inhibited the metabolism of (S)-warfarin in human liver microsomes. Inhibition of the (S)-7-hydroxylase was competitive, with a K_i of 0.21 μ M. The K_i for inhibition of 2C9-mediated metabolism of (S)-warfarin by sulfaphenazole was 0.18 μ M. Therefore, P450 2C9 exhibits the appropriate regioselectivity, stereoselectivity, Michaelis-Menten and inhibition kinetics expected for the human liver P450 isozyme responsible for the major metabolic pathway of the biologically more potent (S)-enantiomer of warfarin. It is concluded that, despite the overall complexity of warfarin metabolism in humans, only those drugs that can effectively inhibit P450 2C9 need to be considered as agents that have the potential to produce clinically significant drug interactions by an inhibitory mechanism.

The metabolism of the carcinogen [3 H]-1-nitropyrene by twelve specific forms of human cytochrome P450 was also investigated. Only CYP3A3 and CYP3A4 demonstrated significant activity in the C-oxidation of 1-nitropyrene. The principal metabolite from both P450 forms was 1-nitropyren-3-ol, produced in at least 4-fold greater abundance than the mixture of 1-nitropyren-6-ol and 1-nitropyren-8-ol, or the K-region dihydrodiols. This is in contrast to the metabolism in many species where 6-ol and 8-ol formations predominate over 3-ol formation. In fact, in rats and rabbits, P450 forms quite distinct from the CYP3A P450s catalyze the majority of the metabolism of this pollutant. The importance of these observations in the overall carcinogenic risk of humans to these chemicals remains to be established. These studies furthermore establish a marked species difference in the metabolism of nitrated polycyclic aromatic hydrocarbons.

To determine carcinogen activation by human P450s, we developed a human lymphoblastoid cell line, designated 3A4/Ho1, which stably expresses human CYP3A4 cDNA. This cell line exhibited testosterone 6 β -hydroxylase activity, produced immunologically detectable CYP3A4 protein and was more sensitive to the cytotoxicity and mutagenicity of the carcinogenic mycotoxin aflatoxin B₁ (AFB₁) than was the parent cell line. The concentration-response for AFB₁ cytotoxicity and mutagenicity in 3A4/Ho1 cells was compared to the responses of isogenic cell lines expressing comparable levels of human CYP1A2 (1A2/Hyg cells) and human CYP2A3 (2A3/Hyg cells). 1A2/Hyg cells were 3- to 6-fold more sensitive than 3A4/Ho1 cells to AFB₁-induced

mutation. 3A4/Hol cells were 10- to 15-fold more sensitive to AFB₁-induced mutation than 2A3/Hyg cells. The differences in mutagenicity were supported by the relative binding of [³H]-AFB₁ to cellular DNA. Cell lines were also developed expressing multiple P450 cDNAs including CYP1A2, CYP2A6, CYP2E1 and CYP3A4. These cells are responsive to a large number of procarcinogens including the nitrosamines and polycyclic aromatic hydrocarbons.

Publications:

Aoyama T, Yamano S, Guzelian PS, Gelboin HV, Gonzalez FJ. Five of twelve forms of vaccinia-expressed human hepatic cytochrome P-450s metabolically activate aflatoxin B₁. Proc Natl Acad Sci USA 1990;87:4790-3.

Crespi CL, Penman BW, Steimel DT, Gelboin HV, Gonzalez FJ. The development of a human cell line stably expressing human CYP3A4: role in the metabolic activation of aflatoxin B₁ and comparison to CYP1A2 and CYP2A3. Carcinogenesis 1991;12:355-9.

Furuya H, Meyer UA, Gelboin HV, Gonzalez FJ. Polymerase chain reaction-directed identifications, cloning and quantification of human CYP2C18 mRNA. Molec Pharmacol (In Press).

Gonzalez FJ, Crespi CL, Gelboin HV. cDNA-expressed human cytochrome P450s: a new age of molecular toxicology and human risk assessment. Mutat Res 1991;247:113-27.

Howard PC, Aoyama T, Bauer SL, Gelboin HV, Gonzalez FJ. The metabolism of 1-nitropyrene by human cytochromes P450. Carcinogenesis 1990;11:1539-42.

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

PROJECT NUMBER
 Z01CP05651-02 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Structure and Function Analysis of P450

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

PI:	Frank J. Gonzalez	Section Chief	LMC	NCI
Others:	Harry V. Gelboin	Laboratory Chief	LMC	NCI
	Kenneth R. Korzekwa	Senior Staff Fellow	LMC	NCI
	Shinji Tamura	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)

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: 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.)

Cytochrome P450s are a group of enzymes capable of metabolizing foreign chemicals of diverse structure. A single form of P450 can oxidize a large number of different compounds and thus a finite number of P450s can deal with numerous chemicals ingested in the diet, in the form of drugs and environmental pollutants, and inhaled from the air. P450s require the enzyme NADPH-P450 oxidoreductase and, in some cases, catalytic activities are potentiated by cytochrome b₅. The structure of mammalian P450s is unknown since none of these membrane-bound enzymes have been crystallized. This contrasts the soluble bacterial P450cam whose substrate-free and substrate-bound three dimensional structures are known. The amino acid residues responsible for catalytic activities and substrate specificities of the mammalian P450s are being elucidated by cDNA expression in conjunction with site directed mutagenesis studies. It is difficult, however, to select potential sites to change amino acid residues by sequence comparisons or by extrapolation of a theoretical mammalian P450 structure based on the bacterial enzyme. We have discovered allelic variants of rat P450s that contain different catalytic activities and only one or a few amino acid differences. These have been exploited to determine those residues responsible for catalytic activities of mammalian P450s towards drugs, chemical carcinogens, and other chemicals.

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

Frank J. Gonzalez	Section Chief	LMC	NCI
Harry V. Gelboin	Laboratory Chief	LMC	NCI
Kenneth R. Korzekwa	Senior Staff Fellow	LMC	NCI
Shinji Tamura	Visiting Fellow	LMC	NCI

Objectives:

- 1) To identify new allelic variants of rat P450 having different catalytic activities.
- 2) To determine which regions of the P450's primary sequence or which amino acids confer substrate specificities of P450s.

Methods Employed:

cDNA libraries are constructed from rat liver poly-A RNA using the vector lambda gt11. The libraries are screened using existing rat cDNAs as probes. The cDNAs are subcloned into plasmid vectors and sequenced using the dideoxynucleotide chain termination strategy. cDNAs are inserted into vaccinia virus and the recombinant viruses are used to infect human hepatoma Hep G2 cells. Levels of P450s are measured by CO-reduced difference spectra. Enzyme assays with full kinetic measurements are carried out by standard protocols using HPLC and fluorescence detection.

Major Findings:

A cDNA coding for an allelic variant of rat CYP2D1, designated CYP2D1v, was isolated that produced a P450 having a 10-fold lower catalytic activity toward the substrate bufuralol when expressed in COS-1 cells. CYP2D1 and CYP2D1v cDNA-deduced proteins differed in sequence by 4 amino acid residues. CYP2D1 has Val, Phe, Arg, and Ile while CYP2D1v has Ile, Leu, Gln and Phe at amino acid positions 123, 124, 173 and 380, respectively. Chimeric cDNAs between CYP2D1 and CYP2D1v were constructed and expressed in hepatoma cells using vaccinia virus. A chimera having the Phe (CYP2D1v) at amino acid 380, with the remaining 3 variant amino acid residues of CYP2D1, was found to have a 17-fold decrease in V_{max} and a 2- to 3-fold decrease in K_m for (+)-bufuralol 1'-hydroxylation when compared to a converse chimera having Ile (CYP2D1) in a background of CYP2D1v sequence. Although this enzyme lacked significant bufuralol metabolism, it was able to carry out debrisoquine 4-hydroxylation. In contrast, the chimera having Ile (CYP2D1) at position 380 was lacking in debrisoquine 4-hydroxylation. Type I difference spectra analysis revealed that both forms could bind debrisoquine with similar spectral dissociation constants. These data demonstrate that the single amino acid substitution Ile³⁸⁰ - Phe differentially decreases the catalytic activity of CYP2D1 toward bufuralol but not debrisoquine.

Publications:

Aoyama T, Nagata K, Yamazoe Y, Kato R, Matsunaga E, Gelboin HV, Gonzalez FJ. Cytochrome b₅ potentiation of P450 catalytic activity demonstrated by a novel vaccinia virus-mediated in situ reconstitution system. Proc Natl Acad Sci USA 1990;87:5425-9.

Hanioka N, Korzekwa K, Gonzalez FJ. Sequence requirements for cytochrome P450IIA1 and P450IIA2 catalytic activity: evidence for both specific and non-specific substrate binding interaction through use of chimeric cDNAs and cDNA expression. Protein Eng 1990;3:571-5.

Matsunaga E, Zeugin T, Zanger UM, Aoyama T, Meyer UA, Gonzalez FJ. Sequence requirements for cytochrome P450IID1 catalytic activity: a single amino acid change (Ile₃₈₀ Phe) specifically decreases V_{max} of the enzyme for bufuralol but not debrisoquine hydroxylation. J Biol Chem 1990;265:17197-201.

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

PROJECT NUMBER
 Z01CP05676-01 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Theoretical Models for Cytochrome P450 Mediated Oxidations

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

P.I. Kenneth Korzekwa Senior Staff Fellow LMC NCI

Others: Dr James Grogan IRTA LMC NCI
 Dr Harry 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, MD 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.)

The ubiquitous superfamily of enzymes, the cytochrome P450s, continue to be the focus of many diverse research efforts. Interest in these heme containing monooxygenases stem from their ability to catalyze the oxidation of a wide variety of lipophilic endogenous (i.e., steroids, prostaglandins and fatty acids) and exogenous (drugs and environmental contaminants) compounds. Because of their importance in drug metabolism and toxicity, predictive models for cytochrome P450 oxidations could be extremely useful. We have previously used molecular modeling techniques to develop a predictive model for cytochrome P450 hydrogen abstraction reactions. Using the p-nitrosophenoxy radical as a model for the P450 active oxygenating species, a linear correlation was observed between ΔH^\ddagger and a combination of $\Delta H R$ and either the modified Swain-Lupton resonance parameter or the ionization potential of the radical formed. The latter relationship gave an estimated standard deviation of the predicted ΔH^\ddagger of 0.8 kcal/mol, suggesting that it may be possible to obtain an estimate of the relative ability for any carbon-hydrogen bond to undergo P450 mediated hydrogen atom abstraction by calculating the relative stability and ionization potential of the resulting radical. We are now in the process of testing this model as well as expanding it to include aromatic and olefinic oxidations. In addition, reactants and products for 54 hydroxylation and desaturation reactions were modeled and used to predict the relative tendency for each reaction to occur. Finally, a model for the aromatase catalyzed formation of estrogen has been developed.

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

Kenneth Korzekwa	Senior Staff Fellow	LMC NCI
James Grogan	IRTA	LMC NCI
Harry Gelboin	Chief	LMC NCI

Objectives:

Since almost any new drug or xenobiotic will serve as a substrate for the cytochrome P450 system, a predictive model for reactions catalyzed by these enzymes would be an extremely useful tool. It can be expected that the ability of a drug or foreign compound to be metabolized by a particular isozyme will be a function of both its binding characteristics (steric factors) and the tendency of various sites within a molecule to undergo oxidation (electronic factors). The first objective of this research is to use theoretical calculations to define the electronic factors which govern the general susceptibility of any given C-H bond toward cytochrome P450 mediated hydrogen atom abstraction. The second objective of this project is to use theoretical calculations to study the mechanisms of other P450 catalyzed reactions, in particular desaturations and aromatizations.

Methods Employed

The semiempirical quantum chemical methods AM1 and PM3 are used for all calculations. All calculations are performed on a Silicon Graphics work station. The UHF Hamiltonian is used for all open shell calculations. Stable geometries were optimized from approximate starting geometries, reaction coordinates were generated by constraining and varying the appropriate oxygen-hydrogen and/or carbon-hydrogen bond lengths. Approximate transition states are optimized with the gradient minimization routines provided by MOPAC. SCF calculations were optimized to $<10^{-8}$ kcal/mol and geometries were optimized to $<10^{-4}$ Angstroms. Most stable geometries and all transition states have been verified by the presence of 0 and 1 negative eigenvalues of the force constant matrix, respectively.

Major Findings:

We presently have two predictive models for P450 mediated oxidations. The first estimates the tendency for a substrate to undergo P450 mediated hydrogen atom abstraction. A small molecule which has thermodynamic properties and transition state symmetry (semiempirical, AM1) similar to the active oxygenating species of P450 is the p-nitrosophenoxy radical. The predictive model uses the calculated (AM1) heats of formation of reactants and products and either ionization potentials or another resonance parameter, i.e., Swain-Lupton R, to predict activation energies for hydrogen atom abstraction by the model radical ($R^2=0.95$, $n=20$). We are presently modeling aromatic and olefinic oxidations in the same manner, and hope to combine models to provide a method for predicting the electronic tendency (neglecting steric effects) for any position of a drug to be oxidized by P450. We are now testing this model to see if a correlation can be found between the predicted oxidation rates and the toxicity of a series of nitriles.

A second model uses the calculated heats of formation of hydroxylated and unsaturated products to predict the relative tendencies for the formation of desaturated and hydroxylated products by the P450 enzymes. We have presently predicted and experimentally verified four new substrates which undergo P450 mediated desaturation.

A study has been completed in which various potential pathways for the third oxidation of aromatase were modeled. The calculated thermodynamic data suggested that this reaction is likely to proceed by abstraction of the 1β -hydrogen atom, followed by heterolytic cleavage of the C_{10} - C_{19} bond. An additional step was also suggested to take place in which a protein nucleophile adds to the aldehyde intermediate to induce enolization of the 2-position.

Publications:

Korzekwa KR, Jones JP, and Gillette JR. Theoretical studies on cytochrome P-450 mediated hydroxylation: a predictive model for hydrogen atom abstractions. J Amer Chem Soc 1990;112:7042-6.

Korzekwa, KR, Trager WF, Smith SJ, Osawa Y, Gillette JR. Theoretical studies on the conversion of estrogens to androgens by aromatase. Biochemistry (In Press).

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

PROJECT NUMBER
 Z01CP05677-01 LMC

PERIOD COVERED

October 1, 1990 to September 30, 1991

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

Studies on the Active Sites and Mechanisms of Cytochromes P450

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

P.I. Kenneth Korzekwa Senior Staff Fellow LMC NCI

Others: Dr Gao Liu Vist. Fellow LMC NCI
 Dr James Grogan IRTA LMC NCI
 Hala Awney Guest Researcher LMC NCI
 Dr Frank Gonzalez Section Chief LMC NCI
 Dr Harry Gelboin Chief LMC NCI

COOPERATING UNITS (if any)

Dr James R. Gillette Chief LCP NHLBI

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SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

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 unrounded type. Do not exceed the space provided.)

The cytochromes P450 are a family of isozymes capable of oxidizing a wide variety of both endogenous and exogenous compounds. Two characteristics of these enzymes make it possible for a limited number of isozymes to metabolize a vast and varied array of chemical compounds. The first is the generally broad substrate and regiospecificity presumably due to relatively nonspecific substrate binding characteristics and multiple binding orientations. The second is a versatile active oxygenating species that is capable of oxidizing a variety of functional groups. The goal of this research is to explore the mechanisms of oxygen activation, substrate oxidation and the topology of the P450 active sites. Methods used in the project include recombinant DNA techniques, determination of enzyme and isotope effect kinetics, and kinetic analysis of both wild type and mutant enzymes. In the past, we have derived several equations for comprehensive kinetic models to describe the observed kinetic isotope effects on cytochrome P450 catalyzed oxidations. These models suggest that the observed isotope effects can provide information on both binding conformations and the amount of uncoupled electron flux that results in water formation. Previous studies on the metabolism of testosterone by several of the expressed P450 isozymes and their chimeric and mutant forms (developed by Dr. Frank Gonzalez) revealed that modification of a few amino acid residues in critical positions can markedly affect the pattern of metabolites. We are now in the process of performing full kinetic analyses, including isotope effect experiments, and stoichiometry experiments to characterize wild-type enzymes and the effect of the mutations on the enzyme mechanisms.

Project Description

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

Kenneth Korzekwa	Senior Staff Fellow	LMC NCI
Gao Liu	Vist. Fellow	LMC NCI
James Grogan	IRTA	LMC NCI
Hala Awney	Guest Researcher	LMC NCI
Frank Gonzalez	Section Chief	LMC NCI
Harry Gelboin	Chief	LMC NCI

Objectives:

Binding Orientations and the Mechanism of Oxygen Activation: Rat cytochrome P450 2A1 is ~90% homologous with P450 2A2 yet they have very different metabolic profiles for testosterone metabolism. Previous studies suggested that both specific and non-specific binding are involved with these enzymes. Twenty-six single point mutants have been prepared by Dr. Frank Gonzalez's group, and we are now in the process of analyzing each of these mutants using kinetic analyses and isotope effects. Analyses of these specific changes in amino acid sequence can provide valuable information concerning the absolute nature of the substrate binding sites for these enzymes as well as the effect of these changes on the efficiency of oxygen activation.

Stoichiometry Studies: The use of stoichiometry can provide information on the efficiency of the P450 isozymes as well as experimental data on the tendency of a functional group to undergo oxidation by cytochrome P450. Uncoupling to hydrogen peroxide formation is apparently in competition with heterolytic dioxygen cleavage and water formation is in competition with substrate oxidation. We are in the process of developing a more sensitive hydrogen peroxide assay in order to assess the extent of its formation with the expressed enzymes. Isotope effects can be used to directly measure the amount of water formation.

Methods Employed:

Enzyme preparations include purified enzymes, microsomal preparations, and cell preparations from the expressed cloned P450s using the vaccinia virus vector prepared by Dr. Frank Gonzalez's group. Stoichiometry is determined by reported methods using a GC-MS method to quantitate hydrogen peroxide. Deuterated substrates are synthesized by standard synthetic methods, and analysis of metabolites is accomplished by HPLC, GC, and GC-MS techniques.

Major Findings:

Binding Orientations and Mechanism of oxygen Activation:

P450 2A1 and P450 2A2, while showing very different metabolic profiles for testosterone, share 90% homology. Previous studies suggested that the differences responsible were located in a region associated with the I and J helices. Kinetic

analyses of the fifteen 2A1 single point mutants associated with that region have revealed that changes in the region of the I helix around the putative oxygen binding site give large changes in K_m and V_{max} . The isotope effect experiments suggest that these mutations also change the efficiency of the enzymes. However, the analyses of all 26 single point mutants shows that no one amino acid is responsible for the differences in regioselectivity of these two isozymes.

Stoichiometry: We have developed an assay to quantitate hydrogen peroxide in the pmol-nmol range. The assay uses horseradish peroxidase to catalyze the metabolism of dimethylaniline to methylaniline. A deuterated internal standard has been prepared and the product is quantitated by GC-MS.

Preliminary studies on the influence of cytochrome b5 on the isotope effect for testosterone metabolism by P450 2A1 suggests that the increased metabolism is due to a decrease in the amount of uncoupling to form water.

ANNUAL REPORT OF

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

October 1, 1990 through September 30, 1991

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 by CPCB encompass a broad range of subject areas, with principal emphasis on: environmental carcinogenesis, including 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 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 Branch also supports the synthesis, acquisition, and distribution of a wide range of chemical standards that are critically needed in the field of carcinogenesis research.

CPCB utilizes a variety of funding instruments to accomplish its objectives. These include the traditional investigator-initiated research project grant (R01); program project grant (P01); First Independent Research Support and Transition (FIRST) award (R29); conference grant (R13); cooperative agreement (U01); contract (N01); Small Business Innovative Research (SBIR) grant (R43/44); SBIR contract (N43/44); Academic Research Enhancement Award (AREA) (R15); Outstanding Investigator Grant (OIG) award (R35); and the Method to Extend Research In Time (MERIT) award (R37). Currently, the Branch administers 477 research grants with an annual budget of approximately 73.95 million dollars.

Grants and contracts administered by the staff of CPCB support six complementary categories of chemical and physical carcinogenesis research and associated resources: Biological and Chemical Prevention; Carcinogenesis Mechanisms;

Nutritional Carcinogenesis (formerly Diet and Nutrition); Molecular Carcinogenesis; Experimental Tobacco Carcinogenesis (formerly Smoking and Health); and Research Resources.

The Biological and Chemical Prevention component is concerned with experimental inhibition of carcinogenesis caused by chemical, physical and biological agents. Efforts are devoted to 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 selected preventive agents, binding proteins and receptors, structure-function relationships, and 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; 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; characterization of carcinogen metabolizing enzymes; and the role of hormones in carcinogenesis.

The Nutritional Carcinogenesis category supports basic studies on the carcinogenic effects of dietary constituents such as lipids, proteins, and macronutrients, as well as specific compounds formed during food preparation or by gut microflora. These myriad classes of chemicals are being studied in animal systems and in humans in vivo and in vitro.

The Molecular Carcinogenesis component focuses on changes in biological macromolecules and 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 the genetics and mechanism of cell transformation and the genetics and regulation of enzymes characteristically associated with the carcinogenesis process.

The Experimental Tobacco Carcinogenesis category supports studies on the toxicology and pharmacology of smoking and tobacco-related exposures. The research is directed toward understanding the nature of the chemicals involved and the reaction mechanisms behind the deleterious effects of tobacco smoke and smokeless tobacco products. A number of studies have used tobacco-specific nitrosamines to induce animal cell-line models of important human cancers such as oat-cell lung and pancreatic carcinomas. The synergistic effect of alcohol and tobacco-derived carcinogens is also under investigation. Both grant and contract mechanisms are used to support these activities.

The Research Resources component is principally concerned with the synthesis of selected chemical carcinogens and certain of their metabolites and their distribution through a repository. The inventory of over 800 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. This component uses the resource contract mechanism to support the synthesis and distribution activities.

During the last year, the Branch awarded one new outstanding investigator grant of 7 years duration to Dr. P. James Whitlock, Jr. (1 R35 CA53887-01), Stanford University. The long-term goal of this OIG application is to understand the molecular mechanism of 2,3,7,8,-tetra-chlorodibenzo-p-dioxin (TCDD, dioxin) action, particularly the mechanisms by which TCDD exposure alters gene expression.

Grants that deal with specific organ sites have been coded in the Branch for the last few years in order to track progress and trends. Together with grants that were already resident in CPCB, some of the current information is as follows: bladder (12) \$1.82 M; breast (46) \$6.15 M; colon (19) \$2.11 M; pancreas (7) \$0.59 M; prostate (5) \$0.73 M; and upper aerodigestive (16) \$2.59.

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 1991 - Estimated)

	No. of Contracts/Grants	\$ (Millions)
Research Contracts	1	0.25
Research Grants	477	73.95
Traditional Research Grants (R01) (356 grants; \$43.68 Million)		
Conference Grants (R13) (13 grants; \$0.05 Million)		
FIRST Awards (R29) (29 grants; \$3.07 Million)		
Program Project Grants (P01) (16 grants; \$10.49 Million)		
Cooperative Agreements (U01) (5 grants; \$1.69 Million)		
Small Business Grants (R43/R44) (6 grants; \$0.75 Million)		
Outstanding Investigator Grants (R35) (9 grants; \$6.85 Million)		
RFAs (R01) (14 grants; \$1.71 Million)		
MERIT Awards (R37) (25 grants; \$5.45 Million)		
AREA Grants (R15) (4 grants; \$0.20 Million)		
Research Resource Contracts	<u>5</u>	<u>1.77</u>
	483	75.97

TABLE II
 CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
 (Contracts and Grants Active During FY 1991)

	CONTRACTS		GRANTS	
	No. of Contracts	\$ (Millions)	No. of Grants	\$ (Millions)
Biological & Chemical Prevention	0	0	72	11.93
Carcinogenesis Mechanisms	0	0	144	20.73
Nutritional Carcinogenesis	0	0	32	3.12
Molecular Carcinogenesis	0	0	218	36.38
Tobacco Carcinogenesis	1	0.25	11	1.79
Research Resources	5	1.77	0	0
TOTAL	6	2.02	477	73.95

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 experimental animals and humans. Agents can derive from naturally occurring products such as foods consumed by man, from chemical synthesis, or from various biological sources. Currently there are 67 grants in this program area with FY91 funding of approximately \$10.24 million, and five cooperative agreements with FY91 funding of approximately \$1.69 million. There are no contracts.

The portfolio consists of 51 Traditional Research Projects (R01s); four Program Projects (P01s); four First Independent Research Support and Transition (FIRST) awards (R29s); one Academic Research Enhancement Award (AREA) (R15); three conference grants (R13s); one Method to Extend Research In Time (MERIT) award (R37); one Outstanding Investigator Grant (OIG) (R35); one Phase 1 Small Business Innovative Research (SBIR) grant (R43); and one Phase 2 SBIR grant (R44). Research currently supported encompasses the following categories: retinoids; antioxidants; natural inhibitors; micronutrients including selenium, vitamin C and vitamin E; carotenoids; protease inhibitors; omega-3 polyunsaturated fatty acids; and miscellaneous areas. Included in the miscellaneous areas category are grants studying protein kinase C inhibitors, vitamin D analogs, isothiocyanates, inhibitors of the arachidonic acid cascade, dehydroepiandrosterone, glucarates, terpenoids, amino acids, dithiolethiones, ellagic acid and derivatives, calcium, immunochemoprevention, and the role of exercise in prevention of the carcinogenic process. The types of studies supported are diverse and include the experimental inhibition of carcinogenesis, 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. A 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 in 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, 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

Vitamin E/Free Radical Protection. Radiation, chemical detoxication/intoxication, and endogenous processes of oxygen metabolism are known to generate reactive free radicals of several types which are injurious to living tissues and important mediators in the carcinogenic process. Vitamin E (α -tocopherol) is a major chemoprotective agent against such oxidative injury by virtue of its radical-trapping abilities. It has exceptional reactivity toward peroxy radicals, effectively suppressing membrane lipid peroxidation at concentrations typically as low as one molecule per 1000 phospholipid molecules. In its initial reaction with peroxy radicals, α -tocopherol reacts to produce a hydroperoxide and the relatively stable tocopheroxyl radical. Sustained antioxidant protection has been postulated to depend on reductive regeneration of α -tocopherol from the tocopheroxyl radical by

ascorbic acid, or by a glutathione-dependent microsomal enzyme reduction of the oxidized tocopherol species. Continuous supply of vitamin E is necessary, however, since the tocopheroxyl radical itself may trap a second peroxy radical to form other products. Recently, the redox chemistry of α -tocopherol has been studied in biomimetic model systems in which an endeavor was made to identify all of the products generated from α -tocopherol upon reaction with the peroxy radical ($ROO\cdot$). The study employed a phosphatidylcholine liposome system to model biological membranes with thermolysis of 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) at 37°C in order to generate peroxy radicals. Reaction products were purified by high-performance liquid chromatography and then characterized by UV-vis spectroscopy, mass spectrometry, and cochromatography using authentic standards. The investigations involved quantitative determinations of the yield of each reaction product, kinetics of product formation, precursor-product relationships of isolated, purified products, and the influence (or lack thereof) of α -tocopherol and peroxy radicals upon the various oxidative steps. Experimental results indicate that two major oxidative pathways exist for peroxy radical oxidation of α -tocopherol in these lipid bilayers. The first oxidative mechanism is consistent with the behavior of α -tocopherol as a radical chain-breaking antioxidant. In this pathway, each molecule of tocopherol consumed traps two peroxy radicals. This occurs by initial (peroxy radical) hydrogen abstraction from the 6-OH constituent of tocopherol to give the tocopheroxyl radical, followed by the addition of a second peroxy radical to the 8a position to give an 8a-(alkyldioxy)-6-tocopherone as a principal product. The predominant fate of this product is to α -tocopherol quinone by simple hydrolysis, with a quite small fraction decomposing by a separate pathway to epoxyquinones. The second product-forming mechanism yields two epoxytocopherones (7,8-epoxy-8a-hydroperoxy-tocopherone and 4a,5-epoxy-8a-hydroperoxytocopherone). In this pathway there is peroxy radical-dependent epoxidation of the tocopheroxyl radical, followed by oxygen addition to the 8a-position (rather than peroxy radical addition at the 8a-position) forming epoxytocopheroxyl peroxy radicals, and then hydrogen abstraction from the milieu to give the epoxyhydroperoxytocopherones. This second pathway results in no net radical trapping. In particular, this pathway can consume tocopherol molecules in a cyclic fashion if the hydrogen donor from the milieu is tocopherol itself interacting with its metabolite. This autoxidation of vitamin E can presumably have a significant probability in natural membranes as well, considering its exceptional reactivity toward peroxy radicals (in this case its own peroxy radical). Indeed, results from various test systems studied thus far suggest that competition between radical-trapping and autoxidation reactions of the tocopheroxyl radical varies with the structure of the attacking peroxy radical and with the reaction environment. Moreover, previous studies by the present group demonstrate that, in homogenous solution, peroxy radicals derived from AMVN react with tocopherol to yield products identical to those found in the lipid bilayers, but with differing relative abundances. An interesting aspect of both the homogenous solution and lipid bilayer studies is that tocopherol dimers and trimers were not found as oxidation products (40).

Dehydroepiandrosterone/Prediction of Cancer Risk in Humans. Dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) are endogenous steroids produced in the adrenal cortex upon stimulation by adrenocorticotrophic hormones and subsequently excreted in the urine. Except for cholesterol, DHEAS is the most abundant steroid in the circulation with levels 300-500 times higher than DHEA. In experimental animals, DHEA and several DHEA analogs have been shown to inhibit/suppress 1,2-dimethylhydrazine-induced colon carcinogenesis in the Balb/c mouse; DMBA-initiated, TPA-promoted skin tumorigenesis in the CD-1 mouse at both the initiation and promotion stages; urethan-induced, and particularly DMBA-induced, pulmonary neoplasia in the

A/J mouse; N-nitrosodiethylamine-induced preneoplastic liver foci in rodents; mouse mammary tumor virus-induced ("spontaneous") mammary carcinogenesis; and epidermal carcinogenesis resulting from repeated applications of DMBA. DHEA has known antiproliferative properties *in vitro*, which have been widely accepted to be a result of its action as a potent inhibitor of glucose 6-phosphate dehydrogenase, the first committed step of the pentose phosphate pathway. It also appears to be an anti-obesity agent, to retard the aging process, and to inhibit the development of atherosclerosis which, similar to carcinogenesis, is now viewed as a proliferative disorder with identifiable initiating and promoting events preceding the proliferative stage. In men and women, circulating serum levels of DHEA and DHEAS peak in early adulthood and drop markedly with age. Results from a number of investigations have suggested that the circulating levels of these hormones are related in some way to the risk of developing breast cancer. However, the exact nature of this relationship has not been defined by these studies because of the differing characteristics of the various populations studied. In a recent study, the serum levels of DHEA and DHEAS were measured by radioimmunoassay in banked sera from 30 postmenopausal women who subsequently developed breast cancer and 59 matched controls without breast or other cancers. Serum samples from both cases and controls were from the Washington County (Maryland) Serum Bank which was formed in 1974 from over 25,000 blood-donating volunteers. All samples used in the study had been donated to the bank at least nine years before the diagnosis of cancer was established. Cases eligible for inclusion in this study were white females free of other cancers (except for nonmelanomatous skin cancer) who had denied taking oral contraceptives or other exogenous hormones at the time of blood drawing, and who had had their last menstrual period at least one year before donating blood to the serum bank. For each case of breast cancer, two controls were selected from the serum bank who were the next younger and next older women who were alive and without diagnosed cancer (except for nonmelanomatous skin cancer) on the date of diagnosis of the index case. These controls were further matched according to month of blood donation, time between last meal and blood drawing, time since last menstrual period, and denial of the use of oral contraceptives or other exogenous female hormones at the time of blood drawing. It was found that serum levels of DHEA were significantly elevated in cases compared to controls, while those of DHEAS were only slightly increased among cases. This observed association of high DHEA levels with development of postmenopausal breast cancer remained after stratification for educational level, time to diagnosis, blood pressure levels and use of blood pressure medication. Since cigarette smoking may have influenced the serum levels of DHEA and DHEAS, an analysis of non-smoking cases and controls was performed. It was again found that cases had a significantly higher mean serum level of DHEA and a slightly higher mean level of DHEAS. The results are taken to demonstrate that a relationship exists between elevated levels of these hormones, particularly DHEA, and the risk of developing post-menopausal breast cancer. The results might be reconciled with the laboratory findings that DHEA administration results in a decreased incidence of cancer in several animal models, including both chemical carcinogen- and virus-induced breast cancer, by noting that the mechanism(s) of DHEA action in these studies is still not clear. Moreover, under some circumstances, DHEA feeding has increased the incidence of lung adenomas, colonic neoplasms or pancreatic lesions in response to carcinogen treatment, and its administration has been associated in one animal model with an increased incidence of ovarian granulosa cell tumors.

A similar study employing specimens from this unique serum bank was performed to assess the relationship between serum levels of DHEA and DHEAS and subsequent development of bladder cancer in humans. Some evidence exists in animal models that

the addition of DHEA to the diet reduces the incidence of papillary nodular hyperplasia in the urinary bladder. In this study, 35 new cases of bladder cancer were identified among the study population, 23 (65%) occurring in men. Transitional cell carcinomas accounted for 86% of the cases. Again, two controls were selected for each case who were living and free from known cancer (with the exception of basal or squamous cell skin cancer) at the time of diagnosis of the cases. Cases and controls were matched for sex, race, age within one year, and time interval (within two hours) between blood collection and the previous meal. In addition, because smoking has been reported to be associated with both DHEA and DHEAS levels and the risk of bladder cancer, smoking histories were used to make appropriate adjustments. In 1974, the Serum Bank collected cigarette smoking histories under the categories of "never smoked," "formerly smoked" and "current smoker" in the ranges of 1-20 or 20+ cigarettes/day. Cigar and pipe smoking were also known. None of the adjustments for smoking altered the observed associations between DHEA and DHEAS levels and the risk of developing bladder cancer. Results indicated that the risk of bladder cancer increased with decreasing serum levels of both hormones. The odds ratios for the middle and lowest tertiles of serum DHEA, with the highest tertile as the reference category, were 2.84 and 4.01, while those for DHEAS were 2.19 and 3.74, respectively. Since cancer may have been present in some cases at the time of blood collection, and since some of the observed effects might be related to such presence, the cases were evaluated further by early diagnosis (within two years of blood collection) and late diagnosis (diagnosed more than two years from the time of collection). The observed protective effects of DHEA and DHEAS against bladder cancer were even stronger upon exclusion of the early diagnosis cases. Similar relationships between serum levels of DHEA and DHEAS and bladder cancer risk were seen for both men and women (57).

Mechanism-Based Model for Chemoprotection Studies. Butylated hydroxytoluene (BHT; 2,6-di-tert-butyl-4-methylphenol), though a known effective anticarcinogen, is paradoxically also a tumor promoter in a variety of tissues, including liver, lung, colon, bladder and thyroid. It is also a weak hepatocarcinogen in male mice, is toxic in both liver and lung, and apparently enhances tumor formation in the progeny of rats that have had high lifetime feeding. The toxic and tumor-promoting activities of BHT are thought to be mediated by its metabolites. Several inhibitors of cytochrome P450 suppress BHT toxicity and a hydroxylated metabolite is more effective than the parent compound as either a tumor promoter or toxin in mouse lung. Toxicity may be mediated through an electrophilic intermediate since agents which deplete glutathione enhance liver and lung damage and elevate BHT covalent binding in these tissues. Structure-activity studies of the 4-methyl position of the BHT molecule indicate that a quinone methide intermediate may be the toxic electrophile. Moreover, the metabolite, butylated hydroxytoluene hydroperoxide (2,6-di-tert-butyl-4-hydroperoxyl-4-methyl-2,5-cyclohexadienone) (BHTOOH) is known to be a tumor promoter in mouse skin and to be much more potent than BHT itself in acute toxicity studies (63). Substantial evidence suggests that free radicals and free radical-mediated processes are involved in the biochemical and biological events of tumor promotion.

In the above regard, BHTOOH is known to be extensively metabolized in mouse keratinocytes to form several free radical intermediates, including phenoxyl, peroxy, alkoxy and alkyl radical derivatives (63). Of these, the primary radical species generated from BHTOOH is the BHT phenoxyl radical. Two molecules of this radical can form a quinol ether intermediate, which spontaneously dismutates to regenerate one molecule of the parent BHT and the quinone methide of BHT

(BHT-QM). This quinone methide (2,6-di-tert-butyl-4-methylene-2,5-cyclohexadienone) is a known reactive electrophile. Electrophiles are recognized to play preeminent roles in the initiation of chemical carcinogenesis by covalently modifying nucleic acids. However, electrophilic species have not been linked to tumor promotion. Consequently, a recent study probed the role of BHT quinone methide (BHT-QM) in tumor promotion, employing BHTOOH (the source of BHT-QM via metabolism to BHT phenoxy radical and subsequent reactions as detailed above) and two analogs of BHTOOH. The analogs were the 4-trideuteromethyl derivative, [²H₃] BHTOOH, and the 4-tert-butyl BHTOOH in which the 4-methyl substituent has been replaced by a tert-butyl group. In the dismutation reaction forming BHT-QM, one of the methyl group 4- α -hydrogens is lost to give the 4-methylene quinone methide compound. Because of the kinetic isotope effect, the deuterated BHTOOH analog forms BHT-QM at a slower rate than the parent BHTOOH, while the tert-butyl analog which has no 4- α -hydrogens is incapable of forming the electrophilic quinone methide intermediate. These theoretical considerations were confirmed by spectrophotometric determination of the relative rates of BHT-QM generation from the three BHT hydroperoxides in an in vitro metabolism system employing hematin, previously shown (63) to be an excellent model of epidermal-mediated metabolism of BHTOOH. The deuterated compound formed the quinone methide at a rate just 40% of that for the parent compound and, as expected, the tert-butyl derivative failed to form QM at all. Further, in both an alkaline ethanol chemical system and with a keratinocyte cytosol preparation, the three BHT hydroperoxides were shown to generate EPR-detectable phenoxy radicals. With the keratinocyte-mediated metabolism, analysis indicated a 2-fold increase in the amount of phenoxy radical generated from the deuterated compound as compared to the parent BHTOOH, and a 270-fold increase in phenoxy radical generated from the tert-butyl compound. Control incubations with heat-inactivated cytosol generated no phenoxy radicals, indicating that metabolism is necessary. Although not sufficient evidence, the capacity of a substance to induce ornithine decarboxylase (ODC) activity in mouse epidermis is widely regarded as a marker of its ability to act as a tumor promoter. Dose-response ODC assays with the three hydroperoxides showed that the t-Bu-BHTOOH did not increase ODC basal activity and that the deuterated compound (at corresponding doses) was less active as an inducer than BHTOOH. Finally, in a two-stage tumor promotion experiment in SENCAR mice (first stage of promotion with the classical phorbol ester tumor promoter phorbol myristate acetate (PMA), second stage with the hydroperoxides), it was found that the t-Bu-BHTOOH was inactive as a tumor promoter at two doses, that the deuterated compound produced a 28% tumor incidence (with papillomas per mouse of about 0.6) at a dose of 20 micromoles per application, and that the parent BHTOOH at this dose gave a papilloma incidence of 64% (multiplicity 1.9 papillomas/mouse). Interestingly, an 8 micromole dose of the parent compound gave a tumor incidence equivalent to that of the deuterated analog at the 2.5-fold higher dose of 20 micromoles per application.

This study is important for several reasons. Many investigations on the peroxide, hydroperoxide and anthrone tumor promoters have centered on their capacity for generating free radicals, with much indirect evidence suggesting that radicals may play a prominent role in mediating tumor promotion. But the actual free radical species formed from promoters in target tissues for carcinogenesis have rarely been characterized, nor are the intracellular targets known. In this study, previous work (63) had established that the phenoxy radical was the primary radical produced from BHTOOH in the target keratinocyte. It was also known that this radical is an intermediate in the formation of the electrophile BHT-QM. The results with the hydroperoxides in this study showed that the ability to act as tumor promoters was directly related to their ability to form the electrophile BHT-QM through the obligatory, intermediate production of phenoxy radicals. The t-Bu analog produced

about 270 times as much phenoxyl radical as the parent BHTOOH compound, but could not generate BHT-QM and was non-tumorigenic. Similarly, the deuterated BHTOOH generated BHT-QM at a rate that was 40% of that for the parent compound and required a 2.5-fold greater dose in promotion to produce the same tumor response. Presumably, equimolar amounts of BHT-QM are generated in the epidermis. These various experimental results provide explicit evidence that reactive intermediates, in general, and electrophiles, in particular, are directly involved in tumor promotion. This observation broadens previously postulated roles for electrophiles in carcinogenesis as participants in initiation and progression to include all stages of carcinogenesis. Furthermore, these studies have now established an elegantly defined system for investigations on the mechanisms of anticarcinogenesis by many types of chemopreventive agent (63).

Natural Inhibitors of Carcinogenesis. Natural sources of chemopreventive agents have included many different types of foods, beverages, herbs and spices. Recently much interest has been shown in the potential of extracts and constituents of tea to inhibit tumorigenesis. This is particularly true of green tea. The importance of defining the potential of this beverage and its constituents in prevention of the development of cancer is very high in view of its enormous consumption throughout the world. In one study, Chinese gunpowder green tea leaves (12.5 g) were extracted twice with 500 ml of boiling water and the extracts combined (1.25% GT extract). This water extract of green tea contained about 4.7 milligrams of green tea solids per milliliter. Analysis by high performance liquid chromatography (HPLC) gave a partial composition of 27.3% total catechins, 8.1% caffeine, and 0.4% theobromine. The following five catechins were found in decreasing order of abundance: (-)-epigallocatechin gallate (EGCG); (-)-epigallocatechin (EGC); (-)-epicatechin gallate (ECG); (-)-epicatechin (EC); and (+)-catechin (C). Administration of 0.63% and 1.25% GT extracts as the sole source of drinking water were then investigated for their capacity to inhibit N-nitrosodiethylamine (NDEA)-induced forestomach and lung tumors in female A/J mice. Carcinogen was administered by oral gavage once a week for 8 weeks and the animals were killed 16 weeks later. This protocol resulted in 8.3 forestomach tumors per mouse and 2.5 lung tumors per mouse in the control group. If the 0.63% and 1.25% GT extracts were administered for the entire 24-week experimental period, forestomach tumors/mouse were decreased by 59% and 63%, respectively. Further, if the extracts were given only during the 8-week period of NDEA administration, inhibitions of 31% (0.63% GT extract) and 58% (1.25% GT extract) were seen. Finally, if the extracts were provided only after the 8-week period of carcinogen administration was completed, forestomach tumors/mouse were still inhibited by 35% and 47% by the two levels in the drinking water. Lung tumor multiplicities were decreased by similar amounts by both extracts under all three conditions of administration: (1) administration of the extracts during the entire 24 week period, 36% and 60% inhibition; (2) administration only during the period of carcinogen administration, 36% and 56% inhibition; and (3) administration only in the post-carcinogen administration period, 52% and 44% inhibition. It is interesting that the extent of tumor inhibition seen at the two organ sites are similar for each of the protocols of extract administration (11).

A second series of studies investigated the capacity of the orally-administered GT extract (1.25%) to inhibit ultraviolet B light (UV-B)-induced red sunburn lesions and UV-B-induced carcinogenesis in the skin of female hairless SKH-1 mice. Administration of 180 mJ/cm² of UV-B once daily for 7 days results in red sunburn lesions of the skin. If, however, GT extract is provided as the sole source of drinking water for 1-2 weeks prior to and during UV administration, the red sunburn lesions are reduced in size and color intensity. The experiment was then expanded

to a tumorigenesis study using similar UV-B irradiation once per day for 10 days followed one week later by twice weekly administration of 12-O-tetradecanoylphorbol-13-acetate (TPA) for 25 weeks. It was found that oral administration of the GT extract in the drinking water for 1-2 weeks prior to, during, and for one week following UV-B irradiation inhibited subsequent skin tumorigenesis by 64-82%. In addition, there was a significant increase in the latency period to tumor appearance. A much smaller inhibitory effect of the GT extract was seen in two similar experiments using CD-1 mice. In two additional experiments, female SKH-1 mice were treated with 200 nmoles of 7,12-dimethylbenz[*a*]anthracene (DMBA) followed 2-3 weeks later by 180 mJ/cm² of UV-B twice weekly for 25-30 weeks. In these experiments, GT extract was given in the drinking water before and during UV treatment (but after DMBA administration). Under these conditions, skin tumor formation in the two experiments was inhibited by 39% and 87%, respectively. The GT extract also caused a substantial decrease in tumor size. These studies on the inhibitory effects of "dietary" green tea on ultraviolet light-initiated, as well as ultraviolet light-promoted, skin tumorigenesis are of some interest in view of present concern about increased risk to human populations from sunlight exposure (11).

An additional series of experiments investigated the chemopreventive potential of the polyphenolic fraction in green tea. For this purpose, green tea leaves were subjected to a series of extractions to yield a dry powder, green tea polyphenol fraction (GTPF), containing substantial amounts of epigallocatechin gallate. The composition of GTPF was analyzed and found to contain EGCG (49.5%), EGC (11.5%), ECG (11.4%), caffeine (7.6%), EC (6.1%), C (0.5%), and gallic acid (0.4%). Application of 0.04, 0.12 and 0.36 mg of GTPF to the ears of mice inhibited TPA-induced inflammation by 8, 36 or 87%, respectively. Similarly, topical application of 0.4, 1.2 or 3.6 mg of GTPF to the backs of mice inhibited TPA-induced ornithine decarboxylase (ODC) activity by 30, 57 or 90%, respectively. Both inflammation and ODC induction are characteristics of TPA-promoted epidermal tumorigenesis. Although neither appears to be obligatory to tumor promotion in general, their potent inhibition here by GTPF (and by GT extracts previously discussed) is an indication of potential activity against TPA-promoted tumorigenesis. Indeed, in mice previously initiated with 200 nmoles of DMBA, topical application of 3.6 mg of GTPF with 5 nmoles of TPA twice weekly for 20 weeks inhibited the average number of skin tumors per mouse by 94% (control group 21 tumors/mouse). The incidence of tumors (fraction of animals with tumor) was also powerfully inhibited from 90% in the control group to 24% in the GTPF-treated group. A small increase in latency was also seen, and interestingly, a decreased level of epidermal hydrogen peroxide.

