CONF-8811202--

DE89 012820

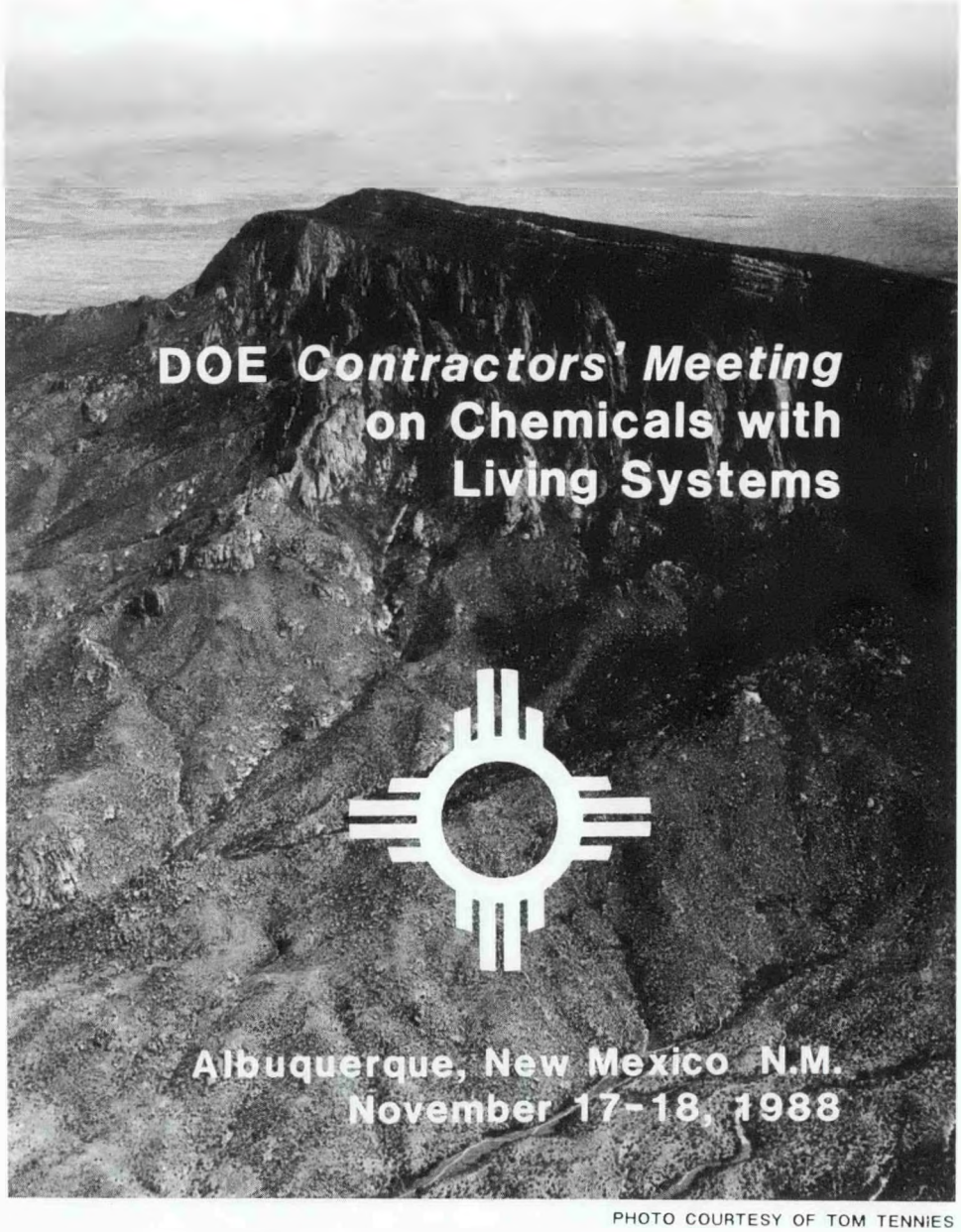


PHOTO COURTESY OF TOM TENNIES

Organized and sponsored by. . .
Office of Health and Environmental Research,
U.S. Department of Energy, and
Lovelace Inhalation Toxicology Research Institute

FOREWARD

The Department of Energy's Office of Health and Environmental Research (OHER) held its Fourth Annual Contractor's Meeting, "Interactions of Chemicals with Living Systems" on November 17 and 18, 1988. There were thirty-four attendees at the workshop. The thirty-three presentations, which were from six DOE laboratories and seven universities, focused on current research findings and explored opportunities for future research.

The meeting covered a wide spectrum of disciplines and research supported by OHER. It provided a forum at which scientists from diverse, but related, areas summarized current OHER research activities. This successful meeting provided an exceptional opportunity for OHER-supported scientists to exchange information and make new contacts for potential collaboration in research. This document contains an abstract of each presentation and a brief summary of each of the three sessions that were held.

Robert W. Wood, Ph.D.
Acting Associate Director for
Health and Environmental Research
Office of Energy Research

This meeting was hosted by the Inhalation Toxicology Research Institute (ITRI), which is operated for the U. S. Department of Energy by the Lovelace Biomedical and Environmental Research Institute under contract No. DE-AC04-76EV01013. Dr. Charles H. Hobbs, Assistant Director and Linda L. Burton (505-844-2435) were responsible for organization of the meeting at ITRI.

The OHER/DOE Program Manager for the Chemical Toxicity Program is:

Dr. James R. Beall, Ph.D.
Toxicologist, Health Effects Research
Division, ER-72 GTN
Office of Health and Environmental
Research, Office of Energy Research
U. S. Department of Energy
Washington, D.C. 20545
301-353-4507

DOE CONTRACTORS MEETING ON INTERACTIONS OF
CHEMICALS WITH LIVING SYSTEMS

ALBUQUERQUE, NEW MEXICO

NOVEMBER 17-18, 1988

TABLE OF CONTENTS

SESSION I (cont.)

SESSION II

SESSION II (cont.)

SESSION III

SESSION III cont.

EXECUTIVE SUMMARY

The Office of Health and Environmental Research (OHER) of the Department of Energy (DOE) has a programmatic need to determine the potential health and environmental effects associated with energy production and use. This need is addressed through a limited number of fundamental research projects directed at understanding effects of chemicals and radiation on biological systems. To ensure appropriate communication among investigators and scientific disciplines that these research studies represent, OHER has sponsored a series of workshops. These workshops provide for exchange of ideas and information, stimulate improved collaboration, and form a basis for future research planning.

This document provides a compilation of activities at the Fourth Annual DOE/OHER Workshop, "Interactions of Chemicals with Living Systems." This workshop was held at the Lovelace Inhalation Toxicology Research Institute in Albuquerque, New Mexico, on November 17 and 18, 1988. There were thirty-four attendees and thirty-three submitted presentations which represent work being sponsored by OHER at six DOE laboratories,(1) and seven universities.(2) The format of the meeting included both platform and poster presentations. The workshop consisted of three sessions entitled:

Session I Metabolism and System Interactions

Session II Effects on Cellular Systems and Differentiation

Session III Chemical Interactions with DNA

This document includes abstracts of thirty-three papers that were submitted for presentation at the workshop. A brief summary of the research presented in each of the three sessions precedes the abstracts for that session.

Session I included eleven papers relating to either the metabolism of chemicals or the noncarcinogenic responses of cells and organs following exposure to chemicals or their metabolites. These studies emphasized the wide variety of expertise and techniques needed to investigate the mechanisms of chemical toxicity.

Session II included twelve papers relating to the effects of chemicals on cellular systems and cell differentiation. These studies emphasized that in order to initiate biological integration, chemicals must interact with specific target molecules, including proteins and nucleic acids.

Understanding these interactions is essential to identifying the mechanisms by which chemicals may cause health effects, such as cancers in people.

Session III included ten papers relating to chemical interactions with DNA. The papers presented in this session emphasize the importance of the amount and types of interactions produced in DNA following chemical exposure and the relation of these interactions to a carcinogenic response.

1. Argonne National Laboratory, Battelle Pacific Northwest Laboratory, Lawrence Berkeley Laboratory, Los Alamos National Laboratory, Lovelace Inhalation Toxicology Research Institute, and Oak Ridge National Laboratory.

2. Johns Hopkins University, Massachusetts Institute of Technology, Michigan State University, New York University, University of California at Los Angeles, University of Chicago, and University of Vermont.

S E S S I O N I

METABOLISM AND SYSTEM INTERACTIONS

DOE CONTRACTORS MEETING
November 17-18, 1988

SUMMARY OF SESSION I

METABOLISM AND SYSTEM INTERACTIONS

By Dr. Alan R. Dahl
Lovelace Inhalation Toxicology Research Institute

Toxicants exert their effect following definable pathways available to the organism. There are only a limited number of such responsive pathways by which an organism can react to a chemical. Thus, potentially, a general understanding of how toxicants produce effects can be achieved by carefully examining the organism's capacity to metabolize toxicants and studying the responsive interactions between various cells and organs when exposed to toxicants or their metabolites.

Using human mammary epithelial cells, Tischler and Bartley (Lawrence Berkeley Laboratory) were able to show that cyclooxygenase probably does not play an important role in activating benzo(a)pyrene-7,8-diol to the benzo(a)pyrene-9,10-epoxide, the ultimate carcinogen. In addition, it was shown that in these cells the oxidation of benzo(a)pyrene could be induced by pretreatment with benzo(a)pyrene, but this treatment did not induce the metabolism of the 7,8-diol to the 9,10-epoxide. Some products of benzo(a)pyrene metabolism inhibited the oxidation of the benzo(a)pyrene-7,8-diol to the ultimate carcinogen. This has important implications in interpreting data from *in vitro* systems where such products are likely to build up, compared to *in vivo* systems where they would likely be kept at low levels.

Metabolizing enzymes may interact in complex ways with hormones or hormone mimes, such as diethylstilbestrol. Recent studies by Springer and Zangar (Battelle-Pacific Northwest Laboratory) suggest that neonatal exposure to diethylstilbestrol alters the susceptibility of animals to tumors and possibly other diseases and that cytochrome P450-dependent pathways may be involved in the mechanism for this effect. Studies by Mitchell *et al*. (Lovelace ITRI) with polychlorinated biphenyls showed complex interactions of the inducing agent with the enzymes in the mouse liver and lung. The induction of specific enzyme activities was not uniform but differed for different substrates and for different tissues.

The toxicity of metals is receiving continuing attention, but the mechanisms of toxicity are not, at this point, well established. The observation that postmenopausal women exposed to cadmium experience calcium loss associated with the Itai-Itai disease, may be explained by an interaction of the cadmium and postmenopausal hormone depletions (Bhattacharyya et al. Argonne National Laboratory). Research with ovariectomized mice indicated that cadmium may mimic the action of parathyroid hormones by activating osteoclasts responsible for the breakdown of the mineral matrix of bone. The amount of cadmium present in the blood of smokers suggests that the cadmium from cigarettes may be, at least in part, responsible for the decreased bone mineral loss and incidences of fractures and tooth loss in women who smoke.

A second metal studied was beryllium. To study chronic beryllium disease, an immune-mediated granulomatous disease with pulmonary manifestations, a dog model was developed. It was found that the local pulmonary immune responses in the dogs are similar to those of humans to a closer degree than are those of rodents. Moreover, dogs and man are the only species which have been shown to have beryllium specific immune responses localized to the lung after inhalation of beryllium (Haley et al., Lovelace ITRI).