Selenoproteins: Possible Role in Chemoprevention. Selenium (Se) is now established as an essential nutrient and a potent anticarcinogenic agent. Selenium compounds inhibit chemical carcinogen-induced tumorigenesis in a broad spectrum of epithelial tissues as well as the growth of mammalian cells in cell culture. However, the mechanisms of action underlying the chemopreventive and growth inhibitory effects of selenium are not understood. In this regard, it has been proposed that the action of proteins which bind selenium may play an important role in its chemopreventive effects. The presence of such proteins has been demonstrated in numerous mammalian organs by several laboratory groups. These proteins can be labeled with trace amounts of ⁷⁵Se in vivo or in cell culture. Analysis of the patterns of selenium-labeled proteins by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) shows that selenium is retained in a limited number of proteins. The physiological functions of these proteins are unknown. Recently, two ⁷⁵Se-labeled proteins were purified from mouse liver cytosol and used to raise polyclonal

antibodies (44). The proteins have molecular weights of 56 and 14 kd. The antibody to the 56 kd liver protein recognized the cellular 56 kd protein in a variety of organs (gastrointestinal, male and female endocrine) from mice and rats, but did not recognize a 57 kd plasma protein that was labeled with ⁷⁵Se. The antibody to the 14 kd liver protein, however, recognized only the protein in mouse and rat liver. The 14 kd protein (SLP-14) was subsequently demonstrated to be mouse liver fatty acid binding protein (LFABP) (44).

In recent work, since the function of liver protein 56 kd (SLP-56) was unknown, the gene was cloned from a mouse liver cDNA library using the antibody specific for the 56 kd protein. The cloned cDNAs represented the complete message. The correct reading frame was verified by alignment of the deduced amino acid sequence with that of peptides sequenced from the purified protein. The primary sequence has not been reported previously since homologous DNA sequences were not found in Gen Bank. The most important aspect of the nucleotide sequence was the absence of any in-frame TGA codons. An in-frame TGA codon represents the amino acid selenocysteine, which is characteristic of the selenoproteins glutathione peroxidase (GSH-Px) and formate dehydrogenase. The absence of any TGA codons implies that SLP-56 may interact with selenium in a manner different from GSH-Px, the prototypic selenoprotein. The amino acid sequence is unremarkable, being slightly enriched in glycine, leucine, aspartic/asparagine and glutamic/glutamine. There are nine cysteines. No protein kinase C recognition sites are present, but one potential N-linked glycosylation site exists. Secondary structure analysis indicates that the protein has little (3%) α -helical structure and extensive (59%) β -sheet structure. Hydropathy analysis suggests the presence of a five, randomly spaced, short hydrophobic areas. The pattern was similar to a soluble protein rather than a membrane-spanning protein, an interpretation consistent with previous cell fractionation studies. The present data indicate that SLP-56, like SLP-14/LFABP, represents a class of proteins that bind Se in a manner, presently unknown, but distinct from eukaryotic GSH-Px and prokaryotic formate dehydrogenase. Not only does it lack a TGA codon for selenocysteine in the appropriate reading frame, but other studies show that cellular levels of SLP-56 are not induced or dependent upon dietary Se concentration. These data suggest that selenium may act to modulate the function of the proteins. Although the function of SLP-56 is not known, others have shown that the concentration of LFABP is elevated in liver hyperplastic nodules and mitotic liver cells. Further, LFABP avidly binds several arachidonic acid metabolites that are thought to be important in growth regulation of the liver. In addition, proteins highly homologous to LFABP have been isolated from bovine mammary gland and fibroblasts and have been identified as growth inhibitory factors. Moreover, selenium binds avidly to only a few proteins (five or six separate proteins) in a wide spectrum of organs. These considerations have led to an important new hypothesis for the mechanism of action of selenium in the inhibition of cell growth and in anticarcinogenesis, namely, that selenium binds to, and modulates the function of, specific growth regulatory proteins. This property is different and distinct from selenium's function as an essential nutrient. In the latter case, for example, the actual presence and function of GSH-Px is dependent upon selenium (44).

Retinoic Acid Receptor Proteins. Retinoic acid (RA) has profound effects upon the proliferation and differentiation of many hematopoietic cells. It stimulates the clonal growth of normal human myeloid and erythroid precursors in vitro, induces differentiation of acute myelogenous leukemia (AML) cells blocked at a fairly mature stage of differentiation, and inhibits clonal growth of the HL-60 and KG-1 human acute myeloid leukemia cell lines in culture. Moreover, RA inhibits the growth of the neoplastic blast cells harvested from some patients with acute myelogenous

leukemia. The mechanisms underlying the effects of RA on differentiation and development are not yet well defined. Much interest in this regard has been generated by the recent discovery of retinoic acid receptor (RAR) molecules and their identification as members of the steroid/thyroid hormone/vitamin D superfamily of receptors. These protein receptor molecules contain discrete DNA-binding and ligand-binding domains. They are thought most likely to mediate their manifold responses by binding their largely lipid-soluble ligands (such as RA), which changes their affinities for DNA, then binding to specific regulatory sequences in DNA, leading to alterations in gene expression. At least three species of RAR are known: RAR- α , RAR- β , and RAR- γ . Several of these have been cloned. A recent study employed a human RAR- α cDNA probe to examine the expression of RAR- α mRNA in a variety of hematopoietic cells blocked at different stages of differentiation. It was found that all hematopoietic cells expressed RAR- α mRNA of two species (3.4 and 4.5 kb), including KG-1 (myeloblasts); HL-60 (promyelocytes); ML3, THP-1, and U937 (myelomonoblasts and monoblasts); K562 (erythroblasts); and S-LB1 (T-lymphocytes immortalized by human T-cell leukemia virus type 1). In another series of experiments, steady-state levels of RAR- α mRNA were not affected by induction of terminal differentiation of HL-60 to either granulocytes (by DMSO or RA) or macrophages (by 1,25-dihydroxyvitamin D3 or TPA). Furthermore, both actively proliferating and resting lymphocytes from the same individuals expressed equal levels of RAR- α mRNA. In addition, transformed cells from three non-hematopoietic human cell lines (hepatocellular carcinoma, adenocarcinoma of the lung, osteogenic sarcoma) also expressed RAR- α mRNA as did normal human embryonic lung fibroblasts. Taken together, these data suggest that the level of expression of RAR- α mRNA is not related to cellular proliferation. It was also found in other experiments that the level of expression of RAR- α mRNA was not inducible by its ligand retinoic acid in either HL-60 cells or in S-LB1 immortalized T-lymphocytes. The half-life of this receptor mRNA, determined by measuring decay of message after addition of actinomycin D, was short (0.7 hour). Consistent with this finding is the observation that RAR- α mRNA is superinducible in three different cell lines upon cycloheximide inhibition of protein synthesis. These results on RAR- α regulation are consistent with the results of others, which have shown differential expression and ligand regulation of RAR- α and RAR- β genes in a hepatoma cell line. In this case, RA induced accumulation of RAR- β , but not RAR- α mRNA. A similar half-life of RAR- α message was also reported in these hepatoma cells (35).

BIOLOGICAL AND CHEMICAL PREVENTION

GRANTS ACTIVE DURING FY91

<u>INVESTIGATOR/INSTITUTION/GRANT NUMBER</u>	<u>TITLE</u>
1. AWASTHI, Yogesh C. University of Texas Medical Branch 5 R01 CA27967-12	Mechanism of Anti-Carcinogenic Effect of Antioxidants
2. BAILEY, George S. Oregon State University 5 R01 CA34732-07	Mechanisms of Inhibition of Chemical Carcinogenesis
3. BANERJEE, Mihir R. University of Nebraska Lincoln 5 R01 CA25304-09	Chemical Carcinogenesis Mammary Gland Organ Culture
4. BELL, Robert M. Duke University 5 U01 CA46738-04	Protein Kinase C Inhibitors As Chemopreventive Agents
5. BIRT, Diane F. University of Nebraska Medical Center 5 R01 CA48028-03	Inhibition of Tumor Promotion by Sphingoid Bases
6. BYUS, Craig V. University of California Riverside 5 R01 CA45707-03	Nutritional Modification of Cancer Cell Growth
7. CASSADY, John M. Ohio State University 5 R01 CA38151-07	Chemistry of Novel Natural Inhibitors of Carcinogenesis
8. CAVE, William T., Jr. University of Rochester 5 R01 CA30629-09	Promotion of Breast Cancer Lipid Hormone Interactions
9. COHEN, Leonard A. American Health Foundation 5 R01 CA47326-03	NMU-Induced Mammary Cancer and Omega-3 Fatty Acids
10. COHEN, Leonard A. American Health Foundation 5 R01 CA48741-03	Voluntary Exercise as Means of Mammary Cancer Prevention
11. CONNEY, Allan H. Rutgers The State Univ New Brunswick 1 R35 CA49756-01A1	Cancer Cause and Prevention Research
12. CONNOR, Michael J. University of California Los Angeles 5 R01 CA47758-02	Retinoid Metabolism and Skin Carcinogenesis

26. HALL, Alan K.
University of Medicine & Dentistry of NJ
5 R01 CA49422-03
Molecular Actions of Retinoids
in Neoplastic Cell Growth
27. HECHT, Stephen S.
American Health Foundation
5 U01 CA46535-04
Isothiocyanates and Nitrosamine
Carcinogenesis
28. HILL, Donald L.
Southern Research Institute
5 P01 CA34968-06
Development of Chemopreventive
Retinoids
29. IP, Clement C. Y.
Roswell Park Memorial Institute
2 R01 CA27706-11
Selenium Metabolism and
Chemoprevention
30. IP, Clement C. Y.
Roswell Park Memorial Institute
5 P01 CA45164-03
Mechanism of Selenium Chemo-
Prevention of Carcinogenesis
31. IP, Margot M.
Roswell Park Memorial Institute
5 R01 CA35641-06
Eicosanoids and Mammary Cancer
32. KENNEDY, Ann R.
University of Pennsylvania
3 U01 CA46496-03S1
Cancer Prevention by Protease
Inhibitors
33. KENSLER, Thomas W.
Johns Hopkins University
5 R01 CA39416-06
Mechanisms of Anticarcinogenesis
by Dithiolthiones
34. KLINE, Kimberly
University of Texas Austin
5 R29 CA45422-03
Micronutrients in Immuno-
modulation and Cancer
35. KOEFFLER, Phillip H.
University of California Los Angeles
5 R01 CA33936-09
Action of Retinoids on Myeloid
Leukemia
36. KOEFFLER, Phillip H.
University of California Los Angeles
5 U01 CA43277-05
Vitamin D and Preleukemia/
Leukemia--Cancer Chemoprevention
37. KOOP, C. Everett
Coordinating Council For Cancer Research
1 R13 CA54094-01
Int Conf on Prevention:
Facts, Maybes, and Rumors
38. LAM, Luke K. T.
LKT Laboratories, Inc.
2 R44 CA47720-02A1
Natural Chemopreventive Agents

39. LAM, Luke K. T.
LKT Laboratories, Inc.
1 R43 CA52378-01A1
Proinhibitors of Chemical
Carcinogenesis
40. LIEBLER, Daniel C.
University of Arizona
5 R29 CA47943-03
Vitamin E Turnover and Chemical
Toxicity
41. LIEHR, Joachim G.
University of Texas Medical Branch
5 R01 CA44069-05
Prevention of Estradiol-Induced
Tumors by Vitamin C
42. LUTZ, Charles A.
Mills College
1 R15 CA47579-01
Role of Nitroxyl in the
Ascorbate-Nitrite Reaction
43. MC CORMICK, David L.
IIT Research Institute
5 R01 CA40874-06
Arachidonic Acid Metabolism and
Cancer Chemoprevention
44. MEDINA, Daniel
Baylor College of Medicine
5 R01 CA11944-19
Biology of Mouse Mammary
Preneoplasias
45. MEHTA, Rajendra G.
IIT Research Institute
5 R01 CA34664-06
Hormone and Retinoid Interaction
in Mammary Tissue
46. MILNER, John A.
Pennsylvania State University-Univ Park
5 R01 CA44567-04
Effects of Dietary Selenium on
the Initiation of DMBA
47. MITSCHER, Lester A.
University of Kansas Lawrence
5 R01 CA43713-03
Novel Antimutagenic Compounds
48. MORDAN, Lawrence J.
University of Hawaii at Manoa
5 R01 CA51498-02
Retinoid Effects on Ca²⁺ and
Mitogenesis in Carcinogenesis
49. NEWELL, Guy R.
U of Texas MD Anderson Cancer Center
1 R13 CA54776-01
Conference: Prevention of
Aerodigestive Tract Cancer
50. NILES, Richard M.
Boston University
5 R01 CA32543-08
Regulation of Growth and
Differentiation by Retinoids
51. PACKER, Lester
University of California Berkeley
5 R01 CA47597-03
Membrane Reactions of Vitamin E

52. PFAHL, Magnus
La Jolla Cancer Research Foundation
5 R01 CA50676-02
Retinoic Acid Receptor in
Development and Disease
53. PROUGH, Russell A.
University of Louisville
5 R01 CA43839-05
Inhibitor Effects on Mono-
oxygenase Function
54. REDDY, Bandaru S.
American Health Foundation
5 U01 CA46589-04
Chemoprevention of Colon Cancer
by Organoselenium
55. REINERS, John J., Jr.
University of Texas System Cancer Center
5 R01 CA34469-08
Inhibition of Chemical Carcino-
genesis by Interferon
56. RUSSELL, Robert M.
Tufts University
1 R01 CA49195-01A3
Intestinal Metabolism of
 β -Carotene
57. SCHWARTZ, Arthur G.
Temple University
1 R01 CA52500-01A1
Chemoprevention of Skin Tumors
by DHEA Analogs
58. SHANKEL, Delbert M.
University of Kansas
1 R13 CA54695-01
Conf on Mechanisms of Anti-
mutagenesis/Anticarcinogenesis
59. SILBART, Lawrence K.
University of Michigan at Ann Arbor
5 R01 CA47132-03
Mucosal Immune Response to
Aflatoxin B1
60. SINHA, Dilip K.
Roswell Park Memorial Institute
5 R01 CA36139-06
Protection against Mammary
Carcinogenesis by Pregnancy
61. STEINBERG, Mark L.
City College of New York
1 R01 CA52915-01
Protease Inhibitor Effects in
Epithelial Transformation
62. STONER, Gary D.
Medical College of Ohio at Toledo
5 R37 CA28950-10
Carcinogenesis Studies in
the Esophagus
63. TALALAY, Paul
Johns Hopkins University
5 P01 CA44530-05
Novel Strategies For Chemo-
protection against Cancer
64. THOMPSON, Henry J.
AMC Cancer Research Center
5 R01 CA49212-03
Antioxidants and Breast Cancer
Prevention

- | | |
|---|--|
| 65. THOMPSON, John A.
University of Colorado at Boulder
5 R01 CA41248-06 | Bioactivation of Dietary Phenols
by Hemoproteins |
| 66. THRAVES, Peter J.
Georgetown University
1 R01 CA52945-01 | Protease Inhibitors and
Radiation Transformation |
| 67. TROLL, Walter
New York University
1 R01 CA53003-01 | Anticarcinogenesis by Nicotin-
amide a Protease Inhibitor |
| 68. VERMA, Ajit K.
University of Wisconsin Madison
5 R01 CA42585-05 | Inhibition of Ornithine
Decarboxylase by Retinoic Acid |
| 69. WALASZEK, Zbigniew
University of Texas System Cancer Center
5 R01 CA47342-03 | Mechanism of Glucarate
Inhibition of Mammary Cancer |
| 70. WANG, Sho-Ya
State University of New York at Albany
5 R29 CA44955-05 | Differentiation of Terato-
carcinoma Cells: Regulation |
| 71. WARGOVICH, Michael J.
University of Texas System Cancer Center
1 R01 CA52006-01A1 | Calcium Inhibition of Colon
Cancer Progression |
| 72. YAVELow, Jonathan
Rider College
5 R01 CA47762-03 | Anticarcinogenic Mechanism of
Proteinase Inhibitors |

SUMMARY REPORT

CARCINOGENESIS MECHANISMS

The Carcinogenesis Mechanisms component of the Branch includes studies on the (1) etiology of neoplasia in poikilothermic, aquatic animals; (2) metabolism, toxicity, physiological disposition, and mechanisms of action of carcinogens and their metabolites; (3) syntheses of both known and suspect carcinogens or the development of derivatives for molecular structure-activity relationships; (4) development of carcinogenicity/mutagenicity testing procedures; (5) development of organ and cell culture systems and whole animal biological models for use in carcinogenesis studies; (6) 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; (7) genetics and regulation of enzymes characteristically associated with carcinogenesis; (8) identification of biochemical and molecular markers and properties of cells transformed by carcinogens; and (9) hormone-related biochemistry of cancer and cancerous hosts. In FY 91 144 grants were supported totaling \$20.73 million. There are no contracts in this area.

Grants Activity Summary

Bladder RFA: A Request for Applications (RFA) for "The Identification of Genetic Alterations Involved in Bladder Carcinogenesis" was issued by the National Cancer Institute in 1988. In response to this RFA, seven applications were received and one was funded. Proto-oncogene expression by cultured urothelial cells prepared from the bladders of male F344 rats that had been treated with N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide (FANFT) or N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) were examined by the RFA awardee. C-Ha-ras transcripts were detected in all cultured urothelial cells prepared from the carcinogen-treated rats and in normal urothelial cells. However, the transcript levels were severalfold higher in carcinogen-treated cells than in normal cells. Increased expression of p21, as determined by immunohistochemical techniques, was also observed in all the original bladder tissues from which the cultures were derived. C-myc transcripts were detected in the cells from carcinogen-treated rats but not in normal cells. The presence of myc product in hyperplastic urothelial lesions and carcinomas of original bladder tissues was confirmed by immunohistochemical methods. Transcripts of *mos*, *erb B*, *Ki-ras*, *abl* and *src* were not detected. Since increased expression of c-myc and c-Ha-ras were present in both transplantable and non-transplantable cell lines, and the expression of p21 occurs in preneoplastic cells, this suggests that elevated expression of these two genes may be an early genetic event during bladder carcinogenesis in the rat and that further alteration of these two genes, or mutation of additional genes, may be required for the completion of malignant transformation (138).

Fish PA: A Program Announcement (PA) for "Studies on Cancer Etiology in Finfish and Shellfish" was issued in 1989. The PA was jointly sponsored by the Division of Cancer Etiology of the National Cancer Institute (NCI) and the Extramural Program of the National Institute of Environmental Health Sciences (NIEHS). In response to this PA, 38 applications were received by the first round deadline of February 1, 1990. These were reviewed by a special study section in June 1990. A total of eight proposals were funded, four by NCI and four by NIEHS. Subsequently, 10 additional applications were received for the second and third rounds combined. These applications were assigned by the Division of Research Grants to regular study sections. One of these proposals received NCI funding. One of the PA awardees was

first funded by an RFA mechanism in 1986. This team has developed a colony of medaka, and has perfected the care, feeding, and handling of these fish which mature rapidly, have a short life cycle, and respond to a number of carcinogens when the latter are injected or added to the feed. They have formulated a medaka test diet made from purified ingredients which allows positive experimental control over nutrients and chemical carcinogens to be tested. Comparative studies have been done between the medaka and rainbow trout. In the new proposal, the investigators will determine the efficiency of carcinogen activation and DNA adduct formation/DNA repair in trout hepatocytes, biliary epithelial cells, and medaka liver. They will also establish biochemical actions of growth factors and tumor promoters in hepatocytes and biliary epithelial cells (48).

Polycyclic Aromatic Hydrocarbons: Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants resulting from incomplete combustion of fossil fuels, and their wide range of action in animals suggests that they may be responsible for some cancer induction in man. The biological properties of PAHs, such as mutagenicity, carcinogenicity, and covalent binding to cellular macromolecules, require metabolic activation to an electrophilic species. Metabolic activation of PAH may occur by two main pathways: monooxygenation or two-electron oxidation; and one-electron oxidation. Monooxygenation affords oxygenated metabolites by direct attack of an "oxene-like" activated oxygen generated by P-450 monooxygenase. One-electron oxidation produces radical cations. In an aromatic system a radical cation is formed by removal of one pi-electron, whereas one n-electron oxidation of a phenol or amine, followed by rapid loss of a proton, yields a radical.

Covalent binding of chemical carcinogens to DNA is thought to be the first critical step in the initiation of the tumor formation process. The ultimate goal of a great deal of research in chemical carcinogenesis is to elucidate the structure of various DNA-carcinogen adducts and to establish their biological significance. When benzo(a)pyrene [B(a)P] is activated by one-electron oxidation to its radical cation, B(a)P binds at C-6 to the C-8 of deoxyguanosine (dG), forming 8-(benzo(a)pyrene-6-yl)deoxyguanosine. This adduct, which is relatively stable in DNA, has been identified in vitro. B(a)P radical cation also forms an adduct with B(a)P bound at C-6 to the N-7 of guanine 7-[benzo(a)pyrene-6-yl]guanine [B(a)P-N7Gua]. This adduct is rapidly lost from DNA by depurination. When rats were treated with [¹⁴C]B(a)P by intraperitoneal injection, about 0.02% of the administered dose of B(a)P was excreted as B(a)P-N7Gua in feces and urine within 5 days. Chloroform extracts of the urine and feces were analyzed by high-pressure liquid chromatography (HPLC). The structure of the adduct was established by cochromatography with electrochemically prepared B(a)P-N7Gua and by fluorescence line narrowing spectrometry. This study represents the first demonstration that B(a)P-N7Gua is formed in vivo in animals treated with B(a)P (14).

According to proponents of two-electron oxidation pathways, the bioactivation of PAHs involves initial epoxidation at several sites, followed by hydrolysis and subsequent epoxidation to generate highly reactive electrophilic bay-region diol-epoxides. The formation of deoxyribonucleoside adducts in mouse epidermal DNA following topical application of [³H]dibenz(a,j)anthracene (DB(a,j)A) or unlabeled DB(a,j)A coupled with fluorescence detection was reported in one grant. A single topical application of 400 nmol per mouse [³H]B(a,j)A yielded a total binding value of 9.92 ± 1.45 pmol/mg epidermal DNA. However, after digestion of DNA samples to deoxyribonucleosides only $8.64 \pm 0.4\%$ of the total radioactivity eluted in the methanol phases from short Sephadex LH-20 columns. The water phases obtained from

the short LH-20 columns were further analyzed by ion-exchange chromatography to determine the nature of the radioactivity associated with this material. These analyses revealed that the majority of the original DNA-associated radioactivity was tritium exchanged into normal deoxyribonucleosides and deoxyribonucleotides. HPLC of the methanol phases from the LH-20 step revealed the presence of both deoxyguanosine (dGuo) as well as deoxyadenosine (dAdo) adducts formed from the anti- and syn-3,4-diol-1,2-epoxides of DB(a,j)A. The major DNA adduct formed in mouse epidermis was identified as the (4R,3S)-diol-(2S,1R)-epoxide bound through trans addition to the exocyclic amino group of dGuo, although substantial relative amounts of the corresponding dAdo adduct were also detected. In addition, a K-region 5,6-oxide-dAdo adduct was identified in HPLC chromatograms based on cochromatography with an authentic marker adduct. The current data suggest that the bay region anti-diolepoxide is an ultimate carcinogenic metabolite of DB(a,j)A (20).

In newborn mice bay region diolepoxides of PAH generally are more tumorigenic than their parent PAH. In order to evaluate the mechanism(s) responsible for this behavior, one MERIT awardee has investigated the binding of metabolites of [³H]B(a)P, [³H]5- and [³H]6-methylchrysene (5-MeC and 6-MeC) and their corresponding dihydrodiols, and bay region diolepoxides, to pulmonary and hepatic DNA in male and female newborn mice, and compared the results with their tumorigenic activities. Groups of one-day old mice were treated with the appropriate compounds in DMSO by i.p. injection. HPLC analysis of DNA hydrolysates obtained 24 hours after treatment indicated that levels of diolepoxide-DNA adducts following treatment with (±)-[³H]7,8-dihydroxy-7,8-dihydroB(a)P and (±)-[³H]anti-7,8-dihydroxy-9,10-epoxy,7,8,9,10-tetrahydroB(a)P are 5- and 10-fold higher than those formed from [³H]B(a)P. Major products (70-80%) released from enzymatic hydrolysis of DNA following treatment with [³H]B(a)P, [³H]5-MeC, and [³H]6-MeC were unidentified polar compounds. Levels of these unknown products were lower, and formation of diolepoxide-DNA adducts higher, when test compounds were changed from parent PAH to the corresponding dihydrodiols and diolepoxides. Comparison of these results with those of tumorigenesis studies indicates a correlation between formation of B(a)P-diolepoxide-DNA adducts and induction of tumors in newborn mouse lung, but not in liver. These observations are consistent with the high sensitivity of the newborn mouse lung towards the tumorigenic effects of bay region diolepoxides. Previous studies from this laboratory have demonstrated that 1R,2S-dihydroxy-3S,4R-epoxy-1,2,3,4-tetrahydro-5-MeC (5-MeC-1R,2S-diol-3S,4R-epoxide) is a potent lung tumorigen, while the corresponding diolepoxide of 6-MeC had no effect in newborn mice. From the results of the present study, it is estimated that at equimolar doses the formation of diol-epoxide-DNA adducts from 5-MeC-1R,2S-diol-3S,4R-epoxide would be at least 20-fold greater than from the corresponding diolepoxide of 6-MeC in newborn mouse lung. Thus, the higher tumorigenic activity of 5-MeC-1R,2S-diol-3S,4R-epoxide compared to the corresponding diolepoxide of 6-MeC could partially be due to its high reactivity with pulmonary DNA (45).

Xylene is an aromatic hydrocarbon solvent of industrial and environmental significance. It is produced in large quantities, used in many industrial processes and found as a constituent of gasoline, paint, adhesives, inks and cleaning agents. Commercial xylene comprises three structural isomers, ortho-, meta-, and para-, with m-xylene being present in the largest amount (45-70%). There exists epidemiological evidence that painters exposed to organic solvents like xylene have a higher risk of lung cancer. Alterations in the pulmonary biotransformation of B(a)P and other PAHs could greatly influence the toxicity and subsequent carcinogenicity of these compounds in lung. P-Xylene and m-xylene administration inhibit rat pulmonary microsomal detoxication of B(a)P, as evidenced by decreased aryl hydrocarbon

hydroxylase activity. This appears to be due to the ability of xylene isomers to decrease cytochrome P450 content in rat lung, while liver and kidney P450 levels remain unchanged or slightly increased. Studies of B(a)P metabolism revealed that p-xylene decreased production of the nontoxic metabolite 3-hydroxy-B(a)P, while formation of toxic B(a)P diols was unaltered. This indicates that p-xylene produces a shift in the metabolic balance of this compound away from detoxication and towards production of more toxic, mutagenic species. One grantee examined the ability of m-xylene to alter the pulmonary microsomal metabolism of B(a)P. M-Xylene increased formation of B(a)P mutagenic bay region diols, B(a)P-7,8-diol (66%) and B(a)P-9,10-diol (56%) by rat pulmonary microsomal preparations, while formation of individual B(a)P phenols and quinones was unaltered. M-Xylene administration produced a decrease in cytochrome P450IIB1 activity as measured by pentoxy- and benzyloxyresorufin O-dealkylation, while cytochrome P450IA1 activity, expressed as ethoxyresorufin O-dealkylation, was unaltered. Pulmonary microsomal epoxide hydrolase activity was also unaltered by m-xylene. This demonstrates that m-xylene alters the relative contribution of P450 isozymes to B(a)P metabolism resulting in inhibition of B(a)P detoxication and increased production of toxic metabolites (117).

Alkylating Agents: Covalent binding of chemical carcinogens to cellular macromolecules is a critical step in the initiation of the carcinogenic process. Most chemicals require metabolic activation to electrophilic intermediates before reacting with cellular nucleophiles. In the case of hydrazine derivatives, their tumorigenic properties are usually ascribed to biotransformation to cationic alkylating metabolites. DNA alkylation by carbon-centered radicals is possible on chemical grounds. A few in vitro studies have indicated that chemically generated methyl radicals in acidic medium, or photochemically generated α -hydroxycarbon radicals, attack guanine and adenine bases by substituting at position 8 of purine rings. DNA alkylation at C-8 has not been demonstrated in vivo, probably because of a lack of appropriate standards and/or loss of the DNA adducts by depurination. Methylhydrazine oxidation promoted by horseradish peroxidase-H₂O₂ or ferricyanide led to the generation of high yields of methyl radicals and the formation of 7-methylguanine and 8-methylguanine upon interaction with calf thymus DNA. In one grantee's laboratory, methyl radicals were identified by spin-trapping experiments with α -(4-pyridyl-1-oxide)-N-tert-butyl nitrene and tert-nitrosobutane. The methylated guanine products were identified in the neutral hydrolysates of treated DNA by HPLC analysis and spiking with authentic samples. The structure of 8-methylguanine, a product not previously reported in enzymatic systems, was confirmed by HPLC chromatography, UV absorbance, and mass spectrometry. The formation of 8-methylguanine suggests a possible role for carbon-centered radicals as DNA-alkylating agents (13).

Ingested nitrate and nitrite have been shown to contribute to endogenous, N-nitroso compound formation in man and experimental animals. N-nitroso compounds have long been suspected of contributing to higher levels of gastric cancer in various populations. Reconstructive gastric surgery to treat ulcers is accompanied by a change in bile reflux, gastritis and an increased incidence of gastric cancer in humans. To evaluate possible reconstructive surgery and gastric cancer, the surgically altered domestic ferret was used as an experimental model. The aim of the study was to determine if surgery would alter the stomach in a way which would increase gastric nitrite concentration, and thereby enhance the likelihood of gastric N-nitroso compound formation. Three groups of ferrets were studied: one control group and two groups of surgically altered ferrets, one to simulate maximal bile reflux (MABR), and the other to model minimal bile reflux (MIBR). Each group's

response to an exogenously administered dose of sodium nitrite did not differ significantly with respect to rate of gastric nitrite absorption, with half-lives in the 13-minute range. Permeability of gastric mucosa to nitrite did not differ between controls and MIBR ferrets. Mean doubling time of gastric nitrate appeared slowed in surgically altered ferrets. Mean rate of gastric emptying was the same in the three groups, but appeared delayed initially in MIBR ferrets. Thiocyanate concentration, pH, and HCl in 1c secretion, all parameters which have been shown to affect gastric nitrite processing, did not differ significantly between groups. Gastric mucosal endoscopic biopsies obtained at 6-month intervals showed no clear difference in degree of mucosal dysplasia. These findings indicate that the increased incidence of cancer following gastric, reconstructive surgery may not be related to increased gastric N-nitroso compound formation (131).

Chlordecone (Kepone) has been extensively studied for its toxicity in male production workers who were exposed to large quantities of this organochlorine pesticide. Concern that these workers might be at increased risk of developing liver cancer prompted one grantee to test chlordecone in a two-stage rat model of hepatocarcinogenesis. Dose-response experiments showed that in non-initiated rat liver the hepatocarcinogenic effects of long-term chlordecone administration became undetectable at concentrations in the same range as those measured in human biopsies taken from exposed workers who exhibited no liver effects. Although the toxicity of chlordecone in women has never been studied, a dramatic sex difference in the incidence of malignant liver tumors caused by chlordecone promotion was found in rats. Frank hepatocellular carcinomas were observed in up to 63% of female rats whose livers were previously "initiated" with a subcarcinogenic dose of diethylnitrosamine given 24 hours after partial hepatectomy, and then "promoted" by 27 weeks of chlordecone administration. In contrast, none of the comparably treated males had malignant liver tumors, even after 44 weeks of "promotion" with chlordecone. Females in the diethylnitrosamine-initiated/chlordecone-promotion groups also contained γ -glutamyltranspeptidase-positive "preneoplastic" hepatocellular foci that were more abundant and larger than those observed in comparably-treated males. Moreover, because similar levels of chlordecone were measured in the livers of both sexes at the end of the experimental period, the development of hepatocellular carcinomas in the diethylnitrosamine-initiated female rats appeared to be due to their increased sensitivity to the promotion treatment (124).

N-(2-Chloroethyl)-N'-alkyl-N-nitrosoureas (Cl-ENU) are some of the few clinically useful antineoplastic agents employed in the treatment of human brain malignancies. The predominant position for DNA alkylation by Cl-ENU is at N7-G, a major groove site, with other monofunctional modifications also found at O⁶-G, N3-C, N3-T, and the phosphodiester backbone. The O⁶-(2-chloroethyl)-G adduct, which slowly cyclizes to N1,O⁶-ethano-G and then undergoes nucleophilic attack by an N3-C on the complementary strand, is the precursor for the cytotoxic interstrand cross-link between N1-G and N3-C. This cross-link is thought to be responsible for Cl-ENU anticancer activity. One grantee performed the synthesis and characterization of a series of compounds that contain an N-alkyl-nitrosourea functionality linked to DNA minor groove binding bi- and tripeptides (lexitropsins or information-reading peptides) based on methylpyrrole-2-carboxamide subunits. The lexitropsins (lex) synthesized have either a 3-(dimethylamino)propyl or propyl substituent on the carboxyl terminus. The preferred DNA affinity binding sequences of these compounds were footprinted in ³²P-end-labeled restriction fragments with methidiumpropyl-EDTA•Fe(II), and in common with other structural analogues, e.g., distamycin and netropsin, these nitrosoureas recognize A-T-rich runs. The affinity binding of the

compound with the dimethylamino terminus, which is ionized at near-neutral pH, appeared stronger than that observed for the neutral dipeptide. The sequence specificity for DNA alkylation by (2-chloroethyl)nitrosourea-lex dipeptides (Cl-ENU-lex), with neutral and charged carboxyl termini, using ^{32}P -end-labeled restriction fragments, was determined by the conversion of the adducted sites into single-strand breaks by sequential heating at neutral pH and exposure to base. The DNA cleavage sites were visualized by polyacrylamide gel electrophoresis and autoradiography. The alkylation of DNA by Cl-ENU-lex was compared to that by N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea (CCNU), which has no DNA affinity binding properties. While all the Cl-ENU compounds generate DNA breaks as a consequence of the formation of N7-alkylguanine, the Cl-ENU-lex compounds induced, in a time- and dose-dependent fashion, intense DNA cleavage bands at adenine, cytosine, and thymine residues associated with affinity binding sites. These non-G cleavages induced by Cl-ENU-lex were inhibited by the coaddition of distamycin at concentrations that did not affect G-alkylation break sites. CCNU, even at much higher concentrations, does not generate any similar detectable lesions at non-G sites. Therefore, linking the Cl-ENU moiety to minor groove binders is a viable strategy to qualitatively and quantitatively control the delivery and release of the ultimate DNA alkylating agent in a sequence-dependent fashion (32).

Enzymes and Markers of Tumor Cells: Research included in this sub-grouping involves studies on cancer marker proteins or enzymes which are specific for one or more tumor types. Although most human colon cancers are thought to originate in adenomas, some suggest that the more aggressive cancers develop in small flat adenomas that frequently show severe dysplasia and are difficult to recognize endoscopically. The normal-appearing mucosa resected with human colonic carcinomas for the presence of aberrant crypts after staining with methylene blue was examined by one grantee. Strips of colonic mucosa from 22 consecutive patients with colon cancer were evaluated. Aberrant crypts (AC) were identified for the first time in human material in 4 of 12 resections from the right colon, 0 of 1 from the transverse colon, and 9 of 9 from the left colon. There were 10-fold more AC in the left colon (0.33 ± 0.18 AC/sq cm) than in the right colon (0.033 ± 0.063 AC/sq cm). The AC varied from single altered glands to plaques of 30 crypts. The mean percent of mucosa altered was $0.064 \pm 0.14\%$ for the right colon and $0.49 \pm 0.71\%$ for the left colon. The mean percent of mucosa altered in all 22 cancer patients was $0.23 \pm 0.50\%$ compared with $0.002 \pm 0.005\%$ in control (autopsy) patients without colon cancer. AC were marked with permanent ink, embedded in methacrylate, and evaluated histologically in 2-4 μm sections. All AC analyzed from carcinogen-treated rats had decreased hexosaminidase activity, while most AC evaluated from human colons had increased hexosaminidase activity. Aberrant crypts appear to be the earliest identifiable lesions in colonic mucosa at risk for cancer. Aberrant crypts are postulated to be putative precursors of colon cancer, but their role in the pathogenesis of the disease remains to be established. Increased concentration of aberrant crypts in the colonic mucosa from Gardner's syndrome supports this hypothesis (104).

A number of studies in experimental animals and humans have suggested that alterations in the activity of protein kinase C (PKC) may be involved in the malignant transformation process. To determine whether such alterations in this kinase were present before the development of 1,2-dimethylhydrazine (DMH)-induced colon cancers, rats were given s.c. injections of this procarcinogen (20 mg/kg body weight/week) or diluent for 10 or 15 weeks. Animals were sacrificed after these time periods and colonic epithelium was harvested from each group. The activity and distribution of PKC in the cytosolic and membrane fractions of these preparations,

as well as 1,2-diacylglycerol mass and phosphoinositide turnover, were then examined and compared in the presence and absence of 10 nM 1,25-dihydroxycholecalciferol, an agent which has previously been found to influence these biochemical parameters in the normal rat colonic epithelium. The results of these studies demonstrate that (a) the percentage of PKC activity in the membrane fraction was significantly greater in DMH-treated animals compared to their control counterparts at 10 and 15 weeks; (b) the total PKC activity was similar at 10 weeks, but markedly reduced in the colonic mucosa of the DMH-treated group at 15 weeks; (c) 1,2-diacylglycerol mass and phosphoinositide turnover were increased in the colonic mucosa of rats administered this carcinogen at both time points; and (d) in control, but not in DMH-treated animals, in vitro addition of 1,25-dihydroxycholecalciferol increased PKC activity, 1,2-diacylglycerol mass, and phosphoinositide turnover at each of the times studied. Based on these findings, it would appear that alterations in PKC activity may play a role in the malignant transformation process of the colon in animals administered DMH (9).

The major process involved in the bioactivation of chemical carcinogens is oxidation by P450 enzymes. Thirty or more different forms of P450 exist within each animal species, each with at least some distinct elements of catalytic specificity and regulation. Understanding which of these P450s activate and detoxicate each chemical carcinogen would be of use in ascertaining the importance of P450s, as well as the risk of each chemical to humans. The role of human cytochrome P450IIE1 (P450IIE1) in the oxidation of a number of suspect carcinogens was examined by one OIG awardee using a variety of approaches including: (1) selective inhibition of catalytic activity in human liver microsomes by diethyldithiocarbamate, which was found to be a selective mechanism-based inactivator of P450IIE1; (2) correlation of rates of different catalytic activities with each other and with chlorzoxazone 6-hydroxylation, an indicator of P450IIE1, in human liver microsomes; (3) demonstration of catalytic activity in reconstituted systems containing purified human P450IIE1; and (4) immunoinhibition of catalytic activity in human liver microsomes with rabbit anti-human P450IIE1. The results collectively indicate that P450IIE1 is a major catalyst of the oxidation of benzene, styrene, CCl₄, CHCl₃, CH₂Cl₂, CH₃Cl, CH₃CCl₃, 1,2-dichloropropane, ethylene dichloride, ethylene dibromide, vinyl chloride, vinyl bromide, acrylonitrile, vinyl carbamate, ethyl carbamate, and trichloroethylene. Levels of P450IIE1 can vary considerably among individual humans--the availability of chlorzoxazone as a noninvasive probe of human P450IIE1 and of disulfiram (oxidized diethyldithiocarbamate) as an inhibitor may facilitate discernment of the in vivo significance of human P450IIE1 as a factor in the bioactivation and detoxication of these carcinogen suspects. Further, many investigations with diethyldithiocarbamate, disulfiram, and ethanol in humans and experimental animals may be interpreted in light of mechanisms involving P-450IIE1 (36).

Arylamine chemicals in industry, the environment, and from other sources are thought to be responsible for initiation of human cancers, particularly in the bladder. A model for biotransformation of arylamines to carcinogenic species suggests a sequential N-oxidation to N-hydroxyarylamines catalyzed by microsomal oxygenases, followed by N-acetylation/deacetylation pathways to yield proximate carcinogens capable of enzyme or acid catalyzed reaction with DNA of the bladder epithelial cells. The relative rates of these metabolic activation/bioactivation reactions, although not fully defined, play critical roles in the susceptibility of selected target organs and tissues to arylamine-induced carcinogenesis. Recently a diverse array of acetyl transfer reactions has been identified in arylamine metabolism catalyzed by cytosolic N-acetyltransferase (NAT) enzymes present in liver and many

extrahepatic tissues, including bladder. N-acetylation capacity is genetically regulated in humans and other mammalian species via two major codominant alleles at a single gene locus. The proportion of slow acetylators varies somewhat with ethnic origin, but is more than 50% in most populations. Of particular interest have been human epidemiological studies indicating increased incidence of bladder tumors and increased invasiveness of bladder tumors among slow acetylators. The higher incidence of slow acetylators with bladder cancer is particularly evident in human populations with documented exposures to arylamine carcinogens. One grantee partially purified and characterized NAT activity in inbred hamster urinary bladder cytosols of defined acetylator genotype. Acetylator gene-dose response relationships were observed for the N-acetylation of p-aminobenzoic acid, p-aminosalicylic acid, and the arylamine carcinogens 2-aminofluorene, 4-aminobiphenyl, and β -naphthylamine in hamster bladder cytosol. Partial purification of hamster bladder cytosol by anion-exchange fast protein liquid chromatography yielded two NAT isozymes that catalyzed the N-acetylation of each of the arylamine substrates. The catalytic activity of the first isozyme was acetylator genotype-dependent (polymorphic), whereas the second isozyme appeared to be acetylator genotype-independent (monomorphic). Catalytic activities between homozygous-rapid, heterozygous, and homozygous-slow acetylator genotypes were compared with respect to both initial rates and apparent maximum velocities. Comparison of homozygous-rapid and -slow acetylator bladder cytosol showed that the apparent V_{max} for 2-aminofluorene NAT activity was significantly higher in rapid than slow acetylators (6-fold in cytosol, 50-fold in the polymorphic NAT isozyme). These results suggest a key role for a polymorphic NAT isozyme, regulated by the acetylator genotype and expressed in urinary bladder cytosol, in the initiation of bladder cancer via arylamine carcinogens (46).

During tumor progression, micrometastases at their earliest stages have been difficult to analyze qualitatively or quantitatively because of a lack of suitably sensitive markers to discriminate small numbers of tumor cells from normal tissue cell populations. For the study of micrometastases at their earliest stages, one grantee transfected the *lacZ* gene, which codes for β -D-galactosidase in *Escherichia coli*, into BALB/c 3T3 cells transformed by the Ha-ras oncogene of a human EJ bladder carcinoma. These cells were subsequently injected into 6-week-old, female athymic NCR-NU nude mice by several routes. With chromogenic detection of the product of the *lacZ* gene (a heterologous gene not observed in animal cells) by use of 6-bromo-4-chloro-3-indoyl- β -D-galactopyranoside, the tumor cells implanted in the lungs minutes after intravenous injection were easily identified by the intensely blue staining of the cells harboring the *lacZ* gene. The number of lung-associated tumor cells remained constant for several hours after intravenous injection but then decreased to a stable level by 24 hours. At most sites of lung invasion, multiple tumor cells, rather than single cells, were identified. This finding suggests that cooperation among multiple cells may be important in the early stages of micrometastasis development. Within several days, a few foci of micrometastases were expanding by proliferation and/or migration of individual tumor cells among host lung cells. These results confirm that the *lacZ* gene is an ultrasensitive histochemical marker for analyzing both qualitatively and quantitatively the earliest stages of micrometastasis development in the lung and in other organs where micrometastases may ensue (104).

Transforming growth factor type- β (TGF- β) is a peptide which was initially identified by its capacity to induce phenotype transformation of rat fibroblasts. More recently it has been found to be synthesized by a variety of normal adult and embryonic cells as well as by neoplastic cell populations. In addition, essentially

all cells have specific membrane, high-affinity, TGF- β -receptors. It now appears highly likely that TGF- β plays a critical role in the regulation of normal cell growth and/or differentiation. As rat tracheal epithelial cells progress from a normal to a neoplastic phenotype, there are systematic changes in their ability to produce and activate latent TGF- β , as well as systematic changes in their response to this growth factor. Using a TGF- β radioreceptor binding competition assay, it was found that normal proliferating rat tracheal cells in early primary culture produced latent TGF- β . With the emergence of terminally differentiated cell populations active TGF- β was also detected in the conditioned medium. When normal cells were cultured under conditions allowing for continued proliferation, no active TGF- β was detected in the conditioned medium. Colonies of proliferating epithelial cells in 4-6 week primary cultures or subculturable tracheal cell lines did not produce detectable levels of active or latent growth factor. With neoplastic progression there was likewise a change in response to active TGF- β . Normal tracheal cells in primary culture were highly sensitive to growth-factor-induced decreases in thymidine uptake as well as to the induction of terminal differentiation. Proliferating epithelial cells in late (4-6 weeks) primary cultures and preneoplastic, subculturable cell lines were often as sensitive as normal cells to the growth factor-induced decline in thymidine uptake. However, none of these altered populations was induced to differentiate in the presence of TGF- β (133).

Genetics and Properties of Tumor Cells: The types of research covered in this sub-grouping include studies on gene expression, gene analysis and characteristics of cells associated with carcinogenesis. The c-Ki-ras and N-ras oncogenes have been characterized in aflatoxin B₁ (AFB₁)-induced hepatocellular carcinomas. Detection of different proto-oncogene and oncogene sequences and estimation of their frequency distribution were accomplished by polymerase chain reaction cloning and plaque screening methods. Two c-Ki-ras oncogene sequences were identified in DNA from liver tumors that contained nucleotide changes absent in DNA from liver of untreated control rats. Sequence changes involving G:C to T:A or G:C to A:T nucleotide substitutions in codon 12 were scored in three of eight tumor-bearing animals. Distributions of c-Ki-ras sequences in tumors and normal liver DNA indicated that the observed nucleotide changes were consistent with those expected to result from direct mutagenesis of the germ-line protooncogene by AFB₁. N-ras oncogene sequences were identified in DNA from two of eight tumors. Three N-ras gene regions were identified, one of which was shown to be associated with an oncogene containing a putative activating amino acid residing at codon 13. All three N-ras sequences, including the region detected in N-ras oncogenes, were present at similar frequencies in DNA samples from control livers as well as liver tumors. The presence of a potential germ-line oncogene may be related to the sensitivity of the Fischer rat strain to liver carcinogenesis by AFB₁ and other chemical carcinogens (131).

Experiments were conducted by another MERIT awardee to determine whether point mutations activating K-ras or H-ras oncogenes, induced by the procarcinogen DMH, were detectable in preneoplastic or neoplastic rat colonic mucosa. Rats were injected weekly with diluent or DMH at 20 mg/kg body weight for 5, 10, 15, or 25 weeks, then killed and their colons dissected. DNA was extracted from diluent-injected control animals, histologically normal colonic mucosa from carcinogen-treated animals, and from carcinomas. Ras mutations were characterized by differential hybridization using allele-specific oligonucleotide probes to polymerase chain reaction-amplified DNA, and confirmed by DNA sequencing. Although no H-ras mutations were detectable in any group, K-ras (G to A) mutations were found in 66% of DMH-induced colon carcinomas. These mutations were at the second

nucleotide of codons 12 or 13, or the first nucleotide of codon 59 of the K-ras gene. The same type of K-ras mutations were observed in premalignant colonic mucosa from 2 out of 11 rats as early as 15 weeks after beginning carcinogen injections when no dysplasia, adenomas, or carcinomas were histologically evident, suggesting that ras mutation may be an early event in colon carcinogenesis (9).

Cytochrome P450IA1 catalyzes the mixed-function oxidation of a wide variety of endogenous and exogenous polycyclic aromatic compounds. Many of these, including the potent environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and the carcinogen B(a)P, induce the expression of the cytochrome P450IA1 gene (CYP1A1) by both transcriptional and posttranscriptional mechanisms. Three nuclear factors--the Ah receptor, Xf1, and Xf2--bind sequence specifically to the Ah response elements or xenobiotic response elements (XREs) of the cytochrome P450IA1 gene. Using a series of synthetic oligonucleotides with systematic alterations in the XRE core sequence, the interactions of these factors with the Ah response element XRE1 were compared by three independent methods: methylation interference footprinting; orthophenanthroline-Cu⁺ footprinting; and mobility shift competition experiments. These studies established that: (1) all three factors interact sequence specifically with the core sequence of XRE1; (2) the pattern of contacts made with this sequence by the Ah receptor are different from those made by Xf1 and Xf2; and (3) although Xf1 and Xf2 can be distinguished by the mobility shift assay, the sequence specificities of their interactions with XRE1 are indistinguishable. Further characterization revealed the following additional differences among these three factors: (1) Xf1 and Xf2 could be extracted from nuclei under conditions quite different from those required for extraction of the Ah receptor; (2) Xf1 and Xf2 were present in nuclei of untreated cells and did not respond to polycyclic compounds, such as TCDD and β -naphthoflavone, while nuclear Ah receptor was undetectable in untreated cells and rapidly increased in response to TCDD; (3) inhibition of protein synthesis did not affect the TCDD-induced appearance of the Ah receptor but substantially decreased the constitutive activities of Xf1 and Xf2, suggesting that the Ah receptor must be present in untreated cells in an inactive form than can be rapidly activated by polycyclic compounds, while the constitutive expression of Xf1 and Xf2 depends on the continued synthesis of a relatively unstable protein; (4) the receptor-deficient and nuclear translocation-defective mutants of the hepatoma cell line Hepal, which are known to lack nuclear Ah receptor, expressed normal levels of Xf1 and Xf2, suggesting that the former factor is genetically distinct from the latter two; and (5) a divalent metal ion, probably Zn²⁺, is known to be an essential cofactor for the Ah receptor but was not required for the DNA-binding activities of Xf1 and Xf2. Together, these findings indicate that the Ah receptor is distinct from Xf1 and Xf2, while the latter two activities may be related. Because the DNA-binding domains of these three factors overlap substantially, their binding to XREs is probably mutually exclusive, which suggests that the interplay of these factors with Ah response elements may be important to the regulation of CYP1A1 gene transcription. The results of preliminary transfection experiments with constructs harboring XREs upstream of the chloramphenicol acetyltransferase gene driven by a minimal simian virus 40 promoter are consistent with this hypothesis (26).

Cytochrome P450IA1 (Cyto-P450IA1) is the isozyme most closely associated with aryl hydrocarbon hydroxylase (AHH). At least two distinct high-affinity binding proteins may regulate its expression, the 4S protein that primarily binds PAHs and the 8S Ah receptor that binds TCDD and like congeners. A study was conducted to investigate ligand-binding characteristics of the 4S- and 8S- binding proteins before and after separation from liver cytosol in the presence and absence of sodium molybdate.

Liver cytosol and 4S- and 8S- receptor-enriched fractions from livers of male Sprague-Dawley rats (AHH-responsive), and from C57BL/6N (AHH-responsive) and DBA/2N and AKR/N (AHH-nonresponsive) mice served as sources of these proteins. Competitive binding studies were performed using 10 nM [³H]-B(a)P or [³H]-TCDD in the presence and absence of a 200-fold excess of B(a)P, 3-methylcholanthrene (3-MC), and tetrachlorodibenzofuran (TCDBF). Specific PAH-binding activity was assayed by using either sucrose density gradient analysis or a hydroxylapatite assay. Results indicate that before and after the separation of liver cytosol into 4S and 8S fractions, ligand-binding characteristics were relatively unaltered for the 4S protein in comparison to that for the Ah receptor, particularly in the presence of molybdate. The 4S protein had high affinity for B(a)P and 3-MC but very low affinity for TCDBF; the 8S protein had high affinity for TCDBF, lesser affinity for 3-MC, and low affinity for B(a)P. In the presence of sodium molybdate, the Ah receptor fractions were significantly stabilized, whereas the 4S protein was relatively unaffected. After the separation of Ah receptor fraction from liver cytosol in the presence of molybdate, 3-MC consistently bound to a greater extent. These results affirm the existence of two distinct PAH-binding proteins (10).

Mammary Cell Carcinogenesis Studies: Study of the role of chemical carcinogens in human mammary cancer requires (1) an understanding of the mechanism by which normal cells become malignant and (2) the availability of a reproducible, quantitative system for transforming mammary epithelial cells by exposure to carcinogens. Development of an in vitro system for mammary carcinogenesis studies in humans is the goal of five grantees. One of the investigators has been successful in growing single human mammary epithelial cells (HMECs) inside different matrices. The cells grow in three-dimensions and produce colonies of different morphologies and growth rates. The significance of these differences in the susceptibility of the cells to transformation is being tested (95). Development of a model culture system to induce and dissect the processes leading to an unlimited lifespan for HMECs was the goal of another investigator. After conditions for optimal growth of HMECs were determined, SV40 large T-antigen was transfected into the cells and clones were grown out and expanded. Results showed that after 50-55 population doublings, senescent HMECs have greatly altered expression of extracellular matrix components, including proteases and protease inhibitors, when compared to cells obtained from the same patient at lesser population doublings. Such extracellular matrix components may play a role in invasion and metastasis. Experiments are underway to extend these initial findings by characterizing additional HMECs at early and late passages in cell culture to determine if progressive altered expression of extracellular matrix components is part of the normal aging process of HMECs (120). Elsewhere, studies on growth requirements of HMECs revealed that co-culture with mammary fibroblasts results in growth behavior quite different from either cell type alone. Such cultures were studied for expression of milk-related genes in response to hormonal and extracellular matrix stimulation. These investigators now have complete coding sequences for three independent human beta-casein cDNAs and have found that human beta-casein cDNA lacks an exon (exon 3) that is uniformly expressed in other species. The cDNA also has a C-terminal extension of exon 7 that is variably deleted in other species, and is closer to the "ancestral" consensus sequence than the others (41). As part of such studies, two major morphologically distinguishable cell types in primary HMEC cultures were observed; one type was completely deficient in gap junctional intercellular communication (GJIC) (Type I) and the other was competent in GJIC (Type II). Further results indicate that these phenotypes are stem and progenitor cells, respectively, for HMECs. These cells

should be useful for the study of the role of stem cells in neoplastic transformation of human mammary epithelial cells. It was further observed that Type I cells can be induced to differentiate into Type II cells by cholera toxin, which presumably is capable of increasing the cellular cyclic AMP level. Besides induction of differentiation, cholera toxin was also found to extend the cumulative population doubling level for normal HMEC cultures from 44 to 66 in eight passages (15). Lastly, another grantee was the first to report transfection of HMECs with c-Ha-ras oncogene. Insertion of activated c-Ha-ras alone was enough to induce transformation of HMEC. The availability of this experimental system, in which specific phenotypes of malignant transformation have been characterized both in vivo and in vitro, will allow investigators to monitor the effect of carcinogens on primary cultures using these phenotypes as markers. In addition, it will serve to determine whether the carcinogenic effect is mediated by activation of the c-Ha-ras oncogene, as has been demonstrated in other experimental systems (114).

Development of in vitro systems for mammary carcinogenesis studies in rodents was the goal of investigators on a program project and four research project grants. A novel experimental culture system has been established by members of the program project which has resulted in the first demonstration of a critical role for the extracellular matrix and of the direct effects of hormones on the ultrastructural and biochemical differentiation of mouse mammary epithelial cells (MMECs). Employing this culture system, several discoveries have been made, including findings that MMECs can proliferate in vitro in the presence of a variety of different mitogens (hormones, growth factors, lipids, c-AMP, TPA, and lithium). These investigators were the first to consistently induce neoplastic transformations of MMECs in serum-free, defined conditions with chemical carcinogens (94). A traditional research project grantee observed that insulin-like growth factor (IGF) and epidermal growth factor (EGF) independence occurs by separate mechanisms which are very different. Independence of IGFs occurs in the absence of any demonstrable autocrine factor and may occur as a result of a recessive mechanism. Independence of EGF appeared to occur by an autocrine mechanism that may involve a novel growth factor that interacts with the *erbB-2* receptor (25). In another grant, the mouse mammary multistage model was used to understand potential essential alterations in the early stage of neoplastic transformation. Positive achievements have been the ability to reproducibly establish DMBA-exposed MMEC lines in vitro and the discovery that several of these cell lines produce preneoplastic hyperplastic outgrowths in vivo. The treated cells were examined for alterations in growth factor dependence, proto-oncogene expression (those proto-oncogenes that are homologues of hormone receptors), and altered keratin expression as well as morphological types of preneoplasias (84). A MERIT awardee performed an analysis of mouse mammary beta-casein gene transcription in MMEC lines using a series of single-stranded genomic clones that span the locus of the gene. The development of MMEC lines that express the endogenous beta-casein gene in a hormonally- and cell substratum-regulated fashion has facilitated both gene transfer experiments as well as studies of endogenous gene regulation. As part of such studies, lines of transgenic mice carrying beta-casein-*neuN* and *neuT* fusion genes have been established. The *neu* (c-*erbB-2*) oncogene has been implicated in the development of human breast cancer. It is known that a high percentage of breast cancers have been found to contain amplified copies of the *neu* gene and many of these tumors also show a high level of *neu* protein expression. Therefore, in order to investigate how the level and timing of *neu* expression may affect normal mammary development and carcinogenesis, the beta-casein-based expression vector system has been employed to express the rat *neu* oncogene in transgenic mice (112).

Estrogens: The exact biochemical and molecular mechanism(s) of estrogen-induced carcinogenesis is not well understood. Several hypotheses have been proposed for estrogen-induced tumors in hamster kidney, the animal model most intensively investigated at present. Although not widely used, the stilbene estrogen, diethylstilbestrol (DES), has been associated with human and experimental animal neoplasms. DES and natural estrogen-reactive metabolites have been shown to bind irreversibly to microsomal proteins. Direct evidence for oxidation of DES to DES-p-quinone and covalent binding of DES-quinone to DNA both in vitro and in vivo have been described. More recently, the synergistic covalent attack of DES reactive metabolites on the nucleophilic sites of nuclear proteins has been shown (113). Administration of estrogen to hamsters decreases the cytochrome P450 content in the kidney specifically. The decrease occurs in conjunction with decreasing activities of several cytochrome P450 isozymes. It has been suggested that one of the reasons for the decrease in cytochrome P450 may be inactivation of the enzyme by covalent binding of catechol estrogen quinone to the protein (70). The net effect could increase estrogen activity as a consequence of a decrease in metabolism (68). Elsewhere, after considerable research on the synthesis of several estrogen-quinones and estrogen epoxides, it has been reported that these compounds do not interact with DNA through covalent linkage in the hamster kidney. However, some of these compounds do react with proteins irreversibly. Thus, it is possible that estrogen carcinogenesis may not involve adduct formation but rather the formation of free radicals. Additionally, experiments using estrogen quinones and semiquinones in a human mammary cancer cell line have shown DNA damage. Further experiments using cell cultures clearly showed that estrogen quinones induce DNA damage primarily by cleavage of single-strand DNA (1). However, a recent report on the metabolism of Moxestrol in hamster kidney strongly weakens the belief that estrogen metabolism, specifically catechol formation, and redox recycling play significant roles in the tumorigenicity of estrogens. Moxestrol is a potent estrogen in both animals and humans. Its estrogenic potency has been shown to be 10-100 times greater than 17-beta-estradiol and 5 times greater than ethinylestradiol. Importantly, Moxestrol is as carcinogenic as 17-beta-estradiol or DES in producing renal tumors in the hamster kidney (100% incidence). When the oxidative metabolism of radiolabeled estrone and Moxestrol was studied, the quantity of 2-/4-hydroxyestrone found in kidney microsomes of the hamster represented over 14% of the total metabolites formed, whereas similar microsomal incubations with Moxestrol yielded no or negligible amounts of catechol intermediates. Since these results show that 94-97% of Moxestrol remained unmetabolized compared to 64% of estrone, the mechanism of action of estrogen carcinogenicity still needs to be elucidated. On this basis, it has been suggested that estrogens are non-genotoxic carcinogens (68).

Additional studies on estrogen carcinogenicity involved the development of a multistage carcinogenesis system for the mouse cervico-vaginal epithelium. Squamous cell carcinomas of the cervico-vaginal epithelium can be induced in DMBA or MNNG treated animals after promotion with either TPA or estrogens, but not by DMBA or MNNG alone. Six cervical tumors induced by DMBA-estrogen combination were tested for the presence of Ha-ras gene as an indicator of mutation. Five out of the six tumors showed the presence of a mutation (16).

Treatment of Noble rats for 16 weeks with estradiol-17-beta (E_2) and testosterone (T) caused dorsolateral prostate (DLP) dysplasia, a putative preneoplastic lesion, while the ventral prostatic lobe (VP) of the Noble rats is refractory to treatment of E_2 and T. Organ culture of prostatic tissue showed that normal DLP tissue favors the conversion of estrone (E_1) to E_2 , while the VP favors E_2 to E_1 metabolism. Following E_2 and T treatment for 16 weeks, the dysplastic DLP demonstrated a higher

uptake of E_2 and reduced metabolism of this steroid. These findings indicate that the bioavailability of the active estrogen, E_2 , in the DLP is greater than that in the VP, partially explaining why DLP is more susceptible to estrogen stimulation. Furthermore, dysplastic DLP tissue seems to have an enhanced ability to retain E_2 (65). Another demonstration of the role of E_2 in the Noble rat prostate neoplasia was reported by an investigator who made the discovery that a 100% incidence of invasive carcinoma was seen with the combined treatment of E_2 and T in the Noble rat. These tumors were microscopic in size and required long-term treatment with exogenous hormones (8). In other experimental animal studies, cloning from hormone-induced leiomyosarcomas of cDNAs containing retroviral long terminal repeats (LTR) suggested a new approach to investigating the mechanisms of sex steroid induction of reproductive tract tumors in hamsters. The Syrian hamster reproductive tract has a well-defined susceptibility for estrogen-androgen induced carcinogenesis. A specific requirement for both estrogens and androgens in the induction process was observed. A retrovirus was identified in epithelial cells and semen of hamster vas deferens and epididymis. Tests were run to evaluate the role of sex steroids and growth factors on transcriptional regulation and cultures of hamsters and mouse tissue which were transfected with LTR-reporter gene constructs (96).

Other Agents: This subgrouping consists of grants investigating a variety of agents, compounds, or factors which are not covered in the other subcategories, or with mixtures of agents from more than one category. One project has been concentrating on I-compounds which 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. To characterize their properties, I-compounds were compared with carcinogen-DNA adducts in liver, kidney and skin of three rodent species. Weanling female Sprague-Dawley rats, ICR mice and Syrian hamsters were fed Teklad LM485 chow diet for three months and raised concurrently and strictly under the same environmental conditions. Animals of each species were treated topically with 24 $\mu\text{mol/kg}$ dibenz[a,j]acridine per day for three days, then by gavage once with a mixture of safrole and 7,12-dimethylbenz[a]anthracene (60 and 80 $\mu\text{mol/kg}$ respectively), or with one of the individual carcinogens. Liver, kidney and skin DNA from carcinogen-exposed (23 hours after treatment) and unexposed animals was analyzed by the monophosphate version of the ^{32}P -postlabeling assay. Although each of the three carcinogens produced qualitatively identical major adduct patterns in all samples examined, I-compounds in untreated animals showed distinct species- and tissue-dependent profiles. Among the three species, rats displayed the highest I-compound levels but the lowest adduct levels in both liver and kidney. These findings demonstrate fundamental differences between I-compounds and carcinogen-DNA adducts, and support the hypothesis that I-compound formation is primarily related to species-specific, i.e. genetically determined, normal metabolic activities rather than exposure to environmental genotoxic carcinogens (106).

Neoplastic transformation of cells is believed to be a multistep process, beginning with an alteration in the genetic material of a somatic cell. This somatic mutation hypothesis arises from correlations between specific locus mutation induction and cancer induction by chemical and physical agents. Additional support for the involvement of mutations in neoplastic transformation comes from the reporting of an association between point mutations and the process of carcinogenesis. For example, cellular oncogenes, such as *ras* and *neu*, can be activated by single-point mutations. Deletion or mutational inactivation of recessive tumor suppressor genes also plays an essential role in the genesis of several tumor types, including retinoblastoma and Wilms' tumor. Thus, short-term tests for specific locus mutation induction can be used to identify potential carcinogens based on their mutagenic activity.

Specific locus mutation induction by chemical and physical carcinogens has been documented in human diploid fibroblasts. However, since 85-90% of all human cancers originate from epithelial cells, it is important to determine if mutagenesis is also cell type- or organ-specific. For these reasons a study was performed to examine tissue- and locus-specific mutation induction by UV radiation in human mammary epithelial cells (HMEC). Primary HMEC from normal tissue, as well as immortalized HMEC derived from normal HMEC, were cultured under identical conditions and exposed to 10J/m² ultraviolet (UV) radiation (254 nm peak wavelength), which produced approximately 50% mean survival in all cell strains and lines tested. UV radiation was found to induce mutations at the Na⁺-K⁺ ATPase locus as determined by ouabain-resistance in both normal and immortalized HMEC. Mutation frequencies measured in these cells following UV exposure were similar to those reported for human diploid fibroblasts. In addition, mutation induction was investigated at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus in normal and immortalized HMEC. Induced mutations at the HPRT locus as determined by 6-thioguanine resistance in normal primary HMEC were not observed following UV-exposed immortalized HMEC. This study has demonstrated a similar frequency of UV-induced specific locus mutations at the Na⁺-K⁺ ATPase locus in human normal mammary epithelial cells as has been reported for human normal fibroblasts. The data also suggest caution in the use of immortal cell lines in genotoxicity testing; tissue-specific characteristics associated with the organ of origin may be overridden by the process of immortalization of the immortal phenotype (33).

Tenascin, originally described as myotendinous antigen, is a recently characterized extracellular matrix glycoprotein. It is intimately involved in tissue interactions during fetal development and oncogenesis. Tenascin appears to mediate epithelial/mesenchymal interactions during kidney, tooth, and mammary gland development by inhibiting, in part, the binding of cells to fibronectin. Such tissue interactions are equally as important for normal embryonic development as they are for the altered growth seen in neoplastic states. In order to investigate the involvement of tenascin in epithelial growth and malignancy, one grantee examined, immunohistochemically, its specific distribution pattern in the human uterus. During the proliferative phase of the menstrual cycle, this antigen was found as a sharp band around the endometrial glands. The immunoreactivity persisted until the early postovulatory phase of the menstrual cycle, but was not detectable in the glandular or stromal compartment during this later secretory stage, instead endometrial arterioles were immunostained. In marked contradistinction, when antibodies directed against tenascin were applied to sections of endometrial adenocarcinoma, almost the entire extracellular space stained, whereas the neoplastic cells themselves were nonreactive, whatever the degree of tumor differentiation. In precancerous proliferative lesions of the endometrium, tenascin's presence was variable. It was detectable around some superficial glands demonstrating cystic hyperplasia and around all deeply situated glands at the endometrial/myometrial interface. In cases of adenomatous hyperplasia, tenascin immunolocalized throughout the extracellular space of the stroma and the staining intensity was increased as the hyperplasia became more atypical. The results suggest that tenascin may be a stromal marker for epithelial proliferative states including those associated with malignancies of the endometrium (121).

C-nitroso aromatics, products of metabolic reduction of nitroaromatic environmental pollutants and peroxidative metabolism of carcinogenic N-arylhydroxamic acids, are potent direct mutagens for bacterial and mammalian cells. The genotoxicity of C-nitroso aromatics has been ascribed to enzymatic and/or non-enzymatic reduction to the hydroxylamines which form arylamine-type adducts with DNA. Damage to DNA by

C-nitroso aromatics may also result from products of their reaction(s) with other cellular components such as lipids. The possibility that the interaction of C-nitroso aromatics with polyunsaturated fatty acids (PUFA) causes lipid peroxidation was investigated through determination of conjugated diene and malodialdehyde (MDA) formation after anaerobic/aerobic vs. aerobic incubations of nitrosobenzene (NOB) or 2-nitrosofluorene (2-NOF) with linoleic, linolenic or arachidonic acid or methyl linolenate. Anaerobic incubation of NOB or 2-NOF with linolenic acid at the molar ratio of 1:1 for 24 hours yielded ~5.5-13% of the PUFA as conjugated diene which appeared stable upon exposure to air. Interaction of PUFA and 2-NOF or NOB yielded MDA, the amounts of which were significantly greater when 24 hour anaerobic preceded 1-6 hour aerobic incubation. Furthermore, the difference in the amounts of MDA resulting from 24 and 0 hour anaerobic incubations were significantly greater when the molar ratio of 2-NOF (or NOB) to PUFA was increased (2.0 > 1.0 > 0.5). Superoxide dismutase or catalase had no effect on the yields of MDA following either anaerobic/aerobic or aerobic incubations of PUFA and 2-NOF. EDTA (1 or 10 μ M) had no effect on the yields of MDA from aerobic incubations, but it decreased the amounts of MDA (by ~30 or 60%, respectively) from anaerobic/aerobic incubations. The data suggest that inhibition by EDTA is due to chelation of trace iron, which, following anaerobic interaction of PUFA and 2-NOF, might be reduced to Fe^{2+} and contribute to enhanced lipid peroxidation. Thus, adduction of C-nitroso aromatics to PUFA yields radical species which directly, and/or via reaction with trace iron, leads to lipid peroxidation. The lipophilicity of C-nitroso aromatics suggests that this process may be of consequence in their mutagenicity/carcinogenicity (80).

CARCINOGENESIS MECHANISMS

GRANTS ACTIVE DURING FY91

INVESTIGATOR/INSTITUTION/GRANT NUMBER

TITLE

- | | |
|--|---|
| 1. ABUL-HAJJ, Yusuf J.
University of Minnesota of Mnpls-St Paul
5 R01 CA41269-03 | Macromolecular Binding of
Estrogens and Carcinogenesis |
| 2. ALBERTINI, Richard J.
University of Vermont and St Agric College
5 R01 CA30688-09 | Direct Mutagenicity Testing
in Man |
| 3. BAIRD, William M.
Purdue University West Lafayette
5 R37 CA28825-11 | Modifiers of Chemical Carcino-
genesis in Cell Culture |
| 4. BARCELLOS-HOFF, Mary H.
University of California Berkeley
1 R29 CA51841-01A1 | Stromal Influence on Expression
of Preneoplasia |
| 5. BARTSCH, Helmut
World Hlth Org Intl Agcy Res on Cancer
5 R01 CA47591-03 | Bacteria-DNA Damage in Stomach
and Bladder Cancers |
| 6. BEATTIE, Craig W.
University of Illinois at Chicago
5 R01 CA45355-03 | Estrogen and MNU Synergism
in Carcinogenesis |
| 7. BOSLAND, Maarten C.
New York University
5 R01 CA43151-06 | Chemical Induction of Prostatic
Adenocarcinomas |
| 8. BOSLAND, Maarten C.
New York University
1 R01 CA48084-01A3 | Hormonal vs Chemical Carcino-
genesis in the Rat Prostate |
| 9. BRASITUS, Thomas A.
University of Chicago
5 R37 CA36745-08 | Colonic Epithelial Cell Plasma
Membranes |
| 10. BRESNICK, Edward
Dartmouth College
5 R01 CA36106-08 | Polycyclic Hydrocarbon Meta-
bolism and Carcinogenesis |
| 11. BUHLER, Donald R.
Oregon State University
5 R01 CA22524-12 | Pyrrolizidine Alkaloid
Toxicity Metabolism, and
Binding |
| 12. CARR, Brian I.
University of Pittsburgh at Pittsburgh
5 R29 CA44602-05 | TGF β Receptors in Hepato-
carcinogenesis |

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| 13. CAVALIERI, Ercole L.
University of Nebraska Medical Center
5 R01 CA44686-03 | Radical Cations of PAH in
Carcinogenesis and Metabolism |
| 14. CAVALIERI, Ercole L.
University of Nebraska Medical Center
5 P01 CA49210-03 | Mechanisms of 7,12-Dimethyl-
benzanthracene Carcinogenesis |
| 15. CHANG, Chia-Cheng
Michigan State University
5 R01 CA50430-03 | Transformation of Human Breast
Epithelial Cells in vitro |
| 16. CONTI, Claudio J.
University of Texas System Cancer Center
5 R01 CA47105-02 | Multistage Carcinogenesis of the
Cervix and Vagina |
| 17. CUCHENS, Marvin A.
University of Mississippi Medical Center
5 R01 CA33111-06 | Carcinogenesis of B-Lymphocytes
in Rat Peyer's Patches |
| 18. CURPHEY, Thomas J.
Dartmouth College
1 R01 CA51106-01A1 | Solid-Phase Electrophile
Trapping Agents |
| 19. DETRISAC, Carol J.
IIT Research Institute
5 R29 CA40501-05 | Transitional Cell Carcinoma: in
vitro/in vivo Correlates |
| 20. DIGIOVANNI, John
University of Texas System Cancer Center
5 R01 CA36979-08 | Role of DNA-Binding in Skin
Tumor Initiation |
| 21. DUFFEL, Michael W.
University of Iowa
5 R01 CA38683-07 | Aryl Sulfotransferase in Drug
and Xenobiotic Metabolism |
| 22. EATON, David L.
University of Washington
5 R01 CA47561-03 | Species Differences in
Glutathione-S-Transferase |
| 23. EL-BAYOUMY, Karam E.
American Health Foundation
5 R01 CA35519-08 | Nitroaromatics: Carcinogenicity
and Metabolism |
| 24. ELMETS, Craig A.
Case Western Reserve University
5 R01 CA48763-03 | Skin Cancer--Immunotoxic
Mechanisms |
| 25. ETHIER, Stephen P.
University of Michigan at Ann Arbor
5 R01 CA40064-06 | Growth Factor Independence
in Mammary Neoplasia |

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|---|--|
| 26. FAGAN, John B.
Maharishi International University
5 R01 CA38655-06 | Cytochrome P-450 Gene Structure
and Regulation |
| 27. FIALA, Emerich S.
American Health Foundation
5 R01 CA31012-09 | Disposition of Hydrazines--
Species and Strain Effects |
| 28. FISHBEIN, James C.
Wake Forest University
1 R01 CA52881-01 | N-Alkyl-N-Nitro-Nitroso-
guanidines and Alkane Diazotates |
| 29. FLESHER, James W.
University of Kentucky
3 R01 CA45823-03S1 | Bioalkylation in Chemical
Carcinogenesis |
| 30. GLUSKER, Jenny P.
Institute for Cancer Research
5 R01 CA10925-41 | Application of Crystallographic
Techniques |
| 31. GOLD, Avram
University of North Carolina Chapel Hill
5 R01 CA47965-03 | Pathways of Activation and DNA
Adducts of Cyclopenta PAH |
| 32. GOLD, Barry I.
University of Nebraska Medical Center
5 R01 CA29088-07 | Activation and Transportation
of Nitrosamines |
| 33. GOULD, Michael N.
University of Wisconsin Madison
5 R01 CA30295-10 | Mammary Carcinogenesis--
Interspecies Comparisons |
| 34. GREENBERGER, Joel S.
University of Massachusetts Medical Sch
5 R37 CA39851-08 | Stem Cell Age and X-Ray
Chemotherapy Leukemogenesis |
| 35. GRISHAM, J. W.
University of North Carolina Chapel Hill
2 R37 CA29323-10 | Clonal Analysis of Carcino-
genesis in vitro |
| 36. GUENGERICH, F. Peter
Vanderbilt University
5 R35 CA44353-04 | Enzymic Activation of Chemical
Carcinogens |
| 37. GUENTHNER, Thomas M.
University of Illinois at Chicago
5 R01 CA34455-07 | Toxicologic Implications of
Multiple Epoxide Hydrolases |
| 38. GUENTHNER, Thomas M.
University of Illinois at Chicago
5 R01 CA46129-03 | Xenobiotic-Metabolizing Enzymes
in Human Lung |

39. GURTOO, Hira L.
Roswell Park Memorial Institute
5 R01 CA25362-11
Genetics of Aflatoxin Metabolism
Role in Carcinogenesis
40. GUSTAFSSON, Jan-Ake
Caroline Institute
5 R01 CA42054-03
Hormonal Regulation of Liver
Carcinogenesis
41. HAM, Richard G.
University of Colorado at Boulder
5 R01 CA30028-09
Human Mammary Cell Growth and
Function in Defined Media
42. HANAUSEK-WALASZEK, Margaret
University of Texas System Cancer Center
1 R01 CA54296-01
Oncofetal Protein Expression
in Hepatocarcinogenesis
43. HANKINSON, Oliver
University of California Los Angeles
5 R01 CA28868-11
Carcinogen Activation and
Screening in Variant Cells
44. HARVEY, Ronald G.
University of Chicago
5 R01 CA36097-08
Mechanism of Carcinogenesis of
Polycyclic Hydrocarbons
45. HECHT, Stephen S.
American Health Foundation
5 R35 CA44377-04
Metabolic Activation of
Carcinogens
46. HEIN, David W.
University of North Dakota
7 R01 CA34627-07
Pharmacogenetics of Drug and
Carcinogen Metabolism
47. HENDRICKS, Jerry D.
Oregon State University
5 R01 CA44317-04
Neoplasia--Tumor and Mechanism
Studies
48. HINTON, David E.
University of California Davis
2 R01 CA45131-05
Mechanistic Hepatocarcinogenesis
in Fish
49. HIXSON, Douglas C.
Rhode Island Hospital Providence
5 R01 CA42715-06
Cellular Origins of Liver Cancer
50. HOLLENBERG, Paul F.
Wayne State University
5 R37 CA16954-16
Hemoprotein-Catalyzed
Oxygenations of Carcinogens
51. HUNT, John M., Jr.
University of Texas Hlth Sci Ctr Houston
5 R01 CA37150-06
Alloantigens as Probes for
Hepatocarcinogenesis

52. IVERSEN, Patrick L.
University of Nebraska Medical Center
5 R01 CA49135-03
Gene Specific Inhibition of
Cytochrome P-450 Isoforms
53. JAMES, Margaret O.
University of Florida
5 R01 CA44297-04
Carcinogen Biotransformation by
Aquatic Invertebrates
54. JEFCOATE, Colin R.
University of Wisconsin Madison
2 R01 CA16265-16A1
Metabolism of Polycyclic
Hydrocarbons and Carcinogenesis
55. JENSEN, David E.
Temple University
5 R01 CA31503-08
N-Nitroso Compound Detoxifi-
cation
56. JIRTLE, Randy L.
Duke University
5 R01 CA25951-11
Survival and Carcinogenesis
in Transplanted Hepatocytes
57. KANG, Jae O.
University of New Hampshire
1 R15 CA54443-01
Mechanism of DMH-Induced
Carcinogenesis
58. KAUFFMAN, Frederick C.
Rutgers the State Univ New Brunswick
5 R01 CA20807-12
Pharmacology of Carcinogen
Activation in Intact Cells
59. KING, Charles M.
Michigan Cancer Foundation
5 R37 CA23386-14
Mechanistic Approaches to
Carcinogenesis
60. KLEIN-SZANTO, Andres J.
Fox Chase Cancer Center
5 R01 CA44981-05
Carcinogenesis of Xeno-
transplanted Human Epithelia
61. KOKKINAKIS, Demetri M.
Northwestern University
5 R01 CA42983-03
DNA Damage Induced by
Pancreatropic Nitrosamines
62. KOREEDA, Masato
University of Michigan at Ann Arbor
5 R01 CA25185-12
Synthesis and Properties of
Arene Oxides and Analogs
63. KRAUTER, Kenneth S.
Yeshiva University
5 R01 CA39553-07
Induction of Gene Expression
by Chemical Carcinogens
64. LAWSON, Terence A.
University of Nebraska Medical Center
5 R01 CA43646-03
Alkylation and Mutagenesis
by Pancreas Carcinogens

65. LEAV, Irwin
Tufts University
5 R01 CA15776-14
Prostatic Differentiation and
Sex Hormone Metabolism
66. LEE, Mei-Sie
Michigan Cancer Foundation
5 R01 CA37885-06
Metabolic Activation of
Unsubstituted Hydroxamic Acid
67. LEVINE, Walter G.
Yeshiva University
5 R01 CA14231-17
Role of Metabolism in the
Biliary Excretion of Drugs
68. LI, Jonathan J.
Washington State University
5 R01 CA22008-14
Estrogen Carcinogenicity and
Hormone-Dependent Tumors
69. LI, Jonathan J.
Washington State University
1 R13 CA55109-01
1st International Symposium on
Hormonal Carcinogenesis
70. LIEHR, Joachim G.
University of Texas Medical Branch
2 R01 CA43232-05A1
Prevention of Estrogen-Induced
Tumors by Chemical Means
71. LINZ, John E.
Michigan State University
1 R01 CA52003-01A1
Aflatoxin B-1 Biosynthesis in
Aspergillus parasiticus
72. LISTOWSKY, Irving
Yeshiva University
2 R01 CA42448-04A2
High Affinity High Capacity
Steroid and Carcinogen Binder
73. LOEPPKY, Richard N.
University of Missouri Columbia
5 R37 CA26914-11
Carcinogenesis: Nitrosamine
Formation and Inhibition
74. LONGNECKER, Daniel S.
Dartmouth College
2 R01 CA47327-04
Transgenic Mouse Models of
Pancreatic Carcinogenesis
75. LOTLIKAR, Prabhakar D.
Temple University
2 R01 CA31641-07A2
Modulation of Mycotoxin Carcino-
genesis by Glutathione
76. LU, Lee-Jane W.
University of Texas Medical Branch
5 R01 CA44163-04
DNA Damage During Pregnancy
77. LUDLUM, David B.
University of Massachusetts Medical Sch
5 R01 CA47103-03
The Role of DNA Adducts in Ethyl
Enoxide Exposure

78. LUK, Gordon D.
Wayne State University
5 R01 CA51206-02
ODC as a Therapeutic Target--
Gene Function Analysis
79. MAGEE, Peter N.
Temple University
5 R37 CA43342-05
Formation and Metabolism of
Nitrosamines in Pigs
80. MALEJKA-GIGANTI, Danuta
University of Minnesota of Mnpls-St Paul
5 R37 CA28000-12
Mammary Carcinogenesis by N-
Substituted Aryl Compounds
81. MANGOLD, Bonnie L. K.
University of Connecticut Storrs
5 R29 CA48972-02
Sulfotransferase Inactivation
by Carcinogens
82. MARCHOK, Ann C.
Oak Ridge National Laboratory
5 R01 CA42798-05
Preneoplastic Markers in
Specific Lesion Cell Populations
83. MCCORMICK, J. Justin
Michigan State University
5 R01 CA21289-13
In vitro Transformation of
Human Cells
84. MEDINA, Daniel
Baylor College of Medicine
5 R01 CA47112-04
Early Events--Chemical
Carcinogen Induced Mammary Tumor
85. MEHTA, Rajendra G.
IIT Research Institute
5 R01 CA47329-03
Nutrients and Growth Modifier
Interaction--Mammary Cancer
86. MELIKIAN, Assieh
American Health Foundation
5 R01 CA43910-03
Mechanism of Catechol Co-
carcinogenesis with BAP
87. MICHALOPOULOS, George K.
Duke University
5 R01 CA30241-11
Cell Culture and Transplantation
of Human Hepatocytes
88. MILO, George E.
Ohio State University
5 R01 CA25907-09
Neoplastic Transformation of
Human Epithelial Cells
89. MIRVISH, Sidney S.
University of Nebraska Medical Center
5 R01 CA35628-07
Nitrosamine Metabolism in
the Esophagus
90. MITCHELL, Ann D.
Genesys Research, Inc.
2 R44 CA45903-02
L5178Y Mouse Lymphoma TK Locus
Host-Mediated Assay

91. MORRIS, Rebecca J.
University of Texas System Cancer Center
5 R29 CA45293-04
Epidermal Stem Cells in
Two-Stage Carcinogenesis
92. MORSE, Daniel E.
University of California Santa Barbara
1 R01 CA53105-01
Oncogenes and Tumors in Marine
Invertebrate Larvae
93. NAIRN, Rodney S.
University of Texas System Cancer Center
5 R01 CA44303-04
Genetic Determinants of
Carcinogenesis
94. NANDI, Satyabrata
University of California Berkeley
2 P01 CA05388-31A1
Mammary Tumorigenesis in Rodents
95. NANDI, Satyabrata
University of California Berkeley
5 R01 CA49374-03
Transformation of Human Breast
Epithelial Cells in vitro
96. NORRIS, James S.
Medical University of South Carolina
5 R01 CA52085-02
Hormonal Carcinogenesis--
Mechanisms
97. OSTRANDER, Gary K.
Oklahoma State University Stillwater
1 R29 CA54590-01
Glycolipid Changes during
Chemical Hepatocarcinogenesis
98. PAULI, Bendicht U.
Cornell University Ithaca
5 R01 CA48642-03
Carcinogenesis Testing of
Sintered Porous CoCrMo Implants
99. PENNING, Trevor M.
University of Pennsylvania
5 R01 CA39504-06
Dihydrodiol Dehydrogenase and
PAH Metabolism
100. PEREIRA, Michael A.
Environmental Health Research and Testing
5 R44 CA45110-03
Initiation - Promotion Bioassay
in Mouse Liver
101. PHILLIPS, David H.
University of London
5 R01 CA21959-14
Mechanisms of Activation of
Polycyclic Hydrocarbons
102. POUR, Parviz M.
University of Nebraska Medical Center
5 R01 CA35042-06
Prevention of Nasal Cavity
Tumors by Castration
103. POUR, Parviz M.
University of Nebraska Medical Center
5 R01 CA42133-03
Transplacental Induction of
Pancreatic Cancer

117. SCHATZ, Robert A.
Northeastern University
5 R01 CA47671-03
Solvent and Ethanol Interaction
on BAP Metabolism
118. SCHMALE, Michael C.
University of Miami
1 R01 CA53313-01
Cancer in Damsel fish--Immunology
and in vitro Models
119. SHARMA, Minoti
Roswell Park Memorial Institute
5 R01 CA46896-03
Fluorescence Postlabeling Assay
For DNA Damage
120. SHAY, Jerry W.
University of Texas Hlth Sci Ctr at Dallas
5 R01 CA50195-03
In vitro Transformation of Human
Mammary Epithelial Cell
121. SIEGAL, Gene P.
University of Alabama at Birmingham
5 R29 CA45727-06
Chemical Progression and
Inhibition of Neoplasia
122. SIKKA, Harish C.
State Univ of New York Coll at Buffalo
1 R01 CA53197-01
Metabolism of Acetylamino-
flourene by Rainbow Trout
123. SINCLAIR, Peter R.
Dartmouth College
5 R01 CA25012-13
Liver Cell Cultures for Study
of Carcinogen Activation
124. SIRICA, Alphonse E.
Virginia Commonwealth University
2 R01 CA39225-07
Hepatic Oval Cells in Culture
and in vivo
125. SOLT, Dennis B.
Northwestern University
5 R01 CA34160-07
Sequential Analysis of Oral
Carcinogenesis
126. SOROF, Sam
Institute for Cancer Research
5 R01 CA05945-27
Ligand-Protein Complexes, Growth
and Carcinogenesis
127. STAMBROOK, Peter J.
University of Cincinnati
5 R01 CA36897-06
Mammalian Cell Assay for
Mutagenesis and Carcinogenesis
128. STAMBROOK, Peter J.
University of Cincinnati
5 R01 CA48118-03
Genotoxicant-Induced Deletion
and Rearrangement
129. STROBEL, Henry W.
University of Texas Hlth Sci Ctr Houston
5 R01 CA42995-04
Human Large Bowel Cancer and
Cytochromes P-450

130. SUDILOVSKY, Oscar
Case Western Reserve University
5 R01 CA35362-06
DNA Content of Dysplastic Lesions in Human and Rat Liver
131. TANNENBAUM, Steven R.
Massachusetts Institute of Technology
2 P01 CA26731-12
Endogenous Nitrite Carcinogenesis in Man
132. TAYLOR, John-Stephen A.
Washington University
5 R01 CA40463-07
DNA Photolesion Structure-Activity Relationships
133. TERZAGHI-HOWE, Margaret
Oak Ridge National Laboratory
5 R01 CA34695-08
Cell Interactions: Expression of Preneoplastic Markers
134. TOLBERT, Laren M.
Georgia Institute of Technology
5 R01 CA43806-03
Bio-Oxidation of Arylalkyl Hydrocarbons
135. TUKEY, Robert H.
University of California San Diego
5 R01 CA37139-08
Cytochrome P-450 Genes and Chemical Carcinogenesis
136. UNDERWOOD, Graham R.
New York University
5 R01 CA47599-03
Mechanistic Studies of Arylamide Carcinogens
137. WANG, Ching Y.
Michigan Cancer Foundation
5 R01 CA23800-13
Mechanisms of Bladder Tumorigenesis
138. WANG, Ching Y.
Michigan Cancer Foundation
5 R01 CA49783-03
Genetic Alterations Involved in Bladder Carcinogenesis
139. WEBER, Wendell W.
University of Michigan at Ann Arbor
2 R01 CA39018-06A2
Acetylation Pharmacogenetics: Arylamines and DNA Damage
140. WEYAND, Eric H.
Rutgers the State Univ New Brunswick
5 R29 CA49826-03
Initiation of Mammary Carcinogenesis in the Rat
141. WHITLOCK, James P., Jr.
Stanford University
5 R37 CA32786-08
Carcinogen-Metabolizing Enzymes: Action in Variant Cells
142. WILLIAMS, Gary M.
American Health Foundation
1 R13 CA53160-01
Cancer Mechanisms: Implications For Risk Assessment

143. YANG, Chung S.
Rutgers the State Univ New Brunswick
5 R37 CA37037-08

Metabolic Activation of
N-Nitrosamines

144. YANG, Shen K.
U.S. Uniformed Services Univ of Hlth Sci
5 R01 CA29133-09

Metabolic Activation of
Monomethylbenz Anthracenes

SUMMARY REPORT

CHEMICAL RESEARCH RESOURCES

The Branch currently has five resource contracts, totaling \$1.77 million, which make up the Chemical Research Resources program. Support is provided to synthesize and make available to the scientific community research quantities of chemical carcinogens, mutagens, their metabolites and adducts as well as experimental chemopreventive agents. These compounds are used primarily as authentic reference standards for chemical carcinogenesis and mutagenesis studies and to ascertain carcinogen metabolic pathways, activation, and molecular mechanism(s) of action. Since the desired derivatives are generally unavailable commercially, or are extremely expensive, and researchers requiring these compounds are often not equipped to synthesize these compounds themselves, a large number of well-characterized radiolabeled and unlabeled derivatives have been synthesized under these contracts. The contract laboratories have some areas of specialization (i.e. fecapentaenes, nitrosamines, PAH diolepoxides, etc.) but in general the work is fairly evenly distributed. The reference standards and metabolic substrates labeled and unlabeled are available for purchase at a nominal price by researchers around the world, and are ordered through the two repository contracts. Since some of the compounds are extremely difficult to make, the "payback charge" for all compounds partially subsidizes their preparation thereby enabling the program to make available a much wider range of compounds. Funds generated by compound sales are used to defray the cost to the Government on all five contracts.

Repository Activities: Midwest Research Institute (MRI) operates the Chemical Carcinogen Reference Standard Repository (N01-CP-05621), supplies unlabeled standards, and handles the logistics of coordinating repository inventory needs with resynthesis activity at the other laboratories. This contract was recompeted during the current fiscal year. Chemsyn Science Laboratories (CSL) operates the Radiolabeled Chemical Carcinogen Reference Repository (N01-CP-61037) and supplies radiolabeled standard polycyclic aromatic hydrocarbons (PAHs), metabolites of PAHs and aromatic amines, retinoids, and other compounds of interest. Because of their highly unstable nature, CSL also supplies unlabeled PAH diolepoxide optical enantiomers.

In order to effectively serve the carcinogenesis research community, the repositories are required to (1) procure chemical samples, (2) provide for their safe and stable storage until requested by users, (3) repackage to meet user needs, (4) ensure the uniformity of all samples, and (5) ship samples to users along with materials comparable to Material Safety Data Sheets. These materials contain appropriate analytical documentation and characterization of chemical, physical and toxicological data, as well as safe handling and storage instructions. All of these operations must be performed in a safe manner and in compliance with current NIH guidelines and Occupational Safety and Health Administration (OSHA), Nuclear Regulatory Commission (NRC), International Air Transport Act (IATA), Resource Conservation and Recovery Act (RCRA), 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 for synthesis, thus allowing for the projection of future needs.

Of comparable importance to the program's major goal of assuring 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 synthesis or receipt at

the repositories, to the user's receipt 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 repository personnel and to the environment, compliance with all transportation regulations, and the user's safety upon receipt of the materials. This aspect of the program was recently highlighted at a symposium on repositories at the annual American Chemical Society meeting in Atlanta, Georgia.

Shipments made from the repositories are sent air freight by commercial carrier. Although most of the compounds stocked by the repositories are known carcinogens, some are not, and many may still be controversial, at least with regard to their effect on humans. However, to stock both carcinogens and noncarcinogens, and to provide different handling, packaging and shipping procedures for each, presents too many opportunities for error which could lead to possible personnel exposure, mislabeling at the repository, or accidental release of carcinogens into the environment. Consequently, all compounds entrusted to the repositories are regarded as potential carcinogens and are handled, packaged and labeled accordingly. This precaution is not necessarily meant to imply that every sample is a carcinogen, only that the compound is intended for use in research involving chemical carcinogens. The procedure does imply however that, unless the recipient has information to the contrary, a repository compound should be treated as a carcinogen.

The current inventory of the Chemical Carcinogen Reference Standard Repository at MRI consists of 809 unlabeled chemicals. Six new chemicals were added to the inventory this year and five were deleted. Twenty-four samples of chemicals were also procured to replace depleted or decomposed stocks.