The response of the lungs to inhalants has been studied with regard to toxicity to the immune system (Bice and Muggenburg, Lovelace ITRI) and with regard to the recruitment of inflammatory cells in the lungs (Harkema et al. Lovelace ITRI). Studies by Bice have identified the immune cells at risk to inhaled chemicals while those by Harkema et al. have shown, using ozone as the irritant, that lung recruitment of neutrophils may so deplete the supply of circulating neutrophils as to prevent influx of neutrophils into the nose in response to an irritant gas. In related studies, Henderson et al. (Lovelace ITRI) found that higher dose rates of particulate matter, in this case carbon black particles, may overwhelm the clearance capacity of the lung. Higher dose rates increase the inflammatory response in the system's study.

Patterns of respiratory tract deposition for particles varies with particle size. The deposition patterns for ultrafine particles (those less than 1/10 μm in diameter) has not been well studied. Studies by Yeh et al. (Lovelace ITRI) showed that smaller particles are substantially deposited in the nose: up to 70% for particles less than .01 μm in diameter.

ACTIVATION AND DETOXICATION OF INHALANTS

A. R. Dahl

Lovelace Inhalation Toxicology Research Institute
Albuquerque, NM 87185

Virtually all xenobiotic metabolizing enzymes found in the liver are also found in the respiratory tract. These enzymes are localized in specific sites within the nose and lung. As a rule, the olfactory epithelium of the nasal cavity and the Clara cells of the lung bronchioles contain the highest activities, but exceptions to this rule do occur.

Xenobiotic metabolizing enzymes found in the respiratory tract include aldehyde dehydrogenases, flavin-containing monooxygenase, rhodanese, glutathione S-transferases, UDP-glucuronyl transferases, carboxylesterases, and epoxide hydrolase. Some of these enzymes are responsible for the activation of inhaled materials to toxic metabolites. Chief among these are the cytochromes P-450. Other enzymes are most often associated with detoxication of either inhalants themselves or their metabolites. Still other enzymes, not strictly xenobiotic metabolizing enzymes, repair lesions to DNA and thus prevent mutation and further toxic consequences. Determining the localization of the various enzymes, their substrate specificities and the relative contributions of activation, detoxication, and repair enzymes can lead to predictions of the site of toxicity within the respiratory tract and elsewhere in the body.

Knowledge of xenobiotic metabolism in the respiratory tract contributes in important ways to information on the toxicity of inhalants. Since there are arguably millions of chemical compounds that might be inhaled, it is not possible to determine the toxicity of each using standard toxicology protocols. When a standard toxicology protocol is used, foreknowledge of the probable sites of toxicity can help pinpoint the specific tissues to be examined histologically. In addition, understanding species variability in the distribution of xenobiotic metabolizing enzymes within the respiratory tract refines our capacity to extrapolate results from animal experiments to expected toxicity in humans.

The accurate prediction of specific sites of toxicity requires detailed knowledge of the nature of the reactive metabolite and of the distribution of activating and detoxicating enzymes. A metabolite that is chemically

inert will not react with cell macromolecules and, therefore, won't cause any damage. One that is slightly reactive, with a half-time in a physiological system of a few hours, may be translocated from its site of production to other regions of the body. The tissues affected may depend more upon the distribution of the metabolite than upon its site of production. Route of administration can, however, still affect the toxicity of the compound. For example, a compound that is metabolized in the respiratory tract mostly to a reactive metabolite may be more toxic when administered by inhalation than when administered by the oral route, if the liver produces less of the reactive metabolite.

Metabolites with half-lives on the order of a few minutes may be translocated short distances from the site of production and affect tissues in the vicinity of the activating tissue as well as those in the activating tissue. Inhaled benzo(a)pyrene is metabolized to a large extent in the nasal mucosa of the Syrian hamster and produces tumors in the nose as well as in the esophagus, which is exposed to metabolites produced in the nose.

Metabolites that have half-lives of a few seconds may survive long enough to escape the cell of origin, but probably not the tissue of origin. Thus, the target tissue for these compounds will be the same as the activating tissue. Dimethylnitrosamine is metabolized in the olfactory mucosa of rats and produces tumors in that tissue.

Some reactive metabolites are so labile that they do not escape from the cell of origin or even from the enzyme that produces them. Dihydrosafrole inactivates nasal cytochrome P-450, but apparently has no other toxic effects. It is metabolized by P-450 to an orthoformate that decomposes to a carbene. The carbene binds to the heme iron of the P-450 or decomposes before it can affect other parts of the cell.

Knowledge of the relative reactivity of a reactive metabolite is necessary in the prediction of target tissue toxicity. Equally necessary is knowledge of the capacity of various cell types to produce reactive metabolites and to detoxicate them once produced. Advances in our knowledge of site specific locations of activating and deactivating enzymes activities have involved mainly rabbit and rat nasal and lung tissue. Specific isozymes of P-450 have been identified at various locations. These can be used to explain or predict the toxicity of inhaled materials.

The capacity of a cell to repair damage also needs to be evaluated in any predictive scheme. Both the rat nasal respiratory and olfactory tissues

contain O^6-methylguanine DNA methyl transferase. The cellular location of the activity is not known, but, if it occurs to a greater degree in one cell type than in another, differences in sensitivity to the toxic effects of alkylating agents might easily result.

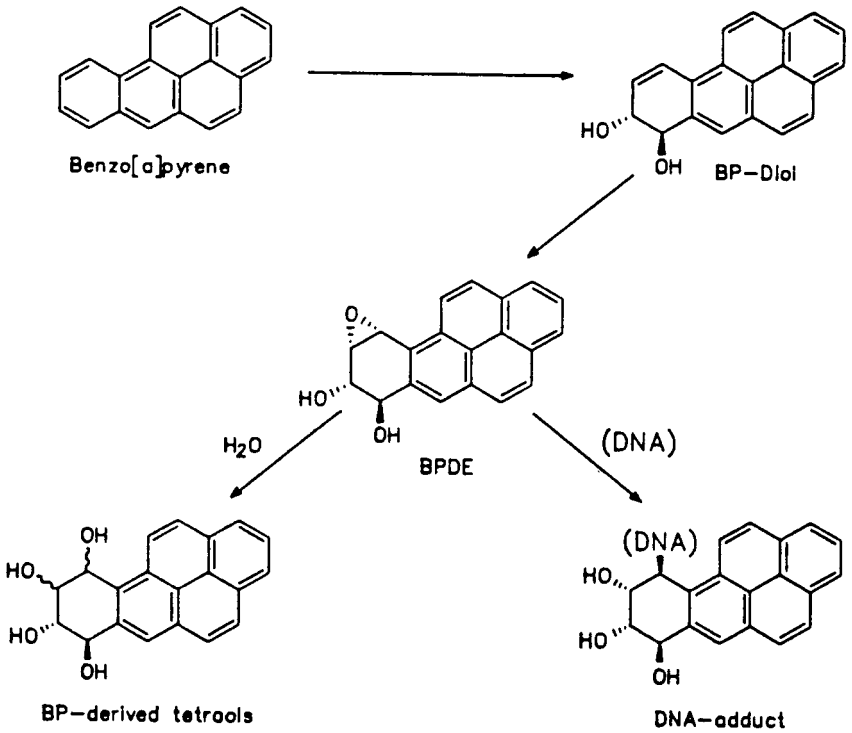
Research on the xenobiotic metabolism activities in the respiratory tract is bringing this important area into ever sharper focus. Observations of toxic effects from inhalants can be explained. The toxic effects in continuing studies can be predicted. By examination of potential activation pathways and consideration of the stabilities of reactive metabolites, assessments of specific cells and tissues at risk can be made. Research is still in early stages, however. Relationships between activation, detoxication, and repair enzyme activities need to be examined. Relationships among the animal species commonly used in inhalation studies need to be closely examined and then compared to humans. This research is progressing and to date allows for good guesses regarding toxicity of inhaled materials. These guesses should become even better as more data regarding respiratory tract xenobiotic metabolism become available. (Research supported by the U.S. Department of Energy, Office of Health and Environmental Research under contract No. DE-AC04-76EV01013.)

THE METABOLISM OF BENZO[a]PYRENE-7,8-DIHYDRODIOL IN HUMAN MAMMARY EPITHELIAL CELLS: MECHANISTIC IMPLICATIONS

Allan Tischler and Jack Bartley

Lawrence Berkeley Laboratory
Berkeley, CA 94720

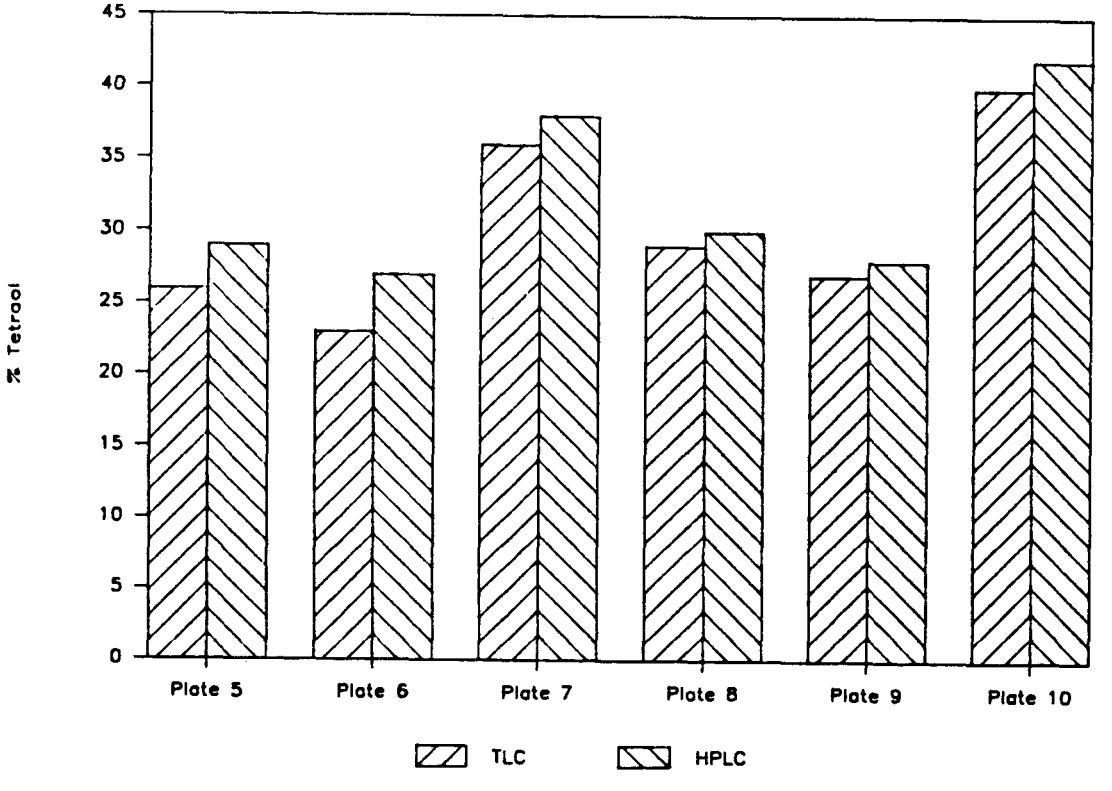
This laboratory has been interested in the activation of benzo[a]pyrene (B[a]P) in human mammary epithelial cells (HMECs) for some time. We have previously described B[a]P metabolism in HMECs from numerous sources and under various conditions and found that HMECs readily activate B[a]P to the "ultimate carcinogen," BPDE[1]. More recently, we have focused our attention on the nature of the key step, the conversion of benzo[a]pyrene-7,8-dihydrodiol (BP-diol) to BPDE.



Possible mechanisms for this conversion include cytochrome P-450s (the same or different than the first step), co-oxidation via cyclooxygenase[2], some other oxygenase or nonenzymatic conversion via alkyl hydroperoxides[3]. We have found that indomethacin, a potent and selective inhibitor of cyclooxygenase, does inhibit ^{3}H-B[a]P metabolism, but only at high concentration (50 μl) and apparently nonspecifically- all ^{3}H-B[a]P-derived products are inhibited.