A total of 222 requests were received for chemicals other than fecapentaenes during the fifth year of the contract. Of these, 157 were received from organizations in the United States and 65 were from foreign organizations. Of the total, 129 requests came from academia, 58 from industry (including independent research organizations), and 35 from government facilities. This was a marked increase in all categories except government.

From 225 chemicals, 932 aliquots were shipped during the fifth year of the contract, an increase of over 17%. Over 225 chemicals were analyzed at least once during this period. Four chemicals were found to be unusable because excessive levels of impurities were detected. Ultraviolet/visible and nuclear magnetic resonance (NMR) spectroscopy, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) were the most frequently used methods of analysis.

A new edition of the catalog for the NCI Chemical Carcinogen Reference Standard Repositories, listing both labeled and unlabeled compounds, has been produced and distributed. For the first time since 1983 the pricing structure has been changed. In order to simplify paperwork and control bookkeeping costs, shipping and handling have been included in the purchase price and a standard shipping charge of \$250 has been established for foreign requests. Approximately 200 catalogs have been distributed during the fifth year of the MRI contract.

Because of multiple requests for physical and chemical properties information on polycyclic aromatic hydrocarbons and their metabolites, it was decided that spectral and other data would be published as a series of handbooks in conjunction with the Branch's PAH Symposia activities. The format was determined after consultation with

the NCI Project Officer and other NCI principal investigators/resource contractors. Preparation of Volume 1 of the PAH handbook series has been completed, including collection of all analytical data, evaluation, and production of camera-ready copies. Negotiations are underway for the printing of the handbook.

Twelve chemicals were procured to support NCI's in vitro testing program. Analytical data sheets were obtained from the supplier (eight chemicals) or analyses were performed (four chemicals) to ascertain sample purity. Coded aliquots of eighteen samples were provided to the in vitro toxicology contractor along with hazard summaries that allow for safe handling in the event of a spill or accident. These compounds have been selected as part of the NCI's chemical selection process whereby suspect chemicals with insufficient data are screened in the Ames *Salmonella typhimurium* and mouse lymphoma short-term mutagenicity tests. Positive findings are used to support the nomination of compounds to the National Toxicology Program for further in vivo evaluation. The most notable of these to date have been a number of naphthenate soaps, alkyl amines, acrylates, unsaturated organosilanes and a nitrosamine found in commercial sunscreens, N-Nitroso-N-methyl-p-aminobenzoic acid-2-ethylhexyl ester. These are in various stages of manuscript preparation and have been entered in the NCI Chemical Carcinogen Research Information System (CCRIS) database.

Two sets of nitrosamine standards were prepared, analyzed and shipped to all participating laboratories in the United States Department of Agriculture (USDA) Nitrosamines in Bacon Program.

Approximately \$520,000 has been received for over 2300 samples sent to requestors from the repository at MRI during the five-year contract period. These charges represent cost recovery for packaging and shipping and a prorated cost for chemical synthesis. The funds received by the contractors are subtracted from the total monthly operation cost of the contract prior to invoicing the NCI (1).

CSL maintains the radiorepository for the NCI which presently has an inventory of 92 radiolabeled (^3H , ^{14}C) polynuclear aromatic hydrocarbons and their metabolites. A portion of the synthesis, and packaging and shipping costs, are recovered from radiorepository users by a payback mechanism similar to that used by the MRI repository. During the current reporting period, a total of 97 radiolabeled and 31 unlabeled compounds were delivered to 76 different investigators at a total billed cost of approximately \$42,700 (5).

Synthesis Activities: 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. These contractors develop suitable routes for the unequivocal organic synthesis of compounds designated by the NCI Project Officer, and develop methods for production of adequate amounts of well-characterized compounds of high purity (generally greater than 98%).

Compounds are analyzed by a combination of techniques to assess purity and confirm structure. These may include ultraviolet, fluorescence, and/or infrared spectrophotometry; nuclear magnetic resonance; and gas chromatography/mass spectrometry. The activity has focused primarily on DNA and glutathione adducts of carcinogenic epoxides and diolepoxides, specifically on the indeno[1,2,3-c,d]pyrene and dibenz[a,l]pyrene metabolites, as well as on the replenishment of stocks of depleted and decomposed compounds. The major objective of two of the contractors, Chemsyn Science Laboratories (CSL) and the American Health Foundation, is the synthesis and

purification of NCI-selected nonlabeled and labeled (^3H , ^{14}C) polynuclear aromatic hydrocarbon (PAH) 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 (>98%) for distribution as metabolite standards through the NCI Chemical Carcinogen Reference Standard Repository discussed above. They are characterized by a combination of analytical techniques including TLC, IR, UV, NMR, GC, melting point, boiling point and elemental analysis.

The synthesis, purification and characterization of the following 32 compounds was completed during the current reporting period at CSL (5). They are now available at the repository.

Tritiated PAHs and related compounds:

[G- ^3H]Benz[a]anthracene
[G- ^3H]Benz[b]fluoranthrene
[G- ^3H]Benzo[a]pyrene
[7- ^{14}C]Benzo[a]pyrene
[G- ^3H]Benzo[e]pyrene
7,12-Dimethyl[ring G- ^3H]benz[a]anthracene
[G- ^3H]Indeno[1,2,3-c,d]pyrene
3-Methyl[ring-G- ^3H]cholanthrene
Dibenz[a,e]pyrene
Cyclopenta[c,d]pyrene

Dihydrodiols:

(±)-trans-7,8-Dihydro[1,3- ^3H]benzo[a]pyrene-7,8-diol
(+)-7S-trans-7,8-Dihydro[7- ^{14}C]benzo[a]pyrene-7,8-diol
(+)-7S,trans7,8-Dihydro[1,3- ^3H]benzo[a]pyrene-7,8-diol
(-)-7R-trans-7,8-Dihydro[1,3- ^3H]benzo[a]pyrene-7,8-diol

Phenols:

3-Hydroxy[ring-G- ^3H]benzo[a]pyrene
7-Hydroxy[ring-G- ^3H]benzo[a]pyrene
9-Hydroxy[ring-G- ^3H]benzo[a]pyrene

Diolepoxides:

(±)-r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro-[1,3- ^3H]-benzo[a]pyrene (anti-BPDE- ^3H)
(±)-r-7,t-8-Dihydroxy-c-9,10-epoxy-7,8,9,10-tetrahydro-[1,3- ^3H]-benzo[a]pyrene (syn-BPDE- ^3H)
(-)-7S-r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro-[1,3- ^3H]-benzo[a]pyrene (- anti-BPDE- ^3H)
(+)-7R-r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro-[1,3- ^3H]-benzo[a]pyrene (+ anti-BPDE- ^3H)
(+)-7R-r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro-[7- ^{14}C]-benzo[a]pyrene (+ anti-BPDE- ^{14}C)
(+)-7R-r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene (anti-BPDE)
(-)-7S-r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene (- anti-BPDE)

Epoxide:

(±)-[G- ^3H]-4,5-Dihydrobenzo[a]pyrene-4,5-oxide

Quinone:

[1,3-³H]benzo[a]pyrene-7,8-dione

Hydroxyalkyl hydrocarbons:

7-Hydroxymethyl-12-methyl[ring-G-³H]benz[a]anthracene
12-Hydroxymethyl-7-methyl[ring-G-³H]benz[a]anthracene

Fluorenes:

2-Acetylamino[ring-G-³H]fluorene
N-Hydroxy-2-amino[ring-G-³H]fluorene
N-Acetoxy-2-acetylamino[ring-G-³H]fluorene
2-Amino-3-methyl[5-³H]imidazo[4,5-f]quinoline

Miscellaneous:

4-Nitro [5-³H]quinoline-1-oxide
8-methoxy-1,2,11,12-tetrahydrochrysene-4(³H)-one

The syntheses of the following 14 compounds are currently in progress:

Glutathione conjugates:

8-S-(7-Hydroxy-7,8-dihydrobenzo[a]pyren-8-yl)glutathione
7-S-(8-Hydroxy-7,8-dihydrobenzo[a]pyren-7-yl)glutathione
7-S-(8-Hydroxy-7,8,9,10-tetrahydrobenzo[a]pyren-7-yl)-glutathione
10-S-(r-7,t-8,9-Trihydroxy-7,8,9,10-tetrahydro-benzo[a]pyren-10-yl)
glutathione

Hydroxyindeno pyrenes:

7-Hydroxyindeno[1,2,3-c,d]pyrene
9-Hydroxyindeno[1,2,3-c,d]pyrene
10-Hydroxyindeno[1,2,3-c,d]pyrene
12-Hydroxyindeno[1,2,3-c,d]pyrene

Dihydrodiols:

(±)-trans-7,8-Dihydroindeno[1,2,3-c,d]pyrene-7,8-diol
(±)-trans-9,10-Dihydroindeno[1,2,3-c,d]pyrene-9,10-diol
(±)-trans-1,2-Dihydrodibenz[a,l]pyrene-1,2-diol
(±)-trans-11,12-Dihydrodibenz[a,l]pyrene-11,12-diol

Miscellaneous:

(±)-13,14-Dihydrodibenz[a,e]pyrene-13,14-oxide
8-Nitrofluoranthene

The other contract for the preparation of derivatives of polycyclic aromatic hydrocarbons is with the American Health Foundation (2). The focus during this reporting period has been on the synthesis and stability determination of authentic DNA (N²-deoxyguanosyl-3'-phosphate) adducts of the benzofluoranthenes, benzo[a]pyrenes, methylchrysenes and chrysene for use in the ³²P-postlabeling assay of Randerath. They have attempted to synthesize these adducts directly from the diolepoxides of benzo[a]pyrene and deoxyguanosine monophosphate salts, but the original reaction with calf thymus DNA and enzymatic hydrolysis has proven the most efficient. The isolation of 8-(N²-deoxyguanosyl-3'-phosphate)-8,9,10-trihydroxy-8,9,10,11-tetrahydro-benzo[k]fluoranthene showed this compound to readily decompose to the corresponding tetrahydroxyl compound. Other adducts seem to be more stable. Other compounds synthesized include the following:

trans-1,2-Dihydroxy-1,2-dihydronaphthalene
2-Hydroxybenzo[b]fluoranthene
3-Hydroxybenzo[b]fluoranthene
2-Methylbenzo[b]fluoranthene
Fluoranthene-1,2,3,10b-tetraol
Fluoranthene-2,3,-diol-1,10a-epoxide
trans-2,3-Dihydroxy-1,10b-epoxy-1,2,3,10b-tetrahydrofluoranthene
trans-2,3-Dihydroxy-1,10a-epoxy-1,2,3,10b-tetrahydrofluoranthene
7R,8S,9S-trihydroxy-10R-(N²-deoxyguanosyl-3'-monophosphate)-7,8,9,10-tetrahydro-
benzo[a]pyrene
5,8-Dimethylchrysene
5,9-Dimethylchrysene
5,10-Dimethylchrysene
5-Methylchrysene
5-Methylchrysene-1,2-dione
6-Methylchrysene-1,2-dione
6-Methylchrysene-1,2-diol-3,4-epoxide
Chrysene-1,2-dione
1,2,3-trihydroxy-4-(N²-deoxyguanosyl-3'-monophosphate)-1,2,3,4-tetrahydrochrysene

A second, very exciting development is the resolution of the four racemic diolepoxides of benzo[a]pyrene by high pressure liquid chromatography using semi-preparative Pirkle Type 1-A columns.

The final two contractors, Chemsyn Science Laboratories and SRI International, prepare a wider variety of compounds for the repository program. These include selected aromatic amines, steroid derivatives, nitrosamines, physiologically active natural products and other parent polynuclear aromatic hydrocarbons. The contractors also provide resynthesis, purification, and characterization of selected metabolites (e.g., quinones, dihydrodiols, epoxides, diolepoxides and phenols) of PAHs already available in the repository but in need of replenishment. The products include both unlabeled and labeled (³H and ¹⁴C) compounds at a purity level of >98%.

Under the second synthesis (non-repository) contract (4), CSL has completed the synthesis, purification and characterization of the following PAH compounds:

Phenols:

Benzo[a]pyrene-9-ol

Sulfates:

Benzo[a]pyrene-7-sulfate

Benzo[a]pyrene-9-sulfate

Epoxide:

(+)-4,5-Dihydro[G-³H]benzo[a]pyrene-4,5-oxide

Diolepoxides:

(±)-r-7,t-8-Dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene

(±)-r-9,t-10-Dihydroxy-t-7,8-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene

Dihydrodiols:

(+)-trans-7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene

(-)-trans-7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene

Tetrols:

(±)-r-7,t-8,c-9,t-10-Tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene

The other contractor, SRI International (3), has completed synthesis of the following compounds: fecapentaene fecal mutagens, both labeled and unlabeled, including fecapentaenes-1, -12 and -14; N-nitroso-N-methyl-p-aminobenzoic acid, 2-ethylhexyl ester, the nitrosamine contaminant occasionally found in some sunscreens; N-hydroxy derivatives of the food mutagens IQ, methyl IQx, and PhIP; and the miscellaneous PAH metabolites benzo[a]pyrene-1-0-sulfate, trans-2,3-dihydro-fluoranthene-2,3-diol, and 3-hydroxy-benzo[a]pyrene glucuronide. A synthesis study of 1,2-dihydroindeno[1,2,3-c,d]pyrene-1,2-epoxide has also been performed. The synthesis of the sunscreen nitrosamine was developed by Food and Drug Administration chemists and was modified and used to produce sufficient N-nitroso compound to carry out mutagenesis testing (approximately 18g). The primary synthetic contaminant 4-N,N'-dimethyl-amino-3-nitrobenzoic acid, 2-ethylhexyl ester was also synthesized to determine its mutagenicity as the heretofore reported mutagenicity of the parent compound was not demonstrable. As part of a request by NIH researchers for synthesis of potential food mutagen metabolites, the N-hydroxy food mutagens mentioned above have been converted to the nitro analogues and reduced to the unstable N-hydroxy compound in varying yields, ranging from 6% for IQ to 80% for methyl IQ. Purification is by preparative reverse-phase HPLC. The current activity includes the synthesis of aflatoxin B₁ epoxide by a new one-step synthesis and its use to prepare DNA adducts.

RESEARCH RESOURCES
CONTRACTS ACTIVE DURING FY91

<u>INVESTIGATOR/INSTITUTION/GRANT NUMBER</u>	<u>TITLE</u>
1. GRAVES, Steven Midwest Research Institute N01-CP-05621	Chemical Carcinogen Reference Standard Repository
2. HECHT, Stephen S. American Health Foundation N01-CP-61041	Synthesis of Derivatives of Polynuclear Aromatic Hydrocarbons
3. REIST, Elmer J. SRI International N01-CP-71108	Synthesis of Selected Chemical Carcinogens
4. RUEHLE, Paul H. Eagle-Picher Industries, Inc. N01-CP-71007	Synthesis of Selected Chemical Carcinogens
5. WILEY, James C. Eagle-Picher Industries, Inc. N01-CP-61037	Synthesis of Derivatives of Polynuclear Aromatic Hydrocarbons

SUMMARY REPORT

EXPERIMENTAL TOBACCO CARCINOGENESIS

The Experimental Tobacco Carcinogenesis component of the Branch includes 15 grants and one Interagency Agreement. Support of these grants is continuing for research directed toward understanding the nature of the chemicals involved and the reaction mechanisms behind the deleterious effects of tobacco smoke and smokeless tobacco products. The long-term goals of the program have been to (1) identify smoking- and smokeless tobacco-related cancers; (2) analyze major smoke and tobacco components; (3) understand the factors involved in initiation and promotion of cancers resulting from the use of tobacco products; (4) determine the mechanism and activity of tobacco-specific nitrosamines; (5) develop concepts leading to reduced smoke toxicity; (6) monitor the major smoke and tobacco components in new types of cigarettes; (7) assess active and passive risk through the identification of biomarkers; and (8) study the addictive nature of smoking and the factors involved in cessation.

Epidemiological studies have demonstrated a causative association between cigarette smoking and cancer of the lung, upper digestive tract, pancreas, renal pelvis and bladder. Smoking has also been associated with cancer of the liver and cervix, and with leukemia. Epidemiological support for the adverse health effects from the use of smokeless tobacco are less numerous but nevertheless are quite convincing. To date, 45 carcinogens have been identified in tobacco smoke including myriad polynuclear aromatic hydrocarbons; tobacco-specific N-nitrosamines (TSNAs); aromatic amines such as 2-toluidine, 2-naphthylamine and 4-aminobiphenyl; some volatile aldehydes such as acrolein, formaldehyde and acetaldehyde; benzene; butadiene; acrylonitrile; and vinyl chloride among others.

The eventual mitigation of the adverse health effects initiated by tobacco, and a decrease in the habitual use of all tobacco products, are the ultimate goals of the Government's multi-faceted research program. Past efforts toward this goal have included the identification of smoking-related diseases and the chemical analysis of major whole smoke components as well as their in vitro and in vivo metabolic products. The particulate matter which induces malignant tumors of the respiratory tract contains about 3500 different chemical compounds with nicotine the most abundant single compound at 0.1 to 0.2 mg per cigarette. A number of these structurally diverse compounds have been synthesized to verify their identification and have been further characterized by in vivo and in vitro mutagenesis and carcinogenesis assays. The work has moved beyond the study of polycyclic aromatic hydrocarbons and their metabolically activated ultimate carcinogenic forms, the arene oxides and diolepoxides. The genotoxicity of nicotine and related nitrosamines is an active area of current research. In addition, the toxicological and pharmacological aspects of these chemical smoke components and the study of smokeless tobacco products are recent research topics.

Use of tobacco products is the single most important risk factor for cancer in the United States. In 1982 the Surgeon General's report entitled "The Health Consequences of Smoking" estimated that 30% of all cancer mortality was related to smoking. Based on the 1989 Surgeon General's report, it is now believed that 50-90% of lung, oral, laryngeal, and esophageal cancer deaths in the United States are associated with smoking. The International Agency for Research on Cancer (IARC) Working Group (1986) determined that the occurrence of malignant tumors of the oral and pharyngeal regions is causally related to smoking various forms of tobacco, and

that cigarette smoking also causes malignant tumors in a number of other tissues. The American Cancer Society's prospective studies on the risk of lung cancer from smoking indicates a 20- and 12-fold higher rate among male and female smokers, respectively, than among non-smokers.

The prevalence of cigarette smoking among men in the U.S. has declined in recent years from over 50% in 1965 to less than 35% in 1988. Among women, the decrease has been much less pronounced, from just over 30% to around 27%. Part of the reason for this is because smoking rates are actually increasing among female high school students and working class women. If this trend continues, the smoking rate for women overall is projected to equal and perhaps surpass the rate for males by the mid 1990's. There are still approximately 50 million smokers in the United States and hundreds of millions of smokers worldwide. In fact, in less developed areas of the world, smoking frequency rates are continuing to increase dramatically.

Use of smokeless tobacco in the United States has shown a dramatic increase which can be partially attributed to the attempts by smokers to quit or cut down on smoking. In 1985 over 12 million Americans used smokeless tobacco products, half of those at least once a week. Unfortunately, a very marked increase in usage has been occurring among teenage and young adult males. Snuff usage has increased 15-fold, while chewing tobacco usage has quadrupled. Epidemiological research has shown a significant carcinogenic risk from the use of these products in the development of oral, nasal, pharyngeal and esophageal cancer, in addition to the development of nicotine dependence, tooth loss, gingival recession, and periodontal disease. 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. Some of these findings have been reproduced in rats chronically exposed to snuff in a surgically created lip canal (2,3).

The 1985 IARC monograph and the 1986 U.S. Surgeon General's report have concluded that oral use of snuff causes oral cancer. Its use has also been associated with tumors of the nasal cavity, pancreas, kidney and bladder. The cancerous and precancerous effect of smokeless products is primarily believed to be caused by the action of nicotine-derived nitrosamines.

The interaction of tobacco-derived chemicals with other dietary components, such as alcohol, is an increasing area of research interest and is based upon epidemiological evidence for such synergistic interactions. Two recently awarded grants addressing this topic have found that ethanol stimulates levels of P450-dependent nicotine and nitrosamine metabolic activation to compounds with significant carcinogenic potential (6,8).

Nicotine, a biomarker for tobacco and smoke exposure, is the critical pharmacological agent for maintenance of tobacco dependence and continued usage. It is the most abundant (90-95%) alkaloid present in tobacco products, comprising 0.5-5% of the tobacco, by weight. Commercial cigarettes, smoked under standard laboratory conditions, deliver up to 3 mg of nicotine and related alkaloids in mainstream smoke and up to 20-fold higher levels (per equivalent weight) in sidestream smoke. As a tertiary amine, nicotine can react with nitrite during smoking to form nitrosamines which can be detected in mainstream and sidestream smoke at levels of 30-770 ng per cigarette. In smokeless tobacco products, the levels of tobacco-specific nitrosamines (TSNAs) have been shown to be greater than 1000 ppm. TSA levels in snuff are substantially higher than in smoking tobacco.

Daily consumption has been estimated to exceed 400 µg. Human exposures calculated for prolonged snuff usage (40 years) approach the levels of TSNA's known to cause tumors in rats.

Studies conducted under a program project grant on tobacco carcinogenesis (2,3) have revealed that tobacco contains nine carcinogenic N-nitrosamines and two carcinogenic nicotine-derived N-nitrosamino acids, 4-(methylnitrosamino)-4-(3-pyridyl)-butyric acid (iso-NNAC) and 4-(methylnitrosamino)-4-(3-pyridyl)-propionic acid (NMPA).

The most abundant tobacco N-nitrosamino acid, NMPA, occurs at 2-65 µg/g tobacco. The major nitrosamines formed from nicotine are N-nitrosoornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). These potent carcinogens occur at up to 150 and 15 ppm respectively, and induce lung and nasal cavity tumors in rats and hamsters, tracheal tumors in hamsters, and lung tumors in mice. In several cases the organ specificity is independent of the route of exposure, e.g., NNK in rats causes lung tumors by all routes except via drinking water as well as eliciting pancreatic tumors, while after subcutaneous injection, nasal cavity and liver tumors predominate. In vitro, cultured explants of human tracheobronchial and lung tissue metabolically activate NNK, inducing hyperplasia and metaplasia comparable to early changes seen in the bronchial epithelium of smokers (2,3). In the surgically created lip canal model, nicotine and several of the TSNA's are leached out of the snuff sample during the exposure period but levels are too low to directly demonstrate TSNA- or methyl-DNA-adduct formation. Metabolism of NNN and NNK under these conditions parallels that described in other tissues, although here the data strongly support separate enzymes activating each compound (2).

Another group of investigators has shown that NNK also transforms human pancreatic explants, leading to the development of ductal meta- and hyperplasia within three months and development of anaplastic growths and transplantable carcinomas after eight months, when promoted with tetraphorbol acetate. The potential use of these models for studying the effects of both chemopreventive and chemotherapeutic agents on pancreatic tumor cancer is incredibly promising (7).

New findings are confirming NNK as a pancreatic carcinogen in vitro and in vivo and data is accumulating to support NNK as a transplacental carcinogen. NNK, a potent respiratory tract carcinogen in adult rodents, is metabolized to an active carcinogen by fetal respiratory tract tissue of Syrian golden hamsters and nonhuman (*Cynomolgus*) primates. It has been demonstrated that the nitrosamine crosses the placental barrier and is rapidly reduced to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). This compound is distributed in the placenta, amniotic fluid, and in the fetal lung and liver. Uptake into the cells utilizes the same receptor type as nicotine in pulmonary neuroendocrine cells; in fact, competition for the receptor has been demonstrated. This may explain why high nicotine concentrations inhibit NNK carcinogenesis. Lung and liver tumors have also been produced in rodent progeny exposed in utero to NNK (10).

NNK or diethylnitrosamine (DEN) alone causes hamster lung adenomas and adenocarcinomas derived from Clara cells. The most malignant type of lung tumors are neuroendocrine-derived small cell or oat cell cancers, the most common lung cancers in smokers. These tumors have never before been induced experimentally. A grantee has now shown that nitrosamine exposure at increased or decreased oxygen levels can lead to formation of hamster neuroendocrine lung tumors similar to human carcinoids, and small cell lung tumors can be produced by combining a stimulus to pulmonary neuroendocrine cell hyperplasia (hyperoxia) with NNK or DEN. In the presence of

60-70% oxygen (hyperoxia condition), administration of DEN or NNK leads to the development of neuroendocrine tumors in two stages: (1) the transformation of focal alveolar type II cells into neurocrine cells and (2) the development of neurocrine tumors from these foci. Clara and type II cells appear to be absent in the mature tumor, which may be characterized by the presence of dense-core secretion granules and neuroendocrine peptide markers. H-ras oncogene amplification has also been noted in the tumors. The DEN-induced tumors show increased levels of mammalian bombesin (MB) and immunoreactive calcitonin in tumor and lung tissues with increased MB in the sera as well. MB, which acts as an autocrine growth factor in neuroendocrine lung tumors, has been shown to be localized in alveolar type II cells. NNK-induced tumors, which show an identical pathogenesis, have not yet been examined at this level of detail, but do appear to contain MB. The Clara cell changes noted occur fairly early in the treatment regimen, on the order of a few months, and with these markers the system may serve as the first animal model for studying the pathology, neuroendocrinology and molecular biology of lung carcinogenesis in vivo in a short term bioassay system. This model is now being used in the study of lung tumor chemopreventive agents.

From NNK/hyperoxia-induced neuroendocrine tumors, two continuous cell lines have been established which demonstrate dense-cored neuroendocrine secretion granules in vitro and express the peptide markers neurotensin, calcitonin-related peptide, cholecystokinin and MB. Cell proliferation is stimulated by nicotine and bombesin involving specific membrane receptors. The investigation of these cell lines as models for studying tumor biology has just begun (11).

TSNAs are metabolically activated by alpha-hydroxylation forming unstable alpha-hydroxynitrosamines. This pathway is at least in part P450-dependent in both the liver and lung. The specific P450 isozyme has been shown to be ethanol inducible. These alpha hydroxyl compounds decompose to diazohydroxides which react with cellular components. Surprisingly, the reaction is inhibited competitively by nicotine (8). With NNK, the metabolic alpha-hydroxylation at the methylene group yields methyl diazohydroxide and leads to 7-methylguanine, O⁶-methylguanine and O⁶-methylthymine in the DNA of target organs both in vivo and in vitro. O⁶-Methylguanine is known to cause miscoding of DNA and oncogene activation; in fact, NNK induces lung tumors in mice with activated K-ras oncogene. In addition, it has been reported that in lung adenocarcinomas of smokers, the K-ras gene is activated by point mutations in codon 12, resulting in part from O⁶-methylguanine-induced miscoding.

The second pathway of NNK and NNN metabolism proceeds via alpha-hydroxylation at the NNK methyl group or the 2' position of NNN. This leads to the DNA and protein binding intermediate 4-(3-pyridyl)-4-oxobutyl-diazohydroxide, whose reaction with hemoglobin has become a highly sensitive marker for human dosimetry of TSNA, useful in epidemiological studies and in estimation of exposure to environmental tobacco smoke. The clearance time for this marker is tied to the biological half life of hemoglobin which is measured in weeks. The half life of cotinine in urine or plasma is approximately two days, while nicotine is measured in hours, making these compounds good markers for recent smoke exposure as well. Analytic methodology for these compounds is simpler, however, than for the hemoglobin adduct. Micro scale analytical techniques have also been developed to demonstrate DNA binding of nitrosamines in specific tissues of individual rodents. Prior to this time, pooled animal tissues were required. HPLC separation of tissue DNA on diethyl-amino-ethyl (DEAE) ion exchange columns have identified binding of NNK to nasal mucosal tissue double stranded DNA at a rate of 0.2 pmol per μ mole guanine.

A gas chromatography-negative ion chemical ionization-mass spectrometry method has been developed to detect 4-hydroxy-1-(3-pyridyl)-1-butanone released from hemoglobin or DNA following exposure to NNN or NNK. This has been used to quantitate exposure in smokers, nonsmokers and smokeless tobacco users and has been validated against a radiochromatography method. Levels of up to 7 fmol/mg DNA have been detected in lung samples from autopsy, demonstrating these adducts in humans directly for the first time. Work is currently underway to correlate lung tissue levels with those found for hemoglobin adducts, the latter being orders of magnitude less invasive.

The TSNA 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the reduction product of NNK, is a strong animal carcinogen, producing pancreatic and lung tumors in rats. Two minor alkaloids, anabasine and anatabine, are also nitrosated during tobacco cultivation and curing. N'-nitrosoanabasine shows only weak activity, inducing rat esophageal tumors. N'-nitrosoanatabine (NAT), iso-NNAC (the oxidation product of NNA), and NMPA have recently been bioassayed for carcinogenicity. NMPA proved to be highly carcinogenic, inducing lung adenomas and adenocarcinomas in strain A mice, while iso-NNAC and NAT were inactive. Iso-NNAC was also negative in the primary rat hepatocyte DNA-repair assay. In vitro studies under physiological conditions have shown that iso-NNAC can be formed from the major nicotine metabolites, cotinine and 4-(methylamino)-4-(3-pyridyl)butyric acid (cotinine acid), implying that iso-NNAC is endogenously formed indirectly from nicotine. If this can be confirmed in vivo, iso-NNAC may serve as a marker for the endogenous formation of TSNA's in smokers, since the compound is not found in smoke. Since the compound is excreted unchanged, urinary measurements for the compound may suffice. Studies are continuing in rats and mice to examine these possible relationships.

A parallel project involves examination of the oral carcinogenesis of chewing betel-quid with and without tobacco and areca nut. The IARC has reported that over 200 million people worldwide practice this habit. These practices are linked with positive carcinogenic response in both humans and the hamster. In addition to the TSNA described above, chewing betel-quid with tobacco leads to the formation of arecoline derived N-nitrosoguvacine and N-nitroso-guvacoline as well as the highly carcinogenic 3-(methylnitrosamino)-propionitrile (2,3).

NNK metabolism and genotoxicity in fetal tissues have been well-characterized in culture. Carbonyl reduction, alpha-hydroxylation, and pyridine N-oxidation have all been observed when fetal trachea and lung explants were cultured in vitro with [³H] NNK and metabolites released in the culture media were analyzed by HPLC. Significant increases in chromosomal aberrations have been noted in explanted tracheal epithelium from in utero-exposed hamster fetuses. Micronucleus induction was also observed in fetal liver and bone marrow polychromatic erythrocytes albeit at a lower level of activity compared with adult tissue. Analyzed by light and electron microscopy, morphological changes suggestive of neoplastic transformation have also been detected when tracheal explants were incubated with NNK in vitro, but less marked changes were observed in lung explants. This data correlates well with the metabolism data in this system which has demonstrated that fetal tracheas metabolize NNK to a greater extent than fetal lungs. Methylation at the O⁶ and O⁷-guanine sites of lung DNA and the pyridyloxobutylation of protein have also been observed. These data originally supported the theory that NNK should be a potential transplacental carcinogen. Indeed, preliminary data in the hamster support this (10) as does a completed study in mice by another research group. In the latter study, transplacental exposure to NNK in A/J, C3B6F1, and Swiss outbred mice showed lung tumors in female A/J progeny, and liver tumors in male C3B6F1 and Swiss progeny. The response is, however, much stronger in newborn animals (2).

The primary objective of work under an interagency agreement with the Department of Energy (12) is to identify and demonstrate chemical markers of environmental tobacco smoke (ETS) that may be used for source apportionment and estimation of 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 physiological fluids. Epidemiological studies have incriminated environmental smoke exposure as a risk factor for lung cancer in nonsmokers. It has been estimated that nonsmokers who are married to smoking spouses have a 30% increased risk of developing lung cancer compared with nonsmoking spouses. Some groups have considered the data inconclusive due to the lack of apparent quantitative correlation with ETS exposure, even though compounds resulting from the combustion of tobacco and the TSNAs, which are known carcinogens, are inhaled as constituents of ETS and retained by nonsmokers. Development of highly sensitive chemical and biochemical methods to quantitate exposure and uptake of tumorigenic agents from ETS will increase the sensitivity of epidemiological study methods.

A number of advanced analytical methods have been evaluated for their utility in the characterization of smoke samples. Fourier transform mass spectrometry appears to have the ability to perform exact mass measurements and thus to identify high molecular weight constituents in mainstream smoke samples, without the need for extensive sample clean-up or chromatographic separation. Ion trap mass spectrometry provides a very high-speed, sensitive and selective analytical approach to the determination of nicotine and cotinine in air and urine samples, with no sample preconcentration or other preparation. Both compounds can be determined in urine simultaneously with detection limits down to 0.01 $\mu\text{g}/\text{ml}$ using a sample of 1 μl . The method requires only 3-5 minutes per sample, which allows over 100 samples to be analyzed per day. The method permits sampling of experimental animals repeatedly with no risk of mortality, thus allowing careful study of nicotine pharmacokinetics.

Additional activities currently in progress involve evaluating the utility of ITMS for the determination of ETS constituents. Ambient nicotine has been successfully determined in ETS by thermal desorption at concentrations ranging from 1-300 $\mu\text{g}/\text{m}$ ITMS. The approach is extraordinary because it allows analysis time of ten minutes or less, at the same time permitting quantitation of 0.5 ng and less of collected nicotine. The methodology can be used for simultaneous multicomponent analysis as well.

Additional work has involved studies of levels of benzo(a,l)pyrene and the food mutagens IQ, methyl IQ, methyl IQx, and PhIP in tobacco and smoke condensate and diesel exhaust particulate. These compounds, if present in more than trace amounts, may help explain the apparent lack of additivity of carcinogenicity when the various fractions are assayed. The activity of benzo(a,l)pyrene is over 400-fold greater than that of benzo(a)pyrene. Extensive work-up of multigram samples of both cigarette smoke condensate and diesel exhaust have been underway and benzo(a,l)pyrene has been tentatively identified in smoke, based on absorption and fluorescence spectra. However, the low content, 26 ng/g of smoke condensate, has not allowed structural confirmation to date. Any compound in diesel exhaust is below the 25 ng/g detection limit.

EXPERIMENTAL TOBACCO CARCINOGENESIS

GRANTS ACTIVE DURING FY91

<u>INVESTIGATOR/INSTITUTION/GRANT NUMBER</u>	<u>TITLE</u>
1. GUTTENPLAN, Joseph B. New York University 5 R01 CA44986-03	Mutagenic Components in Smokeless Tobacco
2. HOFFMANN, Dietrich American Health Foundation 5 P01 CA29580-10	Experimental Tobacco Carcino- genesis
3. HOFFMANN, Dietrich American Health Foundation 5 R01 CA44161-03	Bioassay of Snuff for Carcino- genic Activity in Rats
4. MARSHALL, Milton V. University of Texas Hlth Sci Ctr San Ant 5 R01 CA33069-07	Xenobiotic Metabolism in the Cigarette Smoking Baboon
5. MARSHALL, Milton V. University of Texas Hlth Sci Ctr San Ant 5 R01 CA49401-03	Tobacco Products and Oral Cavity Cancer
6. MC COY, George D. Case Western Reserve University 5 R01 CA49384-02	Oral Cancer: Etiological Role of Tobacco and Alcohol
7. MICHL, Josef Health Science Center at Brooklyn 3 R01 CA22682-12S1	An In Vitro Model of Pancreas Carcinogenesis
8. MUFTI, Siraj I. University of Arizona 5 R01 CA51088-02	Models of Tobacco-Alcohol Related Carcinogenesis
9. POMERLEAU, Ovide F. University of Michigan at Ann Arbor 2 R01 CA42730-07	Smoking and the Effects of Nicotine in Women
10. SCHULLER, Hildegard M. University of Tennessee Knoxville 5 R01 CA42829-05	Transplacental Carcinogenicity of NNK
11. SCHULLER, Hildegard M. University of Tennessee Knoxville 5 R01 CA48014-03	Characterization of Induced Neuroendocrine Lung Cancer

CONTRACT ACTIVE DURING FY91

INVESTIGATOR/INSTITUTION/GRANT NUMBER

TITLE

12. GUERIN, Michael
Department of Energy
Y01-CP-60513

Collection, Separation and
Elucidation of the Components
of Cigarette Smoke and Smoke
Condensates

SUMMARY REPORT

MOLECULAR CARCINOGENESIS

The Molecular Carcinogenesis component of the Branch includes 218 grants with FY91 funding of approximately \$36.38 million. There are no contracts in this area. The currently active grant portfolio consists of 167 R01 (Research Project) grants, 16 R29 "FIRST" (First Independent Research Support and Transition) awards, 2 R15 "AREA" (Academic Research Enhancement Award) grants, 1 R44 "SBIR" (Small Business Innovative Research) grant, 8 P01 (Program Project) grants, and 6 R35 "OIG" (Outstanding Investigator Grant) awards. In addition, 11 grants have been approved as R37 "MERIT" (Method to Extend Research In Time) awards. Also included are 7 R13 (Conference) grants. Research supported by this component focuses on (1) the role of exocyclic DNA adducts in carcinogenesis (10 grants), (2) characterization of carcinogen-macromolecular interactions (20 grants), (3) changes in biological macromolecules and cell functions as a result of carcinogen or cocarcinogen exposure (13 grants), (4) mechanisms of carcinogen-induced mutagenesis and genetic damage (24 grants), (5) the role of DNA repair in carcinogenesis (24 grants), (6) genetics and mechanisms of cell transformation (60 grants), (7) the role of oxygen radicals in carcinogenesis (19 grants), and (8) identification and properties of tumor promoters and mechanisms of tumor promotion (48 grants). Of the total number of grants in this component, 21 focused on various organ systems of interest to the NCI. There are 8 projects focused on breast cancer, 7 on bladder cancer (including 1 Program Project on human bladder cancer), 4 on colon cancer and 1 each on nervous system and upper aerodigestive tract cancers. Expanded descriptions of individual subject areas, along with examples of research accomplishments, are provided below.

Grants Activity Summary

Exocyclic Adducts in Carcinogenesis: In order to stimulate basic research on a class of important compounds which have the capability of forming exocyclic nucleic acid adducts, and to focus on the role of exocyclic adducts in carcinogenesis, the National Cancer Institute issued in, 1986 and 1987, a Program Announcement entitled "Biological Role of Exocyclic Nucleic Acid Derivatives in Carcinogenesis." As a result, interest in this area of research has increased with the involvement of many other investigators. Several currently funded studies are focused on the reaction of bifunctional carbonyl compounds such as crotonaldehyde, acrolein and malonaldehyde with nucleotides and with DNA, both in vitro and in vivo. The potential mechanisms of activation to mutagenic and carcinogenic compounds are also being studied. The ability to detect exocyclic nucleotide adducts in DNA from biological samples requires extremely sensitive detection methods. Several projects are working on the development of such methods. These include development of specific ³²P-postlabeling procedures, development of monoclonal antibodies specific for the major nucleic acid adducts of bifunctional carbonyl compounds (i.e., malonaldehyde, acrolein, methyl glyoxal) in DNA and bioanalytical methodologies such as ¹³C-NMR and coupled liquid chromatography-mass spectrometry systems. Other studies are investigating the in vitro mutagenic potential or replication fidelity of three potential exocyclic adducts (ethenoguanine, ethenoadenine and ethenocytidine) using oligonucleotides or DNA carrying site-specific lesions. Studies on the effect of exocyclic lesions on duplex DNA structure using structural NMR techniques and computational methodologies are also being supported. One program project grant supports integrated studies to examine the relationship of molecular structure to biological

function as it relates to the chemistry and biology of exocyclic DNA adducts. In addition, part of the research of two different OIG awardees is in this subject area.

Acrolein and crotonaldehyde are α , β -unsaturated carbonyl compounds and common pollutants in the human environment. Acrolein has been shown to be a metabolite of the widely used chemotherapeutic agent cyclophosphamide and is responsible for the bladder toxicity associated with the use of cyclophosphamide. Previously, acrolein was shown to be mutagenic in *Salmonella* and human fibroblast test systems. It has also been demonstrated that crotonaldehyde is mutagenic in *Salmonella* and induces liver tumors in rats. It is a metabolite of the hepatocarcinogen N-nitrosopyrrolidine and crotonaldehyde and related carbonyl compounds have been shown to form endogenously as products of lipid peroxidation. When reacted with DNA in vitro, these compounds form 1,N²-propanodeoxyguanosine adducts. Immunoassay and ³²P-postlabeling methods were used in one study to determine whether acrolein and crotonaldehyde form these adducts in cultured Chinese hamster ovary cells. This laboratory had previously demonstrated that acrolein forms adducts in *Salmonella* and that adduct formation was associated with mutagenicity in the tester strains used. Adduct levels were found to be higher in cells exposed to acrolein than in cells exposed to crotonaldehyde. ³²P-Postlabeling analysis confirmed the presence of adducts in crotonaldehyde-treated cells. When the cells were cultured for 6 hours before DNA isolation, adduct levels were found to be unchanged. Mutagenicity studies performed to determine the biological consequences of these adducts were unsuccessful because of the toxicity of the compounds. The ability of acrolein to induce mutations in mammalian cells appears to depend on the specific cell type in question. For example, in human fibroblasts, acrolein has been shown to be mutagenic only in cells from xeroderma pigmentosum patients and not in cells from normal individuals. The potential role of 1,N²-propanodeoxyguanosine adducts in the toxic, mutagenic and tumorigenic effects of acrolein or crotonaldehyde is not yet clear due to the paucity of studies in which the adduct formation, toxicity and mutagenicity of acrolein has been compared directly. This study and others have clearly demonstrated that 1,N²-propanodeoxyguanosine adducts are formed in vivo under conditions where toxic, mutagenic or tumorigenic events occur. This finding strongly suggests that these adducts play a direct role in mediating at least some of these biological effects, but that further studies are needed to elucidate the biological consequences of 1,N²-propanodeoxyguanosine adducts (34).

Vinyl chloride is a known human carcinogen. The mechanism of action of vinyl chloride involves its metabolic epoxidation to form chloroethylene oxide, which then rearranges to form chloroacetaldehyde. Both compounds react with DNA to form a mixture of mono- and bifunctional adducts with the base residues. The reaction of chloroacetaldehyde with deoxyadenosine and deoxycytidine results in a derivative with an additional five-membered ring that then undergoes dehydration to form 1,N⁶-ethenodeoxyadenosine (etheno-dA) and 3,N⁴-ethenodeoxycytidine, respectively. The reaction with deoxyguanosine leads to the formation of analogous derivatives, and both 1,N²-ethenodeoxyguanosine and N²,3-ethenodeoxyguanosine (etheno-dG) have been previously isolated and characterized. Unlike other identified etheno derivatives found in vinyl chloride exposed rodents, etheno-dG does not sterically interfere with etheno-dG:dT pairing, since the etheno ring is not involved in the formation of the two hydrogen bonds. In order to study how etheno-dG pairs with a specific base in a synthetic DNA, it was necessary to synthesize etheno-dG and etheno-dGTP. The successful synthesis of the very labile 5'-triphosphate of etheno-dG by one laboratory has made it possible to study the base pairing properties of this derivative placed opposite a defined normal base in a 25-base oligonucleotide

template. The studies performed, using a site-directed kinetic assay, showed that etheno-dG:dT pairs, which would be mutagenic, were formed with a frequency 2- to 4-fold greater than the analogous wobble pair, dG:dT. The non-mutagenic pairing, etheno-dG:dC, was shown to occur with a lower frequency than dG:dC, but neither etheno-dG:dT nor etheno-dG:dC constituted a significant block to replication. The frequency of etheno-dG:dT formation was shown to be similar with all polymerases tested. From these studies, it was concluded that these prokaryotic and eukaryotic replicating enzymes apparently recognize the same structural features, and on replication, G to A transition mutations would occur. In contrast to the dG:dT mismatch, which is known to have a specific repair system, etheno derivatives do not appear to be repaired *in vivo*. The relatively high affinity to form a base pair which would result in targeted transitions may be important in the initiation of vinyl chloride-induced cancer (181).

The contribution of 1,N²-ethenodeoxyadenosine (etheno-dA) to mutagenesis is not yet clear and continues to be investigated. Several investigators have sought to test the role of etheno-dA in directing the incorporation of erroneous bases during replication and transcription. Recently, *in vitro* replication and transcription systems have been used to attempt the quantitation of etheno-dA misincorporation. These studies have confirmed earlier results that dG is misinserted on replication, but at a much lower frequency than previously reported. The results suggested that etheno-dA generally does not prevent dT incorporation, but behaves as a bulky lesion that could be bypassed, thus possibly causing a frame-shift mutation. In order to elucidate the error-free bypass and mispairing alternatives of the etheno-dA exocyclic adduct, studies of the structure of the etheno-dA lesion opposite dT and dG in short oligonucleotide duplex segments were undertaken by three laboratories in a collaborative effort. Two dimensional proton NMR and energy minimization studies were undertaken on a nonanucleotide duplex (designated etheno-dA:dT 9-mer duplex) containing etheno-dA positioned opposite dT in the center of the helix. The NMR data and energy minimization calculations were consistent with a nonplanar alignment of etheno-dA (*anti*) and dT (*anti*), with dT displaced toward the flanking dG:dC base pair within the trinucleotide central segment of the etheno-dA:dT 9-mer duplex. Thus, the etheno-dA adduct in an *anti* orientation can be positioned opposite dT in an *anti* orientation in a DNA duplex with the potential steric clash relieved by the noncoplanar alignment of etheno-dA and dT across the lesion site. Similar studies were undertaken using a complementary nonanucleotide duplex (designated etheno-dA:dG 9-mer duplex) containing the exocyclic adduct etheno-dA positioned opposite dG in the center of the duplex. The data obtained with this 9-mer duplex was consistent with a dG (*anti*):etheno-dA (*syn*) alignment stabilized by two hydrogen bonds that could readily be accommodated into the DNA helix without the disruption of the flanking base pairs. This could account for the incorporation of dG opposite etheno-dA during *in vitro* replication by DNA polymerase I and would also explain recent genetic studies which have shown that etheno-dA can induce A to C transversion mutations *in vivo* (54, 151).

Carcinogen-Macromolecular Interactions: Research in this area seeks to identify, quantitate, and characterize carcinogen-nucleic acid adducts. Interest in the identification and characterization of DNA adducts stems from the role that DNA alterations play in the initiation of carcinogenesis. Most of the carcinogens used in these studies are metabolized by cellular xenobiotic metabolizing enzymes to a variety of metabolites of which one or, in some cases, a few are reactive and bind to nucleic acids and/or proteins. Identification and quantitation of the binding species are generally determined by chromatographic and radioisotopic techniques. Other techniques, such as fluorescence line-narrowing spectrometry which has a

detection level of about five adducts per 10^6 bases, are being developed to analyze complex mixtures of DNA adducts. In addition, development of monoclonal antibodies to various carcinogen-nucleic acid adducts has led to the increased use of radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) as sensitive methods of adduct detection.

The levels and persistence of specific DNA adducts are often related to the organ specificity of the carcinogen and may indicate which adducts are biologically relevant. For many carcinogens, such as polycyclic aromatic hydrocarbons, alkyl nitrosamines, N-2-acetylaminofluorene, and aflatoxin B1, the reactive metabolites and identities of the various nucleoside adducts are known. The chemical nature and physical conformation of the adducts are thought to determine an adduct's biological effect. For this reason, several investigators are focusing on chemical and biophysical characterization of carcinogen-DNA adducts and 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 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. 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 made it possible to build molecular models for the most likely conformations. The results of these studies have provided information as to the possible mechanisms by which a carcinogen may cause a mutation or other alteration in DNA structure.

DNA damage is produced upon exposure of cells to carcinogenic chromium(VI) compounds. Chromium(VI) has been shown to induce DNA interstrand cross-links and DNA-protein cross-links in rat and chick embryo liver *in vivo*. It also produces DNA strand breaks, DNA interstrand cross-links, and DNA-protein cross-links in cultured cells. However, several experiments have demonstrated that chromium(VI) does not react significantly with purified DNA *in vitro*, and that chromium(VI) must be activated within the cell in order for DNA damage to occur. Enzymes such as DT-diphosphorase, components of the mitochondrial electron transport chain, and small redox-active molecules such as ascorbate, hydrogen peroxide, and the thiols, glutathione and cysteine, have been shown to reduce chromium(VI) to its active form. Since thiols reduce chromium(VI) readily under physiological conditions, thiols were thought to be involved in chromium metabolism *in vivo*. Several lines of evidence have supported this view. In order to determine whether the nature of the thiol-reducing agent affects the ability of chromium to interact with DNA, one set of experiments assessed the levels of chromium bound to the plasmid pBR322 DNA after reaction with chromium(VI) in the presence of four different thiols. Chromium(V) and chromium-DNA adduct formation was observed and, depending on which thiol was used, chromium binding to DNA and the level of chromium(V) formed differed by several orders of magnitude. Chromium-DNA adducts formed by the reaction of chromium(VI) in the presence of glutathione or cysteine did not lead to DNA conformational changes as detected by agarose gel electrophoretic analysis. In the presence of dithiothreitol or beta-mercaptoethanol, changes in DNA conformation were detected

after reaction with chromium(VI). The effects observed by transmission electron microscopy of chromium-DNA complexes (aggregates of several plasmids and condensation of individual plasmids into compact kinked forms) were thought to be due to cross-linking of DNA induced by chromium metabolites. From these studies, it was determined that the levels of chromium bound to DNA are related to the levels and stability of the chromium(V) species formed in these reactions. Thus, the DNA damage induced by thiol reduction of chromium(VI) may be important in the carcinogenicity of chromium(VI) compounds (211, 212).

One of the most commonly used treatments for psoriasis is daily topical application of 2-5% coal tar preparations, sometimes followed by irradiation with UVB (Goeckerman therapy). Coal tars contain complex mixtures of polycyclic aromatic hydrocarbons (PAHs) produced by the destructive distillation of coal and are well established mutagens and animal carcinogens. A number of studies have confirmed that occupational exposure to coal tar results in elevated risks for cancer in a number of sites. In addition, absorption of PAHs through the skin has been demonstrated. It is now possible to detect and quantitate DNA adducts by a number of methods, such as immunological and ³²P-postlabeling methods. Antibodies developed against benzo(a)pyrene diol-epoxide (BPDE)-modified DNA have been used to quantitate adducts in biological samples obtained from individuals with a number of different occupational and environmental exposures to PAHs. ³²P-postlabeling has also been applied to the detection of adducts in humans. Because it requires smaller amounts of DNA than the immunoassays, this method is ideal for measurement of adducts in small-sized samples such as human biopsies. In one such study, skin biopsies were obtained from coal tar-treated psoriasis patients and non-treated controls. Indirect immunofluorescence staining with antibodies generated against BPDE-modified DNA demonstrated specific nuclear staining in epidermal cells of all biopsies from treated patients, but not from control biopsies from untreated individuals. ³²P-postlabeling analysis of DNA isolated from the biopsies revealed a pattern of multiple adducts in samples from treated patients but not from controls. A larger-scale study using several markers of exposure and a biologically effective dose in this population is currently being carried out as a model for skin exposure to environmental and occupational carcinogens (207).

Several different studies have shown that the interaction of chemicals with genomic DNA seem to be nonrandom in nature. When various DNA sequences were studied in the form of supercoiled plasmids, regulatory regions of genes were found to readily adopt altered DNA conformations as revealed by chemical probes and nucleases. DNA sequences surrounding the immunoglobulin heavy chain (IgH) enhancer have been shown to contain negative regulatory elements which are important for the tissue specificity of the enhancer sequence. Nuclear matrix association regions (MARs) or scaffold-attached regions (SARs) are often found in close vicinity to known enhancer sequences. In one study, an unusual property of DNA sequences located both 5' and 3' of the enhancer region was reported. These sequences correspond to the negative regulatory elements and the MAR sequences of the IgH enhancer. They were shown, even at room temperature, to become entirely base-unpaired over an extended length when subjected to the torsional stress of negative supercoiling. The ability of the sequences to assume a stably unpaired conformation was demonstrated by reactivity with chloroacetaldehyde, which is specific for unpaired DNA bases, and by two-dimensional gel electrophoresis of topoisomers. The sequences located 3' of the enhancer induced base unpairing in the direction of the enhancer. This unpaired, highly A+T rich region progressively expanded to include as many as 200 base pairs when the ionic concentration was decreased or the superhelical density was increased. Mutation by site-specific mutagenesis of an ATATAT motif within a negative regulatory

element located 3' of the enhancer resulted in abolishing the extensive base-unpairing property. That the structural properties of DNA may play a role in the regulation of gene expression was also suggested by the observation that multiple sites within transcriptional regulatory regions, when under torsional stress, readily adopted a DNA structure that deviated from the normal B-form DNA. Results obtained to date suggest that DNA itself plays an active role in biological functions, such as replication and transcription, by utilizing its structural potential, rather than by being just a passive participant serving merely as the sequence information source. It was concluded that the extensive base-unpairing property of negative regulatory elements and matrix attachment regions in the IgH enhancer flanking sequences may also be of functional importance in vivo (106).

Two types of mutagenic lesions, mismatched and modified base pairs, have been shown to occur in DNA. Mismatched base pairs occur mainly as the products of natural processes. They can arise normally during genetic recombination, as incorporation errors during DNA replication, and during the folding of single-strand DNAs. Mismatched base pairs can also occur as the product of the deamination of 5-methylcytosine in DNA to give G:T and after the repair of O⁶-methylguanine (O⁶-meG)-thymidine base pairs by alkyltransferase to give G:T. In addition, modified base pairs occur as the product of environmental damage to DNA by both direct-acting and metabolically activated chemicals. O⁶meG and mismatched bases such as G:T and A:C have been shown to be destabilizing in that they decrease the T_m of DNA significantly. The structures of such modified and mismatched base pairs have been the subject of considerable investigation. The principal methods used have been X-ray crystallography and NMR, which have indicated little or no local helical distortion. However, these techniques are not sensitive to more global distortions of the DNA molecule. In one set of studies, polyacrylamide gel electrophoresis and thermal denaturation were used to examine A:C, G:T, and O⁶-meG:T and O⁶-meG:C mismatches synthesized in place of either of two adjacent G:C base pairs in synthetic DNA duplexes. Substitution for G:C at either position was shown to decrease the stability of the duplex, with O⁶-meG more destabilizing in place of the 5'G than in place of the 3'G. Structural distortion differences, as measured by gel electrophoretic mobility changes, were observed under conditions where stability differences, as measured by T_m changes, were negligible. The structural distortions appeared to be composed of a symmetric component that imparts a frictional drag on the polymer during gel electrophoresis and an asymmetric component or bend that constitutes a more global effect on the conformation of the DNA. Comparisons between these effects at the two adjacent positions showed that the extent of structural perturbation depends on sequence context. The effect of base substitution on DNA electrophoretic mobility was shown to be greatest when the substitutions were all located along one face of the helix. Such effects on the trajectory of the DNA molecule are hypothesized to be factors in how enzymes recognize such lesions in the DNA helix (199).

Changes in Cellular Macromolecules and Cell Functions: Research activities in this 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. Neoplastic cells have been shown to manifest a variety of morphological and biochemical phenotypes which are different from their normal cell counterparts. This heterogeneity and phenotypic instability generated in cancer presumably originates, in part, from 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 modification of nuclear DNA-nuclear protein complexes. There is an increasing volume of evidence demonstrating that the state of DNA

methylation regulates gene expression and is also involved in 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 needed. Several studies are being supported which seek to understand the role of DNA methylation in the control of gene expression and carcinogenesis and the mechanisms involved. 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-aza-cytidine, which are known to affect the transfer of methyl groups to DNA.

Approximately 3-4% 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-aza-cytidine can result in the reactivation of genes on the transcriptionally inactive X chromosome, induction of tissue-specific gene expression, and 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. Several 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. Thus, it is clear that the mechanisms by which methylation signals are recognized have thus far remained obscure and await more definitive isolation and characterization of specific genes in the various systems that have been examined.

Less than 1% of the bases of human DNA are known to be 5-methylcytosine. Methyl groups that occur on both C residues in the double-stranded palindrome CpG have been shown to account for more than 90% of the methylated C residues. Although the CpG sequence is underrepresented by a factor of five in the vertebrate genome, it has been shown to be the site of a disproportionately high number of human germ-line point mutations. Estimates have been made which suggest that 35% of point mutations causing human genetic disorders have occurred at CpG dinucleotides and that over 90% of these were transitions from C to T or the corresponding G to A transitions. CpGs may also be overrepresented among sites of somatic mutation in tumor suppressor genes, such as the p53 or retinoblastoma genes, in which about 40% of reported point mutations have been localized to CpGs. It is hypothesized that methylation of CpGs in normal tissues increases the probability of mutations at such sites due to the ability of 5-methylcytosine to undergo deamination, resulting in a thymine. As a test of this hypothesis, one OIG awardee used ligation-mediated polymerase chain reaction (PCR) genomic sequencing to analyze directly CpGs that have undergone either a germ-line mutation in the low density lipoprotein receptor or somatic mutations in the p53 tumor suppressor genes. The studies demonstrated that all of these CpGs were methylated in the normal human tissues analyzed. The mutations could thus be scored as transitions from 5-methylcytosine to thymine rather than from cytosine to thymine. Methylated cytosines were shown to occur exclusively at CpG dinucleotides. These are markedly underrepresented in the human genome, but are sites for more than 30% of all known disease-related point mutations. It was calculated that about 3.5% of the p53 sequence has CpGs and that these are contributing to 33-43% of the point mutations as transitions from 5-methylcytosine to T. Thus, the

observations made suggest that 5-methylcytosine acts as an endogenous mutagen and that a high percentage of mutations in human tumor suppressor genes may be induced by deamination of methylated cytosines to form thymines (95).

CpG dinucleotides have been shown to be unevenly distributed in the vertebrate genome with bulk DNA mostly CpG deficient. Most of the cytosines in the dinucleotide in this fraction are methylated. The remaining 1% of DNA does not demonstrate CpG suppression or methylation and can be detected by using the restriction enzyme *HpaII*, which cleaves this DNA into small fragments. These DNA sequences are generally called "CpG islands," and are often associated with genes. CpG islands were found to commonly occur in 5' regions of housekeeping and tissue-specific genes and also in the transcribed regions of some tissue-specific genes. A key feature of islands is that they are unmethylated at testable sites in most tissues with the exception of the genes on the inactive X chromosome. In another study from an OIG awardee, *Hpa II/Msp I* analysis and ligation-mediated PCR was used to examine the methylation of the *MyoD1* CpG island in adult mouse tissues, early cultures of mouse embryo cells, and immortal fibroblastic cell lines. This gene is expressed exclusively in skeletal muscle and is an example of a vertebrate determination gene. Its expression is sufficient to convert several cell lines into the myoblast phenotype. Since nuclei in muscle syncytia are postmitotic, the expression of *MyoD1* has the potential to end cellular division. Inactivation of such determination genes is thought to be necessary to establish cellular immortality. The CpG island was found to be almost devoid of methylation at CCGG sites in adult mouse tissues and in low-passage mouse embryo fibroblasts. In marked contrast, the island was found to be methylated in C3H 10T1/2 cells and in six other immortal cell lines examined, demonstrating that methylation of this CpG island had occurred during escape from senescence. The island became even more methylated in chemically transformed derivatives of C3H 10T1/2 cells. These studies demonstrated that CpG islands not methylated in normal tissues may become modified to an abnormally high degree during immortalization and transformation (95).

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. 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.

The established role of O⁶-alkyl-G in mutagenesis and the initiation of carcinogenesis by alkylating agents has obscured the possible similar role of other alkyl derivatives. Over the last decade, there has been increased attention focused on O-alkylpyrimidines as also having biological relevance. Several experiments have indicated that O⁶-alkyl-T was not only mutagenic, but that repair was very slow in mammalian cells and tissues. The presence of O⁶-methyl-T and O⁶-ethyl-T has been correlated with the organ specificity of tumors resulting from N-nitroso alkylating agents. It was of interest to compare the mutation frequency of O⁶-alkyl-G with that of O⁶-alkyl-T in the same system, using site-directed methods for insertion of the desired derivative. For one set of studies, a 25-mer oligonucleotide template containing the relatively labile O⁶-methyl-T at a unique site was successfully synthesized. The sequence used was analogous to that studied previously to

determine the mutation frequency of O⁶-methyl-G in vitro and in vivo. Templates containing O⁶-methyl-T or unmodified T were used in a primer-extension gel assay to determine kinetic parameters for incorporation by *E. coli* DNA polymerase I (Klenow fragment) and *Drosophila melanogaster* polymerase *alpha*-primase complex of dGTP and dATP opposite either O⁶-methyl-T or T. The pairing of O⁶-methyl-T:G was shown to be preferred over that of both O⁶-methyl-T:A and T:G by more than 10-fold. The two polymerases gave almost identical values for the frequency of formation of all pairs investigated, suggesting that the 3' to 5' exonuclease activity of the Klenow fragment does not efficiently edit such pairs. Extension beyond O⁶-methyl-T:G was demonstrated with both DNA polymerases. The kinetic approach utilized in these studies allows quantitation of mutagenic potential in the absence of alkylation repair and additionally provides qualitative data on mutagenesis that are in accord with previous in vivo studies showing that replication of O⁶-methyl-T causes T to C transitions. These and other studies have demonstrated that both O⁶-methyl-G and O⁶-methyl-T preferentially form a type of G:T pair, which leads to transition mutations (180, 54).

DNA replication is normally an extremely accurate process. The high fidelity of DNA synthesis, at least in *E. coli*, is thought to occur through a sequence of mechanisms involving selection of the complementary base by the replicative DNA polymerase, exonucleolytic 3'-5' editing of a noncomplementary base at the growing point, and postreplicative mismatch repair. In eukaryotes, however, the 3'-5' exonuclease did not appear to be a consistent constituent of isolated polymerase *alpha*, since it was found only exceptionally or as a cryptic activity. Recently, however, indirect evidence has been presented that exonucleolytic proofreading occurs during DNA replication. Since fidelity with DNA polymerase *alpha* is known to be controlled mainly by the selection of the complementary base, it has been frequently postulated that alterations in DNA polymerases by chemical carcinogens might promote misincorporation during initiation of the carcinogenic process or during tumor progression. Although the mechanism by which mutations are introduced in mammalian cells is still not known in detail, results indicating that mutagenesis might be controlled at the level of DNA polymerase *alpha* have prompted another OIG awardee to study whether or not an error-prone DNA polymerase *alpha*-primase complex is involved in mutagenesis. In order to investigate whether carcinogens induce alterations of the DNA polymerase *alpha*-primase complex, the physicochemical and catalytic properties of the complexes, and the fidelity of DNA synthesis of DNA polymerase *alpha*-primase complexes from carcinogen-treated and untreated Chinese hamster ovary cells, were compared. Complexes were purified and both DNA polymerizing activities and those of ancillary enzymes, such as RNA primase and exonuclease, were examined. All of the physicochemical and catalytic properties of the complexes measured were identical in the preparations from both carcinogen-treated and untreated cells. The fidelity of DNA polymerase *alpha*-primase complexes measured by the *phi*X174am3 reversion assay was also similar in carcinogen-treated and untreated cells. Thus, a carcinogen-mediated induction of a DNA polymerase *alpha*-primase complex with low fidelity was not observed within the detection limits of the *phi*X174 assay. An ancillary enzyme activity, RNA primase, was detected in both carcinogen-treated and untreated cells. The RNA primase:DNA polymerase *alpha* activity ratio, however, was significantly higher in DNA polymerase *alpha*-primase complexes from carcinogen-treated cells. Using supercoiled or unprimed single-stranded DNAs as templates, these complexes also exhibited at least three times greater velocity of synthesis. Since the binding sites of DNA polymerase *alpha*-primase complexes for deoxynucleotide triphosphates and DNA templates were identical before and after carcinogen treatment of cells, the increased synthesis catalyzed by the DNA polymerase *alpha*-primase

complex from carcinogen-treated cells was thought to be due to a carcinogen-induced alteration of an accessory protein of the complex. The biological significance of the elevated primase activity in carcinogen-treated cells is not yet clear. It was hypothesized that the chemical carcinogen used in the study might induce the synthesis and/or posttranslational modification of an initiation factor which subsequently alters the frequency of initiation of DNA replication (123).

Mechanisms of Mutagenesis and Genetic Damage: The projects that are being supported in this subject area seek to understand how mutations and DNA/chromosome damage are generated by carcinogenic chemicals. The spectrum of mutations produced in a defined cellular gene following exposure of cells to chemical and metal carcinogens is being determined in several laboratories. The defined genes include hypoxanthine-guanine phosphoribosyl transferase (HPRT) and dihydrofolate reductase (DHFR). Additionally, specifically synthesized oligonucleotides of a defined base sequence are being used to examine the molecular mechanism of base-pair substitution and frame-shift mutagenesis. Base sequence specificities of the interactions of mutagens with oligonucleotides are being determined in other studies and correlated to their mutagenic activity. Newer studies in this area have focused on the use of specific genes cloned into plasmids as targets for the mutagenic action of various chemical carcinogens. For example, the c-Ha-ras oncogene cloned into a plasmid and the lac gene introduced into M13 phage DNA are being used as target genes to assess the mutagenic action of chemicals such as benzo(a)pyrene diolepoxide (BPDE), N-acetoxy-2-acetyl-aminofluorene (AAF), and other aromatic amines. In addition, the construction of plasmids containing a single site-specific lesion has allowed investigators to study the mutagenic effect of a specific type of DNA lesion in cells. DNA sequence analysis and analysis of the effects of lesions on the fidelity and extent of DNA synthesis have been used to determine the mechanisms of mutagenesis.

The reaction of cells with a variety of mutagens and carcinogens results in the production of adducts in DNA that can block DNA synthesis in vitro. In order to produce either mutations or a transformed cell, it is understood that the DNA synthetic machinery must replicate past the lesion. One possible way that this bypass might be accomplished is by translesion synthesis, in which a nucleotide is added opposite an adducted base and the added nucleotide then serves as a primer to further elongation. At least three steps are known to be involved in the process of translesion synthesis. In order to study the factors involved in translesion synthesis in vitro, one MERIT awardee chose a pair of DNA adducts differing only by an acetyl group. The reaction of DNA with N-acetoxy-2-acetylaminofluorene leads to the formation of N-(deoxyguanosin-8-yl)-2-acetylaminofluorene (AAF-G) adducts. Unrepaired AAF-G lesions in prokaryotic DNA have been shown to be lethal and an absolute block to DNA synthesis in vitro. AAF-G adducts in the DNA of bacteriophage M13 can be converted to N-(deoxyguanosin-8-yl)-2-aminofluorene (AF-G) adducts in situ by treatment with 1.0 M NaOH. The conversion was accompanied by a dramatic increase in transfection activity of the samples that was correlated with measured deacetylation of the AAF-G adduct. These substrates (AAF-G/AF-G), with adducts at identical places in the DNA, were used to study bypass synthesis by a variety of polymerases [T7 DNA polymerase, an altered T7 DNA polymerase from which the 3' to 5' exonuclease has been genetically removed (Sequenase 2), T4 DNA polymerase and *E. coli* DNA polymerase I.] All polymerases were blocked at AAF-G lesions. Sequenase 2 was shown to add nucleotides opposite the AAF-G lesion but to be unable to catalyze further elongation. T7 DNA polymerase was unable to bypass AAF-G adducts, whereas Sequenase 2 bypassed the lesions readily. The data obtained support the hypothesis that the elongation step is rate limiting in synthesis past lesions. The low 3' to 5'

exonuclease activity of the polymerases allows the priming nucleotide opposite the altered template site to remain in position long enough for elongation past particular adducts (190).

Induction of mutations in mammalian cells by chemical carcinogens is considered to play an important role in the multistep process of carcinogenesis. As part of an investigation into the mechanisms by which normal human cells are transformed into malignant cells by carcinogens, mechanisms of mutation induction by racemic 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) were examined in one laboratory. In previous studies, a shuttle vector carrying a *supF* target gene was treated with BPDE and structurally related carcinogens and allowed to replicate in human cells. An analysis of the kinds of mutations induced indicated that the majority were G:C to T:A transversions, but that each agent produced its own unique spectrum of mutational "hot spots." For comparative purposes, an endogenous gene of diploid human cells, the hypoxanthine (guanine) phosphoribosyltransferase (*HPRT*) gene, was used as the target for mutagenesis. Individual populations of diploid human fibroblasts were treated with BPDE or left untreated, and progeny cells were then selected for resistance to 6-thioguanine. The *HPRT* gene sequence from 29 unequivocally independent mutants from BPDE-treated populations and 13 from control populations was directly determined from amplified cDNA copies. Results showed that 23 of the 29 BPDE-induced mutants examined contained a single base pair substitution, with four exhibiting two base pair substitutions. Eight out of 13 control mutants exhibited base pair substitutions with the remaining four missing a complete exon. Thirty of the 32 base pair substitutions in the BPDE-induced mutants were shown to involve G:C base pairs that were primarily G:C to T:A transversions. The majority (89%) of the base pair substitutions observed in the mutants from the control population, however, were shown to involve an A:T base pair, primarily A:T to G:C transitions. Base substitutions were observed throughout the coding region of the gene, but 41% of those seen in mutants from the BPDE-treated population and 44% of those from the untreated population were located in the first half of exon 3. This is the hypothesized region that codes for the catalytic sites of the enzyme. In the majority of point mutations examined, the affected guanine was located on the non-transcribed strand. From this and other data, this bias of potentially mutagenic lesions was attributed to the selective removal of BPDE adducts from the transcribed strand of the human *HPRT* gene before these lesions could be converted into a mutation (128).

Benzo(c)phenanthrene (B(c)PH) is a weakly tumorigenic polycyclic aromatic hydrocarbon that is metabolized to diastereomeric pairs of bay-region epoxides. The diol-epoxides, although only minor metabolites of B(c)PH, have been shown to be exceptionally tumorigenic and mutagenic, with the most potent being the diastereomers with trans stereochemistry. DNA adducts derived from each of the configurational B(c)PH diolepoxide isomers have been characterized and shown to occur at exocyclic amino groups of guanine (N-2) and adenine (N-6). The mutagenic specificity of the optically active (-)-*anti*-dihydroxy-2-epoxide of B(c)PH diolepoxide (B(c)PHDE) has been investigated using the *supF* shuttle vector system and shown to cause mostly G:C to T:A and A:T to T:A transversions. The *DHFR* locus in cultured Chinese hamster ovary cells has been used as a target to study spontaneous and carcinogen-induced mutations at the DNA level. In one study, racemic B(c)PH was used for mutagenesis studies in which the induced sequence changes were determined in 38 isolated *DHFR* mutants by polymerase chain reaction amplification and direct DNA sequencing. Base substitutions accounted for 78% (30/38) of the mutations. The other mutations observed included four frameshift and four complex mutations. The preferred type of mutation was shown to be transversions (A:T to T:A and G:C to T:A), which occurred

in 69% of the analyzed mutants. A purine was found on the 3' side of the putative adduct site in every mutant. Mutations were favored at sequences AGG, CAG, and AAG (the underlined base being the target). Surprisingly, 42% of the mutations were shown to create mRNA splicing defects, especially at splice acceptor sites for each of the five introns of the *DHFR* gene. These splice acceptor sites contain polypyrimidine tracts upstream of the AG, with poly(A) runs on the opposite strand. B(c)PH may recognize some aspect of DNA structure in these regions corresponding to pre-mRNA splice sites. In general, splicing mutations are known to generate deletions in the specified protein and thus are likely to cause a null phenotype. For tumor suppressors, production of a null phenotype may be necessary for loss of growth control. The propensity of a mutagen to attack splice sites may thus magnify its potency as a carcinogen (81, 207).

In other laboratories, analysis of mutations induced in human or other mammalian cells at the DNA sequence level is being approached by developing recombinant DNA shuttle vectors composed of various viral sequences that allow the plasmid to be replicated in human or mammalian cells. For this purpose, the simian virus 40 (SV40) early region, the Epstein-Barr virus (EBV) oriP element, or some other sequence have been used. Including sequences derived from the bacterial plasmid, pBR322, permit the plasmid to also replicate in *E. coli*. Finally, some selectable target genes for mutant selection are also included. In addition, retrovirus based shuttle vectors containing a selectable gene are being developed for these types of studies. Studies supported 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.

Transformation of normal cells into tumorigenic cells is recognized to be a multi-step process with at least one of the steps involved in the development of cancer being the generation of mutations. 1,6-Dinitropyrene (1,6-DNP) has been shown to be one of the most mutagenic of the common environmental contaminants in certain bacteria and mammalian cell assays, and it is strongly tumorigenic in experimental animals. For this reason, it was of interest to investigate the mechanisms by which this carcinogen induces mutations. For this purpose, a comparison was made of the frequency and spectrum of mutations induced when a shuttle vector with a *supF* target DNA sequence (pS189) carrying covalently bound residues of structurally related carcinogens, replicates in human 293 cells. The mutagenic effects of N-hydroxy-1-amino-6-nitropyrene, a partially reduced derivative of 1,6-DNP, was investigated. Results were compared with what had been previously found in the same assay with N-hydroxy-1-aminopyrene, the partially reduced derivative of 1-nitropyrene. ³²P-post-labeling was used to show that only a single adduct was formed from exposure to N-hydroxy-1-amino-6-nitropyrene. Results showed a linear increase in number of adducts per plasmid as a function of applied concentration and also in frequency of *supF* mutants as a function of adducts per plasmid. Upon comparison of the frequency of mutants induced when plasmids carrying residues of 1,6-DNP replicated in human cells, with that induced by 1-nitropyrene residues, the former was shown to be 1.8 times more mutagenic than the latter. Both carcinogens induced mainly base substitutions, primarily G:C to T:A transversions; but 1,6-DNP produced a significant fraction of -1 frameshifts, with most of these located in a unique run of five Gs in the gene. Polymerase termination reactions indicated that 1,6-DNP adducts were formed at every guanine, but not elsewhere in the gene. The "hot spots" for adduct formation were not perfectly correlated with "hot spots" for mutation induction.

This indicated that the ultimate biological effect of the chemical depends not only on the number of adducts originally formed, but also on processes such as DNA repair and on the structure of the neighboring bases at the adduct site (128).

There is evidence which points to genetic recombination as a mechanism for the loss of wild-type alleles of critical genes involved in the pathogenesis of several types of human cancers. Because homologous recombination may be involved as one step in the carcinogenesis process, an investigation was made of the ability of a series of six chemical carcinogens and UV radiation to induce recombination between two herpes simplex virus type 1 thymidine kinase (*Htk*) genes carried on a plasmid stably integrated in the genome of a *tk*-deficient mouse L-cell line. Each *Htk* gene had been inactivated by an 8-base-pair *XhoI* linker inserted at a unique site, so that only by undergoing a productive recombinational event between the two nonfunctional genes could a functional *Htk* protein be produced. Using this system, it was shown that each of the carcinogens tested induced a dose-dependent increase in the frequency of homologous recombination and that the increase was directly related to the number of residues covalently bound to the DNA of the cells. In order to study the role of nucleotide excision repair in the induction of intrachromosomal homologous recombination in mammalian cells, a plasmid containing a substrate for recombination was introduced into three human cell lines that differed in their repair capacity, and the frequency of recombination induced by UV radiation or by 1-nitropyrene was compared. One strain had a normal capacity for nucleotide excision repair, the second exhibited an intermediate rate of repair, and the third, derived from a patient with xeroderma pigmentosum, had no ability to repair UV- or 1-nitropyrene-induced DNA damage. The endogenous *tk* genes in these cell strains had been inactivated. In all three strains, UV and 1-nitropyrene induced dose-dependent increases in the frequency of recombinants. The doses required to cause a specific increase in recombination in repair-deficient strains, however, were shown to be 10-30 times lower than the dose required for the cell strain with a normal capacity for repair. Results obtained strongly suggest that the unexcised DNA lesions, rather than excision repair per se, stimulate intrachromosomal homologous recombination. Southern blot analysis of DNA from representative recombinants indicated that, in all cases, one of the two *Htk* genes had become wild type (*XhoI* resistant). The majority (90%) retained the *Htk* duplication, which is consistent with the nonreciprocal transfer of genetic information (gene conversion). These results suggest that the increased risk of cancer in xeroderma pigmentosum patients may also reflect increased recombination (129).

Role of DNA Repair in Carcinogenesis: This subject area includes studies on the characterization of DNA damage produced by bulky chemical carcinogens, alkylating agents, ultraviolet light and ionizing radiation; isolation and characterization of proteins responsible for DNA nucleotide excision repair and base excision repair; cloning and characterization of the DNA nucleotide and base excision repair genes; and determination of the role of chromosome structure, location and site of DNA lesions and poly(ADP-ribosylation) in repair of DNA damage. A variety of rodent, 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 in humans 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 on exposed surfaces at an early age, thus establishing a link between genetic damage, defective repair of damage, and cancer in the exposed tissue.

Mutagenicity and carcinogenicity of UV radiation have been attributed to the effects of base modifications formed in DNA as a consequence of this radiation. The most readily formed UV-induced base modifications are cyclobutane pyrimidine dimers and pyrimidine 6-4 photoproducts. There is evidence, however, that all of the biologic consequences of UV irradiation cannot result from the effects of these lesions. For example, mutations formed at cytosine residues that are not adjacent to other pyrimidines, for example, cannot be ascribed to either of the dimeric photoproducts. These mutations might instead result from UV-induced modification of single cytosine residues, the most readily formed of which is the photohydrate resulting from addition of water to the 5,6 double bond. Experiments using the *E. coli* repair enzyme endonuclease III have indicated that UV irradiation can modify single cytosine residues. This enzyme is known to contain two activities that act sequentially: a DNA glycosylase that catalyzes hydrolysis of the N-glycosyl bond releasing the modified pyrimidine base; and an AP (apurinic/aprimidinic) endonuclease that catalyzes cleavage of the phosphodiester bond of the resulting abasic site. A MERIT awardee had previously demonstrated that cytosine hydrate was formed in DNA, using HPLC to identify the material released from UV-irradiated poly(dG-dC) by endonuclease III. When incubated with the UV-irradiated double-stranded alternating copolymer poly(dA-dU), release of intact uracil hydrate was demonstrated. Cytosine hydrate and uracil hydrate can eliminate water and revert back to cytosine and uracil, respectively, but at different rates. The observations made suggested that hydrates of cytosine in DNA were much more stable than hydrates of free cytosine, deoxycytidine and deoxycytidine monophosphate. In order to prove this hypothesis, measurements of the formation and stability of cytosine hydrate in UV-irradiated poly(dG-dC), and uracil hydrate in UV-irradiated poly(dA-dU), were undertaken. As measured by *E. coli* endonuclease III, it was shown that 2.2% of cytosine residues in the UV-irradiated poly(dG-dC) were converted to cytosine hydrate, while 0.09% were converted to uracil hydrate. When incubated in solution for up to 24 hours after UV-irradiation, cytosine hydrate was shown to be stable at 4°C and to decay at 25, 37 and 55°C with half lives of 75, 25 and 6 hours, respectively. Uracil hydrate produced in irradiated poly(dA-dU) was shown to be stable at 4 and 25°C and to decay with a half-life of 6 hours at 37°C and less than 0.5 hours at 55°C. These experiments demonstrated that UV-induced cytosine hydrate may persist in DNA for prolonged periods of time and that it undergoes deamination to uracil hydrate, which loses water to yield uracil. The formation and stability of these photoproducts in DNA, which could lead to transition and transversion mutations, are thought to have promoted the evolutionary development of the repair enzyme endonuclease III and analogous DNA glycosylase/endonuclease activities of higher organisms, as well as the development of uracil-DNA glycosylase (196, 14).

O⁶-Alkylguanine is a DNA lesion which is induced by simple alkylating agents. It has been shown to be a major mutagenic and carcinogenic lesion because of its preference for pairing with thymine during DNA replication. O⁶-Methylguanine-DNA methyltransferase (MGMT), a unique DNA repair protein present in most organisms, has been shown to remove the carcinogenic and mutagenic adduct O⁶-alkylguanine from DNA through stoichiometric transfer of the alkyl group to a cysteine residue in a suicide reaction. The mammalian protein is known to be highly regulated in both somatic and germ-line cells. The extent of repair which occurs in a cell, therefore, is dependent on the initial amount of alkyltransferase. An inverse correlation between the presence of this repair protein and sensitivity of cells to the actions

of alkylating agents has been demonstrated, but the extent to which other factors and other DNA adducts not repaired by the alkyltransferase also play roles is not well understood. One approach to this problem has been to design experimental methods to modulate alkyltransferase activity and examine the consequences of such modulation on carcinogenesis, mutagenesis and cytotoxicity. Attempts have also been made to deplete alkyltransferase activity by the use of inhibitors. One such inhibitor is O⁶-methylguanine, which was previously demonstrated to decrease the amount of active alkyltransferase in cells by about 80% and thereby increase cell responsiveness to chemotherapeutic chloroethylating agents. However, the clinical potential of O⁶-methylguanine was limited because the high drug doses and long exposure periods required produced only a partial reduction in alkyltransferase activity. From chemical and structural considerations governing the rates of displacement reactions, it was hypothesized that the alkyltransferase would have an increased affinity for O⁶-benzylguanine. In order to test this hypothesis, the effectiveness of O⁶-benzyl-, O⁶-(p-chlorobenzyl-) and O⁶-(p-methyl-benzyl)guanine as depleters of alkyltransferase activity in human colon tumor HT29 cells was examined. O⁶-Alkylguanine DNA alkyltransferase was shown to be rapidly and irreversibly inactivated by exposure to either O⁶-benzylguanine or its p-chlorobenzyl and p-methylbenzyl analogues. This inactivation occurred much more rapidly and required about a hundred-fold less concentration than O⁶-alkylguanine itself. Complete loss of activity in HT29 cells was produced within 15 min after addition of O⁶-benzylguanine to the culture medium with maximal effect at 5 *micro*M. In contrast, at least 100 *micro*M O⁶-methylguanine for 4 hours was needed to get the maximal effect of an 80% reduction in the alkyltransferase. Pretreatment of HT29 cells with O⁶-benzylguanine led to a dramatic increase in the cytotoxicity produced by the chloroethylating chemotherapeutic agents tested. Administration of a 10 mg/kg dose of O⁶-benzylguanine to mice reduced alkyltransferase levels by more than 95% in both liver and kidney. These results indicated that depletion of the alkyltransferase by O⁶-benzylguanine may be used in investigating the role of the DNA repair protein in carcinogenesis and mutagenesis and that this treatment may be valuable in increasing the chemotherapeutic effectiveness of chloroethylating agents (152).

Poly (ADP-ribose) polymerase is a nuclear enzyme that has been shown to play a role in modulating repair of damaged DNA in mammalian cells and is thought to also play a role in DNA recombination and replication. The catalytic activity of the enzyme is dependent upon the presence of DNA strand breaks, suggesting that the polymerase plays a role in the modification of chromatin in the vicinity of DNA damage. Inhibition of the polymerase *in vivo* interferes with normal DNA repair, increases the extent of cellular transformation and DNA amplification, and increases sister chromatid exchanges. Human poly(ADP-ribose) polymerase cDNA has been cloned and the location of the polymerase hybridizing sequences has been mapped to human chromosomes 1 (the active gene), 13 and 14. Using the human cDNA as a probe for isolation of clones of the murine poly(ADP-ribose) polymerase gene, murine sequences homologous to the human poly(ADP-ribose) polymerase cDNA were mapped to a region on mouse chromosome 4 which is associated with both plasmacytoma resistance and efficient chromatin repair. Based on preliminary evidence which indicated the presence of an extraneous polymorphic restriction fragment for murine poly(ADP-ribose) polymerase loci in strains of mice susceptible to plasmacytomas, one grantee investigated the correlations between the restriction fragment length polymorphism of the poly(ADP-ribose) polymerase gene(s) and human Burkitt's lymphoma. No increase was observed in the frequency of polymorphisms on chromosome 1 (containing the active gene) or on chromosome 14 (a pseudogene). However, fragment length polymorphism analysis of poly(ADP-ribose) polymerase sequences on chromosome 13 [either a processed pseudogene or a gene with extensive identity to poly(ADP-ribose) polymerase] revealed that

of 19 DNA samples derived from endemic Burkitt's lymphoma, all contained at least one copy of a rare allele (B). Simple two-allele (A/B) polymorphisms in this poly(ADP-ribose) polymerase-like locus were identified by digestion with a number of restriction enzymes. These restriction fragment length polymorphisms always segregated together, suggesting that they identify a deletion within or close to the poly(ADP-ribose) polymerase sequences on chromosome 13, which was precisely mapped to 13q33-qter. Based on family studies, the A and B alleles were shown to be transferred in a Mendelian codominant fashion. This probe was subsequently used as a linkage marker to study the frequency of this deletion in various tumors, such as B-cell follicular lymphomas, and small cell lung, breast and colorectal carcinomas. In noncancer control populations, the frequency of this deletion was found to be three-fold higher in blacks than in Caucasians. When DNA from various tumors was compared to normal DNA from racially appropriate noncancer controls, the frequency of this deletion was still found to be two- to threefold higher in the tumor DNA. Matched samples provided instances of tumor-specific loss of heterozygosity, but also revealed that the predominant source of this deletion is the germ line. This suggests that the chromosome 13 region neighboring the poly(ADP-ribose) polymerase locus may harbor a gene whose loss may predispose individuals to malignancy (185).

The processes of DNA excision repair in mammalian cells serve to alleviate some of the lethal, mutagenic and carcinogenic consequences of DNA damage. Although the biological consequences of repair are appreciated, the mechanisms are understood only in general terms. Recent discoveries of variable repair activities in different genomic regions have served to emphasize our lack of knowledge of how damage is recognized and repaired in the nucleoprotein chromatin complex, which contains regions of diverse molecular structures and activities. The biological consequences associated with preferential repair of selected genomic regions, such as active genes, underscore the necessity of defining these processes. Further evidence for preferential repair of selected chromatin regions in human cells was obtained by an OIG awardee in studies using xeroderma pigmentosum complementation group C (XP-C) cells. Cultured fibroblasts from XP-C patients exhibit only about 10% of normal DNA repair activity. The cells exhibit sensitivity to UV when colony-forming ability is measured, but show relative resistance compared to other XP strains when maintained as nondividing cells. The limited DNA excision repair in UV-irradiated nondividing XP-C fibroblasts occurred in localized chromatin regions characterized by the generation of large DNA segments (at least 30-70 kb) free of pyrimidine dimers. A genomic fraction enriched for this DNA was isolated on the basis of the larger size of the repaired fragments after UV-endonuclease treatment and was screened for specific genes. More copies per microgram DNA of two transcriptionally active genes, *beta*-actin and dihydrofolate reductase, compared to the remaining DNA were discovered, but an equal number of copies per microgram DNA of an inactive locus, termed 754, was found. By measuring the removal of pyrimidine dimers from specific genomic restriction fragments comprising these sequences, it was confirmed that the active genes were preferentially repaired. Results obtained indicate that a unique set of relatively large chromatin domains are repaired in nondividing XP-C cells, even though most of the DNA remains unrepaired. The repaired domains are thought to contain the active genes. This specific repair could account for the relatively high UV-resistance of nondividing cells. In normal cells, a very rapid repair of a restriction fragment containing the *beta*-actin gene and slow repair of the 754-containing fragment were detected, which indicated that a similar domain-oriented repair process also exists in these cells. The observations made are consistent with the previously discovered rapid repair of active genes compared to bulk DNA. Thus, it was hypothesized that separate damage-recognition systems may exist in

human cells for chromatin domains that contain transcribed regions versus those that contain no transcribed regions. The latter system may be the one deficient in cells from XP-C patients (83).

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease characterized by extreme sensitivity to sunlight and a high predisposition to skin cancers. At least eight distinct genetic complementation groups have been discovered, but currently little is known about the molecular basis for this genetic heterogeneity or the defect in DNA repair that characterizes XP. Attempts to isolate wild-type XP genes by complementation of cells following transfection with total human DNA have not been successful. Phenotypic complementation of XP-A cells following transfection with mouse genomic DNA has been achieved with the resultant isolation of the complementing mouse *XP-A* gene. Phenotypic complementation of XP cell lines was also achieved by introduction of genetically tagged single human chromosomes via micro-cell-mediated chromosome transfer from monochromosomal human-mouse hybrids. Preliminary studies showed that this complementing chromosome contains a translocation involving chromosome 9, and suggested that the *XP-A* gene may be located on the q arm of this chromosome. A combination of chromosome transfer experiments and cytogenetic analysis of the hybrids generated demonstrated that the gene which complements XP-A cells maps to human chromosome 9 at the 9q22.2-q34.3 region. Southern blot analysis confirmed the presence of distal chromosome 9q sequences. This localization was considered to be especially interesting because the ABO blood group markers have also been located near the distal end of 9q, and an early report suggested linkage between XP and the ABO blood groups. This linkage might be revealed by more selective analysis (68).

Genetic studies in both higher and lower eukaryotes have indicated that nucleotide excision repair of DNA is a complex process involving numerous gene products. The yeast *Saccharomyces cerevisiae* has been a particularly useful eukaryotic model because it is amenable to genetic, molecular and biochemical investigations. Several studies have demonstrated an absolute requirement for at least five genes (designated *RAD1*, *RAD2*, *RAD3*, *RAD4* and *RAD10*) for damage-specific incision of DNA following exposure of cells to UV radiation. All five RAD genes have been isolated by molecular cloning and extensively characterized. An examination of the predicted amino acid sequence of the cloned *RAD10* gene, as well as searches of several data bases for homology with other proteins, have not suggested a biochemical function for Rad10 protein. Understanding the role of this protein in nucleotide excision repair is therefore predicated on its purification and biochemical characterization. In further studies with the *RAD10* gene, one major and three minor transcriptional start sites were mapped. The locations of these sites relative to the translational start codon were similar to those previously identified in the yeast *RAD2* gene. The cloned *RAD10* gene was then tailored into expression vectors for overexpression in *E. coli* and yeast. In contrast to *RAD2*, *RAD10* was not inducible following exposure of cells to the DNA damaging agent 4-nitroquinoline 1-oxide. Native Rad10 protein and two different Rad10 fusion proteins were rapidly degraded in most *E. coli* strains. Following overexpression of the cloned *RAD10* gene in yeast, however, native Rad10 protein was purified to greater than 95% homogeneity. A catalytic function has not yet been identified for the purified protein. *RAD10* cells contained fewer than 500 molecules per cell, which is similar to the levels of the UvrA, UvrB, and UvrC nucleotide excision repair proteins in *E. coli*. Elucidation of the precise role of this protein in nucleotide excision repair in yeast must await purification and characterization of the five Rad proteins of interest (67).

Genetics and Mechanisms of Cell Transformation: Studies in this subject area test the somatic cell mutation hypothesis of cell transformation and seek 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. Moreover, 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 by one grantee. This may yield a model analogous to human retinoblastoma and Wilms' tumor. Another grantee is developing a mouse model for susceptibility to hepatocarcinogenesis. It has been shown that C3H/HeJ male mice are 20- to 50-fold more susceptible to 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), which affects the growth control of both normal and preneoplastic liver cells. In order to further address the question of which specific genes are involved in the process of tumor formation, liver tumors from various rodent strains with characteristic differences in their susceptibility to hepatocarcinogenesis were analyzed for the presence of mutations at codon 61 of the c-Ha-ras gene. By using the polymerase chain reaction and allele-specific oligonucleotide hybridization, dC to dA transversions at the first base and dA to dT transversions or dA to dG transitions at the second base of c-Ha-ras codon 61 were detected in 20 to 60% of spontaneous or carcinogen-induced liver tumors of the C3H/He, CBA, CF1 and B6C3F1 mouse strains, all of which are highly susceptible to hepatocarcinogenesis. However, no such mutations were found in any of 31 liver tumors in the insensitive C57BL/6J and BALB/c mouse strains or in any of 35 liver tumors in the comparatively resistant Wistar rat. Further, no positive results were obtained from additional analyses of c-Ha-ras codon 12 mutations in liver tumors from the three insensitive rodent strains. In early precancerous liver lesions, c-Ha-ras codon 61 mutations were found in 13-14% of lesions in the sensitive C3H/He and B6C3F1 mouse strains, but not in any of 34 lesions in the insensitive C57BL/6J mouse. These results indicated that there is a close correlation between the mutational activation of the c-Ha-ras gene in liver tumors of the different rodent strains and their susceptibility to hepatocarcinogenesis. The mutations appeared to provide a selective growth advantage, leading to clonal expansion of the mutated liver cell population in livers of sensitive but not insensitive strains. However, the observation that even a certain percentage of liver tumors from the sensitive strains do not possess mutations in the Ha-ras gene suggested that additional, currently unknown pathways of tumorigenesis must exist. Activation of other dominant transforming genes or the loss of suppressor genes and inactivation of other genes responsible for maintenance of normal growth and differentiation, are other hypothesized possibilities (155).

Another grantee is conducting studies in pursuit of a heritable mutation leading to the development of gastrointestinal tumors. Production of mice carrying single germline mutations that result in a susceptibility to spontaneous intestinal tumor formation was accomplished by using germline mutagenesis with ethylnitrosourea. The primary phenotype of mice carrying the generated mutation appeared to be the development of multiple adenomas throughout the entire intestinal tract at an early age. These then progressed to adenocarcinomas of the intestine in older mice. A secondary characteristic was development of anemia. The mutant gene was named the multiple intestinal neoplasia (Min) gene. This gene was shown to be transmitted by affected mice to 50% of progeny with an unbiased sex distribution, which is

characteristic of a fully penetrant autosomal dominant trait. Using this model, mapping of the Min locus and identification of linked probes are some of the future experiments that are needed.