In order to better study the BP-diol to BPDE conversion we are currently investigating the use of ^{3}H-BP-diol as the initial substrate in place of ^{3}H-B[a]P. ^{3}H-BP-diol is rapidly and relatively cleanly metabolized to the expected BPDE-derived tetraol mixture. Typically, HMECs are incubated with ^{3}H-BP-diol (400 pmoles/ml medium) for 16 hours before the cells and media are combined and extracted with ethyl acetate:acetone. Metabolism can be measured either by reversed-phase HPLC (50 to 90% methanol gradient) or by straight-phase TLC (ethyl acetate:acetic acid, 9:1). The TLC method involves doping the organic extracts with cold diol and tetraol standards, which allow direct visualization under long-wave UV light. The thin layer method, which is not applicable to following the metabolism of ^{3}H-B[a]P because of the complexity of metabolites formed, appears to be the method of choice for ^{3}H-BP-diol metabolism.

1. The TLC method is much faster. In addition to requiring less time per sample (30 min. vs 100 min.), multiple samples can be run on the same plate.
2. Tetraol conjugates (primarily the BPDE-glutathione adduct) are readily hydrolized at the origin and run with the tetraol standards.
3. The tetraols elute over a much narrower region on TLC (approx. 20% of the total distance) than on HPLC (approx. 50% of the total time), resulting in fewer minor metabolites co-eluting with tetraols.
4. Since products are counted in single scintillation vials, considerably less radiolabel and scintillation cocktail is required. A scanning counter would simplify the process even more.



The results obtained by TLC and HPLC are quite comparable. Data from six different samples are presented above. As expected, the TLC-determined value is close but slightly lower in each case.

Indomethacin, up to 50µl, does not effect ^{3}H-BP-diol metabolism, suggesting that cyclooxygenase does not play a role under our conditions. This does not rule out the possibility that under different conditions, such as in the presence of a promoter like TPA, cyclooxygenase co-oxidation might be operating.

To gain further insight into B[a]P versus BP-diol oxidation, we have investigated the effect of preincubating HMECs with cold B[a]P for 1 to 3 days before inoculation with either ^{3}H-B[a]P or ^{3}H-BP-diol. Such pretreatments consistently enhance ^{3}H-B[a]P metabolism and inhibit ^{3}H-BP-diol metabolism. Thus, it appears that only the first step is B[a]P inducible. The observed decrease in ^{3}H-BP-diol metabolism, which is far greater than can be explained by ^{3}H-BP-diol dilution, suggests that some B[a]P-derived product(s) inhibits BP-diol oxidation. The major B[a]P metabolite, which we have recently identified as 7-hydroxy-benzo[a]pyrene (BP-7-ol), is a prime suspect. We have recently found that BP-7-ol is an extremely potent inhibitor of ^{3}H-BP-diol metabolism (K_I < 25 nM). Such potent inhibition strongly suggests that an enzymatic mechanism is responsible.

Effect of Cold BP Pretreatment on ^{3}H-BP + ^{3}H-Diol Metabolism

Substrate	Pretreatment	Metabolism	Net Effect
^{3}H-BP	-	15%	
^{3}H-BP	+	20%	+33%
^{3}H-Diol	-	43%	
^{3}H-Diol	+	22%	-49%

REFERENCES

1a. J. Bartley, J.C. Bartholomew and M.R. Stampfer, J. of Cell Biochem. 18, 135-48 (1982).

1b. J.C. Bartley and M. Stampfer, Carcinogenesis 6(7), 1017-22 (1985).

2. T.E. Eling, G.A. Reed, R.S. Krauss, R.P. Mason and J.A. Boyd, "Metabolism of Carcinogens by Prostaglandin H Synthease" in Icosanoids and Cancer, ed. by H. Thaler-Dao, Raven Press, New York, 1984.

3. L.J. Marnett, Carcinogenesis 8(10), 1365-73 (1988).

Research was supported by the Director, Office of Energy Research, Office of Health and Environmental Research Division, of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098.

INVOLVEMENT OF CYTOCHROME P450 IN ALTERED IMPRINTING

D. L. Springer and R. C. Zangar

Battelle-Pacific Northwest Laboratory
Richland, WA 99352

The presence of stimuli, such as hormones during normal development, determines the capability of the organism to express and regulate certain genes later in life. This process, known as imprinting, occur during certain sensitive periods of development. Prenatal or neonatal contact with chemicals or radiation may cause altered imprinting, which results in shifts in biochemical pathways. While these shifts may be lethal, in less severe cases they may be manifested as physiological imbalances and may alter the animals susceptibility to disease. For example, it is well known that female offspring from women given the synthetic steroid diethylstilbestrol (DES) developed cervical adenocarcinoma. Although the mechanism for this effect has not been fully identified, there are data indicating that DES exposure disturbed the normal imprinting process and resulted in hormonal and other imbalances which may have contributed to tumor development.

Several recent reports point to the involvement of the cytochrome P450-catalyzed monooxygenase system in altered imprinting. For example, Lamartiniere and Pardo (1988) reported that neonatal exposure of rats to DES resulted in altered hepatic activation and detoxification enzyme activities, including UDP-glucuronyltransferase, epoxide hydrolase and glutathione transferase; several other changes in cytochrome P450-catalyzed activities were also observed when DES animals were challenged as adults with phenobarbital. Bagley and Hayes (1985) reported that neonatal exposure to phenobarbital resulted in significant elevation of total cytochrome P450, increased activities of cytochrome P450 reductase, cytochrome c reductase, ethoxycoumarin-O-deethylase, testosterone glucuronidase and glucuronosyl transferase; the magnitude of these increases ranged from 27 to 94%. In addition, when these animals were challenged with aflatoxin B1, a known hepatic carcinogen, the amount of this compound that was covalently bound to hepatic DNA increased by 1.5 to 2.3 fold over that in corresponding controls. These results were consistent with those of Faris and Campbell (1983).

The above observations indicate that endogenous P450-dependent enzymes are altered by xenobiotic administration during development and that these changes may play an important role in susceptibility to cancer. Altered cytochrome P450 levels may have other important consequences as well, since certain cytochrome P450-dependent enzymes catalyze reactions in the biosynthesis and metabolism of steriods; in fact, several rate limiting or key regulatory steps in these pathways are occupied by specific P450 isozymes. Thus, altered imprinting could change the isozyme patterns as well as the total levels of P450 dependent monooxygenase activities. Since these key issues have not been addressed experimentally, we have initiated studies to determine the relationships between altered imprinting, P450-dependent enzyme activities and steroid biosynthesis and metabolism.

For this work, we treated both male and female neonatal rats with four potential imprinting agents (DES; pregnenolone-16a-carbonitrile, PCN; 7,12-dimethylbenz(a)anthracene, DMBA and phenobarbital) on days 1, 3 and 5 of age. The animals were sacrificed at approximately 150 days of age and randomly assigned to either the group for in vivo aflatoxin B1 binding to hepatic DNA or to the group that was sacrificed for preparation of hepatic microsomes. Although this is a new project, samples have been evaluated for certain changes in cytochrome P450-catalyzed activities and related endpoints. The results indicated that total cytochrome P450 concentrations for males from all treatment groups were not significantly different for controls whereas, for DES treated females, total cytochrome P450 was increased by 10-15%. In addition, binding of aflatoxin B1 to hepatic DNA for males treated neonatally with DES was decreased by 36% relative to corresponding controls; exposure to the other test materials did not result in significant changes in aflatoxin binding.

The primary purpose of this ongoing work is to determine the consequences of neonatal/fetal exposures on cytochrome P450-dependent monooxygenase system at the molecular level; however, it is also important to relate these changes to the intact animal so that the work remains meaningful. Because of this, we evaluated the animals for survival and growth from the time of neonatal treatment until they were sacrificed as adults (at approximately 150 days of age). Few deaths occurred in the PCN, phenobarbital and control groups from the time of the neonatal injection until the final sacrifice; however, deaths occurred in the DES treatment group beginning around 50 days of age and continued, so that by the final

sacrifice, 35% of the animals in this group had died. At the final sacrifice, body weight for DES-treated females were significantly increased (15%) relative to controls. At 84 days of age, body weights for males were significantly decreased for DES- and DMBA-treated animals. These changes in growth suggest that the females have become masculinized and that the males are more feminine-like in terms of their growth patterns.

These data suggest that neonatal exposure to DES may alter the susceptibility of animals to tumor development (and possibly other diseases) and that cytochrome P450-dependent pathways may be involved. In addition, these changes demonstrate the need for further evaluation of these animals, with particular emphasis on aflatoxin binding for females, since their total cytochrome P450 concentrations were 17% higher than those of controls. It will also be important to incorporate other more molecular endpoints, such as oligonucleotide probes for mRNA and antibodies to P450 to identify the isozymes involved. (Research supported by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC06-76RLO1830.)

REFERENCES

1. Bagley, D.M. and J.R. Hayes, 1985, Xenobiotic imprinting of the hepatic monooxygenase system, Biochem. Pharm. 34: 1007-1014.

2. Faris, R.A. and T.C. Campbell, 1983, Long-term effects of neonatal phenobarbital exposure on aflatoxin B1 disposition in adult rats, Cancer Research, 43: 2576-2583.

3. Lamartiniere, C.A. and G.A. Pardo, 1988, Altered activation/detoxication enzymology following neonatal diethylstilbestrol treatment, J. Biochem. Toxicol. 3, 87-103.

SELECTIVE INDUCTION OF CYTOCHROME P-450 ACTIVITIES BY POLYCHLORINATED BIPHENYL IN MOUSE LIVER AND LUNG

C. E. Mitchell, P. J. Sabourin, and A. R. Dahl

Lovelace Inhalation Toxicology Research Institute
Albuquerque, NM 87185

Polycyclic aromatic hydrocarbons and nitroaromatic hydrocarbons are ubiquitous airborne contaminants released into the environment from a number of sources. Both types of compounds include examples of potent mutagens that induce transformation in mammalian cells, and cause cancer in rodents. Although the mechanism(s) by which these compounds exhibit toxicity is unknown, it is clear that the parent compounds must be metabolized prior to exerting toxic effects. The cytochrome P-450 family of enzymes participate in the activation and detoxication of a variety of natural and foreign compounds. They are selectively induced in tissues of many species by chemicals that commonly occur in the atmosphere. The largest number of P-450 isozymes have been isolated from rat and rabbit liver. The isolation and characterization of isozymes from rat lung is less extensive, and even less information is available on the characterization of isozymes in mouse lung. The present study was undertaken to determine the levels and characteristics of cytochrome P-450 isozymes in male $B6C3F_1$ mouse liver and lung after administration of Aroclor-1254 intratracheally or intraperitoneally.

Intraperitoneal administration of Aroclor-1254 increased liver cytochrome P-450, aryl hydrocarbon hydroxylase (AHH), benzphetamine N-demethylase, and nitroreductase activities greater than 2-fold. Total lung cytochrome P-450 was increased 1.9-fold. Lung nitroreductase activity, however, was not affected, and benzphetamine N-demethylase was decreased 1.6-fold.

In contrast to the intraperitoneal route, total liver P-450 was unchanged; AHH and benzphetamine N-demethylase were decreased (> 1.4-fold), and only nitroreductase was increased (1.6-fold) after intratracheal administration of Aroclor. In the lung, cytochrome P-450 and AHH were increased 1.4- and 2.2-fold, respectively, after intratracheal administration of Aroclor-1254. Benzphetamine N-demethylase was decreased 2.1-fold and nitroreductase was not affected.