Several studies are ongoing on the role of specific genes and gene products in chemically induced cell transformation. 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 transformation of cells to malignancy. Identification of different transforming genes from various human tumor cells which were found to be homologous to known viral oncogenes has spurred studies to establish which of the known or potentially novel transforming genes have been activated or play a role in the development of tumorigenesis or cell transformation in various chemically induced animal or cell model systems. Of equal importance are studies to identify which specific tumor suppressor genes, when inactivated, allow cells to continue on the pathway to tumorigenesis. In addition, studies are being conducted to determine whether alteration of specific gene expression plays a role in the development of the transformed state. The genes being examined include several oncogenes, murine leukemia virus proviral sequences, long terminal repeat sequences, and metalloproteinase genes. Model systems currently in use include rat and mouse liver, mouse thymic lymphoma, rat nasal carcinoma, mouse skin carcinoma, rat and mouse bladder carcinoma, rat and mouse mammary carcinoma and in vitro rodent and human fibroblast and epithelial cell transformation systems. The interaction of chemicals and viruses such as murine mammary tumor virus and adenovirus type 5 are being examined by different laboratories to determine their role in carcinogenesis.

A family history of breast cancer may indicate a woman's increased risk for the development of breast cancer. It has been estimated that about 5% of breast cancers have a major genetic component in their etiology. The underlying genetics of this predisposition are complex and not completely understood. In order to investigate genetic mechanisms of breast cancer susceptibility, an inbred rat model was developed by one grantee. Most rat strains have been reported to fall into three groups: high susceptibility (e.g., Wistar-Furth [WF]); intermediate susceptibility (e.g., Fischer 344 [F344]); and resistant (e.g., Copenhagen [Cop]). The WF rat has been shown to contain several independently segregating dominant autosomal susceptibility genes. A single autosomal mammary carcinoma suppressor (MCS) gene that specifically prevents carcinogen-induced and spontaneous mammary carcinomas was discovered in Cop rats, while the F344 was shown to have neither type of genes. The first approach to understand the mode of action of these genes and to develop strategies to clone them was to determine where in the carcinogenic process they were functioning. Both the susceptibility and MCS gene did not alter the very early stages of chemical carcinogenesis. For studies to further describe the biological activity of the Cop suppressor gene, mammary cells from the WF rat, which do not carry the MCS gene, were grafted into ectopic sites in WF X Cop and WF X F344 hybrid rats. These chimeric rats were then treated with 7,12-dimethylbenz(a)anthracene (DMBA) and the development of mammary carcinomas was followed. Results obtained suggested that the main site of MCS activity was within the mammary parenchyma. Ectopic mixed mammary glands composed of a mixture of Cop and WF cells were also examined for susceptibility to DMBA-induced carcinogenesis. The presence of Cop cells did not inhibit tumor development from WF cells in these mixed glands. Graft sites in which palpable tumors did not develop were also examined for focal epithelial lesions. The MCS gene completely inhibited formation of microcarcinomas, but did not affect development of alveolar hyperplasias. If this latter lesion is a precursor for development of microcarcinoma, the data suggest that the MCS gene

acts during the progression stage at a time before development of microcarcinoma and after formation of alveolar hyperplasia. In a subsequent set of studies, the putative suppressor gene *Krev-1* (*rap1A*) which had been recently isolated and characterized, or its related gene *rap1B*, was examined to determine whether they might be involved in this strain-specific resistance. Equal expression of these two genes was found in mammary tissue from both resistant and susceptible rat strains. From these observations and from nucleotide sequence comparisons, it was concluded that it is unlikely that the *Krev-1* or *rap1B* putative suppressor genes are involved in the mammary tumor-resistant phenotype of the Cop rat (75).

Identification of *ras* oncogenes in human and animal cancers including precancerous lesions indicated that these genes participate in the early stages of neoplastic development. However, it is not yet clear if mutational activation of *ras* plays an early role in the carcinogenesis process and acts as an initiating lesion or acts later as a postinitiating event or both. In order to ascertain the timing of *ras* oncogene activation, one grantee devised an animal model system that involved the induction of mammary carcinomas in rats exposed at birth to the carcinogen methylnitrosourea. High resolution restriction fragment length polymorphism analysis of polymerase chain reaction-amplified *ras* sequences revealed the presence of both *H-ras* and *Ki-ras* oncogenes in normal mammary glands two weeks after carcinogen treatment and at least two months before the onset of neoplasia. These *ras* oncogenes remained latent within the mammary gland until exposure to estrogens, demonstrating that activation of *ras* oncogenes can precede the onset of neoplasia. This suggested that normal physiological proliferative processes such as estrogen-induced mammary gland development may lead to neoplasia if the targeted cells harbor latent *ras* oncogenes (193).

Carcinogen-induced transformation of Syrian hamster embryo cells has been widely used as a model for experimental carcinogenesis. However, analysis of the molecular mechanisms of hamster cell transformation has been limited to date. In an effort to expand the understanding of the molecular basis of this system, 22 independently derived Syrian hamster neoplastic cell lines initiated with chemical carcinogens were screened by a FIRST awardee for the presence of dominant transforming sequences using the NIH3T3 DNA transfection system. High molecular weight DNAs from 55% of these cell lines were shown to transform NIH3T3 cells through serial transfection cycles. Results from Southern blot hybridization analyses and $p21^{ras}$ mobility assays indicated the presence of *N-ras* oncogenes in 25% of transfection positive lines that were initiated with sodium bisulfite. Non-*ras* transforming sequences were apparently activated in the remaining 75% of transformed lines. In studies using a battery of 38 probes that included non-*ras* oncogenes known to score as positive in the NIH3T3 assay, as well as other retroviral and mammalian oncogenes, no homolog sequences of hamster origin were detected, even under hybridization conditions which allowed their detection in hamster DNA. These results demonstrated that *ras* activation occurred at a low frequency in hamster neoplastic transformation and strongly suggest that novel transforming sequences are activated. Molecular cloning techniques are currently being used to isolate the transforming sequences (144).

Transformation of human cells involves altered control of cellular genes that precisely regulate normal cellular growth and differentiation. Defective regulation of one or more of these genes might cause cells to be abnormally differentiated and to have an elevated rate of cell proliferation. Consistent with this view, it has been shown that when cells are caused to express sufficiently increased levels of certain normal cellular oncogene products, such as *c-myc*, the cells may be transformed. Investigations of normal human cells treated with chemical carcinogens in culture

shows that there are several stages through which normal cells progress as they proceed to full transformation in vitro. Normal human cells in culture, in contrast to rodent cell lines, have generally been resistant to full transformation when transfected with a single oncogene. This is presumed to be due, in part, to the fact that normal human cells in culture have a finite life span and cease proliferating before they can express a transformed phenotype. Development of extended or unlimited life span as reflected by continuous cell growth in culture appears to be an important, and perhaps the rate-limiting, step in the conversion of normal cells to tumorigenic cells. Therefore, studies by one awardee were designed to enhance the growth of human endometrial stromal cells in culture by transfection of a *c-myc* oncogene that contained a constitutively active promoter. When cells were cotransfected in primary culture with pSVc-*myc*, a plasmid containing a truncated *c-myc* gene regulated by simian virus 40 promoter, and pRSVneo, a plasmid containing a neomycin resistance gene regulated by Rous sarcoma virus promoter, the cells demonstrated properties of transformed cells in vitro. These properties included altered morphology, focus formation, anchorage-independent growth, chromosomal alterations and tumor formation in athymic mice. When these cells were subsequently treated with a direct-acting carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine, they demonstrated higher colony-forming efficiency in soft agar and reduced tumor latency. Cells transfected with pRSVneo alone exhibited some properties associated with neoplastic transformation, including altered morphology and formation of colonies in soft agar. It was presumed that in normal cells transfected with pRSVneo, RSV long terminal repeats activated cellular genes that normally regulate growth of human endometrial stromal cells. Results seen in these studies were thought to resemble examples of insertional mutagenesis seen in other systems. One significant consequence of this finding is its implication for studies that involve gene transfer and drug selection of transfected cells. Drug resistance selection is a common method in gene transfer studies, but these results suggest that drug dose selection may not be an innocuous procedure (97).

Expression of *ras* oncogenes results in pleiotropic changes in morphological, biochemical and growth-related properties. Loss of cell surface fibronectin, decreased adhesion to substratum, increased hexose uptake, loss of density dependent growth, increased anchorage-independent growth, decreased binding to growth factors and increased secretion of "autocrine" growth factors are some of the many changes often associated with cellular transformation. All of these changes indicate that normal cellular gene expression patterns are altered in *ras* transformed cells. In spite of numerous studies on the role of *ras* oncogenes in transformation, to date little attention has been paid to the chronology of cellular events involved in *ras*-induced transformation. In order to evaluate the effect of varied *ras* oncogene expression on the expression of genes and proteins related to morphologic transformation in vitro, one MERIT awardee has used a series of Rat-1 cell lines carrying a Zn-inducible human c-Ha-*ras* oncogene construction (MTrasT24). In response to expression of the *ras* oncogene, at least two different classes of events were shown to occur. These events, referred to as "early" and "late" events, were dependent on distinctively different accumulated levels of the *ras* oncoprotein. Relatively low levels of activated c-Ha-*ras* p21 protein stimulated rapid entry of quiescent (G_0) cells into the cell cycle and resulted in increased steady state *c-myc* and glucose transporter mRNA levels. These were detectable as early as three to six hours after zinc addition. In contrast, morphologic transformation developed more slowly and did not appear until 72-96 hours after Zn^{2+} stimulation in cells with very low basal levels of activated p21 and 24-48 hours in cells with higher basal levels. These observed morphologic changes depended on the accumulation of significant amounts of the *ras* oncoprotein (greater than four to five times the proto-oncogene level) and

were accompanied by large increases in the steady state mRNA levels of transin and TGF- α and decreases in platelet-derived growth factor receptor mRNA, fibronectin protein and mRNA levels. In addition, the level of a novel cytoplasmic protein species (referred to as p29), which is stained by a monoclonal antibody for ras, was shown to be greatly reduced in response to these levels of activated ras protein. Thus, the observed changes in morphology and gene expression induced by rasT24 occurred sequentially and were quantitatively dependent on activated ras expression. These findings provide explicit detail in vitro about how a single oncogene can bring about the multiple changes which are believed to occur during neoplastic progression in vivo (117).

In an effort to better comprehend the processes whereby normal human mammary epithelial cells (HMECs) can be transformed, one MERIT awardee began a search for specific genes whose expression could be relevant to this process. Using subtractive hybridization techniques, this study pursued the selection and identification of gene transcripts that were down-regulated during HMEC transformation. The objective was to identify genes whose activity might be incompatible with or suppress tumorigenicity. A normal diploid breast cell strain, 184, that was derived from a reduction mammoplasty, was compared with a tumorigenic cell line that was derived from the 184 cell after immortal transformation following exposure to benzo(a)pyrene and infection with Kirsten sarcoma virus. One clone identified by this procedure corresponded to a 1.4-kilobase mRNA, designated NB-1, whose expression was shown to decrease about 50-fold in the tumorigenically transformed HMEC cell line. Sequence analysis of the NB-1 cDNA revealed an open reading frame with a high degree of homology to calmodulin. NB-1 expression could be demonstrated by polymerase chain reaction amplification in normal breast, prostate, cervix and epidermal tissues. The presence of NB-1 transcripts varied in primary breast carcinoma tissues and was undetectable in tumor-derived cell lines of breast, prostate or other origins. NB-1 mRNA expression could be down-regulated in cultured HMECs by exposure to reconstituted extracellular matrix material, while exposure to transforming growth factor type β increased its relative abundance. The protein encoded by NB-1 is thought to have Ca²⁺ binding properties and to perform functions similar to those of authentic calmodulin. The strong homology observed between NB-1 and calmodulin, combined with the regulated pattern of expression exhibited by the NB-1 gene, suggest that the NB-1 gene product is a signal transducer that may play a differentiation-specific role in breast tissue. Its possible roles in differentiation and/or suppression of tumorigenicity in epithelial tissues remain to be examined (188).

Prostatic adenocarcinoma is the second most common malignancy of male patients and the second leading cause of cancer death in men in the United States. A high mortality rate in prostate adenocarcinoma patients is apparently due to lack of adequate screening programs for detection of early primary disease, and as a result, about 60% of patients present with evidence of metastases. The fact that in some patients prostate adenocarcinoma is disseminated even before presentation of clinical symptoms, while other patients live many years with localized subclinical disease, shows that transformed prostatic cells have a wide spectrum of biological activities. This variability was hypothesized to be due, in part, to variable expression of proteases believed to be important in invasion and metastasis. Secretion of proteolytic enzymes and their role in invasion and metastasis has been shown for a variety of other human tumors. The presence of these enzymes had not yet been studied in human prostate adenocarcinoma. There is now evidence that a multigene family of metalloproteinases, which include collagenases with specificity for interstitial collagen types I and II or basement membrane type-IV collagen, are involved in tumor invasion. Moreover, it has recently been shown that matrix

metalloproteinase-7 (*MMP-7*), when activated, degrades casein, gelatins of type I, III, IV and V, and fibronectin, and can activate collagenase. For the above reasons, the expression of human *MMP-7*, as well as three other human collagenases, were studied in a series of normal, malignant and metastatic human prostate tissues using northern blot analysis and in situ hybridization techniques. When the northern blots were hybridized with a *MMP-7* cDNA probe, a 1.2-kb mRNA was detected in 14 of 18 prostate adenocarcinomas, one of two metastatic lymph nodes and 3 of 11 normal prostates. The three human prostate cell lines tested did not show any evidence of the *MMP-7* transcript. In situ hybridization studies revealed that the *MMP-7* gene was expressed in epithelial cells of primary prostate adenocarcinoma as well as in invasive and metastatic cells. *MMP-7* expression was also detected focally in some dysplastic glands, but not in stroma. Additional northern blot analysis using probes to human type-IV collagenase, type-I collagenase and stromelysin-I showed that 6 of 10 adenocarcinoma samples and none of four normal samples were positive for type-IV collagenase transcripts. None of the adenocarcinomas and normal tissues was found to express the type-I collagenase and stromelysin-I transcripts at detectable levels. The data obtained from this study suggests that certain metalloproteinases are present in prostatic adenocarcinoma and may play a role in invasion and metastasis. Although a functional role for the *MMP-7* protease in prostatic carcinoma invasion and metastasis has not been established, data showing the differential expression of this metalloproteinase gene during tumor development suggests a role for *MMP-7* expression in metastatic prostatic carcinoma. The planned development of specific antibodies against *MMP-7* will further elaborate differential expression and the role of this metalloproteinase in the development of prostate carcinoma as well as its normal functional role in prostate tissue (15).

Another possible mechanism of cell transformation by chemicals could involve induction of DNA sequence rearrangements, specific DNA recombinational events, chromosome alterations, or involvement of mitochondrial genes, metabolism, or specific proteases. These changes could result in the altered cell growth and other properties characteristic of transformed cells. 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 a series of previous studies, progressive aneuploidy was found to be characteristic of chemically induced mouse skin papillomas from very early stages. The sequential trisomizations of chromosomes 6 and 7 were identified as the primary nonrandom cytogenetic events in this model. Trisomy of chromosome 7 was found in severely dysplastic papillomas and in most squamous cell carcinomas induced by dimethylbenz(a)anthracene (DMBA) and promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA). In this same model, the initiation event was postulated to be a point mutation in one of the alleles of the *Ha-ras-1* gene. This finding, plus the discovery that the *Ha-ras-1* gene resides on mouse chromosome 7, led to the speculation of a possible link between dosage of the mutated gene and generation of trisomy 7 by nondisjunction. Southern blot analysis was used in the reported study to estimate the relative number of mutant versus normal *Ha-ras-1* alleles in mouse skin squamous cell carcinomas induced by DMBA initiation and TPA promotion. DNA for these studies was obtained from short-term tumor cultures in order to eliminate the contribution of stromal and inflammatory cells to the sample. The allelotypic analysis was performed in 25 squamous cell carcinomas by quantitative radio-analysis of the *Xba* I restriction fragment length polymorphism as detected by a *v-Ha-ras* probe, and rehybridization of the Southern blots with probes for chromosomes 7 and 8. Results obtained indicated that trisomy 7 occurred by nonrandom duplication of the

chromosome 7 carrying the mutated Ha-ras-1 allele. This provided further evidence for the putative mechanistic role played by this oncogene not only at the initiation stage, but also as a determinant factor for skin tumor progression (39).

Protease inhibitors have been shown to be effective anticarcinogenic agents in several *in vivo* and *in vitro* model systems. Although the mechanisms by which protease inhibitors suppress carcinogenesis are poorly understood, it is believed that these compounds exert their anticarcinogenic effects by inhibiting cellular enzymes involved in induction and/or expression of the transformed phenotype. This is because they do not influence either the *in vitro* growth of normal or transformed cells or the expression of transformed cells. Protease inhibitors have also been shown to suppress transformation induced by several different carcinogens at nontoxic concentrations, the most effective inhibitors being active in the pmol or nmol concentration ranges. Several lines of evidence have indicated a specificity of action for the active protease inhibitors. For these reasons, one MERIT awardee has employed anticarcinogenic protease inhibitors to detect enzymatic activities which may be involved in malignant transformation. Untransformed and malignantly-transformed mouse embryo fibroblasts were found to contain an enzymatic activity which hydrolyzed the synthetic substrate, Suc-Ala-Ala-Pro-Phe-AMC. This activity was partially purified by ion-exchange and gel-filtration chromatography. It is about 55,000 molecular weight in size, maximally active at neutral pH, associated with subcellular organelles or membranes, and inhibited by EDTA, EGTA, phosphoramidon and 1,10-phenanthroline, but not by phenyl methyl sulfonyl fluoride or pepstatin. This indicated that it was a metalloprotease. Several other protease inhibitors, such as chymostatin, tosylamide 2-phenylethyl-chloromethylketone and Bowman-Birk inhibitor, were also potent inhibitors of the activity. These same inhibitors, induced by x-irradiation or chemical carcinogens, are known to suppress the malignant transformation of mouse embryo fibroblast cells *in vitro*. In contrast, soybean trypsin inhibitor and pepstatin, which did not inhibit this activity, also do not suppress malignant transformation. The apparent localization of protease activity to the membrane/lysosomal subcellular fraction was considered to be interesting since neither the site of action nor the mechanism of anticarcinogenic protease inhibitor uptake by cells is well understood. How this activity interacts with another described neutral protease activity, a cytoplasmic serine protease of about 70,000 molecular weight which hydrolyzes the synthetic substrate Boc-Val-Pro-Arg-AMC, is not yet known. The Suc-Ala-Ala-Pro-Phe-AMC hydrolyzing activity is currently being purified more fully, so that its properties, especially with regard to its endogenous substrates and biological role, can be explored in depth (100).

Additional studies in genetics and mechanisms of cell transformation are designed to test cell cycle specificity of the induction of cytotoxicity, mutagenesis and neoplastic transformation by chemical carcinogens. The quantitative relationship between the level, persistence and species of carcinogen-nucleotide adducts and the concomitant cell transformation frequency are also being determined. There is a substantial amount of information supporting the hypothesis of cell cycle specificity of carcinogenesis. It has been shown that in mouse embryo C3H 10T1/2 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, 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 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.

Role of Oxygen Radicals in Carcinogenesis: The increasing number of projects in this research area reflects the increased attention and interest by investigators in studying the potentially important role of oxygen radicals and other oxidation reaction products in tumor initiation, cell transformation and tumor promotion. Included in this subject area are 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. One grantee is testing the hypothesis that the phorbol ester, TPA, and carcinogenic metals, such as Cd, may exert their effects by generating oxygen radicals. In the area of cancer initiation, ongoing studies are examining the role of oxygen radicals in induction of renal tumorigenesis by estrogens and 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 formation of active oxygen species and, subsequently, oxidized bases in DNA. The roles of the arachidonic acid pathway, participation of inflammatory cells, and formation of polyunsaturated fatty acid peroxides in tumor promotion in mouse skin and cultured cells are also being investigated.

Ionizing radiation and chemical oxidants can be toxic, mutagenic and carcinogenic. The major cause of these effects is thought to be the result of macromolecular damage, mediated in aerobic systems mainly by hydroxyl radicals, which can induce DNA base modifications, protein-DNA cross-links, DNA strand breaks, and apurinic/aprimidinic sites. One of the potential lesions formed is 8-hydroxy-2-deoxyguanosine (8-OHdG), which has been demonstrated to be potentially mutagenic in vitro. This adduct has been detected in DNA in vivo and in vitro following exposure to a range of hydroxyl radical producing sources. In order to develop a system whereby the genetic effects of 8-OHdG could be investigated in vivo, one OIG awardee used T4 RNA ligase to construct a deoxypentanucleotide containing a single 8-OHdG residue (dG-dC-dT-dA-8-OHdG). The methods chosen gave an efficient attachment of 8-OHdG with a 50% final product yield. Both the modified and unmodified pentamer control were covalently incorporated by DNA ligase into a five-base gap at a unique *NheI* restriction site in the otherwise duplex genome of an M13mp19 derivative. The adduct was part of a nonsense codon in a unique restriction site, which facilitated identification and selection of mutants generated by replication of the modified genome in *E. coli*. Both control and adducted pentamers were ligated into the genome at 50% of the maximum theoretical efficiency. Greater than 90% of the site-specific adducted products possessed pentanucleotides that were covalently linked at both 5' and 3' termini. The 8-OHdG lesion in the *NheI* site inhibited cleavage of the site by a 200-fold excess of *NheI*. Transformation of *E. coli* strain DL7 with the uniquely modified single-stranded genome resulted in about 0.5-1.0% of the progeny phage showing the dG to dT transversion mutation at the original position of 8-OHdG. No such mutations were observed from control genomes containing guanine in place of 8-OHdG. The targeted dG to dT mutation observed was only one of many 8-OHdG mutations seen by others in vitro. One possible reason for the differences between in vivo and in vitro data is that 8-OHdG is a likely substrate for repair enzymes that remove it from the genome before it can be encountered by the replication machinery of the cell. An alternative pathway leading to 8-OHdG-induced dG to dT mutations may reflect structural and conformational changes imposed by the adducted purine within the DNA helix. In order to answer these questions, the genetic requirements for 8-OHdG mutagenesis are currently being investigated using *E. coli* strains with

different DNA replication and repair backgrounds. Data obtained from these experiments should provide further details on the mechanisms underlying the type of mutations induced by the adduct in vivo as well as the relative contribution of 8-OHdG to the overall spectrum of radiation- and oxidant-induced mutation (54).

The primary biological consequences of oxygen free radicals may result from their reaction with nucleic acids. Superoxide or hydroxyl free radicals are known to react with and cause DNA damage. The reaction of hydroxyl free radicals with DNA bases has been shown to form several products including 8-OHdG. Quantitative methods which can detect extremely low levels of 8-OHdG were developed by using HPLC with electrochemical detection. Use of this methodology has made it possible to quantitate oxidative damage to DNA in human cells. Advancements in the practical aspects of this analytical approach, with respect to digestion analysis and stability of oxidatively lesioned DNA, have recently been made by the developer of this method. In order to prevent loss of 8-OHdG from oxidatively damaged DNA, storage at -20°C at neutral or acidic pH was required. An independent assessment of hydroxyl free radical flux using salicylate trapping allowed the assessment of competitive radical reactions. Ethanol washing of plastic microfuge tubes prior to DNA enzymatic digestion improved the yield of 8-OHdG and reduced the variability between samples. The highest yield of 8-OHdG was produced using a method involving deoxyribonuclease I, endonuclease, phosphodiesterase and alkaline phosphatase. All of these findings are of importance from a practical standpoint in accurately determining the 8-OHdG content in DNA isolated from biological systems (62).

Reactive oxygen species such as the superoxide radical, hydrogen peroxide and the hydroxyl radical are formed in vivo during aerobic metabolism and during radiation exposure. It has been suggested that oxidative damage contributes 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 positively identified. The postulated importance of endogenously produced oxidative damage to DNA in aging and cancer has prompted efforts to develop methods that measure this damage. Endogenously produced oxidative damage to DNA has been assayed by measurement of the urinary levels of thymine glycol and thymidine glycol using HPLC with UV detection and 8-OHdG using HPLC with electrochemical detection. Immunoassays have been developed for quantitative analysis of specific types of DNA damage products involving bulky alkyl and polycyclic aromatic hydrocarbon derivatives. A similar approach employing polyclonal and monoclonal antibodies raised against oxidized bases of DNA has been used to develop highly sensitive assays that detect the radiation-induced oxidative adducts 8-hydroxyadenine and thymidine glycol. This success prompted an OIG awardee to raise polyclonal antibodies against 8-OHdG. The antibodies were raised in rabbits following immunization with protein carrier-hapten conjugates prepared by covalently linking periodate-treated 8-OHdG to bovine serum albumin or casein. The antibodies were shown to bind 8-OHdG with high affinity and selectivity, thus rendering them suitable for the preparation of immunoaffinity columns that were used to rapidly isolate 8-OHdG and 8-hydroxyguanine (8-OHGua) from urine. The high selectivity of the antibodies for 8-OHdG and 8-OHGua reduced the amount of urinary contaminants previously observed in samples prepared by solid phase extraction. Thus, these antibodies offer an improved method for isolating urinary 8-OHdG prior to analysis and an alternative to the HPLC-electrochemical detection method currently used for quantitation of 8-OHdG in DNA. These antibodies should serve as a useful tool to monitor oxidative DNA damage in individuals (4).

Oxygen free radicals are highly reactive species that have been demonstrated to damage DNA and cause mutations. Progress in studying DNA damage and mutagenesis by oxygen free radicals has been hampered by both the diversity in the types of oxygen free radicals produced in cells and the multiplicity of DNA modifications produced by oxygen free radicals. It has been estimated that at least 35 different base modifications are produced in DNA by oxygen free radicals. As a result, it has been difficult to assign a particular type of oxygen free radical, or a modification in DNA, to a specific type of mutation. In one approach to this problem, an OIG awardee has determined the mutagenic spectrum of oxygen free radicals produced by aerobic incubation of single-stranded M13mp2 DNA with Fe^{2+} . Fe^{2+} -treated DNA was transfected into competent *E. coli*, and mutants within the nonessential *lac Z* α gene for *beta*-galactosidase were identified by decreased *alpha*-complementation. The assay used scores for all 12 types of single-base substitutions, deletions, additions, frameshifts and complex rearrangements at multiple sites within a nonessential gene. The frequency of mutants obtained was 20- to 80-fold greater than that obtained with untreated DNA and greater after the host cells were induced for the SOS "error-prone" response. Sequence data on 94 of the isolated mutants established that mutagenesis resulted primarily from an increase in single-base substitutions. The most frequent mutations observed were dG to dC transversions, followed by dC to dT transitions and dG to dT transversions. The observed clustering of mutations at distinct gene positions suggested that Fe^{2+} /oxygen damage to DNA is nonrandom. This mutational spectrum provided evidence that a multiplicity of DNA lesions produced by oxygen free radicals in vitro are promutagenic and could be a source of spontaneous mutations. The finding that incubation of Fe^{2+} with DNA produces a variety of mutations brings into focus the possibility that intracellular iron is mutagenic and a contributor to pathological processes. Iron has also been found in strong association with cellular DNA and could serve to generate hydroxyl radicals, particularly in the presence of hydrogen peroxide (123).

The ability of vitamin C (ascorbic acid) to act as an antioxidant and a prooxidant is well documented. Its antioxidant activity has been shown to derive from its ability to reduce peroxy radicals that propagate lipid peroxidation or the oxidized form of the naturally occurring antioxidant vitamin E_3 . Its prooxidant activity is a result of its ability to reduce metals (especially Fe^{3+} complexes) to forms that react with oxygen to form initiators of lipid peroxidation. Ascorbate appears to be the principal water-soluble antioxidant in human plasma and is present in some cells at high levels. Thus, it may modulate the oxidant response of cells challenged with surface-active or toxic agents. One such challenge is the production of hydroperoxides through enzymatic oxygenation of unsaturated fatty acids or lipid peroxidation. It was thought that ascorbate would inhibit lipid peroxidation by acting as a radical scavenger or by maintaining high levels of vitamin E. In order to explore this hypothesis, an OIG grantee conducted a series of experiments to determine the effect of ascorbic acid on hydroperoxide-dependent lipid peroxidation in rat liver microsomes. Simultaneous addition of ascorbic acid and organic hydroperoxides resulted in enhanced lipid peroxidation compared to incubation of microsomes with organic hydroperoxides alone. No lipid peroxidation was evident in incubations of microsomes with ascorbate alone. The stimulatory effect of ascorbate on linoleic acid hydroperoxide-dependent peroxidation was evident at all times, whereas stimulation of cumene hydroperoxide-dependent peroxidation occurred after a lag phase of up to 20 min. Addition of EDTA did not inhibit cumene hydroperoxide-dependent lipid peroxidation, but completely abolished ascorbate enhancement of lipid peroxidation. Moreover, linoleic acid hydroperoxide-dependent peroxidation was not significantly inhibited by EDTA, but dramatically reduced ascorbate enhanced lipid peroxidation. Results obtained revealed a synergistic prooxidant effect of ascorbic acid on

hydroperoxide-dependent lipid peroxidation. The inhibitory effect of EDTA on enhanced peroxidation suggested a possible role for endogenous metals mobilized by hydroperoxide-dependent oxidations of microsomal components. It was noted, however, that studies on the effect of dietary ascorbic acid support an antioxidant rather than a prooxidant effect. Based on the results of animal and human studies, there appears to be no compelling support at present for a prooxidant effect of ascorbate *in vivo*. If synergistic stimulation of hydroperoxide-dependent lipid peroxidation by ascorbate does occur *in vivo*, it is thought to take place in localized regions and to an extent that does not have a significant impact on whole body antioxidant status (131).

Some derivatives of nickel, cadmium and cobalt are carcinogenic in humans and/or animals, but their mechanisms of action are not known. Both nickel and cadmium have been shown to be chemotactic for phagocytic cells and to cause inflammatory responses. Accumulating evidence shows that inflammation involves the formation of active oxygen species and that phagocytic cells play a major role. Active oxygen species generated by polymorphonuclear leukocytes have been shown to be mutagenic and carcinogenic. There is evidence that nickel compounds can act as tumor promoters through their ability to disrupt cell-to-cell communication in a manner similar to the potent tumor promoter 12-O-tetradecanolyphorbol-13-acetate (TPA) and to act in a tumor promoting capacity in a two-stage carcinogenesis model. It was shown by one grantee that, similar to TPA and other tumor promoters, the metals studied are capable of stimulating human polymorphonuclear leukocytes, as measured by hydrogen peroxide formation, a known tumor promoter. The most effective stimulators were the carcinogens nickel subsulfide, cadmium sulfide, and nickel disulfide--in that order. Nickel sulfide and cobalt sulfide did not cause statistically significant increases in hydrogen peroxide production. Noncarcinogenic barium and manganese sulfides and sulfates of nickel, cadmium and cobalt were inactive. Enhancement of hydrogen peroxide formation by cadmium sulfide and nickel subsulfide was comparable to that mediated by TPA. Evidence was provided demonstrating that hydrogen peroxide was formed. Superoxide anion radical was also formed when in the presence of nickel sulfide, but not with nickel subsulfide. Since nickel- and cadmium-containing particulates are deposited in the lungs and cause infiltration of polymorphonuclear leukocytes, the ability to activate those cells and induce hydrogen peroxide formation may contribute to their carcinogenicity (65).

There is substantial evidence in mouse skin suggesting that inflammation is an essential component of the phorbol ester tumor promotion stage of multistage carcinogenesis. In order to better understand the significance of this relationship, studies were initiated to identify the principal mediators of the vascular permeability component of inflammation induced by TPA. Histamine is known to produce a variety of physiological changes, which include increasing vascular permeability. Two distinct types of histamine receptors, H₁ and H₂, have been defined in pharmacological terms, based on the antagonism of specific histamine actions by certain drugs. Thus, antihistamines and inhibitors of arachidonic acid metabolism were compared with respect to their anti-inflammatory activity and the correlation of this parameter with their effects on tumor promotion. The H₁ histamine receptor antagonist, diphenhydramine, was shown to suppress TPA-induced vascular leakage when given topically, and to inhibit papilloma development in initiated mice. Inhibition of tumors was also observed when diphenhydramine was given orally. The H₂ antagonist, cimetidine, which was only supplied orally, demonstrated little effect on either TPA-induced vascular permeability or promotion. The lipoxygenase inhibitor, nordihydroguaiaretic acid, also suppressed vascular permeability and was reported by others to inhibit papilloma development. Indomethacin, a cyclooxygenase inhibitor,

did not have an effect on TPA-induced vascular permeability. Data obtained suggested that the increased vascular leakage observed with TPA contributes to tumor development and that this event is mediated by both the H₁ histamine receptors and one or more of the lipoxygenase products of arachidonic acid. Future studies are planned to determine whether agents that are irritants, but not tumor promoters, stimulate production of the same types of inflammatory mediators. This may provide an indication as to the basis for the distinction between irritants that promote and irritants that do not promote tumors (58).

Several types of observations have led to the suggestion that reactive oxygen intermediates are involved in the process of mouse skin tumor promotion and carcinogenesis. Little information is currently available on the specific cellular origin of the reactive oxygen intermediates. Results from several studies suggest that free radical species generated in the skin may be derived from Langerhans cells, as well as inflammatory leukocytes that have infiltrated into the dermis and epidermis following tumor promoter treatment. Recent studies have also suggested that keratinocytes have the capacity to produce reactive oxygen intermediates. Since reactive oxygen intermediates have been implicated in the process of TPA-dependent tumor promotion in mouse skin, the ability of murine epidermal cells to produce intracellular hydrogen peroxide was analyzed using flow cytometry and measurement of 2',7'-dichlorofluorescein (DCFH) oxidation. Epidermal cells isolated from acetone-treated CD-1 mice were relatively homogeneous in cell size and density and oxidized low levels of DCFH. Following TPA treatment of mice, however, two cytokeratin-positive populations of cells were identified that were heterogeneous with respect to size and density. These two TPA-derived cell populations exhibited levels of DCFH oxidation that were time and dose dependent and that were 2- to 10-fold higher than levels of DCFH oxidation by cells isolated from acetone-treated mice. Evidence was presented to show that intracellular hydrogen peroxide was responsible for the enhanced rate of DCFH oxidation in epidermal cells isolated from TPA-treated mice. The ability of mouse epidermal keratinocytes to oxidize DCFH in response to TPA treatment was confirmed by using a cloned keratinocyte cell line. Results obtained suggested that specific subpopulations of keratinocytes produce elevated levels of intracellular peroxides following TPA treatment either *in vivo* or *in culture*. Thus, both keratinocytes and inflammatory leukocytes may be significant sources of reactive oxygen intermediates during phorbol ester-mediated tumor promotion (164).

Properties and Mechanisms of Tumor Promotion: Research in this subject area is focused on projects designed to analyze various cellular, biochemical and molecular activities and pleiotropic effects induced in cells by exposure to tumor promoters. A variety of model systems are being used in these studies, including a heterotopically transplanted rat bladder model, mouse skin and rat liver models, and the use of various rodent and human fibroblast and epithelial cell lines. In one unusual project, a two-stage tumor model in planaria is being established. Phorbol ester tumor promoters are 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. This phorbol ester receptor protein has been identified as protein kinase C (PKC). Results of phorbol ester binding include alterations in membrane phospholipid metabolism and membrane structure and function, alterations in the transport of small molecules such as ions, activation of macromolecular synthesis, and induction or inhibition of terminal cell differentiation by normal or neoplastic cells. In addition, exposure of normal cells to phorbol esters can cause them to mimic the transformed phenotype and to enhance cellular transformation by chemicals and oncogenic viruses. Since the action of the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) may be mediated by phosphorylation

of proteins and lipids, several studies are focused on the purification and characterization of PKC, a calcium and phospholipid-dependent protein kinase whose activity is stimulated by TPA. PKC consists of a large family of proteins encoded by multiple genes, many of which have been cloned. Characterization of the proteins phosphorylated by this enzyme is included in some of these studies. The activation of certain gene expression 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.

In mouse strains there are known to be significant differences in susceptibility to two-stage skin carcinogenesis when phorbol esters are used as promoters. It has been suggested that a primary determinant in the observed strain differences toward two-stage skin carcinogenesis is related to the promotion stage. Although there are strain differences in sensitivity to phorbol ester tumor promoters, it is not clear to what extent such differences extend to other chemical classes of promoting agents. Previous work from several laboratories has demonstrated marked differences in the ability of TPA to induce inflammation and hyperplasia in different stocks and strains of mice. In general, a good correlation between the magnitude of these changes following repetitive treatments, and the degree of susceptibility to phorbol ester-mediated skin tumor promotion, has been observed. However, very similar hyperplastic responses in sensitive and resistant mouse skin following repeated treatments with several non-phorbol ester tumor promoters, including chrysarobin and benzoyl peroxide, have been reported. In order to address several questions regarding cross-sensitivity and/or resistance among mouse stocks and strains to different classes of promoting agents, different mouse stocks and strains with known sensitivity to phorbol ester skin tumor promotion were compared for their sensitivities to skin tumor promotion by a prototypic organic peroxide (benzoyl peroxide) and anthrone (chrysarobin) tumor promoter. Following initiation with either 7,12-dimethylbenz(a)anthracene and/or N-methyl-N'-nitro-N-nitrosoguanidine, groups of mice were promoted with several different doses of each promoting agent. Concurrent tumor promotion experiments examined the responsiveness of two common inbred mouse strains, DBA/2 and C57BL/6. At all doses tested, the phorbol ester-responsive mouse strain DBA/2 was more sensitive to skin tumor promotion by chrysarobin than was C57BL/6, but was less sensitive than both SENCAR and inbred SENCAR (SSIn) mice. DBA/2 and C57BL/6 mice were also similar in their responsiveness to benzoyl peroxide promotion, but again both of these inbred strains were significantly less sensitive than were SSIn and SENCAR mice to this organic peroxide type of skin tumor promoter. Histological evaluations comparing SENCAR and C57BL/6 mice revealed that a major difference between these strains in response to multiple chrysarobin and benzoyl peroxide was in the inflammatory response as measured by edema formation. Unlike TPA, chrysarobin and benzoyl peroxide did not induce dramatic differences in these two mouse lines in the epidermal hyperplasia, as measured by epidermal thickness. Results obtained in this study suggested that there is a common pathway controlling susceptibility to skin tumor promotion by TPA, benzoyl peroxide and chrysarobin. It was implied that different classes of mouse skin tumor promoters may ultimately bring about some similar biochemical and molecular changes leading to the selective clonal expansion of initiated cells in mouse epidermis. Since the promoting activity of TPA is believed to result, in part, through its binding to a cellular receptor, PKC, several aspects of the mouse epidermal PKC system were characterized and compared in phorbol ester-sensitive and -resistant mice. Partially purified

epidermal PKC preparations from both SENCAR and C57BL/6 mice were shown to contain the *gamma*, *beta*, and *alpha*-isozymes of PKC. The distribution of PKC isozymes was similar in both strains. No differences were found in the magnitude or kinetics of TPA-induced translocation and downregulation of total PKC or appearance of Ca²⁺/phospholipid-independent kinase activity between SENCAR, DBA/2 and C57BL/6 mice. These results demonstrated that mouse epidermis contains significant amounts of the three major PKC isozymes that are present in brain, especially PKC-*gamma*. It was concluded from these studies that there were no observed differences between phorbol ester-sensitive and -resistant mice in any aspect of epidermal PKC examined. Thus, the identity of the putative common biochemical pathway(s) remains to be determined (48).

Tamoxifen is a well-tolerated palliative and adjuvant treatment for human breast cancer and requires continuous, long-term administration for optimal therapeutic effectiveness. Using a two-stage model of experimental hepatocarcinogenesis, the carcinogenic potential of tamoxifen was studied by one grantee. The study assessed the effectiveness of tamoxifen both as an initiator and a promoter in hepatocarcinogenesis in female F344 rats. As an initiator, tamoxifen was tested at a single intragastric dose of 40 mg/kg, followed by promotion with 0.05% phenobarbital. From the observed numbers and size of the resulting altered hepatic lesions, tamoxifen was found to lack initiating action at the dose tested. Other groups of animals were initiated with a nonnecrogenic, subcarcinogenic dose of diethylnitrosamine and were fed tamoxifen at either 250 or 500 mg/kg in a purified diet for 6 months. The livers of these animals showed an increase in size and number of altered hepatic lesions compared with those animals that were initiated but not exposed to tamoxifen. This indicated that tamoxifen acts as a tumor promoter in rat liver. The promotion index of tamoxifen, a measure of relative potency, was less than one-tenth that of ethinyl estradiol and more than four times that of phenobarbital, an agent commonly employed as a representative promoting agent in experimental carcinogenesis. Since tamoxifen was demonstrated to lack initiating activity in rat liver at the dose tested, the mechanism of tumor induction by tamoxifen observed in long-term feeding studies may be a result of its promotion of spontaneously initiated hepatocytes. Although carcinogenesis in the rodent cannot be directly extrapolated to humans, these two systems are similar in the natural history of cancer development and in the mechanism of chemically induced carcinogenesis. Thus, rodent studies are useful as model systems to increase our knowledge of the development of cancer in humans and as an aid to risk estimation of an agent for humans. In this regard, the possibility that tamoxifen may act as a tumor promoter in humans as well as in experimental animals should be considered in assessing the relative benefit of long-term administration of tamoxifen to humans (156).

Ethinyl estradiol is a strong promoter of hepatocarcinogenesis and stimulates rat liver DNA synthesis. In studies to determine the mechanism of stimulation of DNA synthesis, ethinyl estradiol has been found to have both indirect and direct effects associated with increased liver growth. The levels of a serum/plasma factor, possibly in the epidermal growth factor (EGF)/transforming growth factor-*alpha* (TGF-*alpha*) family, with activity stimulatory for hepatocyte DNA synthesis, were shown to be enhanced by ethinyl estradiol treatment. This effect was associated with a two-fold increase in surface EGF receptor number. In subsequent experiments the increase in cell surface EGF binding caused by ethinyl estradiol was time dependent, beginning at 8 hr and reaching a plateau at 18 hr. This increased EGF binding was accompanied by a comparable increase in the amount of total EGF receptor protein. Ethinyl estradiol treatment also did not increase the rate of synthesis of the EGF receptor protein, nor did it appear to affect steady-state levels of EGF receptor

mRNA compared to controls. However, ethinyl estradiol treatment did cause an increase in the half-life of EGF receptor protein. Taken together, these results indicated that the ethinyl estradiol-induced two-fold increase in EGF receptor levels, which is associated with the potentiation of responsiveness to EGF, was brought about by stabilization of the receptor protein. In spite of these results, it is still not known whether the effect of ethinyl estradiol on EGF responsiveness is causally related to the actual mechanism of ethinyl estradiol-induced liver growth and its tumor-promoting activity. The answer to these questions will involve additional investigation and perhaps development of new models to study the effects of promoters of hepatocarcinogenesis on well-defined, initiated liver cells (215).

PKC is a calcium- and phospholipid-dependent serine-threonine protein kinase of fundamental importance in signal transduction and growth regulation. The tumor promoter TPA binds to the regulatory domain of PKC and is a potent activator of the enzyme. Once activated, PKC can modulate diverse cellular processes, presumably by phosphorylating specific target proteins. cDNA cloning studies have indicated that PKC belongs to a multigene family consisting of at least six distinct genes. The *beta* gene has been shown to yield two distinct transcripts, designated *beta*₁ and *beta*₂. These seven isoforms are differentially expressed in different tissues, and presumably perform somewhat different physiologic functions. There is evidence that activation of PKC can stimulate cell proliferation and perturb growth control. On the other hand, growth of certain human cell lines is inhibited by TPA, and in some cases this is associated with induction of differentiation. These findings indicate that the effects of PKC (or specific isoforms of PKC) activation on growth and differentiation depends on the particular cell type in which it is expressed. Thus, it was of interest to study the role of PKC in growth control in cell lines where growth is inhibited by TPA, particularly human epithelial cells. In one such study, a MERIT awardee has become interested in testing the possibility that PKC might play a critical role in the origin and growth of human colon cancer. Although this disease is the second major form of cancer in the United States, its precise etiology is not known. Studies by this grantee have previously shown that human colon tumors generally display decreased levels of PKC enzyme activity when compared with normal colonic mucosa. Moreover, bile acid, which has been implicated as a promoter in colon carcinogenesis, was shown to either inhibit or enhance the activity of purified PKC, depending on the Ca²⁺ concentration. These studies and studies from other laboratories demonstrate the complexity of the potential role of PKC in the evolution and growth of human colon tumors. Thus, in order to examine the precise role that PKC plays in human colon cancer, a direct molecular approach was taken. By using a retrovirus-derived vector system, derivatives of the human colon cancer cell line HT29 were generated that stably overexpressed a full-length cDNA encoding the *beta*₁ isoform of rat PKC. Two of these cell lines, PKC6 and PKC7, displayed an 11- to 15-fold increase in PKC activity when compared with the C1 control cell line that carried the vector lacking the PKC cDNA insert. Both of the overexpresser cell lines exhibited striking alterations in morphology when exposed to TPA. They also displayed increased doubling time, decreased saturation density and loss of anchorage-independent growth in soft agar. None of these effects were seen with C1 cells. In contrast to control cells, PKC-overproducing cells also failed to display evidence of differentiation, as measured by alkaline phosphatase activity, when exposed to sodium butyrate. In addition, PKC-overexpresser cells displayed decreased tumorigenicity in nude mice, even in the absence of treatment with TPA. These results provide the first direct evidence that PKC can inhibit tumor cell growth. Thus, in some tumors, PKC might act as a growth-suppressor gene. If PKC is found to have general relevance to the growth of colon tumors, this would suggest a novel approach to colon cancer therapy. It was speculated that new and highly potent

compounds that activate PKC without exerting tumor promoting activity, such as bryostatin, might be used to activate the endogenous PKCs present in colon tumors, thus inducing tumor suppression (208).

The phenomenon of skin tumor promotion has been extensively studied using phorbol esters, such as TPA. Although the exact mechanism of tumor promotion by phorbol esters is not yet known, specific cellular and biochemical responses have been associated with their tumor-promoting action in mouse skin. Promotion response with TPA is presumed to be mediated, in part, by its interaction with PKC. The signaling pathway through the EGF receptor is one of the best-characterized growth controlling mechanisms and is believed to play a central role in the processes of cell proliferation and carcinogenesis. Phorbol ester tumor promoters are known to alter binding of EGF to its cellular receptor and this process is considered to be mediated through activation of PKC. Anthrone tumor promoters, such as chrysarobin, have been shown to have a chemical structure completely different from that of TPA. At optimal promoting doses, the time course and/or magnitude of promotion responses are different from those of TPA. Also, anthrones do not interact directly with the phorbol ester receptor. The studies performed have suggested that anthrone tumor promoters may stimulate alternative signaling pathways in producing their skin tumor-promoting response compared to phorbol esters. These considerations prompted an investigation of the effect of anthrone tumor promoters on the binding of EGF to its receptor on mouse keratinocytes. Chrysarobin was shown to reduce EGF binding to its receptor in primary epidermal cells from SENCAR mice maintained in low Ca^{2+} containing medium. The time course for this effect with chrysarobin was different from that of TPA. Inhibition by chrysarobin was due to a decrease in the number of both high- and low-affinity classes of EGF receptors. TPA, in contrast, caused a rapid inhibition of EGF binding, primarily due to a loss of high-affinity receptors. The mechanism by which chrysarobin inhibited binding of EGF to its receptor did not involve either direct activation or membrane translocation of epidermal PKC, whereas the rapid decrease in EGF binding induced by TPA was consistent with its ability to activate PKC. Structure-activity relationships for EGF binding inhibition by anthrones revealed that inhibition was inversely proportional to chain length at the C_{10} -position, which correlated closely with oxidation rate and skin tumor-promoting activity. Results obtained suggested that oxidation at position C_{10} is at least partially responsible for the inhibition of EGF binding induced by chrysarobin. These studies also support the hypothesis that changes in EGF receptor binding and/or function may play a role in skin tumor promotion by diverse classes of promoting agents (47).

Signal transduction leading to cell growth is a complex process initiated by the interaction of growth-stimulating factors with specific receptors at the cell surface. One of the major classes of growth factor receptors are the ligand-stimulated tyrosine kinases, as exemplified by EGF and platelet-derived growth factor receptors. Once bound to their respective growth factors, these receptors initiate a series of signaling events through tyrosine phosphorylation of interacting proteins, which in turn transmit the signal to the cell nucleus. A major focus of many investigations is establishment of the identity of functionally important intermediates and the mechanisms by which they participate in the signaling process. The EGF receptor is both an activator and a target of phosphorylation by kinases that are believed to be involved in cellular signaling. At least two major sites of phosphorylation on the EGF receptor have been identified: threonine-654 (T654), a target of PKC phosphorylation, which mediates inhibition of the EGF-stimulated tyrosine kinase; and threonine-669 (T669), a residue that is phosphorylated in response to a variety of stimuli including EGF, phorbol esters, and the nonphorbol tumor promoter

thapsigargin. Although the functional consequence of phosphorylating T669 is not currently known, this residue is the only major phosphorylated site on the EGF receptor following treatment with thapsigargin. This phosphorylation inactivates the receptor tyrosine kinase through a PKC/T654-independent mechanism. Microtubule-associated protein (MAP) kinase, originally described as an insulin-stimulated serine/threonine kinase that phosphorylates microtubule-associated protein 2, was similarly phosphorylated as well as stimulated by growth activators, including EGF. In order to determine whether a MAP-type kinase is responsible for T669 kinase activity in EGF-stimulated 3T3-L1 cells, T669 peptide kinase was partially purified and characterized. Results obtained indicate that a MAP kinase phosphorylates the T669 peptide and raises the possibility that this enzyme may participate in a feedback loop, being activated by the EGF receptor and in turn phosphorylating the receptor (169).

The profound biological effects of growth factors and oncogenes on cellular phenotype is thought to be ultimately due to induced changes in the pattern of gene expression. It is envisioned that a cascade of secondary and tertiary messengers induced and/or modified by these agents specifies a pathway that results in a distinctive pattern of gene expression. One grantee attempted to elucidate the mechanism by which a specific growth factor, EGF, modulates the expression of a specific gene, the stromelysin gene. Rat stromelysin belongs to a multigene family of metal-dependent proteinases known as matrix metalloproteinases. Stromelysin expression has been shown to correlate with tumor progression. Initial studies have shown that rat stromelysin mRNA is induced by EGF and that this increase in cytoplasmic mRNA is due to an increase in the rate of transcriptional initiation. In subsequent studies, the necessity of specific secondary (PKC) and tertiary (*c-fos* and *c-jun* protein products) messengers in transactivation of stromelysin gene expression by EGF was examined. Rat-1 fibroblasts exposed to antisense *c-fos* DNA or RNA demonstrated that *c-fos* expression was necessary for complete EGF induction of stromelysin expression. Similar results demonstrating the necessity of *c-jun* protein in EGF induction of stromelysin were obtained. It was also demonstrated that PKC activation is required for EGF induction of stromelysin, since phorbol ester desensitization of PKC abolished the ability of EGF to induce stromelysin mRNA, protein, and promoter activity. In reconstitution experiments, neither *c-fos*, *c-jun* nor PKC activation alone induced significant stromelysin expression. When *c-fos* and *c-jun* were overexpressed, stromelysin was induced to a level similar to that of the growth factor, and stimulation of PKC activity augmented this induction. Data obtained suggested that EGF induction of stromelysin in rat fibroblasts proceeds through a pathway involving *c-fos*, *c-jun* and PKC. It is envisioned that the three necessary components of the signaling pathway converge at the AP-1 site in the rat stromelysin promoter. Several grantees have demonstrated that the Fos-Jun protein complex binds this sequence and activates transcription through this element in the human collagenase promoter. This sequence has also been described as a TPA-responsive element and has been shown to be responsible for TPA induction of gene expression from several genes and synthetic promoter constructs. It was therefore likely that the AP-1 site in the stromelysin promoter is the *cis*-acting DNA element responsible for EGF stimulation of stromelysin gene expression (133).

Gap junctions form transmembrane channels between adjacent cells in organs or tissue cultures and are thought to be involved in tissue homeostasis, morphogenesis, and cell growth control. Recent cDNA cloning studies have revealed that there is a family of related gap junction proteins, the connexins. So far, at least three different mammalian connexin genes have been cloned, connexin 32, connexin 43 and

connexin 26. Connexin expression has been shown to be organ and cell type specific. There is increasing evidence that gap junctional intercellular communication plays an important role in carcinogenesis. Transformed cells in vitro and in vivo have been demonstrated to have a decreased gap junctional intercellular communication capacity among themselves or with surrounding normal cells. In rat hepatocarcinogenesis studies, several laboratories have found that a significant decrease in the major liver gap junction protein, connexin 32, at the mRNA or protein level occurs in preneoplastic nodules and hepatocellular carcinomas induced by chemicals. Results of those studies suggested that the decrease in connexin 32 expression is one of the steps accompanying multistage carcinogenesis of rat liver. Although changes in intercellular junctions had been reported, there were no reports of studies on connexin expression in primary human hepatocellular carcinomas. Consequently the expression of connexin 32 and connexin 43 (the major cardiac gap junction protein) was examined in six surgically removed human hepatocellular carcinoma tissues and surrounding nontumorous livers, using specific rat connexin probes. No decrease in connexin 32 mRNA expression was found in carcinomas compared with the surrounding nontumorous tissue. Morphometric analysis also showed that in most of the carcinomas, the number of gap junction spots stained with connexin 32 antibody was not less than that in the surrounding livers. These results were in striking contrast to the significant reductions in connexin 32 mRNA and protein expression observed in rat primary liver tumors induced by chemicals. On the other hand, all of the six human hepatocellular carcinomas exhibited elevated levels of connexin 43 mRNA, which was expressed at a very low level in the surrounding nontumorous livers. The carcinomas exhibited no detectable amplification of the connexin 43 gene. These results suggested that gap junctional intercellular communication is altered in human hepatocellular carcinomas by molecular mechanisms different from those in rat hepatocarcinogenesis (216).

MOLECULAR CARCINOGENESIS
GRANTS ACTIVE DURING FY91

<u>INVESTIGATOR/INSTITUTION/GRANT NUMBER</u>	<u>TITLE</u>
1. ADAIR, Gerald M. University of Texas System Cancer Center 2 R01 CA28711-10	Expression of Genetic Variation in Cultured Cells
2. ALDAZ, Claudio M. University of Texas System Cancer Center 5 R29 CA48922-03	Chromosomal Abnormalities in Rat Mammary Carcinogenesis
3. ALLFREY, Vincent G. Rockefeller University 5 R01 CA14908-17	Nuclear Proteins in Carcino- genesis of the Colon
4. AMES, Bruce N. University of California Berkeley 5 R35 CA39910-06	Mutagenesis and Carcinogenesis
5. ANANTHASWAMY, Honnavara N. University of Texas System Cancer Center 5 R01 CA46523-03	Mechanisms of Induction of Skin Cancers by UV Light
6. ASHENDEL, Curtis L. Purdue University West Lafayette 5 R01 CA36262-06	Interactions of Tumor Promoters with Receptors
7. AVADHANI, Narayan G. University of Pennsylvania 5 R01 CA22762-14	Mitochondrial Genetic Lesions during Carcinogenesis
8. BAIRD, William M. Purdue University West Lafayette 5 R01 CA40228-07	Molecular Mechanisms of Hydrocarbon-DNA Interactions
9. BARTLES, James R. Northwestern University 1 R01 CA53997-01	Regulation of CE 9 Protein by Peroxisome Proliferators
10. BAXTER, C. Stuart University of Cincinnati 5 R01 CA34446-06	In vivo Immunotoxicology of Tumor-Promoting Agents
11. BEER, David G. University of Kansas Col Hlth Sci and Hosp 5 R01 CA46433-02	Altered Gene Expression during Lung Carcinogenesis
12. BIGGART, Neal W. San Diego State University 5 R29 CA46818-04	Reactive Oxygen-Mediated Mutagenesis by CdCl-2 and TPA

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| 13. BILLINGS, Paul C.
University of Pennsylvania
5 R01 CA45734-05 | Target Proteases of Anticarcinogenic Protease Inhibitors |
| 14. BOORSTEIN, Robert
New York University
5 R29 CA51060-02 | HmUra Mutagenicity and Base Excision Repair |
| 15. BOWDEN, George T.
University of Arizona
5 R01 CA40584-07 | Oncogene Activation during Skin Tumor Promotion |
| 16. BOX, Harold C.
Roswell Park Memorial Institute
5 R01 CA44808-04 | DNA Damage, Promotion and the Prooxidant State |
| 17. BOYNTON, Alton L.
University of Hawaii at Manoa
5 R01 CA39745-06 | Assays for and Mechanisms of Action of Tumor Promoters |
| 18. BOYNTON, Alton L.
University of Hawaii at Manoa
5 R01 CA42942-05 | Tumor Promoters, Second Messengers, and Carcinogenesis |
| 19. BRESNICK, Edward
Dartmouth College
5 R01 CA36679-08 | Regulation of O ⁶ -Methylguanine DNA Methyltransferase |
| 20. BROCKMAN, Herman E.
Illinois State University
1 R15 CA54488-01 | Are "Cryptic Mutagens" Mutagenic in the ARA Test? |
| 21. BROYDE, Suse B.
New York University
2 R01 CA28038-10 | Carcinogen-DNA Adducts Linkage Site and Conformation |
| 22. BUTEL, Janet S.
Baylor College of Medicine
5 R01 CA33369-09 | Carcinogen-Viral Oncogene Interactions in Mammary Cancer |
| 23. BUTLER, Andrew P.
University of Texas System Cancer Center
5 R01 CA46629-03 | Regulation of Ornithine Decarboxylase by Phorbol Esters |
| 24. BUTLER, Andrew P.
University of Texas System Cancer Center
5 R01 CA52468-02 | Gene Regulation in Phorbol Sensitive and Resistant Mice |
| 25. CALOS, Michele P.
Stanford University
5 R01 CA33056-09 | Construction of a Human Artificial Chromosome |

26. CARADONNA, Salvatore J.
Univ of Med/Dent NJ-Sch Osteopathic Med
2 R01 CA42605-06A1
Genetics and Enzymology of
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27. CARTER, Timothy H.
St. John's University
5 R01 CA37761-06
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by a Tumor Promoter
28. CHANG, Chia Cheng
Michigan State University
5 R01 CA21104-13
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Genes in Carcinogenesis
29. CHANG, Ching-Jer
Purdue University West Lafayette
5 R01 CA44416-02
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30. CHEH, Albert M.
American University
1 R15 CA52043-01
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31. CHRISTMAN, Judith K.
Michigan Cancer Foundation
5 R01 CA45028-04
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Alteration in Gene Activity
32. CHRISTMAN, Judith K.
Michigan Cancer Foundation
5 R01 CA50909-02
Cloning the Gene for a Novel
TPA-Induced Protein
33. CHU, Gilbert
Stanford University
5 R01 CA44949-05
Finding the Molecular Defect in
Xeroderma Pigmentosum
34. CHUNG, Fung-Lung
American Health Foundation
5 R01 CA43159-06
Enals in Tumorigenesis
35. CHUNG, Fung-Lung
American Health Foundation
5 R01 CA51830-02
Endogenous Enals in Nitrosamine
Carcinogenesis
36. CLAWSON, Gary A.
George Washington University
5 R01 CA21141-15
Pathology of Chemical
Carcinogenesis
37. COHEN, Samuel M.
University of Nebraska Medical Center
3 R01 CA32513-08S1
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Tumors
38. COHEN, Samuel M.
University of Nebraska Medical Center
5 R01 CA44886-04
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39. CONTI, Claudio J.
University of Texas System Cancer Center
5 R01 CA42157-06
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40. CONTI, Claudio J.
University of Texas System Cancer Center
1 R01 CA53123-01
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41. CRAIGHEAD, John E.
University of Vermont and St Agric College
5 R01 CA36993-07
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42. DAVIDSON, Richard L.
University of Illinois at Chicago
5 R01 CA31781-11
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43. DAVIES, Kelvin J.
University of Southern California
3 R13 CA53492-01S1
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44. DAY, Rufus S. III
Cross Cancer Inst Northern Alberta Ca Prog
5 R01 CA49936-03
Cellular DNA Repair Response
to Methylating Agents
45. DERUBERTIS, Frederick R.
University of Pittsburgh at Pittsburgh
5 R01 CA31680-10
Phospholipid Derived Signals
in Colon Epithelial Growth
46. DIAMOND, Leila
Wistar Institute of Anatomy and Biology
5 R01 CA23413-13
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Differentiation
47. DIGIOVANNI, John
University of Texas System Cancer Center
5 R01 CA37111-08
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Promotion by Chrysarobin
48. DIGIOVANNI, John
University of Texas System Cancer Center
5 R01 CA38871-06
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49. DOVE, William F.
University of Wisconsin Madison
5 R01 CA50585-02
Spontaneous Intestinal Carcino-
genesis in a Mouse Mutant
50. DRINKWATER, Norman R.
University of Wisconsin Madison
5 R01 CA37166-07
Molecular Analysis of
Carcinogen-Induced Mutations
51. DUKER, Nahum J.
Temple University
5 R01 CA24103-09
Molecular Pathology of
Carcinogenic DNA Damage

52. ECHOLS, Harrison
University of California Berkeley
2 R01 CA41655-06
Mutagenesis and its Control
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53. ELLIOTT, Mark S.
Old Dominion University
5 R29 CA45213-05
Modulation of Queuine Levels
with Tumor Promoters
54. ESSIGMANN, John M.
Massachusetts Institute of Technology
5 R35 CA52127-02
Cellular Responses to DNA Damage
55. FAHL, William E.
University of Wisconsin Madison
5 R37 CA42024-07
Carcinogen-Transformed Human
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56. FAUSTO, Nelson
Brown University
5 R01 CA35249-07
Protooncogenes and Cell Lineages
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57. FEINBERG, Andrew P.
University of Michigan at Ann Arbor
5 R01 CA48932-03
Identification of the Earliest
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58. FISCHER, Susan M.
University of Texas System Cancer Center
2 R01 CA34443-07
Role of Arachidonate Metabolites
in Tumor Promotion
59. FISCHER, Susan M.
University of Texas System Cancer Center
5 R01 CA42211-05
Tumor Promoter-Induced Oxidants
from Epidermal Cells
60. FISHER, Paul B.
Columbia University New York
5 R01 CA35675-08
Analysis of Progression of
the Transformed Phenotype
61. FISHER, Paul B.
Columbia University New York
5 R01 CA43208-05
Multifactor Interactions in
Carcinogenesis
62. FLOYD, Robert A.
Oklahoma Medical Research Foundation
5 R01 CA42854-05
Oxygen Free Radicals in
Carcinogenesis
63. FOSTER, Patricia L.
Boston University
5 R01 CA37880-07
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64. FOX, C. Fred
Keystone Center
1 R13 CA54777-01
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65. FRENKEL, Krystyna
New York University
5 R01 CA37858-06
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66. FRENKEL, Krystyna
New York University
5 R01 CA49798-02
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67. FRIEDBERG, Errol C.
University of Texas SW Med Ctr/Dallas
5 R37 CA12428-22
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to Carcinogenesis
68. FRIEDBERG, Errol C.
University of Texas SW Med Ctr/Dallas
5 R01 CA44247-06
DNA Repair and Cancer-Prone
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69. FRIEDMAN, Eileen A.
Memorial Hospital For Cancer and Allied Dis
5 R01 CA50645-02
Tumor Promoter Induced Protein
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70. GALLAGHER, Patricia E.
West Virginia University
5 R29 CA47457-05
Enzymatic Repair of Carcinogenic
Damage to Human DNA
71. GARTE, Seymour J.
New York University
5 R01 CA36342-08
Transforming Genes in Inhalation
Carcinogenesis
72. GEACINTOV, Nicholas E.
New York University
5 R01 CA20851-13
Characterization of Carcinogen
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73. GERSON, Stanton L.
Case Western Reserve University
5 R01 CA45609-03
Prevention of Leukemogenesis
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74. GOLD, Barry I.
University of Nebraska Medical Center
5 R01 CA38976-07
Metabolism and Genotoxicity of
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75. GOULD, Michael N.
University of Wisconsin Madison
5 R01 CA28954-10
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tibility to Mammary Cancer
76. GOULD, Michael N.
University of Wisconsin Madison
5 R01 CA44387-04
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77. GRIFFITH, O. Hayes
University of Oregon
2 R01 CA11695-22
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Properties of Macromolecules

78. GRISHAM, Joe W.
University of North Carolina Chapel Hill
5 R01 CA24144-12
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79. GROLLMAN, Arthur P.
State University New York Stony Brook
5 R01 CA17395-17
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80. GROLLMAN, Arthur P.
State University New York Stony Brook
5 P01 CA47995-02
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81. GRUNBERGER, Dezider
Columbia University New York
2 R01 CA39547-07
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in Mammalian Gene
82. HAMILTON, Joshua W.
Dartmouth College
5 R29 CA49002-03
Effect of Carcinogens on Gene
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83. HANAWALT, Philip C.
Stanford University
5 R35 CA44349-05
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DNA: Role in Oncogenesis
84. HELD, William A.
Roswell Park Memorial Institute
5 R01 CA50675-02
Androgen Conditional Carcino-
genesis in Transgenic Mice
85. HITTELMAN, Walter N.
University of Texas System Cancer Center
5 R01 CA27931-11
Chromosome Aberrations with
Therapeutic Agents
86. HOGAN, Michael E.
Baylor College of Medicine
5 R01 CA39527-07
Mapping Carcinogen Binding
Sites on Genes
87. HOLT, Jeffrey T.
Vanderbilt University
5 R01 CA51735-02
Transcriptional Mechanisms of
Carcinogenesis
88. HUMAYUN, M. Zafri
University of Medicine and Dentistry of NJ
5 R01 CA27735-09
Mutagenesis by Carcinogenes:
A Molecular Approach
89. HUMAYUN, M. Zafri
University of Medicine and Dentistry of NJ
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90. ISAACS, John T.
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91. ITO, Nobuyuki
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5 R01 CA50216-02
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92. IVARIE, Robert D.
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93. JACOBSON, Myron K.
Texas College of Osteopathic Medicine
5 R01 CA43894-06
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94. JAKEN, Susan
W. Alton Jones Cell Science Center
1 R01 CA53841-01
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95. JONES, Peter A.
University of Southern California
5 R35 CA49758-03
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96. KALLENBACH, Neville R.
New York University
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97. KAUFMAN, David G.
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5 R01 CA31733-10
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98. KAUFMAN, David G.
University of North Carolina Chapel Hill
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99. KAUFMANN, William K.
University of North Carolina Chapel Hill
5 R01 CA36906-05
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100. KENNEDY, Ann R.
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5 R37 CA22704-15
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101. KING, Charles M.
Michigan Cancer Foundation
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102. KLEIN-SZANTO, Andres J.
Fox Chase Cancer Center
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103. KLEIN-SZANTO, Andre J.
Fox Chase Cancer Center
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104. KNUDSON, Alfred G.
Institute for Cancer Research
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105. KOESTNER, Adalbert
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106. KOHWI-SHIGEMATSU, Terumi
La Jolla Cancer Research Foundation
5 R01 CA39681-07
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107. KOHWI, Yoshinori
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108. KOLESNICK, Richard N.
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5 R01 CA42385-06
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109. KRAFT, Andrew S.
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110. KRUGH, Thomas R.
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111. KULESZ-MARTIN, Molly F.
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5 R01 CA31101-10
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112. LEADON, Steven A.
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5 R01 CA40453-05
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113. LEBOVITZ, Russell M.
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5 R29 CA49845-03
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114. LEBOWITZ, Jacob
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115. LEBRETON, Pierre R.
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116. LEWIS, James G.
Duke University
5 R29 CA44734-05
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117. LIEBERMAN, Michael W.
Baylor College of Medicine
5 R37 CA39392-08
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118. LIEBERMAN, Michael W.
Baylor College of Medicine
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genic Liver and Kidney Cancer
119. LIEBERMAN, Michael W.
Baylor College of Medicine
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120. LIEHR, Joachim G.
University of Texas Medical Branch
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121. LILLY, Frank
Yeshiva University
5 P01 CA31855-08
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122. LILLY, Frank
Yeshiva University
5 R01 CA52621-02
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123. LOEB, Lawrence A.
University of Washington
5 R35 CA39903-06
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124. LOECHLER, Edward L.
Boston University
5 R01 CA50432-02
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125. LOMBARDI, Benito
University of Pittsburgh at Pittsburgh
5 R01 CA23449-13
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126. MACLEOD, Michael C.
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5 R01 CA35581-08
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127. MAGUN, Bruce E.
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5 R01 CA39360-08
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128. MAHER, Veronica M.
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129. MAHER, Veronica M.
Michigan State University
5 R01 CA48066-03
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143. MULLIN, James M.
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5 R01 CA48121-03
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144. NOTARIO-RUIZ, Vicente
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145. O'BRIEN, Thomas G.
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146. OBERLEY, Larry W.
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147. OYASU, Ryoichi
Northwestern University
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148. OYASU, Ryoichi
Northwestern University
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149. OYASU, Ryoichi
Northwestern University
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150. PATEL, Dinshaw J.
Columbia University New York
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151. PATEL, Dinshaw J.
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152. PEGG, Anthony E.
Pennsylvania State Univ Hershey Med Ctr
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153. PELLICER, Angel G.
New York University
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154. PELLING, Jill C.
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155. PITOT, Henry C.
University of Wisconsin Madison
5 P01 CA22484-14
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169. ROSNER, Marsha R.
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170. ROSSMAN, Toby G.
New York University
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171. SARMA, D. S.
University of Toronto
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Brown University
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175. SHAW, Barbara R.
Duke University
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176. SHAY, Jerry W.
University of Texas SW Med Ctr/Dallas
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177. SHINOZUKA, Hisashi
University of Pittsburgh at Pittsburgh
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178. SHINOZUKA, Hisashi
University of Pittsburgh at Pittsburgh
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179. SHINOZUKA, Hisashi
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Univ of Calif-Berkeley Lawrence Livermore
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Temple University
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183. SLAGA, Thomas J.
University of Texas System Cancer Center
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184. SMART, Robert C.
North Carolina State University Raleigh
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185. SMULSON, Mark E.
Georgetown University
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186. SNOW, Elizabeth T.
New York University
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187. SNOW, Elizabeth T.
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188. STAMPFER, Martha R.
Univ of Calif-Berkeley Lawrence Livermore
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189. STATES, J. Christopher
Wayne State University
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190. STRAUSS, Bernard S.
University of Chicago
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191. STRAUSS, Bernard S.
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192. SUDILOVSKY, Oscar
Case Western Reserve University
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193. SUKUMAR, Saraswati
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194. SUMMERHAYES, Ian C.
New England Deaconess Hospital
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195. SUMMERS, William C.
Yale University
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198. TELANG, Nitin T.
Memorial Hospital For Cancer and Allied Dis
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199. TOPAL, Michael D.
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201. TRUMP, Benjamin F.
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Molecular Epidemiology
202. VERMA, Ajit K.
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210. WENDER, Paul A.
Stanford University
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211. WETTERHAHN, Karen E.
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213. WILLIAMS, Gary M.
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214. WOLF, George D.
University of California Berkeley
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215. YAGER, James D.
Johns Hopkins University
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216. YAMASAKI, Hiroshi
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217. YU, Fu-Li
College of Medicine at Rockford
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218. ZURLO, Joanne
Johns Hopkins University
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SUMMARY REPORT

NUTRITIONAL CARCINOGENESIS

The Nutritional Carcinogenesis component of the Branch has undergone a gradual evolution in emphasis and now supports studies on food mutagens and carcinogens formed during cooking as well as studies on digestive tract mutagens which form as a result of compound biodegradation, biotransformation, etc. Studies are also supported on the etiology of cancer resulting from deficiencies in the availability of various micronutrients, vitamins, metals, etc. The overall program emphasizes 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. The research supported focuses on (1) the role of food mutagens/carcinogens in human cancer causation; (2) the influence of fat source and saturation level on tumor induction; (3) the role of nutrients and micronutrients in carcinogenesis; (4) dietary constituents such as protein and orotate on cancer; (5) the effects of calorie consumption and energy expenditure on cancer causation; (6) dietary lipotropes and cancer; and (7) the role of compounds associated with the gut, including the influence of microflora, in cancer induction.

It has been suggested that certain types of cancer, such as cancer of the stomach, large intestine or other organs, may be attributed to the traditional foods and lifestyles found in certain regions of the world. In December 1984, the National Cancer Institute issued a Request for Applications (RFA) entitled "The Relevance of Mutagens in Human Foods." The purpose of which was to stimulate study of the metabolic fate and carcinogenic potential of known dietary mutagens commonly present in human foods. Mutagens have been associated with the charcoal broiling of steak, boiling of beef broth, frying of hamburger and potatoes, and toasting of bread in addition to being found in many vegetables, alcoholic beverages, spices, coffee and tea. Five specific chemical classes--heterocyclic aromatic amines, carbonyls, hydroxylated flavanoids, fecapentaenes, and endogenous N-nitroso compounds--were identified in the RFA for special consideration. Suggested activities included: in-depth basic studies on mutagens naturally occurring in food, found in feces, or associated with food processing; development of analytical procedures for assaying blood, body fluids, feces and tissues; and laboratory or epidemiological studies on the carcinogenesis of selected compounds, e.g., quercetin and fecapentaenes. Since the RFA was published, nearly three dozen grants have been supported by the program. Progress resulting from a number of these projects is summarized below.

Studies have shown that the cooking of foods, including 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 aminoimidazo-azaarene 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 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP). The mechanism of formation is believed to involve a cyclization of amino acids with creatinine; as the cooking temperature is increased, the quantity of mutagenic compounds increases but the relative ratios of mutagens remain constant. Although these compounds are only found in minute quantities in cooked foods, they have been shown to be very potent mutagens and one, IQ, has shown positive carcinogenic activity in rats (liver and intestine), mice

(liver, lungs, and forestomach), and monkeys (liver). These compounds have been identified in and isolated from human foods and studies to elucidate their activation, metabolism, and mutagenic and carcinogenic activity are currently underway in several laboratories.

Investigators in England have been examining the occurrence of heterocyclic amines in tobacco smoke, cooked food, and vegetable and meat extracts used to flavor commercial fast and frozen foods. The group is also studying absorption and metabolism of these compounds in humans. As part of these studies, the researchers have reported the development of a gas chromatography mass spectrometry assay for the measurement of Trp-P-2, MeIQx and DiMeIQx in fried beef. This assay utilizes capillary column gas chromatography with electron-capture negative-ion chemical ionization (ECNICI) mass spectrometry. Stable isotope-labeled analogues of MeIQx or Trp-P-2 are used as internal standards. Lean beef patties were cooked by heating in a hot frying pan, with no added fat or oil, at a temperature near 200 degrees Centigrade until done, but without charring the exterior. The sample was extracted, derivatized with 3,5-bis-trifluoromethylbenzyl bromide, and analyzed by the ECNICI gas chromatography-mass spectrometry (GC/MS) method. Both MeIQx and DiMeIQx were found to be present in quantities above the 0.2 nanogram limit of detection (1.2-2.4 and 0.5-1.3 ng/g, respectively), but Trp-P-2, detectable at the picogram level, was not seen. Levels in vegetable and meat extracts were comparable, but vegetable extracts had repeatedly higher levels. Bacterial grade extracts were 15-fold higher (36.6 and 6.6 ng/g, respectively) for MeIQx and DiMeIQx. These compounds were not found in raw uncooked beef, confirming that MeIQx and DiMeIQx were formed during the cooking process.

The same procedures utilized above have been used to estimate human exposure to MeIQx following consumption of fried beef. HPLC data in mice has demonstrated rapid elimination of radiolabeled material which was independent of the route of administration. Less than 20% of the dose was excreted unchanged. The urinary metabolites have been identified as hydroxy-MeIQx glucuronide and an N-glucuronide. P450-dependent metabolic activation is believed necessary for mutagenesis and three oxidative metabolites have been detected thus far. Using stable carbon- and nitrogen-labeled standards, and ECNICI GC/MC, urinary clearance within 48 hours was demonstrated in human volunteers. Of the approximately 300-800 ng present in the experimental meat patty, less than 5% was recovered in the urine within 12 hours. 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 risk remains to be determined.

N-Hydroxylation of MeIQx has also been demonstrated in vitro using mouse liver microsomes. However, the compound is rather unstable, at least in aqueous solution. The activated metabolite binds readily to hemoglobin in proportion to the dose administered. Evidence has been presented to indicate that less than one-fifth of the bound MeIQx is clearly identifiable as N-hydroxy derived; this is being further examined using ³²P-postlabeling. These researchers are also developing immunological assay procedures for MeIQx and PhIP, the latter of which is present in cooked meat samples at much higher (10x) levels. PhIP has recently been shown to be an extremely potent mammalian mutagen, though less active than MeIQx in bacterial assays (7).

The aminoimidazo-azaarene (AIA) mutagens have been identified by another research group using two conventional analytical chemical methods, HPLC and negative-ion mass spectrometry, also described above. These methods have proved useful in the

preliminary study of rodent and human metabolism. The methods are not as appropriate for the identification and quantification of AIA mutagens in foods or model systems at very low levels, or in large numbers of samples. To expedite the analysis of model systems and the study of methods to lower cooked mutagen levels in foods, investigators at the Lawrence Livermore National Laboratory have also developed monoclonal antibodies for use in ELISA immunoassays for the AIA mutagens. Anti-bodies have been produced that will uniquely recognize all five of the previously identified AIA mutagens (IQ, MeIQ, MeIQx, DiMeIQx, and PhIP). Samples to be analyzed are cleaned up on HPLC, and the antibodies are sufficiently sensitive to allow detection of AIAs in the part per billion range (levels present in cooked meat). In addition, the antibodies did not react with high levels of structurally similar compounds that might be present in beef extracts, 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. The highest affinity antibody is that produced against PhIP. This antibody will allow the detection and identification of additional PhIP-like compounds. The antibodies have been used to prepare affinity columns and this has led to the isolation of large quantities of the various AIAs directly from beef. Substantial mutagenic activity has also been detected in chicken and beef bouillon, pasta, and cooked cheese products. The highest levels determined to date have been in beef bouillon (8).

The overall metabolism of IQ has also been partially elucidated in rats by another group of investigators. The majority of the primary metabolites have been identified from urine and feces and in vitro as (polar fraction) IQ-N-glucuronide, 5-hydroxy-IQ glucuronide, 5-hydroxy-IQ sulfate and IQ-sulfamate; (non-polar fraction) 7-oxoIQ, 3-N-dimethyl-IQ, 2-N-acetyl-IQ and unchanged IQ. All of these compounds were found in urine and bile, but fecal samples were devoid of 5-hydroxy-IQ glucuronide and the sulfate. An HPLC analytical method has been developed for more detailed and quantitative examination of IQ metabolism but a method to concentrate urinary metabolites (XAD column chromatography) has met with only limited success due to tight binding of 5-hydroxy-IQ to stainless steel columns and injectors. These new assays have been used to study the metabolism of IQ and N-acetyl-IQ, hypothesized to be involved in IQ-induced carcinogenesis of the colon and mammary gland. DNA binding has also been demonstrated with N-acetyl-IQ and IQ itself using the ³²P postlabeling technique (30).

Still another group has identified an additional fecal IQ metabolite, 7-hydroxy-IQ, and its acetyl metabolite. This hydroxy compound is mutagenic in *Salmonella* and has been shown to bind DNA. The compound has been synthesized in large quantity and is currently undergoing carcinogenicity screening in both rats and mice. The investigators have reported that, unlike the observed ring hydroxylation and N-oxidation of other AIAs, PhIP doesn't undergo significant anaerobic bacterial metabolism in fecal extracts from rodents or humans. Because of variable responses of the AIAs in different mutagenicity assays, a battery of bacterial and mammalian tests have been established to avoid missing any potential mutagens (13).

Since the level of liver tumor response to IQ in female mice is twice that of males, the degree of IQ-DNA adduct formation was determined in CDF1 mice following a single oral dose of IQ. Target tissue samples and intestine were assayed using the ³²P-postlabeling assay. Levels of adducts, identified as N-(deoxyguanosin-8-yl)IQ and four other minor components, paralleled the pattern of tumor incidence in females versus males. However, labeling was also high in intestine, a nontarget tissue, implying that additional factors besides DNA alkylation are involved in the

tumorigenesis. IQ-DNA adduct formation in newborn mice was similar to that observed in young adult mice. In addition to binding, an active and effective removal mechanism repairs most but not all of the alkylated sites. The involvement of additional factors was supported by the observation that high levels (20%) of safflower oil in the diet increased the numbers of liver tumors but did not affect DNA adduct levels. The appearance of tumors was observed primarily in animals receiving phenobarbital up to one year after the initiating doses.

IQ has been shown to induce unscheduled DNA synthesis in rat hepatocytes. The formation of DNA adducts of IQ, MeIQx and PhIP have been demonstrated in rhesus monkeys and Fischer 344 rats (IQ and PhIP only) as well as in NIH 3T3 and rat liver epithelial cells. The latter involved preparation of recombinant retroviruses containing the mouse liver P450IA2 gene. These cells, which constitutively express the gene, were shown to metabolically activate IQ and MeIQx thereby leading to high levels of mutation in *Salmonella* TA98 and alkylation of the mammalian cellular DNA. The activation was shown to be P450-dependent by classical methods. Interestingly, cells transduced with viruses containing the P450IA1 gene inhibited the mutagenic activity of IQ but greatly enhanced that of benzo(a)pyrene 9,10 dihydrodiol. These cells will be useful in evaluating the effects of different molecularly defined cytochrome P450s on metabolism, mutagenicity, and carcinogenicity (22).

Other studies supported in the area of Nutritional Carcinogenesis are diverse but can generally be grouped as research that investigates the dietary or nutritional effect(s) of various agents on cancer etiology. Such studies have included the effects of the methylxanthines (caffeine, theobromine, and theophylline), high and low fat diets, different dietary fat sources, caloric restriction, ethanol, metals, etc. Highlights of some of these studies are presented below.

Caffeine (1,3,7 trimethylxanthine) is widely consumed in coffee, colas, tea, and chocolate. It is also used as an additive in many prescription and nonprescription medications. However, the scientific literature concerning 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 methylnitrosourea (MNU) and 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 drinking water on both the initiation and promotion phases of cancer were also examined. Consumption of moderate and high doses of caffeine and caffeinated coffee during initiation significantly reduced the multiplicity of DMBA-induced rat mammary carcinoma and benign fibroadenomas but did not affect the tumor frequency or latency period. There were no observed effects on MNU treated rats. When ingested during the promotion phase, caffeine and coffee had no significant effect on DMBA tumor multiplicity. Similar results were obtained with chemically-defined diets containing standard or high levels of unsaturated fat. The latter studies provide further evidence that the promotion phase may be modulated by high (20%) corn oil diets fed to rats during this phase compared to standard level (5%) diets, 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 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 drinking water resulted in an increase in mammary carcinoma multiplicity but did not affect 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, 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 coffee consumption can significantly influence chemical carcinogenesis in the mammary gland of female rats and mice; however, this effect is dependent upon the dose-level, duration, and time-span of caffeine administration as well as the animal model being 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 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, mammary glands derived from caffeine-consuming mice were more responsive *in vitro* to mammotropic hormone stimulus than were mammas derived from control mice. It has been suggested that one mechanism by which caffeine may modulate rodent mammary tumorigenesis is by altering, systemically, secretion of a hormonal factor or factors affecting the developmental growth of normal and neoplastic mammary epithelium. The mechanisms by which caffeine modulates rodent mammary gland tumorigenesis will be further explored (31).

Several epidemiological studies have demonstrated that a high intake of dietary fat is generally correlated with an increased colonic cancer risk in humans. Recent work by another group of researchers has shown that in the two-stage skin carcinogenesis model with dimethylbenzanthracene (DMBA) and tetraphorbol acetate (TPA), a calorie-balanced but fat-restricted diet markedly increases the tumor latency period, while a high fat diet decreases the latency below control diet. Carbohydrate restriction had a marginal effect on tumor latency but maximal effect on leanness of body mass. The mechanism seems to be linked to changes in levels of protein kinase c (PKC), a cellular TPA receptor, as PKC levels parallel changes in tumor latency. Increased PKC occurs in groups with shorter latency. There also seems to be a decrease in the *c-myc* oncogene product in skin from fat-restricted mice, but further elucidation of these results is necessary (5).

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 polyunsaturated and saturated fats consumed. Sources of dietary fats containing high levels of unsaturated fatty acids, such as corn 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 or coconut oil, had no tumor-enhancing effect. Although 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.

The effects of caloric restriction on mammary and colon tumor formation is also being investigated in a DMBA model. Epidemiological studies have shown a marked linkage with fat intake and human breast cancer development. Comparisons among international cohort groups also show a correlation for colon cancer. These findings have been mirrored in animal studies when high fat or marked caloric restriction was tested. Work by another grantee has generated data linking a calorie-restricted diet with changes in receptor levels specific for tumor growth factor, insulin-dependent growth factor (IGF), and epidermal growth factor (EGF) isolated from tumor tissue directly, in the various dietary groupings. Receptor

mRNAs for EGF were also detected in mammary tumors from the different groups. Caloric restriction also depresses plasma levels of insulin and IGF-I but not IGF-II or EGF. Studies are planned to measure the effect of insulin and recombinant IGF-I on DMBA-induced tumors in calorie-restricted rats. It is expected to increase the tumor latency and bring tumor frequency closer to control levels (14).

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, investigators on one grant 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. It was found that a carbon-carbon double bond adjacent to an oxidized group is the minimal requirement for 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 either by saturation of the hydrocarbon backbone, separation of the oxidized group from the double bond by a methylene group, or by 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. Enzyme involvement with the activation of the fatty acids has been ruled out. Further studies of the metabolic fate of the active compounds are being pursued (6).

A great deal of effort has been expended studying a group of fecal metabolites, the fecapentaenes, which are responsible for about 90% of the mutagenicity observed in human feces. This mutagenic class of compounds is excreted in over 75% of the North American population. The original hypothesis was that these compounds were responsible for human colonic carcinomas. Incubation of bile with feces gives very high levels of mutagenicity, and at least one source for formation is via certain strains of "Bacteroides." However, the feces supply the precursor molecules prior to microbial metabolism. The active compounds, which have been synthesized in bulk for mechanistic and bioassay-type studies, consist of a glycerol molecule with a 12 or 14 carbon pentaenyl group (5 conjugated double bonds) attached to the number one carbon with an ether linkage. These compounds are quite unstable under aerobic conditions, making work on the metabolism of fecapentaenes and identification of the plasmalogen precursors extremely difficult. Epidemiological studies initially supported the hypothesis of colonic carcinoma linkage, as fecapentaene levels in South African populations paralleled colon cancer incidence. Animal studies, however, have failed to show carcinogenesis in a number of test systems. Only an infant mouse study gave weakly positive findings. Further epidemiological studies completed under one grant have now shown that fecapentaenes and their precursor pentaene plasmalogens are excreted in significantly lower amounts in patients with colon cancer. Work is continuing on the study of metabolism of fecapentaenes and their possible chemopreventive role (32).

NUTRITIONAL CARCINOGENESIS

GRANTS ACTIVE DURING FY91

INVESTIGATOR/INSTITUTION/GRANT NUMBER

TITLE

- | | |
|---|---|
| 1. ADRIAN, Thomas E.
Creighton University
5 R01 CA44799-02 | Cholecystokinin Effect on
Pancreatic Growth and Tumors |
| 2. AUSMAN, Lynne M.
Tufts University
5 R01 CA42352-05 | Nutritional Influences on Colon
Cancer in the Tamarin |
| 3. BARCH, David H.
Northwestern University
7 R01 CA40487-06 | Role of Zinc and Ethanol in
Esophageal Carcinogenesis |
| 4. BASEL, Richard M.
Lebensmittel Consulting
1 R43 CA54039-01 | Safer Flame Broil Grill to
Reduce Carcinogenesis |
| 5. BIRT, Diane F.
University of Nebraska Medical Center
5 R01 CA42986-05 | Dietary Fat, Calories, and Two-
Stage Tumorigenesis |
| 6. BULL, Arthur W.
Oakland University
5 R01 CA47912-04 | Hydroperoxide Metabolism by
Colonic Mucosa |
| 7. DAVIES, Donald S.
University of London
5 R01 CA40895-05 | The Metabolic Fate of Mutagenic
Amines in Animals and Man |
| 8. FELTON, James S.
University of Calif-Lawrence Lvrmr Nat Lab
5 R01 CA40811-05 | Quantification of Cooked-Food
Mutagens by Immunoassay |
| 9. GOLDFARB, Stanley
University of Wisconsin Madison
5 R01 CA51549-05 | Biology of the Mouse Hepato-
carcinogenesis |
| 10. HAWRYLEWICZ, Ervin J.
Mercy Hospital and Med Ctr (Chicago)
5 R01 CA50264-02 | Diet Methionine--S-ADO,
Polyamines and Mammary Tumors |
| 11. IP, Margot M.
Roswell Park Memorial Institute
2 R01 CA33240-08 | Lipid Modulation of Mammary
Proliferation and Differenti-
ation |
| 12. ISSENBERG, Phillip
University of Nebraska Medical Center
5 R01 CA43589-03 | Cruciferous Vegetables/
Endogenous Nitrosamine Forms |

- | | |
|---|---|
| 13. KINGSTON, David G.
Virginia Polytechnic Inst and St Univ
5 R01 CA40821-06 | Anaerobic Metabolism of Mutagens
in Human Foods |
| 14. KRITCHEVSKY, David
Wistar Institute of Anatomy and Biology
5 R01 CA43856-03 | Caloric Restriction in Tumor
Promotion: Mechanisms |
| 15. LEA, Michael A.
University of Medicine & Dentistry of NJ
5 R01 CA46442-03 | Nucleotide Metabolism and
Promotion of Carcinogenesis |
| 16. MC COY, George D.
Case Western Reserve University
2 R01 CA32126-04A4 | Role of Ethanol in the Etiology
of Head and Neck Cancer |
| 17. NEWBERNE, Paul M.
Mallory Institute of Pathology Fdn
5 R01 CA40080-05 | Zinc Deficiency and Related
Factors in Esophageal Cancer |
| 18. NEWBERNE, Paul M.
Mallory Institute of Pathology Fdn
5 R01 CA46288-03 | Lipotrope Deficiency and Liver
Cancer in Rats |
| 19. POUR, Parviz M.
University of Nebraska Medical Center
5 R01 CA43550-03 | High Fat Diet in Experimental
Prostatic Cancer |
| 20. REDDY, Bandaru S.
American Health Foundation
5 R01 CA37663-06 | Calories, Energy Expenditure and
Fish Oil in Colon Cancer |
| 21. SARKAR, Nurul H.
Medical College of Georgia
5 R01 CA45127-05 | Effect of Diet on Murine Mammary
Tumorigenesis |
| 22. SCHUT, Herman A.
Medical College of Ohio at Toledo
5 R01 CA47484-03 | Carcinogenesis of Heterocyclic
Amines |
| 23. SELL, Kenneth W.
Emory Univ School of Medicine
1 R13 CA54787-01 | Conference: Research in Human
Cancer--Nutrition and Cancer |
| 24. THOMPSON, Henry J.
Cancer Research Center
1 R01 CA52626-01 | Exercise and Breast Cancer AMC
Prevention |
| 25. TOTH, Bela
University of Nebraska Medical Center
2 R01 CA44075-04A1 | False Morel Hydrazines: Carcino-
genesis and Chemistry |

26. WAINFAN, Elsie
New York Blood Center
5 R01 CA47600-03
Diet Cancer and Oncogene
Expression in Inbred Mice
27. WALKER, Bruce E.
Michigan State University
5 R01 CA39456-03
Transplacental Carcinogenesis
and Diet
28. WATTENBERG, Lee W.
University of Minnesota of Mnpls-St Paul
5 R01 CA50980-02
Carcinogenicity of Fecapentaenes
29. WEISBURGER, John H.
American Health Foundation
5 R01 CA42381-05
Mechanism of the Carcinogen
Aminomethylimidazoquinoline
30. WEISBURGER, John H.
American Health Foundation
5 R01 CA45720-04
Identification of Carcinogens
31. WELSCH, Clifford W.
Michigan State University
5 R01 CA37613-06
Caffeine and Experimental
Mammary Gland Tumorigenesis
32. WILKINS, Tracy D.
Virginia Polytechnic Inst and St Univ
2 R01 CA23857-13
Relationships of Fecal Mutagens
to Colon Cancer

ANNUAL REPORT OF
THE RADIATION EFFECTS BRANCH
CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1990 through September 30, 1991

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), program project grants (P01), small business innovative research (SBIR) grants (R43/44), 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 110 extramural research activities with an annual budget of 19 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 conducted during the years 1951-1963. A working committee consisting of relevant expertise within and outside of the government 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. Extensive efforts have been expended in the identification, recovery, acquisition and analyses of a wide range of data relevant to the required exposure and dose reassessments; these efforts have been carried out with the assistance of staff expertise acquired for this purpose and via interagency agreements. 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 then-existent 3,071 counties within the continental United States for each of the tests.