Cytochrome P-450 isozymes in mouse liver were separated by sequential aminohexyl sepharose, DEAE-cellulose, and hydroxyapatite column chromatography. Solubilized microsomal cytochrome P-450 eluted as two peaks (A and B) from the aminohexyl sepharose 4B column. Further chromatography on DEAE resolved peak A into an additional four peaks (A1-A4) and peak B into an additional four peaks (B1-B4). Of the eight resolved fractions, only five had strong absorbances at 450 nm in the carbon monoxide difference spectrum. The substrate specificities of the cytochrome P-450 fractions were determined. The two sepharose 4B fractions and the five DEAE fractions exhibited different catalytic activities toward a variety of substrates in reconstituted systems consisting of NADPH-cytochrome P-450 reductase, phospholipid, and cytochrome P-450. All fractions were capable of metabolizing all of the substrates tested, but the rate at which the reactions were catalyzed by different fractions differed considerably. The highest AHH activity was found in fraction B2. Nitroreductase activity appeared throughout all fractions. Fraction B1, however, contained approximately two times more nitroreductase activity than any of the other fractions. The highest activity for benzphetamine N-demethylase was obtained with fraction A3. This high activity toward benzphetamine indicates that this cytochrome P-450 fraction is different than the classic P-448 cytochrome that catalyzes AHH activity. However, the specific content (nm cytochrome P-450/mg of protein) and SDS-polyacrylamide gel electrophoretic analyses indicate that further purification of all fractions is necessary for complete substrate/activity determination.

These studies indicate that the route of administration of chemicals affects the induction and repression of form(s) of cytochrome P-450 and this may be an important determinant of organ-targeted chemical toxicity. It will be of interest to determine if the same or a different isozyme pattern is present in mouse lung and if specific isozymes are involved in the metabolism of nitroaromatic compounds. The profile of P-450 isozymes in tissues in the induced and normal states may aid in explaining species differences in metabolism and toxicity. (Research supported by U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.)

CADMIUM-INDUCED BONE LOSS: EFFECTS IN OVARIECTOMIZED MICE AND OSTEOCLAST-LIKE CELLS IN CULTURE

Maryka H. Bhattacharyya, Thomas M. Seed,
David P. Peterson, Elizabeth S. Moretti,
Christine Kuhn, Laura Brice, and Terri Choi

Argonne National Laboratory
Argonne, IL 60439-4833

After the menopause, when ovarian hormone production ceases, women generally lose bone mass at an increased rate for approximately 10 years. This bone loss is greater in some women than others, leading to vertebral fractures in approximately 5-10% of postmenopausal women (1). We sought to determine if cadmium might influence bone loss after the menopause. This line of investigation was pursued because of the unusual finding that 95% of cases of Itai-Itai disease, a particularly severe form of osteoporosis/osteomalacia diagnosed among women in Japan and attributed in part to cadmium exposure, occurred almost exclusively in postmenopausal women, not in younger women, men, or children (2). Although many studies of cadmium-induced bone loss had been conducted with male animals or females with intact ovaries, none asked if the combination of cadmium exposure and ovariectomy (to simulate conditions of postmenopausal hormone depletion) might accelerate the normal loss of bone calcium occurring after ovary removal; these results might explain why Itai-Itai disease did not appear until after the menopause.

We first exposed female CF1 mice to a purified diet containing $CdCl_2$ at either 0.25, 5.0, or 50 ppm Cd starting at 70 days of age. After 12 months of exposure, mice were ovariectomized (OV) or sham-operated (SO). After surgery, they remained on their respective diets for an additional six months before sacrifice. Neither ovariectomy alone nor dietary Cd exposure alone significantly decreased bone calcium content. However, dietary Cd at 50 ppm in combination with ovariectomy caused a striking decrease in the calcium content of mouse bones (3). The mice in this study were quite old (435 days old at ovariectomy; 617 days old at sacrifice) and had been exposed to dietary cadmium for one year before ovariectomy. Our next study was conducted with mice whose skeletons were prelabeled with ^{45}Ca to determine if cadmium exposure would cause an increased release of ^{45}Ca from the skeletons of OV mice immediately after the start of cadmium exposure

without the one-year pre-exposure period. Positive results would indicate that cadmium could act directly on bone rather than indirectly by way of damage to another organ such as the kidney.

In this study, the bone calcium of female CF1 mice was labeled over a one-week period by three intraperitoneal injections of ^{45}Ca. Beginning one month after the first ^{45}Ca injection, all mice were housed individually in stainless steel metabolism cages and given the purified AIN-76A diet with no added cadmium. Two weeks later, mice were either ovariectomized or sham-operated. After another two weeks, one-half of the OV and one-half of the SO mice were given the AIN-76A diet containing 50 ppm Cd. The remaining mice were left on the AIN-76A diet without cadmium. Throughout the study, urine and feces were collected from each animal for multiple sequential 96-h periods to obtain measurements of ^{45}Ca excretion, which in turn provided a measure of ^{45}Ca release from bone.

During the first two periods of excreta collection, which took place prior to both surgery and cadmium exposure, fecal ^{45}Ca excretion decreased with time in mice of all groups; no consistent differences between groups were observed. During the third collection period, which was the first period after ovariectomy, no effect of ovariectomy on fecal ^{45}Ca excretion was found. However, during the fourth and fifth rounds of collection (the second and third rounds after ovariectomy), a clear increase in fecal ^{45}Ca excretion occurred in the OV mice compared to the SO controls. During the fifth round, OV mice excreted 59 ± 7% (mean ± SE, n = 10) more ^{45}Ca into feces than did the SO mice.

After the fifth round of collection, one-half each of the OV and SO mice were switched to diets containing 50 ppm Cd. Cadmium exposure caused an immediate increase in fecal ^{45}Ca excretion in both the OV and SO mice, an increase that was clearly greater in OV than SO mice. In the OV mice, fecal ^{45}Ca excretion during the first collection after the start of cadmium exposure was 26 ± 4% (n = 10) greater than during the collection just before cadmium exposure; by comparison, in the SO mice, the analogous increase was only 6 ± 3% (n = 10). During the subsequent collection periods, fecal ^{45}Ca excretion decreased in all groups, but the increased excretion caused by cadmium consistently remained greater in the OV mice than in the SO controls. Comparisons of the extreme groups in the experiment showed that OV mice exposed to dietary cadmium excreted about twice as much ^{45}Ca into feces as did the SO mice not exposed to cadmium. These results from excreta

analyses were directly reflected in the ^{45}Ca contents of the bones obtained at sacrifice of the mice 6 mo after ovariectomy. The rapid release of ^{45}Ca from bone that occurred after the switch to the cadmium-containing diet supports the hypothesis that cadmium acts directly on bone.

Seed et al. (4) of Argonne recently discovered that canine monocytes, under a select set of culture conditions, fuse together to form giant, multinucleated, osteoclast-like cells (MN-OS), which attach themselves to bone and morphologically, cytologically, and ultrastructurally resemble normal canine osteoclasts in vivo. We incubated canine monocytes with 100-u-thick sections of dog femurs (presterilized by ultraviolet radiation) under culture conditions allowing the monocytes to differentiate into MN-OS cells. Cells were cultured in the presence and absence of 10 nM Cd to determine if this concentration of cadmium might activate the MN-OS cells, causing them to resorb bone. According to Dr. Seed's observations under the phase contrast microscope, on day 7 of incubation the multinucleated cells in the presence of cadmium not only were more numerous than those without cadmium, but also the morphology of the MN-OS cells were distinctly different. The cadmium-exposed cells were larger, more transparent, and had centrally located nuclei, with indications of the microvilli that are characteristic of active osteoclasts.

The results of both the in vivo and in vitro experiments are consistent with the hypothesis that cadmium may increase bone resorption by activating osteoclasts, bone cells that are normally responsible for the breakdown of he mineral and matrix of bone. In this way, cadmium may mimic the action of parathyroid hormone. We are currently investigating this hypothesis further.

Because cadmium concentrations in the blood of smokers are comparable to those in both our in vivo and in vitro studies (5), our results further suggest that cadmium in cigarette smoke in combination with smoking-induced decreases in estrogen levels may be one cause of the decreased bone mineral content, increased incidence of fractures, and earlier tooth loss observed in women who smoke. (Research supported by the U.S. Department of Energy, Office of Health and Environmental Research, under Contract No. W-31-109-ENG-38 and NIH Grant ES-04816.

REFERENCES

1. B.L. Riggs, Pathogenesis of osteoporosis. Am. J. Obstet. Gynecol., 156:1342-46, 1987.

2. K. Nogawa, Itai-itai disease and follow-up studies. In J.O. Nriagu, editor, Cadmium in the Environment, pages 1-37, John Wiley and Sons, New York, NY, 1981.

3. M.H. Bhattacharyya, B.D. Whelton, P.H. Stern, and D.P. Peterson, Cadmium accelerates bone loss in ovariectomized mice and fetal rat limb bones in culture, Proc. Nat. Acad. Sci., 85:8761-8765, 1988

4. T.M. Seed, L.V. Kaspar, F. Domann, G.K. Niiro, and D.A. Le Buis, Developmental and radiobiological characteristics of canine multinucleated osteoclast-like cells generated in vitro from canine bone marrow, Scanning Micros., 2(3):1599-1611, 1988.

5. C.-G. Elinder, L. Friberg, B. Lind, and M. Jawaid, Lead and cadmium levels in blood samples from the general population of Sweden, Environ. Res., 32:220-227, 1983.

EARLY TIME-DEPENDENT CHANGES IN THE TISSUE DISTRIBUTION OF CADMIUM AFTER ORAL VS. INTRAVENOUS EXPOSURE: RELATIONSHIP TO PATHWAYS FOR CADMIUM GI ABSORPTION

Maryka H. Bhattacharyya and Margaret M. Jonah

Argonne National Laboratory
Argonne, IL 69439-4833

Because of the low gastrointestinal absorption of cadmium, intravenous (IV), subcutaneous, or intraperitoneal injections are sometimes used as methods of experimental exposure of test animals in studies of cadmium metabolism and toxicity. However, after a single dose, parenteral routes of exposure produce different cadmium distribution patterns in liver and kidney than those found after oral administration. For example, results from our laboratory show that at 48 to 72 h after a single administration, the ratio of the amount of cadmium in liver to that in kidney is approximately 3:1 for oral and 13:1 for intraperitoneal administration (1,2). These route-dependent differences in deposition patterns indicate possible differences in cadmium metabolism or transport, differences that are already well established by 72 h.

We studied the kinetics of cadmium distribution in tissues and blood fractions in anticipation that, at early times, enough cadmium might be present in blood or tissue fractions to demonstrate route-of-administration-dependent differences in protein binding of cadmium that could explain the differences in tissue distribution. For example, in explanation of the increased deposition of cadmium in the kidney after oral administration, it has been proposed that a significant fraction of cadmium entering the blood from the gastrointestinal tract may be bound to metallothionein, which deposits almost exclusively in the kidney and not the liver (3,4).

For this study, we administered ^{109}Cd by gavage (100 μg Cd/kg) or by intravenous injection (1 mg Cd/kg) to male and female CF1 mice, 80 and 90 days old, respectively. The 100-fold difference in dose was chosen to produce similar chemical concentrations of cadmium in blood in both orally and intravenously treated animals, on the basis of an estimated 1% absorption of the orally administered cadmium. Groups of mice were sacrificed at intervals from 5 min to 15 days after ^{109}Cd administration.