Since the primary exposure of the public to Iodine-131 following the tests 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. It is anticipated that the exposure estimates will be completed within a year.

The Mechanisms of Radiation Damage and Repair Program includes, but is not limited to, studies on molecular and cellular changes resulting from exposure to ionizing and non-ionizing (principally ultraviolet [UV]) 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 UV) 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.

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 108 grants (77) support investigations relating to mechanisms of radiation damage and repair of cellular DNA, 60 of

which investigate the effects of exposure to ionizing radiation and 17 of which study the consequences of exposure to ultraviolet radiation, microwaves and ultrasound. Twenty-four grants and two contracts fund studies in radiation carcinogenesis; research addressing radiation risks and the compilation and assessment of information is the objective of six grants. A Request for Applications (RFA) entitled "Molecular Analyses of Radiation-Induced Genetic Damage" was issued in response to guidance received during a workshop held earlier on the same topic. Twenty-four proposals were submitted in response to the RFA.

TABLE I
 RADIATION EFFECTS BRANCH
 (Extramural Activities - FY 1991 - Estimated)

	No. of Contracts/Grants	\$ (Millions)
Research Contracts	2	0.10
Research Grants	108	19.28
Traditional Research Grants (R01) (77 grants; \$12.16 million)		
Conference Grants (R13) (2 grants; \$0.01 million)		
FIRST Awards (R29) (13 grants; \$1.24 million)		
Program Project Grants (P01) (2 grants; \$2.25 million)		
Small Business Grants (R43/44) (3 grants; \$0.40 million)		
Outstanding Investigator Grants (R35) (2 grants; \$1.33 million)		
MERIT Awards (R37) (9 grants; \$1.89 million)		
AREA Grants (R15) (0 grants; \$0.00 million)		
TOTAL	110	19.38

TABLE II
 RADIATION EFFECTS BRANCH
 (Contracts and Grants Active During FY 1991)

	FY 91 (Estimated)			
	CONTRACTS		GRANTS	
	No. of Contracts	\$ (Millions)	No. of Grants	\$ (Millions)
Radiation Mechanisms and Carcinogenesis	0	0.00	106	19.13
Office of the Chief	<u>2</u>	<u>0.10</u>	<u>2</u>	<u>0.15</u>
TOTAL	2	0.10	108	19.28

Mechanisms of Radiation-Induced DNA Damage and Its Repair: The REB provides support for basic research on the mechanisms of DNA damage and repair, mutagenesis and malignant transformation induced by the various forms of radiation. This program element provides basic information on the mechanisms common to radiation-induced carcinogenesis and mutagenesis and helps to provide a rationale for the carcinogenic effects seen following exposure to radiation. This program also seeks to provide information that has practical significance in attempting to identify cellular and molecular markers for radiation-induced cancer; such markers ultimately may be useful for radiation protection in both the workplace and home environment.

A. Ionizing Radiation: Ionizing radiation interacts with cellular DNA to produce an array of chemical and physical damage to the individual DNA base pairs and to the integrity of the individual DNA strands. The mutations that arise from repair and misrepair of radiogenically damaged DNA likewise include a wide range of effects from localized base changes (point mutations and short deletions) to very large molecular scale deletions/rearrangements involving hundreds of thousands or even millions of DNA base pairs. In quantitative terms, most of the mutations induced by exposure of mammalian cells to ionizing radiation appear to be mainly deletions/rearrangements of substantial lengths of DNA (i.e., kilobase length or greater). Several lines of evidence suggest that unrepaired double strand breaks (dsb) are the main determinants of lethality in irradiated mammalian cells. The radiogenic DNA lesions involved in mutagenesis and cell transformation are less well understood. However, evidence is growing that repair and/or misrepair of dsb may also be important as a cause of mutations and, more generally, as an initial step leading to generalized genetic instability in irradiated mammalian cells.

The mechanisms of repair for radiation-induced DNA damage appear to be complex and involve many components. Excision repair, a major pathway for the repair of the DNA adducts produced by chemical mutagens and non-ionizing radiations (e.g., UV), participates in the repair also of DNA damage produced by ionizing radiations. In addition, major contributions to the repair of radiogenic DNA-damage result from the action of other, less well understood pathways. While the capacity to repair dsb is an important factor in the overall process, there also is evidence for important contributions to DNA repair by processes that appear to operate on large blocks or domains of DNA, recognizing and targeting secondary and tertiary structures that arise during the repair (or subsequent to repair) of primary local lesions (e.g., dsb, damaged basepairs). Processes involved in large-scale events appear to be complex and may involve several steps: (a) repackaging of DNA after repair of local lesions, (b) poorly understood amplification of DNA in response to damage from ionizing radiation, and (c) recombinational reduction of amplified sequences in subsequent cell divisions.

Major themes being addressed in this program element include: (1) studies that deal with the physical and genetic description of the mutations that arise from radiogenically damaged DNA, and (2) cellular and biochemical mechanisms by which cells cope with radiogenically damaged DNA.

One line of experimental work being carried out by several investigators is providing evidence that radiation-induced DNA damage and its repair involves structural and biochemical events involving domains of DNA on a very large molecular scale. One investigator has provided evidence for the preferential formation of DNA damage in the form of x-ray induced DNA-protein cross-links (DPC) and single strand DNA breaks in very large, structurally open or expanded domains of the genome containing transcriptionally active genes. The proteins that contribute to DPC

appear to be a restricted subset of nonhistone proteins which are minor components of the nuclear matrix. During the repair process, the protein linked to DNA in regions of transcriptionally active areas is removed, following which other expanded areas of the genome are bound, and both actively transcribing genes and newly synthesized DNA are preferentially broken and cross-linked with protein in the nuclear matrix. 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 by the structural features of natural (condensed) as opposed to isolated (expanded) chromatin. Recently, additional experiments provided evidence for the importance of chromatin compaction in determining the extent of gamma-radiation-induced DNA-protein cross-links. Chromatin expanded by sedimentation in buffers of decreased magnesium ion concentration becomes more sensitive to gamma radiation. Recondensation of fully expanded chromatin by the addition of magnesium ions markedly reduces the radiosensitivity. The histone content and nucleosome organization of the chromatin are not changed by these procedures; rather, the loss of higher order structure apparently exposes new chromatin regions to attack by radiation-generated radicals. The association of chromatin loops with the nuclear matrix also is not altered by the experimental protocol, although this region appears to be the most sensitive to DPC. The proteins most frequently found cross-linked to DNA are not those generally expected to be cross-linked, i.e., histones and topoisomerase II, but they are a subset of the nuclear matrix proteins which can be identified in highly purified DNA preparations by iodination tracer techniques. These results underscore the complexity of cellular responses and show that the DNA target genome is differentially sensitive to gamma ray exposure throughout its physical length, its position in the cell cycle and whether or not it is in a state of transcription.

In another study, a radiation sensitive CHO (xrs-5) cell line demonstrated significant changes in the structural organization of the nuclear periphery ultrastructure organization and increased resistance to biochemical digestion of chromatin to hydrolytic enzymes (e.g., DNase I) which appear to be correlated with the radiation sensitive phenotype in this cell line.

In other work, biochemical and genetic evidence has been obtained that DNA repair may be dependent on the secondary and tertiary structure of large transcriptional domains of at least 50-80 kilobases (kb) containing both transcriptionally active and inactive genes. The work provides evidence that the tertiary structure of chromatin domains can be factors in DNA repair of radiation-induced genetic damage and that repair may not necessarily be dictated by local transcriptional activity of individual structural genes alone.

Genetic and biochemical evidence also has been obtained implicating large-scale genetic effects as the predominant outcome of DNA damage from ionizing radiation. In one ongoing study, an investigator has used selectable hemizygous thymidine kinase (TK) genes in special CHO cell lines which are available in cells of different DNA repair capacities. Use of this system allows the detection of deletion mutations that extend into flanking regions of DNA and remove the essential genes and prevent detection by conventional X-chromosome based assays for mutagenesis. This investigator determined that the great majority of the x-ray induced TK mutations (i.e., conversion of TK+/- heterozygotes to the TK-/- double mutations) resulted from the loss of the entire TK locus and considerable flanking regions of DNA. A significant proportion of these deletion mutations were large interstitial deletions involving losses of several gene-equivalents of genetic material as shown by the concomitant loss of enzymatic activity in the neighboring,

non-essential galactokinase (gk) gene. The frequency of large-scale deletions was strongly affected by the cell's DNA-repair capacity showing 5-fold increase in the frequency of large-scale deletions in a dsb DNA-repair deficient mutant and more than a 3-fold increase in an excision-repair deficient mutant relative to that observed in the wild-type CHO parental cell line. Thus, biochemical defects in at least two DNA-repair pathways resulted in an increased frequency of radiation-induced deletions. The same investigator exposed these cell lines to alpha radiation from radon and its decay products, a treatment shown to induce irreparable DNA lesions which are independent of the DNA repair pathways. The percentage of induced TK mutants showing extensive deletions in these cells was similar to the highest frequencies observed for the DNA-repair-deficient strains exposed to x-rays. This leads to the interesting possibility that the presence of unrepaired radiation-induced DNA lesions is a sufficient condition for an increase in the frequency of multilocus deletion mutations.

Using a similar approach, a second investigator has made elegant use of a specially constructed cell line consisting of a human-hamster (CHO) cell line with a single human chromosome containing more than 100 physically mapped genes and DNA segments as markers for mutational events. This particular cell line (A1) can detect deletion mutations ranging in size from a few base pairs to as many as one hundred million base pairs by scoring for the disappearance of linked markers of known physical size. Since the A1 CHO cell line retains its wild-type complement of chromosomes, large-scale multilocus deletions of genetic material on the human chromosome are not lethal to the cell. This system was used to obtain quantitative data on the distribution lengths of deletions induced by several types of ionizing radiation delivered to cells over a range of doses and dose rates. Evidence so obtained showed that each of the several forms of ionizing radiation (x-rays, gamma rays, protons and neutrons) were considerably more mutagenic (>100-fold) than were estimates based on events occurring in single genes (e.g., HPRT). The majority of mutations created by ionizing radiations were very large interstitial deletions involving losses of several million base pairs of DNA. Large-scale deletions were favored over a wide range of doses (as low as 50 cGy) delivered at either high or low dose rates. It is noteworthy that these large radiation-induced deletions did not resemble either spontaneous mutations (mostly small deletions) or mutations induced by chemical mutagens that form DNA adducts and cause mainly point mutations or frameshifts.

One investigator is investigating the biochemical and genetic basis for DNA repair in a series of CHO DNA-repair deficient mutants selected by their sensitivity to ionizing radiation. None of these newly isolated DNA-repair mutants belonged to previously known genetic complementation groups for the repair of radiogenic lesions in CHO cells suggesting the discovery of new genes and increased complexity associated with the repair of radiogenic DNA damage in CHO cells. The kinetics of dsb rejoining was studied in one of the newly isolated mutants (XR-1). Cell extracts of the wild-type CHO cells demonstrate two kinetic components of dsb repair, one fast and one slow. The XR-1 mutant was shown to possess a wild-type fast repair component but lacked the kinetically slow component of dsb repair. Loss of the kinetically slow repair component was correlated with an increase in the frequency of chromosome aberrations in the XR-1 mutant. In addition, the XR-1 mutant was unable to repair dsb with blunt ends and only slowly rejoins dsb with staggered ends. This investigator suggests that XR-1 has at least two pathways involved in the slow component of dsb repair and that it is the efficiency of slow dsb repair that is the critical determinant of large-scale chromosomal aberrations. This work is contributing a growing body of evidence indicating that many pathways

cooperate in the repair of DNA damage induced by ionizing radiations and that, in some cases, they may differ from the more extensively studied mechanisms for DNA repair such as excision repair.

The complex and overlapping nature of the number and relationships between the various DNA-repair pathways is illustrated in work done by the same investigator who demonstrated that partial (leaky) mutants for poly(ADP-ribose)polymerase with about 25% of the wild-type enzyme activity showed increased sensitivity to killing by gamma rays, radiomimetic chemical mutagens, as well as a number of other chemical mutagens and DNA alkylating agents.

Using a similar approach, another investigator showed a high degree of cross-sensitivity between UV- and gamma-ray sensitive CHO mutants to both chemical mutagens and gamma-rays. One of the variant strains studied in detail (UV19) was found to be sensitive to UV, x-rays, mitomycin C, and alkylating agents. The common sensitivity of this mutant (and of several other radiation-sensitive mutants) to diverse mutagenic agents suggests that UV19 may lack a DNA-repair function that is not involved in repair of individual lesions to DNA but may function at another level in the repair process (e.g., unfolding of higher order chromatin structures in preparation for lesion repair or repair repackaging of DNA into higher order structures after repair). This work illustrates a growing trend in finding mutants defective in DNA repair that appear to fall outside the standard paradigms of excision repair and strand break repair in mammalian cells and suggests that the "classical" method of selection for defective mutants may yield new information on the repair of DNA damage to the highly complex mammalian chromosome.

In an important study on the relationship of dsb to the mammalian cell cycle, it was demonstrated that induction of dsb per dose of ionizing radiation (x-rays) was constant and that repair of dsb lesions proceeds with biphasic (fast and slow) kinetic components throughout the cell cycle. The repair time-constants were estimated for both the fast and slow components and the rates of dsb rejoining for the slow component was shown to be highly correlated with the appearance of chromosome breaks as demonstrated by premature chromosome condensation. These results (similar to the phenotype of the XR-1 CHO mutant described above) suggest that failure of the cell to carry out the slow repair component of dsb increases the frequency of chromosome breakage and may also lead to cell lethality. The relationship of dsb repair to induced mutations is unknown. However, as discussed above, the correlation of irreparable dsb induced by alpha irradiation and the observed increase in deletion and rearrangement type mutations suggests that the slowly repaired dsb lesions may be important both in mutagenesis and as determinants of cell lethality.

In another study, expression of specific DNA-repair or DNA-repair-associated genes damaged by exposure to ionizing radiation was followed throughout the mammalian cell cycle. The repair gene mRNA patterns were distinguishable with the mRNA levels for two genes (ERCC1 and XRCC1) exhibiting complex variation throughout the cell cycle and the mRNA level for a third gene (ERCC2) remaining essentially constant. These findings represent the first successful attempt at analysis of the expression of DNA repair genes specific for DNA damage from ionizing radiation. It is of interest that the biochemical and enzymatic mechanisms by which the gene products of these DNA repair genes are largely unknown and still under investigation in a number of laboratories. This work illustrates a growing trend in the use of gene probes to follow the expression and function of DNA repair genes in relation to the mammalian

cell cycle and repair of DNA-damage and demonstrates that such analyses can proceed in the absence of a detailed functional or mechanistic knowledge of genes involved.

The possible risk of genetic damage and radiation-induced cancer due to chronic low dose-rate exposure to ionizing radiation is an important issue in radiation biology. In one study using the Chinese hamster model it was determined that low dose radiation from internally deposited gamma-ray emitters followed by exposure to a single acute external dose of 2 Gy of gamma-rays resulted in greater than expected cytogenetic damage in the form of sister chromatid exchanges (SCE) produced in the liver. The number of SCEs observed was more than twice the expected response based on an additive model and suggests that low-level exposure from internally deposited gamma-emitting radionuclides can significantly alter the cells responsiveness to subsequent acute doses of ionizing radiation. Continuing studies are investigating the combined effects of exposure of the animal to low doses of internally deposited gamma- or alpha-emitters followed by acute external doses of gamma rays. The results thus far are part of a growing body of evidence that exposure of mammalian cells to low levels of ionizing radiation results in a significant increase in the capacity of the mammalian cell to respond (albeit with a number of phenotypes) to subsequent, higher doses of ionizing radiation. It is too early to know whether the various phenotypes (e.g., increased resistance to radiation) observed in cells preexposed to low doses of ionizing radiation and then challenged with much higher acute doses that have been observed in this and other studies are related by underlying biochemical and genetic mechanisms. However, the use of the genetic and molecular techniques described above for the analysis of DNA repair may provide a means for analyzing the mechanisms involved.

For decades the mechanism(s) by which ionizing radiation actually kills cells or induces malignant transformation has remained elusive. Even the direct interaction of x-rays or gamma rays with DNA remains controversial. The presently prevailing hypothesis that random interaction of x-rays with DNA results in electrons and holes (solitons) which migrate through the stacked bases of DNA until they are selectively trapped by thymine and guanine bases has now been challenged. An investigator using innovative quantitative electron spin resonance microtechniques recently has shown that direct deposition of energy from ionizing radiation to DNA may not be random; it appears to be selectively captured by the cytidine nucleoside. This suggests that because there is a structural/chemical specificity for the direct capture of this deposited energy, as opposed to general interaction with organic matter, it may be feasible to design and synthesize specific drugs that would significantly limit either the initial capture of the energy or to chemically "repair" the damaged site.

The problem of how x-rays kill cells has recently been studied by two investigators using the same technique but with different cell models. It is generally believed that cell death results largely from unrepaired DNA double strand breaks, but the evidence is controversial and not conclusive. Two particularly controversial areas include (1) the proximity required before the cell's response to two single strand breaks on opposite strands of DNA is indistinguishable from its response to a double strand break, and (2) the number of double strand breaks required for lethality. One of the investigators has recently developed a mathematical model that predicts the relationship between clonogenic cell survival, unrepaired DNA double strand breaks and chromosome aberrations. The use of viscoelastometry, a very sensitive technique for measuring DNA conformation (double stranded versus single stranded DNA) and molecular weight (DNA strand breaks), enabled this investigator to observe that the mammalian chromosome is circular. The average molecular weight of the chromosomal DNA was found to be 3×10^{10} daltons (5×10^7 base pairs); this agrees

with the independently estimated size determined from radiation target theory and the D₃₇ double strand break yield following exposure to 3 Gy of x ray. The significance of these results is that technically it is now feasible to establish whether or not DNA double strand breaks account for cell lethality, to test the proposed model and, if successful, to resolve an important question.

A second investigator used viscoelastometry to study DNA double strand breaks in the radiation resistant eukaryote *Tetrahymena pyriformis*. This protozoan shows over 85% cell survival after split doses totalling 100 Gy (10 krad) x-irradiation. Unlike other eukaryotic cells studied to date, repair of DNA double strand breaks showed complex kinetics indicating several waves of repair over a period of a few hours followed by a significantly more substantial decline in the number of double strand breaks than observed in other eukaryotic cells. This kinetic pattern would repeat itself several times during the two day post-irradiation incubation of cultures. Further characterization of this unusual response to x-irradiation damage indicated the following additional results: (1) Caffeine has little effect on the repair process or growth of the organism indicating that the repair response probably does not involve the post-replication repair pathway studied in mammalian cells. (2) Repair is drastically inhibited by the addition of 3-aminobenzamide, an inhibitor of the enzyme polyriboadenosinediphosphate synthetase, but the direct addition of NAD⁺ or the precursor tryptophan did not markedly alter the inhibition of or stimulate repair processes. This indicates that repair processes involving poly rADP synthase probably have a major role in the radiation resistance of *Tetrahymena*, a role significantly greater than that observed in mammalian cells. (3) Topoisomerase II involvement and protease activated repair processes were not likely major contributors to repair because novobiocin and antipaine had only minor effects. (4) A small but significant increase of protein synthesis in the first ten hours after irradiation and the appearance of a discrete size of DNA molecular fragments post-irradiation which later disappear suggest that inducible DNA repair events and DNA replication are related to the facile repair of the radiation-damaged DNA in the organism. The significance of these studies is that they provide an approach to understanding the poly rADP synthase involvement in the mammalian DNA repair pathway which so far has eluded clarification, and suggests that it may be possible to modulate DNA repair prior to radiotherapy protocols so as to increase the efficiency of this mode of cancer treatment.

The unrecognized presence of experimental artifacts is a ubiquitous problem in science. Artifacts can both conceal important information needed to draw the correct conclusions or they can introduce information which confounds or "overcomplicates" the correct conclusion. An investigator engaged in developing highly sensitive monoclonal antibody-based ELISA assays and electrochemical techniques to quantify specific purine and pyrimidine base lesions in DNA at the femtomole concentration (10^{-15} molar) found that routine procedures for DNA purification and analysis taken from widely used molecular biology laboratory manuals introduced significant levels of DNA base lesions. These lesions were of the oxidative type which also are introduced by ionizing radiation and include 8-oxoguanine, thymine glycol and dihydrothymine. This is important because of the growing need to determine the levels of spontaneous or background mutations, their relationship to specific lesions in the DNA and the identification of agents that induce these lesions at low or chronic doses.

The concept that the type or category of DNA damage, i.e., its chemical specificity, is the overriding component that determines its deleteriousness to the cell is now being challenged by the competing concept that the most important component is not

the type of damage but the "density" of damage, i.e., multiple damaged sites near one another in DNA are significantly more deleterious than the same amount of damage randomly distributed throughout the DNA. Recently an investigator proposing this alternative view has marshalled quantitative evidence indicating that various lesions close together can lead to double strand breaks either spontaneously or by cellular repair/misrepair processes. For example, it was shown that the location of previously believed "innocuous" or easily repaired lesions such as single strand breaks and apurinic or apyrimidinic sites, when near one another, can easily lead to double strand breaks which are far more detrimental to the cell. This idea is particularly relevant to two situations. First, different ionizing radiations deposit energy in different concentrations and in different "volume" sizes. Thus, energy deposited in higher concentrations and/or in a larger continuous volume is likely to be more detrimental because damaged sites will tend to be closer together. Second, in cellular states in which DNA segments are in an extended conformation, damaged sites are more likely to be closer together for a given radiation energy deposition because the DNA without the highly condensed chromosome structure is more accessible to a given amount of the reactive agents produced compared with compact DNA; this increased sensitivity of the extended state of DNA is important for cells that are proliferating and replicating DNA in the S phase of the cell cycle and for the segments (genes) in DNA which are being transcribed into messenger RNA in any phase of the cell cycle. This work has important implications for providing an experimentally testable mechanistic hypothesis for relating the genetic toxicity of ionizing radiation to the physical state of DNA and the mammalian cell's position in the cell cycle at the time of insult.

The amount and complexity of the known genetic information involved with DNA repair and the maintenance of its structural integrity grows each year. There are at least 35 genes associated with the processes of repair in yeast. Some of these genes seem to operate in complex ways, such as RAD3 having helicase, ATPase and damage recognition activities, while other genes seem to operate in simple ways, such as the glycosylases and insertases which simply cleave damaged or insert undamaged bases, respectively. Recently, two gene functions with important consequences have been observed. First, the RAD9 gene has been cloned and sequenced and shown to be required for the G_2 arrest of yeast cells in response to DNA damage. This G_2 arrest appears to be a characteristic early response shared by all eukaryotic cells whose DNA is damaged by ionizing radiation and is thought to be important because it maximizes the repair time and, therefore, the survivability of cells that have sustained substantial genomic damage and are about to undergo cell division. Conversely, eukaryotic cells that are defective in showing the radiation-induced G_2 arrest (e.g., certain yeast and human cell lines) are highly susceptible to killing by ionizing radiation. Second, in other work done by the same investigator, the very complex recB gene which controls most recombination and repair of x-ray-induced DNA double strand breaks and x-irradiation frameshift mutagenesis also has an essential function in long deletion mutagenesis (i.e., highly characteristic of mutations induced by ionizing radiations). These long deletions appear to be a consequence of the recB gene activity, so defective recB organisms do not undergo long deletion mutagenesis. This component of mutation/repair processes is especially significant because long deletion mutagenesis is thought to be an essential process for cancer initiation and progression in mammals.

B. Ultraviolet Radiation: The Radiation Effects Branch is concerned with mechanisms of ultraviolet radiation mutagenesis and malignant transformation. Information for this pervasive and potent environmental carcinogen is useful to provide both insight into the mechanisms of carcinogenesis and the rationale for the

carcinogenic effects seen following exposure to radiation. Also, UV radiation 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.

In recent years RNA has been implicated in unexpected and important cellular functions including transcriptional control and autonomous catalytic activities. Another unanticipated function has been proposed by an investigator this past year in which RNA is the agent by which human cells detect and initiate the response to UV exposure. He has discovered several small molecular weight nucleolar RNA species, U1 thru U5, whose synthesis is extremely sensitive to UV light inhibition. One species in particular, U1, is about 20 times more sensitive to UV radiation than any other known cell effect including general DNA transcription and DNA replication inhibition. There are two types of inhibition of these RNA species by UV and they appear to be separate reactions: an immediate inhibition which requires about 60 J/m² and a delayed inhibition of one to two hours which requires only 7 J/m². Evidence that the reactions are separate mechanisms includes: (1) the fact that the inactivation curves are different; (2) increasing doses of UV results in an increased inhibition resistant fraction in the immediate inhibition reaction but not in the delayed inhibition reaction; (3) species U2 through U5 show similar heterogeneity, even as to the inhibition of synthesis of regions of the RNA molecules. Even though these inhibitions are likely to be involved in the elicitation of repair responses, the ability to repair cyclobutane dimers is not implicated in the inhibition itself because cells that don't repair these lesions, xeroderma pigmentosum cells, group A, also show the same UV responses. The investigator's observation that these RNA species bind to ribosomal RNA and the idea that specific classes of proteins are synthesized on specific groups of ribosomes suggest that translational control can be exerted with both great sensitivity and specificity by these novel RNA molecules.

An investigator has now cloned a human DNA repair gene from a genomic cosmid expression library by using hybridization subtraction techniques to isolate the gene that is responsible for xeroderma pigmentosum, complementation group A. While characterizing the gene he found that the isolated gene only partially complemented the group A genetic defect and it showed no complementation in other groups. To further establish that this is the appropriate gene, the investigator attempted to map the gene to chromosome 9, where a gene involving group A complementation had been shown to reside. However, the results indicated this activity resided on chromosome 8 instead. Based on this data, together with the data of partial complementation with chromosome 9, the investigator concluded that at least two genes are responsible for the genetic defect responsible for xeroderma pigmentosum, group A. These results provide molecular support for a hypothesis proposed in 1985 called co-recessive inheritance. This hypothesis postulates that xeroderma pigmentosum genetic defects are expressed only if the individual is homozygous or hemizygous for defective alleles at more than one of a specific set of genetic loci.

The study of ionizing radiation carcinogenesis strongly suggests that production of large DNA deletions is associated with efficient induction of cancer. However, UV is an effective carcinogen but is not generally associated with production of large DNA deletions. While studying the relationship between the processing of DNA damage and genetic recombination, an investigator showed that a common genetic process could result in large deletion induction by UV. By using gene transfer techniques and mammalian cells defective in a gene involved with homologous recombination, the

investigator could directly determine whether a specific DNA damaging agent invokes the function of a specific gene product. In this case the investigator asked if mammalian cells damaged with UV light invoke the process of homologous recombination for increased survival. It was found that UV light stimulated homologous recombination, presumably by the specific DNA damage, and that when the homologous recombination pathway was deficient nonhomologous recombination occurred resulting in large DNA deletions. Thus, UV carcinogenesis may come about by first damaging the recombination pathway and then inducing large deletions, analogous to ionizing radiation, which bring about the multiple changes necessary for malignant transformation.

For years controversy has engulfed the field of UV damage regarding the identification of the various lesions, how deleterious they are to the cell and what repair pathways handle which lesions. Finally, an investigator has assuaged some important aspects of the confusion by simply demonstrating that a substantial fraction of the differences arise from comparing different species, different end points and different conditions. For example, he found that both the (5-6)-cyclobutane pyrimidine dimer and the pyrimidine (6-4)-pyrimidone are mutagenic; previously several reports indicated that the (6-4) was highly mutagenic, whereas the (5-6) was, at most, weakly mutagenic. This investigator found that the relative roles of these two lesions depends on: (1) the end point studied; when the lesions are introduced into shuttle vectors containing the aprt or gpt genes, both the (5-6) and the (6-4) lesion are highly mutagenic; (2) the UV fluence used; as fluence is increased the relative mutagenicity of the (5-6) lesions appears to decrease as the lesion concentration approaches equilibrium (the inducing lamp also cleaves the (5-6) dimers at a somewhat slower rate), whereas the mutagenicity of the (6-4) lesions appears to increase because they accumulate linearly; thus, the relative amounts differ with different UV light fluences; (3) the cell lines and specie used; as expected, different established cell lines from the same specie (e.g., human HeLa and low passage diploid keratinocytes) or different species (e.g., Chinese hamster cells and insect cells) differ in specific end points and have different repair capacities which must be taken into consideration.

Efforts to clone DNA repair genes have been very slow and difficult. A major block has been an adequate selective system for the gene of interest coupled with techniques for DNA isolation. An investigator has recently devised an innovative general procedure using frog cell lines to select human genes. After isolating solar radiation sensitive frog cell lines the investigator transfects human genomic DNA from repair proficient cells into the sensitive frog cells and isolates radiation resistant cell lines. The presence of human DNA in the resistant lines can be easily tested by using a probe to Alu sequences which are specific to and distributed widely in the human genome. Further, the human DNA is readily recovered and cloned by polymerase chain reaction using Alu primers and the amplified DNA cloned in an appropriate vector. The cloned DNA can then be introduced back into sensitive frog cells to ensure its identity and the gene can be readily characterized.

Radiation Carcinogenesis: This program includes basic studies on the molecular mechanisms of radiogenic cell transformation and cancer as well as research that is oriented towards practical radiation protection and selected epidemiological studies. 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. Major program areas

include studies of the molecular mechanisms of radiation-induced cell transformation, including the role of mutations and 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 cell cycle dependence of radiation-induced neoplastic transformation continues to be an important area of research in radiation biology. One investigator has made an important contribution to this area of research by showing that a human hybrid cell line (HeLa X skin fibroblasts) is highly and coordinately sensitive to both gamma-ray induced killing and the induction of neoplastic transformation, particularly in the G₂ and M phases of the cell cycle. This work has continued with the characterization of the cellular and molecular changes which parallel radiation (neutron or gamma-ray) induced transformation in this cell line. At the cellular level, radiation-induced expression of intestinal alkaline phosphatase was shown to be uniformly associated with tumorigenicity in the human hybrids. At the molecular level, a consistent pattern of damage to chromosome 11 was observed near the locations of a putative suppressor gene and the regulatory gene for intestinal alkaline phosphatase expression. These results may have important implications on the possible role of suppressor genes in the etiology of radiogenic cancer in human cells and they may provide a biochemical and genetic marker of potential importance for detecting human radiogenic cancer.

The effects of acute low-doses of ionizing radiation (e.g., less than 5 rads of x-rays) on the subsequent response of human lymphocytes to high acute doses of radiation (e.g., greater than 100 rad x-rays) which result in cell killing and transformation has been studied. Depending on the individual donor, pretreatment of lymphocytes with a small radiation exposure resulted in a spectrum of effects from no effect, to either a decreased (protective) effect or an increased (synergistic) effect on cell killing when subsequently challenged by higher doses of ionizing radiation. Unexpectedly, the low-dose exposure alone resulted in detectable cell killing in a subpopulation of cells from certain donors suggesting genetic variability in DNA-repair from apparently normal individuals in terms of their capacity to respond to low doses of ionizing radiation. These results provide further evidence that low dose exposures to ionizing radiation can alter an individual's capacity for repair of DNA damage that is caused by a second, much higher challenge dose of ionizing radiation and suggest the existence of inducible DNA repair functions in humans exposed to low doses of ionizing radiations.

In another study, evidence was obtained indicating a high level of radiation resistance in two human cell lines transformed with a plasmid containing the SV40 early (T) region. The radioresistance was associated with the loss of genetic material also found on chromosome 11. Partial deletions of chromosome 11 associated with radioresistance appeared to be located in the same chromosomal regions as genes that may be involved in the control of senescence in these cultured human cells.

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 characterize the genetic components associated with the susceptibility of the mouse to x-ray-induced leukemia. Results have been obtained showing that the *ril-1* locus on chromosome 15 is a critical genetic determinant of the susceptibility of the mouse to leukemia induced by x-rays. Physical distances in the chromosomal region containing this locus have been mapped with restriction fragment polymorphisms within the immediate vicinity of the locus, and a preliminary

molecular map of the region has been constructed with physical distances of 60 kilobases to several million DNA base pairs (corresponding to genetic distances of one to several centimorgans based on recombination). Numerous congenic mouse strains have been identified which have different alleles of the *ril-1* gene which therefore can be used for relating the gene linkage data to biochemical and molecular level events during radiation-induced leukemogenesis in an animal model.

In another project, the interaction between gamma rays and the chemical mutagen methylnitrosoguanidine (MNNG) was investigated in cultured mouse cells (C3H10T1/2). The preliminary data indicate that oncogenic transformation and mutation frequencies for a dose of 1 Gy gamma ray 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 UV plus the chemical carcinogen, dimethylbenzanthracene. This and the previous project 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 induces melanogenesis, one of the body's major protective mechanisms against photocarcinogenesis induced by non-ionizing radiation. The mechanisms of melanogenesis are being studied using human epidermal melanocytes in culture. Results have shown that a diacylglycerol (DAG) analog (1-oleoyl-2-acetyl-glycerol [OAG]), a protein kinase C (PKC) activator, stimulated melanogenesis without affecting cell growth, and that the stimulation of melanogenesis could be blocked by PKC inhibitors. No evidence was obtained for the involvement of a cyclic adenosinemonophosphate (cyclic AMP) pathway in melanogenesis by these human cells. New protein synthesis appeared to be required for the OAG to stimulate melanogenesis. These data are of considerable interest as the cyclic AMP pathway has been implicated in murine cell melanogenesis by many lines of evidence, but to date it has not been possible to establish a role for the cyclic AMP pathway in human melanogenesis. This work 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. Evidence also was obtained that tyrosinase, considered to catalyze a rate limiting step in human melanogenesis, may be regulated at both the cellular levels of transcription and translation. This work indicates the need for molecular analyses in order to compare results obtained from animals from those obtained from humans, and suggests that it may be possible to stimulate the protective tanning in human skin without exposure to potentially carcinogenic ultraviolet radiation.

Comparative life-span exposure studies with rodents and dogs have been carried out in order to provide additional information that may permit extrapolation and scaling of the effects of radiation-induced damage observed in experimental animals to humans. The effects of ionizing radiation on life shortening of the beagle dog are being studied as a function of protracted whole-body gamma irradiation, exposing identified groups of animals to various total doses at several dose rates. The results of these studies thus far indicate that (a) there is no evidence for a dose rate effect, (b) the incidence of death from tumors is proportional to total dose, (c) the time of fatal tumor onset is reduced as the total dose is increased, and (d) an increased total dose increases the number of nonfatal tumors. This study, one part of a large multifaceted program funded by another agency, is now complete, although analyses of the data remain in progress. The final results of this

comprehensive study are expected to provide an expanded and more rational experimental data base upon which more realistic assessments of the late effects of protracted whole-body irradiation in man can be formulated.

Radiation-induced lymphomas in the mouse possess a high molecular-weight glycoprotein oncofetal antigen (OFA) that appears to be a radiation-induced marker for cancer. The 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 post-natal or adult tissues. In a continuing study an investigator has used the RFM mouse model for producing a high incidence of x-ray promoted T-cell lymphomas resulting from fractionated whole body exposure (4 x 1.75 Gy irradiation). Lymphoma incidence begins at two months post-irradiation and 70% of the mice develop primary tumors by six months post-irradiation. Monoclonal antibodies specific for OFA were used to show that OFA expression began 5-8 weeks post-irradiation. Parallel histological analysis of the same specimens of the thymus and spleen failed to reveal any evidence for neoplasia 5-6 months post-irradiation. These results confirm and extend earlier work and strongly suggest that OFA can be used as an early detection marker for radiation-promoted lymphomas which will faithfully predict which mice will develop malignancy months before conventional histological techniques demonstrate this cancer. In other work on this project, the gene encoding OFA has been cloned and sequenced from a mouse cDNA library and is currently being used to generate antigenic epitopes of recombinant OFA protein for producing additional antibodies for OFA. The OFA has no recognized DNA sequence homology with any other identified human or rodent proteins, and is tumor specific but not tumor-type restricted since it is expressed in all three stem lines giving rise to leukemia in mammals. No OFA has been detected in normal human or animal tissues to date. Future work will focus on the validation of OFA as an early marker for radiogenic leukemia in this animal model and will begin to address the possibility of OFA as marker for human cancer.

The identification of molecular markers correlated with exposure to the various forms of ionizing radiation continues as a major objective of a study with the rat skin model. Previous work on this project showed that amplified and rearranged c-myc oncogenes are associated with advanced skin carcinomas induced by x-irradiation and that amplification of c-myc was seen in the late stages of tumor development and not as an early marker for this type of radiogenic cancer. The amplification of c-myc in these studies was strongly correlated with tumor size and time of tumor development. More recently, this investigator has completed a study of c-myc amplification in 70 tumors induced by high linear energy transfer (high-LET) neutrons for comparison with the results from earlier studies of x-ray (low-LET) induced rat skin tumors. In contrast to the results for x-irradiation, there was virtually no amplification of c-myc and no relationship between tumor size, growth rate and time occurrence for the high-LET induced skin tumors. These results strongly suggest differences in the molecular mechanisms of radiation carcinogenesis induced by low- and high-LET irradiation. The molecular-level comparisons of tumors induced by low- and high-LET radiation is continuing in this study, in part consisting of DNA sequencing of activated ras mutations after polymerase chain amplification of targeted DNA sequences. This project illustrates comparative research on the possible mechanistic differences of high-LET and low-LET forms of ionizing radiation which is of importance for improving the basis of radiation protection standards.

In a related study using the mouse skin model, ionizing radiation was shown to be a more effective agent in causing tumor progression than it was as an initiator of tumorigenesis. Histological examination of x-ray-induced tumor tissues revealed 4

distinct tumor types: (1) benign papillomas, (2) squamous cell carcinomas (SCC), (3) basal cell carcinomas (BCC), and (4) fibrosarcomas. A benign papilloma cell line (308) was used to follow molecular events during the progression to frank malignancy. In this work a malignant variant 308 line (308 10Gy5) derived by x-irradiation of the parental 308 cell line showed correlations between the expression of gene transcripts for stromelysin (a protease), metallothionein II A and the protooncogenes c-fos and c-jun as the papilloma cell progressed to malignancy. Transient infection studies with a chimeric mouse stromelysin promoter sequence indicated that the stromelysin promoter sequence had become constitutively expressed during progression to malignancy suggesting a molecular rearrangement in location of the stromelysin structural gene placing it under the control of a new promoter in the genome during progression to cancer. In a second line of research at least three different non-ras type dominant transforming genes were shown to be induced in SCC by x-irradiation. Future work will elucidate the number and type of dominant oncogenes and other genes that may be involved in the progression to radiogenic cancer in this animal model. The ability to divide the process of carcinogenesis into distinct multiple stages in this model system will facilitate mechanistic studies on the molecular pathways involved in radiation-induced carcinogenesis.

In humans, squamous cell carcinomas of the head and neck are known to be heterogeneous in their responsiveness to radiation therapy. Previous work has implicated the raf oncogene as contributing to the radiation resistance of these tumors. However, the radiation resistance phenotype appears to be a complex response due to several cellular factors. In recent work, cellular proteins from three radiation-resistant human cell lines were analyzed by two-dimensional electrophoresis. Computer-aided quantitative analysis of the gels showed that six proteins from the radioresistant cell lines were expressed 200- to 500-fold above comparable levels in radiation-sensitive cell lines. Another set of between four to six proteins were overexpressed 10- to 50-fold in the radiation-sensitive cells compared to their expression in radiation-resistant cell lines. These data suggest that the response to ionizing radiation in these human tumor cell lines may be mediated by several structural genes coding for different proteins that are formed specifically in response to DNA damage induced by ionizing radiation. Moreover, the data suggest that the relative expression of damage repair pathways may be different in cells that are sensitive versus those operating in cells that are resistant to killing by ionizing radiation. This work is continuing with the use of subtraction cDNA libraries and protein microsequencing techniques in an attempt to obtain DNA and protein sequence information about the genes and gene products involved in radioresistance and radiosensitivity for (a) comparison with known protein and gene sequences from human cells, and (b) construction of probes for measuring expression of cellular proteins associated with radiation sensitivity and resistance in human cells under various exposure conditions.

In a related study, possible correlations have been demonstrated between the raf oncogene and radiation resistance in human tumor cell lines from the head and neck. In previous work, evidence was obtained that the raf oncogene is functionally important in maintaining the radioresistance of the SQ20B human cell line. Efforts are continuing to use the polymerase chain reaction to amplify DNA segments that flank the tumor-derived c-raf-1 DNA sequences in an attempt to determine if the raf oncogene in the radiation resistant cell lines has been rearranged to new genomic locations or otherwise modified differently with respect to DNA sequence than is the raf protooncogene in radiosensitive cell lines. One possibility that will be examined is that fragile sites are located in or near the tumor c-raf-1 gene and undergo transformation along with activation of the raf oncogene.

The linkage between epidemiology and molecular biology is evident in a study of approximately 5000 patients who received x-ray irradiation during childhood mostly as part of treatment of thyroid disease and who are being followed in order to detect the development of secondary cancers. In this work, it was found that the incidence of parathyroid tumors and hyperthyroidism was increased 2.9-fold ($P < 0.05$) compared to the general population. The prevalence of thyroid cancer among patients with parathyroid tumors was also found to be higher than among the remainder of the irradiated population. It was concluded that patients with a history of head and neck irradiation should have their parathyroid glands evaluated on a regular basis. The frequency with which coincident hyperthyroidism and thyroid neoplasms were found in this population provides further evidence that some people may have enhanced radiation-susceptibility. Work on thyroglobulin, a tumor marker useful in monitoring the treatment of thyroid cancer, included the determination of the chemical bond linking chondroitin sulfate to the thyroglobulin molecule and significant differences in the anionic complex-carbohydrate structures from normal and neoplastic thyroid tissues.

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 (NTS) between 1951 and 1963. A leukemia case-control study consists of 1,177 cases and 5,330 matched controls. The exposure of each person to fallout, primarily Cesium-137, was estimated based upon each person's location of residence and age at the time of the tests, lifestyle and occupation. The overall finding of the leukemia study was a weak dose-related association (for all forms of leukemia, all ages and time periods combined) that was not statistically significant. However, a highly significant dose-related trend was found in a subgroup comprised of those who died from acute leukemia within ten years of exposure and were under age 20 at the time of exposure. The study concluded that a small but indistinguishable excess of leukemia in Utah may be attributed to radioactive fallout from the NTS. The thyroid study consists of a follow-up of 4,818 children who were examined for thyroid disease during the 1960s as a part of a Public Health Service study. Of that number, 2,473 were located, eligible and willing to be interviewed and clinically re-examined for thyroid disease 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 radiiodine 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 2,473 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. The completion of the analyses of data from the thyroid study is expected during the present reporting period.

RADIATION EFFECTS
GRANTS ACTIVE DURING FY91

<u>Investigator/Institution/Grant Number</u>	<u>Title</u>
1. ALDERFER, James L. New York State Department of Health 5 R01 CA 39027-07	Effects of Light on Nucleic Acids
2. ANANTHASWAMY, Honnavara University of Texas 5 R01 CA 51015-02	Cellular & Molecular Basis for Basal Cell Nevus Syndrome
3. APOSHIAN, Vasken H. University of Arizona 2 R01 CA 49252-06	Polonium-210 Radiation and Mutagenesis
4. ASHMAN, Charles R. University of Chicago 2 R01 CA 45336-04	Specificity of Ionizing Radiation Mutagenesis
5. BALCER-KUBICZEK, Elizabeth K. University of Maryland at Baltimore 5 R01 CA 50629-03	Neoplastic Transformation by Fission Neutrons and Gamma Rays: Dose-Rate Effect
6. BASES, Robert E. Yeshiva University 5 R01 CA 36492-06	X-Ray Damage and Repair of Primate Cell Alpha-DNA Sequences
7. BEDFORD, Joel S. Colorado State University 5 R37 CA 18023-17	Dose and Time Factors in Cellular Radiosensitivity
8. BEDFORD, Joel S. Colorado State University 5 R01 CA 49501-03	Radiation Cytogenetics
9. BERNHARD, William A. University of Rochester 5 R37 CA 32546-16	Solid State Radiation Chemistry of Nucleic Acid Bases
10. BERNHARD, William A. University of Rochester 1 R13 CA 51705-01	Workshop on "Early Effects of Radiation on DNA"

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| 11. | BOWDEN, George T.
University of Arizona
5 R01 CA 42239-06 | Radiation-Induced Skin
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| 12. | BOX, Harold C.
Roswell Park Memorial Institute
5 R37 CA 25027-24 | Transfer Mechanisms in
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| 13. | BOX, Harold C.
Roswell Park Memorial Institute
5 R01 CA 46838-03 | Analysis of Radiation
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| 14. | BRENT, Thomas P.
St. Jude Children's Research Hospital
2 R01 CA 14799-16A1 | Enzymes and Reactions for
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| 15. | BROOKS, Antone L.
Battelle-Pacific Northwest Laboratory
5 R01 CA 45590-04 | Radiation-Induced Damage
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Tel Aviv University
1 R01 CA53139-01 | Molecular Basis of Xeroderma
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| 18. | CHOU, Wen-Gang
University of Rochester
5 R01 CA 51064-02 | Radiation-Associated Gene
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University of South Alabama
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| 23. | CROUSE, Gray
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| 31. | EVANS, Helen H.
Case Western Reserve University
4 R37 CA 15901-17 | Mutants and Altered
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| 32. | FOX, Fred
University of CA, Los Angeles
1 R13 CA 51707-01 | Conference on Ionizing
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| 33. | FRANKLIN, William
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5 R29 CA 52025-02 | Novel Enzyme Acting on
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| 34. | FUKS, Svi
Sloan-Kettering Cancer Center
5 R01 CA 52462-02 | FGF Induced Radiation
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Dana-Farber Cancer Institute
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50. KANTOR, George J.
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5 R01 CA 49498-03 Mechanisms of Cellular and
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Vanderbilt University
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Medical College of Virginia
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Tufts University
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99. WALLACE, Susan S.
University of Vermont
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University of Vermont
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| 102. WARD, John F.
Univ. of California, San Diego
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| 103. WEBER, Christine
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Johns Hopkins University
5 R01 CA 39543-06 | X-Ray Induction of Cellular
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| 106. YAROSH, Daniel
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2 R44 CA 52401-02 | UV-DNA Repair & Mutagenesis
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| 107. YASUI, Linda S.
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| 108. ZAIN, Sayeeda B.
University of Rochester
5 R01 CA 46625-03 | C-abl Oncogene in Radiation-
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CONTRACTS ACTIVE DURING FY91

<u>Investigator/Institution/Contract Number</u>	<u>Title</u>
109. FRITZ, Thomas E. Department of Energy Y01-CP-50503	Late Effects of Protracted Irradiation in Dogs
110. STEVENS, Walter University of Utah N01-CO-23917	Assessment of Leukemia and Thyroid Disease in Relation to Fallout in Utah

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