Results show that cadmium deposition patterns in liver and bone were

similar for both modes of administration. Unexpectedly, cadmium deposition in the kidney was also similar at early times and did not become distinctly different until after 8 h. In mice receiving oral cadmium, kidney ^{109}Cd levels were initially low and increased almost fourfold between 30 min and 72 h after administration. In contrast, after IV injection, mice showed kidney levels that were also initially low but remained low from 30 min to 72 h after injection. Consequently, the ratio of the ^{109}Cd content of liver to that of kidney was 14-15:1 for female mice at 30 min after cadmium administration independent of mode of administration. At 72 h, however, differences in kidney:liver ^{109}Cd ratios similar to those reported in the literature were observed: 15:1 of the IV mice and 4:1 for the gavage mice.

These results are consistent with the existence of two pathways for cadmium deposition after oral exposure. At early times, cadmium may enter the blood from the intestinal cells, bind to albumin, and accumulate primarily in liver, the same as the pathway that has been identified after IV administration (5,6,7). At later times, however, absorbed cadmium may be released from the intestinal cells bound to metallothionein and deposit preferentially in the kidney. Because cadmium bound to metallothionein causes kidney damage at very low dosage levels, the different pathways of deposition after oral vs. intravenous exposure may in part explain why acute parenteral cadmium exposure causes liver toxicity while chronic oral exposure causes renal toxicity.

This work was supported by the U.S. Department of Energy, Office of Health and Environmental Research, under Contract No. W-31-109-ENG-38.

REFERENCES

1. M.H. Bhattacharyya, B.D. Whelton, and D.P. Peterson, Gastrointestinal absorption of cadmium in mice during gestation and lactation. I. Short term exposure studies, Toxicol. Appl. Pharmacol., 61:335-342, 1981.

2. A.L. Cahill, D. Nyberg, and C.F. Ehret, Tissue distribution of cadmium and metallothionein as a function of time of day and dosage, Environ. Res., 31:54-65, 1983.

3. M.G. Cherian, R.A. Goyer, and L.S. Valberg, Gastrointestinal absorption and organ distribution of oral cadmium chloride and cadmium-metallothionein in mice, J. Toxicol. Environ. Health, 4:861-868, 1978.

4. M. Nordberg, Studies on metallothionein and cadmium, Environ. Res., 15:381-404, 1978.

5. S.E. Bryan and H.A. Hidalgo, Nuclear ^{115}cadmium: uptake and disappearance correlated with cadmium-binding protein synthesis, Biochem. Biophys. Res. Comm., 68:858-866, 1976.

6. R.J. Cousins, Absorption, transport, and hepatic metabolism of copper and zinc: Special reference to metallothionein and ceruloplasmin, Physiol. Revs., 65:238-309, 1985.

7. T. Kjellstrom and G.F. Nordberg, Kinetic model of cadmium metabolism, in Cadmium and Health: A Toxicological and Epidemiological Appraisal: Exposure, Dose, and Metabolism Vol I, pages 179-197, CRC Press, Inc., Boca Raton, FL, 1985.

A CANINE MODEL OF BERYLLIUM-INDUCED GRANULOMATOUS LUNG DISEASE

P.J. Haley, G.L. Finch, J.A. Mewhinney
F.F. Hahn, M.D. Hoover, and D.E. Bice

Lovelace Inhalation Toxicology Research Institute
Albuquerque, NM 87185

Chronic beryllium disease (CBD) of man is a systemic immune-mediated granulomatous disease with predominantly pulmonary manifestations. Despite the recognition of beryllium as the etiologic agent, the pathogenesis of the disease remains obscure. Because CBD presents histologic lesions in the lung similar to other granulomatous lung diseases, such as sarcoidosis, assays that would allow definitive diagnosis have been sought. The observations that CBD occurs in only 3% of individuals exposed to beryllium, and that affected individuals develop beryllium-specific immune responses in blood and lung have suggested that an immunological assay, such as lymphocyte proliferation, might allow more accurate diagnosis of the disease. While such beryllium-specific immune responses have been identified in lung lymphocytes from humans, similar results in laboratory animals exposed to beryllium have not been observed. More importantly, data from human patients with CBD indicate that peripheral blood lymphocyte reactivity alone may not accurately reflect either the predilection for, or the progression of, CBD. In fact, it appears that localized lung lymphocyte reactivity to beryllium is central to the development of CBD, and that sampling of lung lymphocytes is necessary for diagnosis in humans, as well as for experimental manipulation in laboratory animals.

Alveolar lymphocytes obtained by bronchoalveolar lavage (BAL) and peripheral blood lymphocytes from 20 dogs exposed once by inhalation to low and high concentrations of beryllium oxide (BeO) calcined at one of two different temperatures, 500°C or 1000°C, were tested in vitro for sensitivity to $BeSO_4$. Lymphocyte responses to $BeSO_4$ and phytohemagglutin (PHA) were performed in triplicate using a standard lymphocyte stimulation assay. The concentrations of $BeSO_4$ were 1.0, 10, and 100 μM; and of PHA were 0.5 and 1.0 μg/ml. The stimulation index (SI) for each sample was calculated as a ratio of counts per min in experimental cultures divided by control cultures. A stimulation index of greater than 4 was considered a positive response. Lymphocytes were obtained from dogs at 3,6,7,11,15,18 and 22 months after exposure.

Percentages of lung lymphocytes in dogs with high ILBs of 500°C-treated BeO had a marked increase at 3 mo, which declined slowly through 22 mo (Fig. 1). Dogs with low ILBs of 500°C-treated BeO also had an increase in percent lymphocytes at 3 mo which returned to control levels more rapidly. Total lung lymphocytes were likewise elevated in animals exposed to 500°C-treated BeO with the high ILB group again showing the greatest increases at 3 mo with a progressive decrease thereafter (Fig. 2). Stimulation indices (SI) of blood and BAL lymphocytes demonstrated marked individual variation with positive responses to $BeSO_4$ observed more frequently in blood (14 of 16 dogs) than in lung; however when blood and BAL samples were positive in the same animal, the BAL response was consistently greater. Positive BAL lymphocyte responses were observed at 6 and 7 months after exposure, with peak responses occurring at 7 months followed by a rapid decline (Fig. 3). Peak BAL SI values ranged from a high of 64 at 6 months to a low of 6 at 7 months. Positive BAL SI were seen only in dogs with the high initial lung burden (ILB) of 500°C BeO. Positive blood SI were observed at 7,15,18, and 22 months after exposure in some but not all dogs with high and low ILBs of 500°C and 1000°C BeO (Fig. 4). A positive dose-response to 10 and 100 μM $BeSO_4$ for both lung and blood samples was observed. Lymphocytes from lung and/or blood of control dogs did not respond to *in vitro* cultivation with $BeSO_4$. *In vitro* culture of lung lymphocytes with a non-specific mitogen, PHA, resulted in four positive responses, only two of which also had positive responses to $BeSO_4$.

Dogs with positive lymphocyte stimulation indices in BAL samples usually, but not always, had positive indices in blood as well. Eleven of 16 dogs had a combination of negative BAL SI accompanied by positive blood lymphocyte SI.

These data indicate that a single exposure of dogs to an aerosol of BeO can result in beryllium-specific immune responses by alveolar lymphocytes consistent with those observed in humans with chronic beryllium disease, with the caveat that the responses appear to resolve. It also appears that inhalation exposure to beryllium produces nonspecific inflammatory changes characterized by pulmonary infiltration by large numbers of lymphocytes. In most cases, these lymphocytes are not beryllium-specific and rapidly leave the lung. In a few individuals, a subset of beryllium-specific lymphocytes persists within the lung and mediates beryllium-specific cell mediated immune responses. It is postulated that beryllium-induced lung lesions are mediated by this beryllium-sensitized subpopulation of lymphocytes.

The canine model described here offers distinct advantages over other animal models of CBD that include: (1) granulomatous lung lesions in the dog after inhalation of an occupationally relevant dose of beryllium, (2) the ability to repeatedly and sequentially sample lung cells by BAL in a single animal, (3) local pulmonary immune responses of dogs are more similar to those of humans than are those of rodents, and (4) dogs and man are the only species shown to have beryllium-specific immune responses localized to the lung after inhalation of beryllium. (Research sponsored by the U.S. Department of Energy Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.)

FIGURE LEGENDS

Figure 1 Percent lymphocytes of cells recovered by bronchoalveolar lavage of dogs with either high or low ILB, and 500°C- or 1000°C-treated BeO. Each symbol represents a single animal and is the same symbol for the same dog in all figures except for • which represents the mean for the control animals (N = 4). Bars = standard error for control values.

Figure 2 Total lymphocyte numbers in bronchoalveolar lavage samples from dogs with either high or low ILB, and 500°C- or 1000°C-treated BeO. Each symbol represents a single animal and is the same symbol for the same dog in all figures except for • which represents the mean for the control animals (N = 4). Bars = standard error for control values.

Figure 3 Stimulation indices after culture with 100 μM $BeSO_4$ of cells obtained by bronchoalveolar lavage of dogs with either high or low ILB, and 500°C- or 1000°C-treated BeO. Each symbol represents a single animal and is the same symbol for the same dog in all figures.

Figure 4 Stimulation indices of peripheral blood lymphocytes after culture with 100 μM $BeSO_4$ of dogs with either high or low ILB, and 500°C- or 1000°C-treated BeO. Each symbol represents a single animal and is the same symbol for the same dog in all figures.

Figure 1

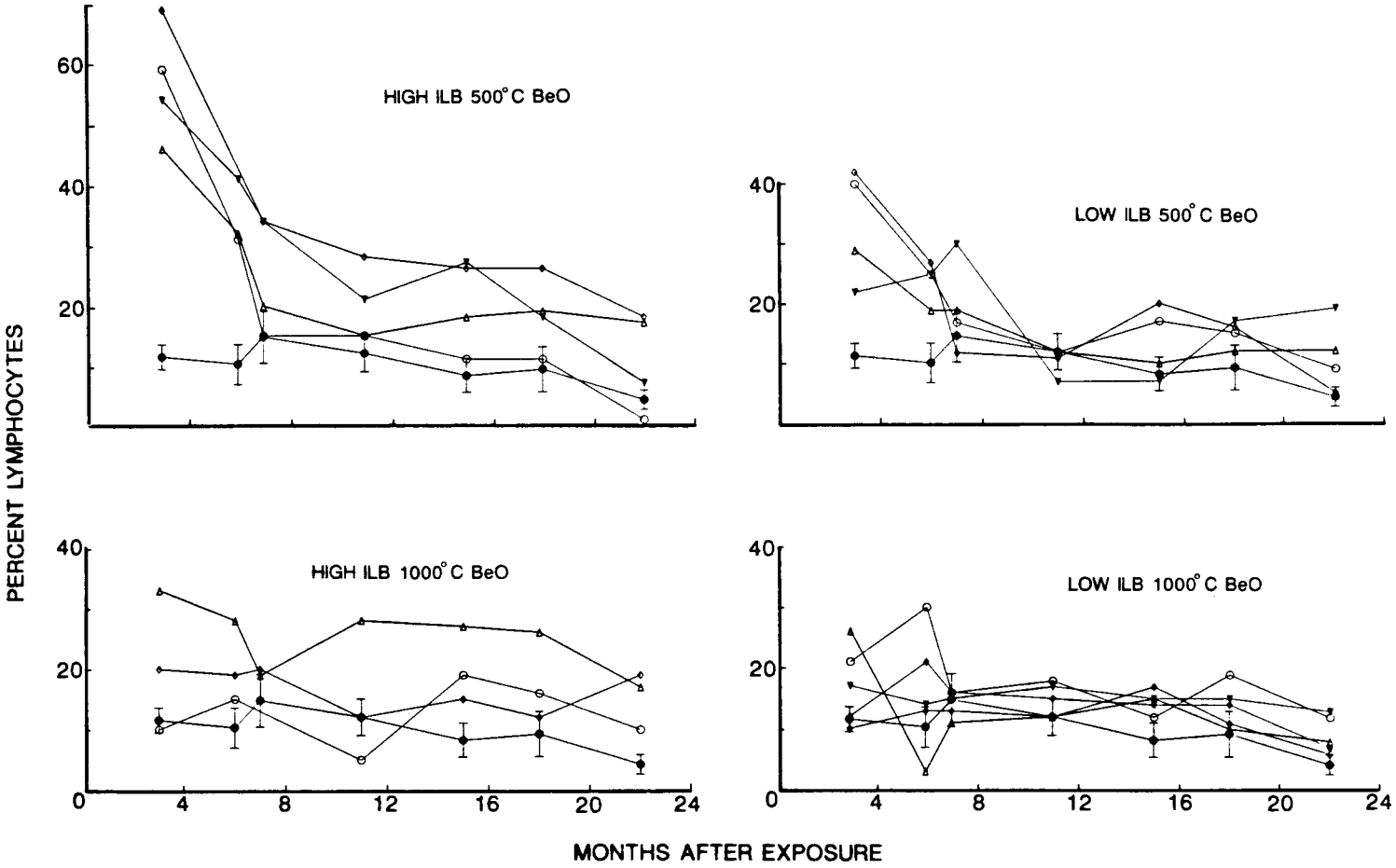

HIGH ILB 500°C BeO
LOW ILB 500°C BeO
HIGH ILB 1000°C BeO
LOW ILB 1000°C BeO
PERCENT LYMPHOCYTES
MONTHS AFTER EXPOSURE
0
20
40
60
4
8
12
16
20
24

Figure 2

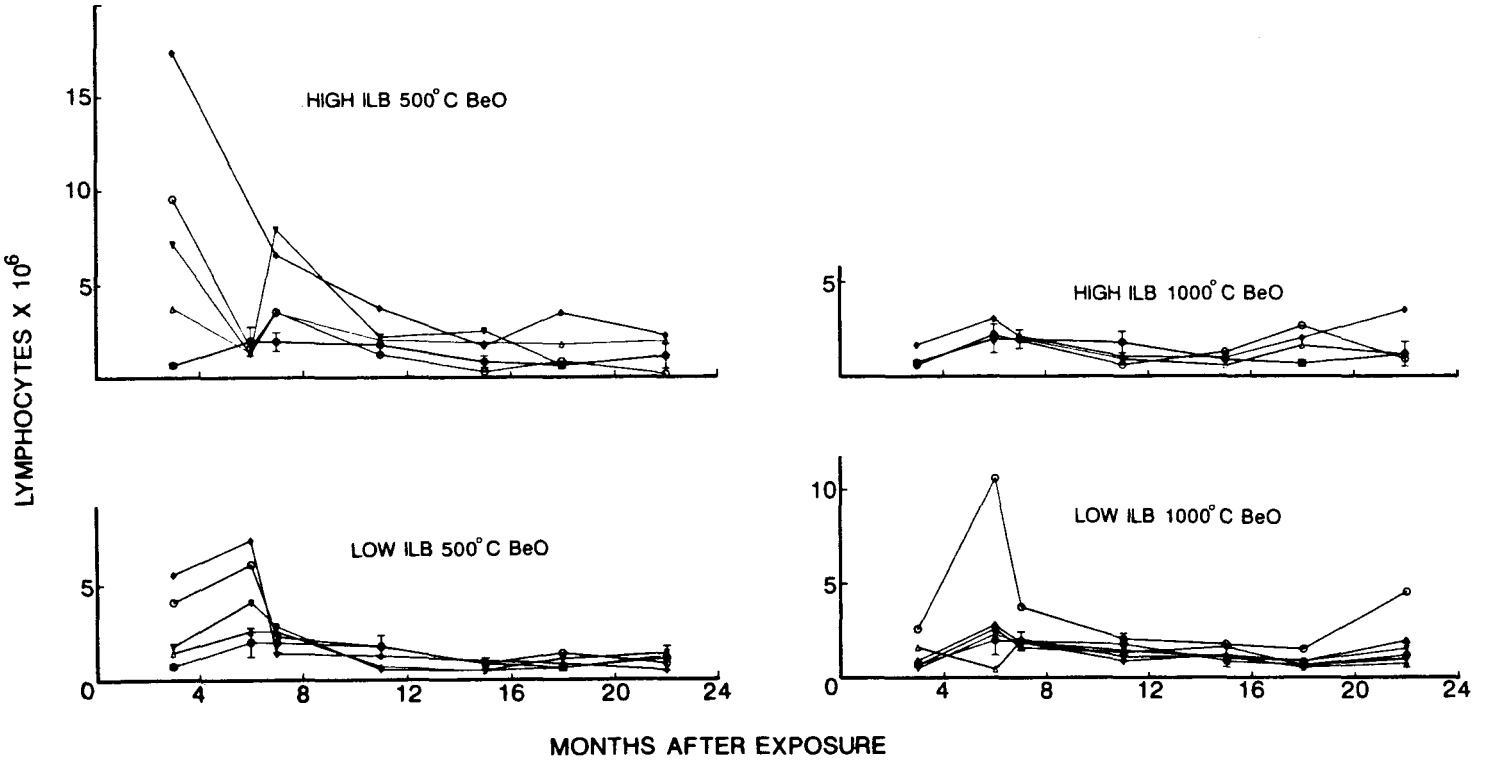

HIGH ILB 500°C BeO
HIGH ILB 1000°C BeO
LOW ILB 500°C BeO
LOW ILB 1000°C BeO
LYMPHOCYTES X 10⁶
MONTHS AFTER EXPOSURE
0
4
8
12
16
20
24
5
10
15

Figure 3

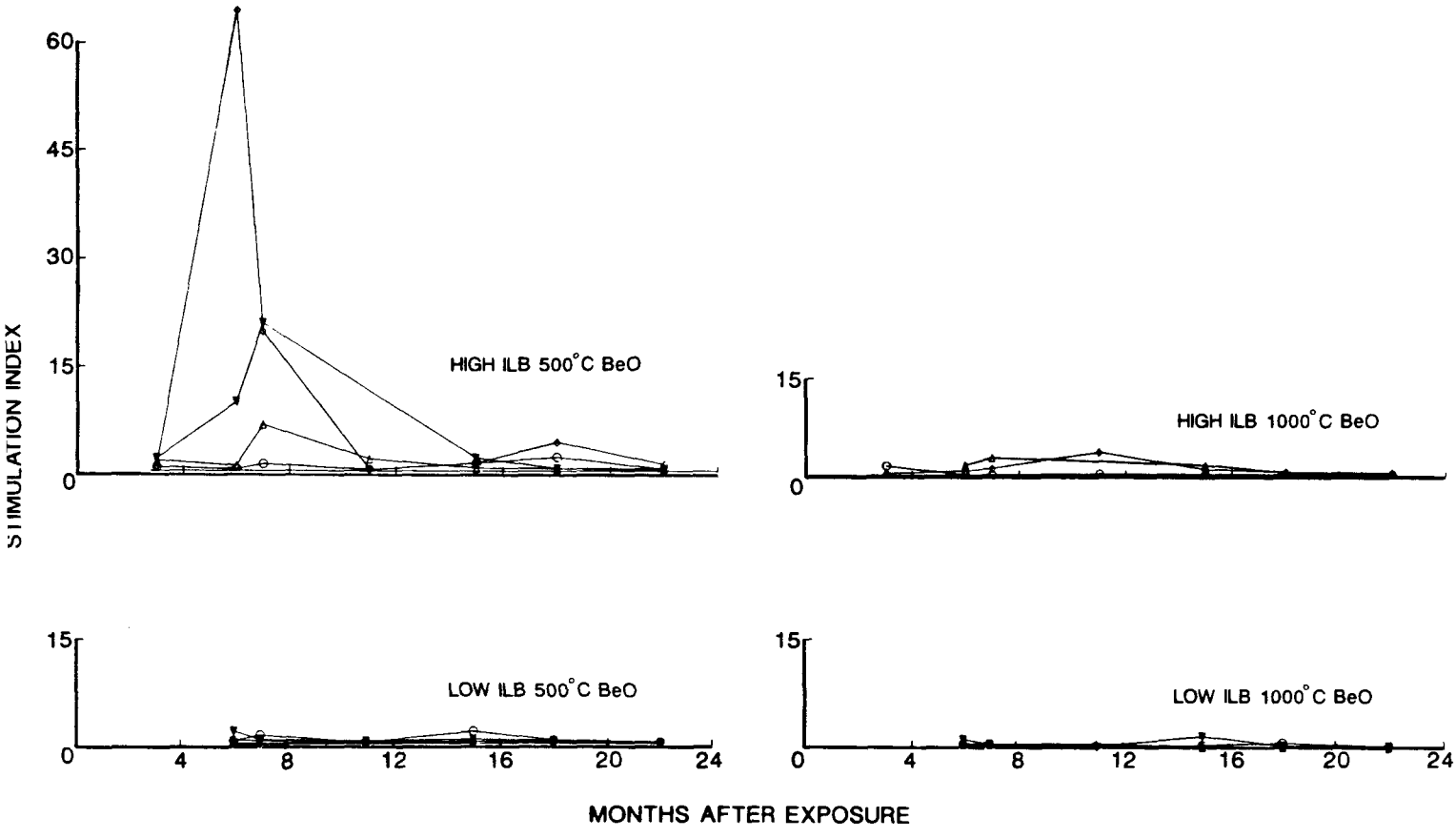
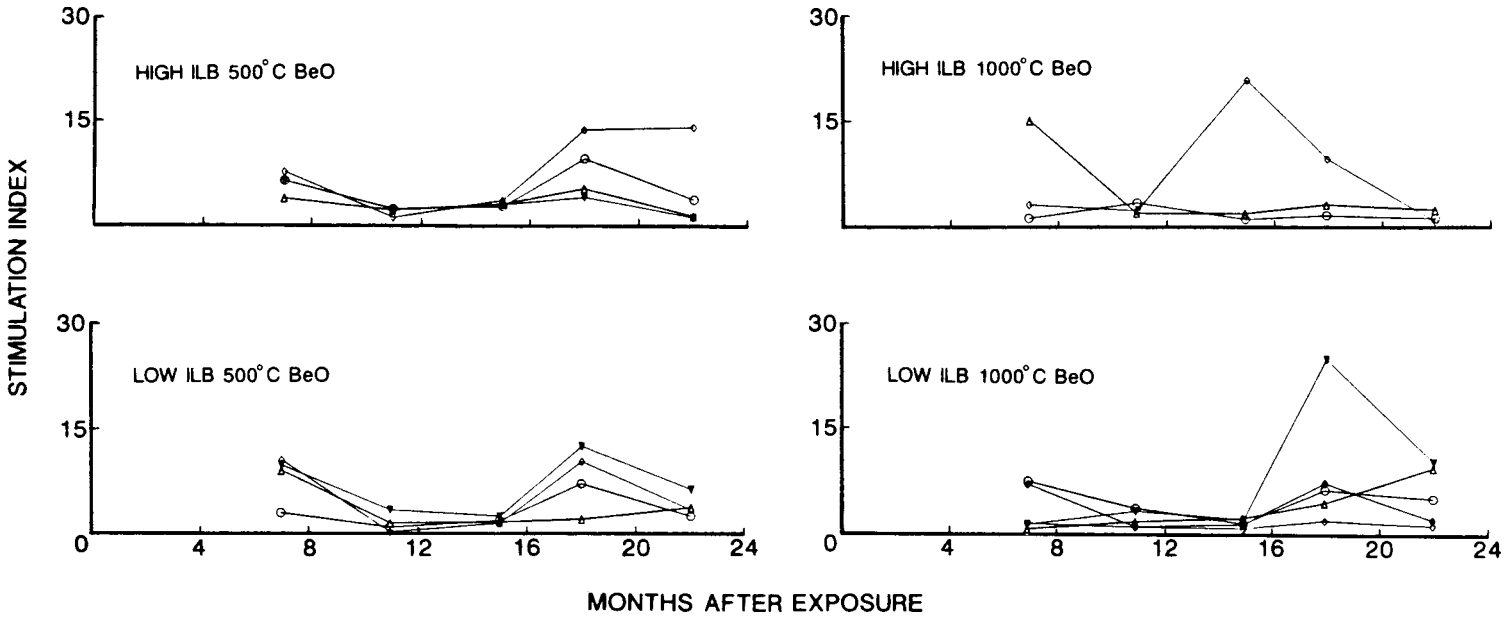

STIMULATION INDEX
60
45
30
15
0
HIGH ILB 500°C BeO
HIGH ILB 1000°C BeO
LOW ILB 500°C BeO
LOW ILB 1000°C BeO
4
8
12
16
20
24
MONTHS AFTER EXPOSURE

Figure 4


HIGH ILB 500°C BeO
HIGH ILB 1000°C BeO
LOW ILB 500°C BeO
LOW ILB 1000°C BeO
30
15
0
4
8
12
16
20
24
STIMULATION INDEX
MONTHS AFTER EXPOSURE

INDUCTION AND MAINTENANCE OF LOCAL IMMUNE RESPONSES IN THE LUNG

D. E. Bice and B. A. Muggenburg

Lovelace Inhalation Toxicology Research Institute
Albuquerque, NM 87185

The inhalation of pollutants in the environment and in certain occupations can result in serious lung disease. Inhaled pollutants may suppress pulmonary defenses resulting in an increase in lung infections or possibly lung cancer. In contrast to suppressed lung immunity, immune responses in the lung to inhaled antigens or chemicals (e.g. beryllium) can lead to hypersensitivity lung disease. An understanding of the basic mechanisms responsible for the induction of immunity in the lung and how local lung immunity is regulated is essential to understand how immune defenses may be suppressed by inhaled pollutants, or how hypersensitivity lung disease is induced.

The evaluation of immunity induced by the localized deposition of antigen into selected airways of the lungs of dogs and primates has provided details of the mechanisms involved in development of pulmonary immunity. The size of these animals allows the instillation of antigens into selected lung lobes by the use of a fiberoptic bronchoscope, while saline or other control materials are instilled into lung lobes of the same animal. At intervals after a primary immunization or after antigen challenge (e.g. 3, 5, 7, 10, 12, and 14 days), the animals are anesthetized and the control and immunized lung lobes are lavaged by the instillation and withdrawal of saline through the fiberoptic bronchoscope. The number of immune cells and level of specific antibody present is evaluated in lymph nodes, blood, and in lavage fluid from control and immunized lung lobes.

In primary antibody responses, antigen is cleared from the lung to the lung-associated lymph nodes, possibly within neutrophils and/or alveolar macrophages (1, 2). There are several lung-associated lymph nodes in the thoracic cavity, and large numbers of antibody-forming cells (AFC) are found only in those lymph nodes that receive lymphatic drainage from the lung lobe instilled with antigen (3). Large numbers of specific AFC and antibody are released via the efferent lymphatics into the blood (4). Although the mechanisms are not understood, antibody and AFC present in the blood accumulate predominantly in the immunized lung lobes (4). We have carried

out studies to determine if mediators of inflammation may be responsible for recruitment of lymphocytes and AFC into the immunized lung lobe (5). The accumulation of immune cells into the lung is not antigen specific, and cells produced in any lymphoid tissue can enter into immunized lung lobes (6, 7). Lymphocytes recruited into the lung mature to plasma cells and produce antibody locally, increasing the titer above that entering the lung by transudation from the vasculature (8, 9).

Our data indicate that immune memory cells are also recruited mainly into lung lobes exposed to antigen (10 11). Additional antigen challenges result in intense antibody responses only in the immunized and challenged lung lobes (10, 11). Immune memory cells in the lung respond to antigen challenges for at least 180 days after the last antigen exposure. Data from Cynomolgus monkeys indicate that the cells responding to antigen challenge are in the interstitial tissues of the lung.

In addition to memory cells, AFC can also be found in the lung at long times after antigen exposure. The immunized and control lung lobes of dogs that were exposed to antigen three years earlier were lavaged to determine the levels of specific antibody in lavage fluid and to evaluate for cells producing antibody. There was significantly elevated antibody in the immunized lung lobe compared to the control. In addition, cells from the immunized lung lobe produced significantly more antibody after being cultured for seven days than observed in cultures of cells from the control lung lobe. These data indicate that exposure of lung to antigen induces a localized immune response that is maintained for at least three years and possibly for the life of the animal. It seems likely that immune memory and long-term production of antibody in the lung is essential in pulmonary defense to infectious agents and possibly in the recognition and removal of cancer cells. In addition, a loss of control of these immune cells could also lead to hypersensitivity lung diseases.

The identification of the tissues and cells responsible for the development of lung immunity helps identify immune cells at risk to inhaled chemicals. Both immune memory cells and AFC are present in the alveoli and interstitial lung tissues for long times after immunization. Therefore, these immune cells are at risk to inhaled pollutants. Because the effects of inhaled pollutants on the functions of memory cells or AFC in the lung have not been evaluated, studies are needed to determine if inhaled

pollutants alter the immune functions of these cells. (Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.)

REFERENCES

1. Harmsen A.G., Muggenburg B.A., Snipes M.B., and Bice D.E., Science 230, 1277-1280; 1985.

2. Harmsen A.G., Mason M.J., Muggenburg B.A., Gillett N.A., Jarpe M.A., and Bice D.E., J. Leukocyte Biol. 41, 95-103, 1987.

3. Bice D.E., Harris D.L., and Muggenburg B.A., Exp. Lung Res. 1, 33-40; 1980.

4. Bice D.E., Harris D.L., Hill J.O., Muggenburg B.A., and Wolff R.K., Am. Rev. Respir. Dis. 122, 755-60; 1980.

5. Bice D.E., King-Herbert A.P., Morris M.J., Hanna N., and Haley P.J., Am. J. Cell. Molec. Biol. (submitted)

6. Bice D.E., Degen M.A., Harris D.L., and Muggenburg B.A., Am. Rev. Respir. Dis. 126, 635-9; 1982.

7. Hillam R.P., Bice D.E., and Muggenburg B.A., Immunology 55, 257-61; 1985.

8. Bice D.E., Gray R.H., Evans M.J., and Muggenburg B.A., J. Leukocyte Biol. 41, 1-7; 1987.

9. Harmsen A.G., Bice D.E., and Muggenburg B.A., J. Leukocyte Biol. 37, 483-92; 1985.

10. Mason M.J., Bice D.E., and Muggenburg B.A., Am. Rev. Respir. Dis. 132, 657-60; 1985.

11. Bice D.E., and Muggenburg B.A., Am. Rev. Respir. Dis. 138, 565-571; 1988.

COMPARISON OF ACUTE OZONE-INDUCED NASAL AND PULMONARY INFLAMMATORY RESPONSES IN RATS

J. R. Harkema, J. A. Hotchkiss, J. D. Sun, and R. F. Henderson

Lovelace Inhalation Toxicology Research Institute
Albuquerque, NM 87185

INTRODUCTION AND EXPERIMENTAL DESIGN:

Ozone is a major irritant gas in photochemical smog. The centriacinar pulmonary lesion induced by acute and chronic inhalation of O_3 has been extensively characterized in several laboratory animals, including nonhuman primates. Despite the fact that the nasal cavity has been shown to absorb 40 - 70% of inhaled O_3 in dogs and 50% in rabbits and guinea pigs, the response of the upper respiratory tract to O_3 exposure has been less extensively studied. Recently, it has been shown that both short- and long-term exposure to ambient levels of O_3 can induce significant epithelial lesions in the nasal cavity of monkeys. Short-term alterations following O_3-exposure included an influx of neutrophils into the nasal surface epithelium. Humans may have a similar acute response to O_3, since subjects exposed briefly to 0.5 ppm O_3 had increased numbers of nasal neutrophils for at least 22 h after exposure. Since an influx of neutrophils is an acute cellular response to epithelial injury, changes in the number of inflammatory cells recovered by nasal lavage may be a useful indicator of acute O_3 toxicity. Although the nasal cavity reduces the amount of inhaled O_3 reaching the lung, and both upper and lower respiratory tract epithelia are damaged by exposure to O_3, to our knowledge, there is only one brief report in the literature that has examined the simultaneous lesions induced in the nose and lungs of animals exposed to O_3.

The present study was designed to compare the effects of acute O_3 exposure in the nose and lungs of rats. Specifically, we compared the cellular inflammatory response in the nasal cavity and lower respiratory tract by means of nasal and bronchoalveolar lavage and morphometric quantitation of neutrophils within the nasal mucosa and pulmonary terminal bronchiole-alveolar duct regions.

METHODS:

A total of 108 female F344/N rats (12-18 wks; 280 to 400 g) were obtained from the Inhalation Toxicology Research Institute's colony. Rats

were exposed for 6 hr to 0.0 (air controls), 0.12, 0.8 or 1.5 ppm O_3 (equivalent sea level concentrations; 0.0, 0.10, 0.66, 1.2 ppm) in whole-body inhalation chambers. Groups (n=6) of rats from each ozone-exposure concentration group were euthanized immediately after (0 hr), or 3, 18, 42, or 66 hr following the end of exposure. The nasal cavity and right lung of each rat was lavaged with saline and then the nasal cavity and entire lung were fixed and prepared for light microscopy. Total and differential cell counts were performed on cells recovered by bronchoalveolar and nasal lavage. The nasal cavity neutrophilic response was estimated by quantitating the number of pavementing neutrophils (i.e., neutrophils in contact with the luminal surface of an endothelial cell) within blood vessels of the region of nasal turbinate that parallels the nasal septum. Terminal bronchioles from centriacinar regions were analyzed for infiltrating PMN by quantitating the number of intramural PMN nuclear profiles per unit length of alveolar and bronchiolar basal lamina.

RESULTS:

At 18 hr after exposure, increased numbers of neutrophils, as compared to controls, were recovered from nasal lavage fluid (NLF) from rats exposed to 0.12 ppm O_3, but there was no change in the number of neutrophils recovered from bronchoalveolar lavage fluid (BALF) at any time after exposure. Rats exposed to 0.8 ppm O_3 had more neutrophils in NLF than controls immediately after exposure, but no concomitant increase in BALF neutrophils. However, as the number of neutrophils in BALF increased (maximum at 42 hours), the number of neutrophils recovered from NLF decreased (minimum at 42 hours). Rats exposed to 1.5 ppm O_3 had no significant increase in nasal neutrophils in NLF, but had greatly increased numbers of neutrophils in BALF 3, 18, and 42 hours after exposure. The number of neutrophils recovered by nasal and bronchoalveolar lavage accurately reflected the tissue neutrophil response at sites within the nasal cavity and lung that were injured by acute ozone exposure.

DISCUSSION:

This study demonstrates that a single 6-hr exposure to O_3, at a concentration equal to the current Air Quality Standard (0.12 ppm) or only slightly above peak excursion levels reached in certain metropolitan areas (0.8 ppm), induces an acute inflammatory response within the nasal cavity of

rats. The inflammatory lesion was restricted to the anterior portion of the nasal cavity and was characterized by an influx of neutrophils within the medial tips and lateral surfaces of the nasal and maxilloturbinates covered by nonciliated, cuboidal epithelium with scant epithelial mucosubstances. The O_3-induced inflammatory lesion observed in this study is similar to that seen in nonhuman primates exposed to a similar (0.15 ppm) concentration of O_3. We have shown that the relative numbers of neutrophils recovered by nasal and bronchoalveolar lavage accurately reflect the tissue neutrophil response at sites within the nose and lung injured by acute inhalation of O_3. Our results suggest that at high O_3 levels (0.8 and 1.5 ppm), the acute nasal inflammatory response is attenuated by a simultaneous, competing, inflammatory response within the centriacinar region of the lung. To our knowledge, this is the first study to examine the simultaneous effects of a range of O_3 concentrations of the upper and lower respiratory tract epithelium. After acute exposures to ambient levels of O_3, alterations in cellular, and perhaps biochemical, parameters within the nose may provide sensitive indicators of O_3 exposure. In contrast, nasal cellular inflammatory responses after exposure to higher O_3 levels, may underestimate the effects of O_3 within the lung. (Research supported by U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.)

THE EFFECT OF DOSE RATE ON THE INFLAMMATORY RESPONSE OF THE LUNG TO INHALED PARTICLES

R. F. Henderson, R. K. Wolff, J. R. Harkema and J. L. Mauderly

Lovelace Inhalation Toxicology Research Institute
Albuquerque, NM 87115

Total suspended particulate (TSP) matter in the work place is regulated by time weighted averages (TWA) of exposures over an 8-hr period (OSHA, 1987). The use of TWA for evaluating exposures assumes that the pulmonary toxicity of particles inhaled over an 8-hr period will be the same whether the particles are inhaled at a high concentration over a short period of time or are inhaled at a lower concentration over the whole 8-hr period. Recent observations at the Lovelace Inhalation Toxicology Research Institute (ITRI) indicate that the rate at which particles are deposited in the lung may influence the inflammatory response to the particles and the rate of clearance of the deposited particles from the lung.

METHODS:

Male F344/N rats were exposed to the same concentration times time product (C x T) of carbon black over a 12-week period, with one group of rats exposed to episodic high concentrations of the particles and a second group exposed to a more continuous, low level of particles. The carbon black was Cabot Elftex 12. Lung burdens of particles and the lung inflammatory response in the two groups were compared.

Rats in the episodically-exposed group were exposed nose-only to 98 mg carbon black/m^3 air, 4 hr/day, 1 day/week for 12 weeks. Rats exposed in the more continuous mode were exposed whole body to 11 mg carbon black/m^3 air; 7 hr/day, 5 days/week for 12 weeks. Lung burdens of carbon black were determined after 2, 4, 8 and 12 weeks of exposure by a method based on the light extinction produced by the particles (Henderson et al., 1987). The pulmonary inflammatory response was quantitatively evaluated by markers of an inflammatory response in bronchoalveolar lavage fluid (BALF) (Henderson, 1988) and qualitatively evaluated by histopathology.

RESULTS:

The actual C x T product achieved by the more continuous exposure was 90% of that achieved by the episodic exposure. In the episodic exposures,

the particles had a bimodal size distribution with 70% of the particles in the small mode [mass median diffusion diameter (MMDD) of 0.09 μm with a geometric standard deviation (σ_g) of 2.5] and the rest in a larger mode [mass median aerodynamic diameter (MMAD) of 1.6 μm with a σ_g of 2.5]. The aerosol characteristics of the more protracted exposures were similar with 70% of the particles in the small mode (0.12 MMDD, σ_g=2.3) and the rest in the larger mode (2.0 MMAD, σ_g=2.5).

The lung burdens rose more rapidly in the episodically exposed rats after 4 weeks of exposure than in the slowly exposed animals. Final lung burdens were 2.8 ± 0.6 and 3.8 ± 0.6 mg soot/g lung for the more continuous and the episodic exposure regimens, respectively, indicating a slightly higher accumulation in episodically exposed animals.

The inflammatory response measured in bronchoalveolar lavage fluid at the end of the 12-week exposure was much greater in the episodically exposed rats. There was only a mild inflammatory response in the rats exposed by the more continuous regimen, as indicated by increased neutrophils (10x) and acid protease activity (1.5x). The inflammatory response in episodically exposed animals was much greater, with BALF increases in pulmonary macrophages (2x), neutrophils (100x), protein (3x) and several enzyme activities [lactate dehydrogenase (3x), beta glucuronidase (10x), acid protease (2x) and alkaline phosphatase (2x)]. The histopathology confirmed the differences in the inflammatory response. Control values for BALF from animals exposed by the two regimens were not statistically different.

DISCUSSION:

The animals exposed to approximately the same C x T product of inert particles had two differences in response. The episodically exposed rats had slightly higher lung burdens at the end of the exposure and a much greater pulmonary inflammatory response. The higher dose rate may have overwhelmed the ability of the lung to clear particles in a normal fashion, resulting in an increased inflammatory response. The increase in inflammatory response after the high dose rate exposures may have been partly due to the different exposure systems used (nose-only for the high dose rate, whole-body for the lower dose rate), but the values for the BALF parameters from control animals did not differ between the two control groups. The data indicate that further studies should be done with animals exposed to inert dust particles at different dose rates, but using the same

exposure systems, to determine if the effects reported here can be confirmed. If so, the use of time weighted averages for setting of permissible particle levels in industrial settings may not always be appropriate. (Research supported by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.)

REFERENCES

1. Occupation Safety and Health Administration (OSHA) (1987). Code of Federal Regulation 1910.1028.

2. Henderson, R. F., J. J. Waide, J. L. Mauderly, and R. O. McClellan (1987). A rapid method for determining soot content of lungs in diesel-exposed rodents. J. Appl. Toxicol. 7:357-360.

3. Henderson, R. F. (1988) Use of bronchoalveolar lavage to detect lung damage. In "Toxicology of the Lung," (D.E. Gardner, J. D. Crapo and E. J. Massaro, eds.) Raven Press, NY, pp. 239-268.

DEPOSITION OF ULTRAFINE PARTICLES IN THE HUMAN NOSE AND MOUTH

H. C. Yeh, Y. S. Cheng, Y. Yamada*, and D. L. Swift**

Lovelace Inhalation Toxicology Research Institute
Albuquerque, NM 87185

*National Institute of Radiological Sciences, Chiba-shi, Japan
**Johns Hopkins University, Baltimore, MD 21233

The regional respiratory deposition of inhaled particles has been recognized as a critical factor in the evaluation of health effects of exposure to airborne material. The upper airways have usually been considered as a filter, preventing or limiting exposure to the more distal respiratory tracts. However, a substantial fraction of inhaled particles may deposit in the nose and mouth and these regions of the respiratory tract may be tissues at risk for health effects. Information on the aerosol deposition pattern in this region is needed to estimate the initial deposited dose.

Previous studies of upper airway deposition have emphasized the role of inertial deposition of particles > 0.5 μm in aerodynamic diameter (AD). For ultrafine particles less than 0.2 μm in diameter, only a few studies have been reported. George and Breslin[1] reported 60 to 80 % deposition efficiency for unattached radon progeny in the nasal airways at 50 to 3 L/min inspiratory flow. Recently, we reported aerosol deposition in a human nasal cast for ultrafine particles ranging from 0.005 to 0.2 μm at constant inspiratory and expiratory flow rates between 4 to 50 L/min[2,3]. The deposition efficiency increased with decreasing particle size and flow rate with expiratory phase having a slightly higher deposition efficiency. However, no studies have been performed on the oral deposition of particles in the ultrafine size range. This report describes an experimental study on the deposition of ultrafine particles in a human oral cast and the results are compared to our previously reported nasal deposition.

A clear polyester resin cast of the upper airways of a normal human adult was used. It was made from the same negative master of the cast used for a previous study[3]. Entrance to the nasal airway was sealed and a 10-mm diameter entrance was open to the oral cavity. The pressure drop across the oral airway between the entrance and the trachea was measured by an inclined manometer at flow rates between 2 and 50 L/min. The experimental methods

have been reported elsewhere[2]. Monodisperse NaCl aerosols ranging from 0.0046 to 0.2 μm were used in the study. The tested flow rates were 4, 20, and 40 L/min. Both inspiratory and expiratory modes were used for deposition study.

The results of measured pressure drop across the oral cast as compared to that of the nasal cast are shown in Fig. 1, which indicates that the pressure drop was slightly lower in oral breathing than in nasal breathing. Figure 2 shows deposition efficiency plotted as a function of particle size and flow rate for inspiratory condition. Deposition efficiency increased with decreasing particle size for particles between 0.0046 μm and 0.2 μm. A weak trend toward decreasing efficiency with increasing flow rate was also detected. Particle deposition for the inspiratory flow was higher than that for the expiratory flow.

Comparing oral and nasal deposition data obtained previously showed that deposition efficiencies in both oral and nasal airways were essentially the same for the inspiratory flow (Fig. 3). However, lower oral deposition efficiencies were observed for the expiratory flow (Fig. 4). This trend was true at all three flow rates tested. (Research sponsored by the U.S. Department of Energy Office of Health and Environmental Research Under Contract No. DE-AC04- 76EV01013.)

REFERENCES

1. George, A. and A. J. Breslin. Deposition of Radon Daughters in Humans Exposed to Uranium Mine Atmospheres, Health Phys. 17: 115-124, 1969.

2. Cheng, Y. S., Y. Yamada, H. C. Yeh, and D. L. Swift. Diffusion Deposition of Ultrafine Aerosols in a Human Nasal Cast, J. Aerosol Sci. (1989, in press)

3. Yamada, Y., Y. S. Cheng, H. C. Yeh, and D. L. Swift. Inspiratory and Expiratory Deposition of Ultrafine Particles in a Human Nasal Cast, Inhalation Toxicol. Premier Issue, 1-11, 1989

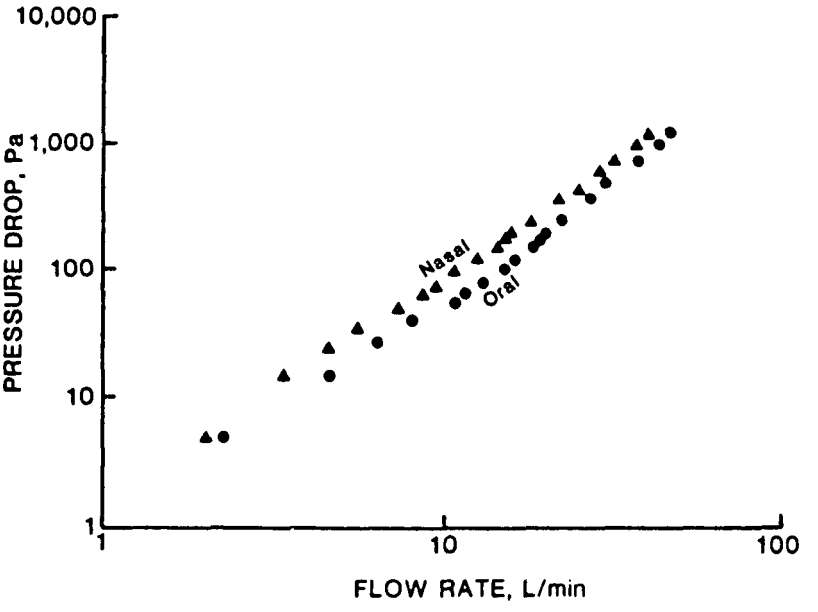


Figure 1. Comparison of pressure drop across the oral and nasal cast.

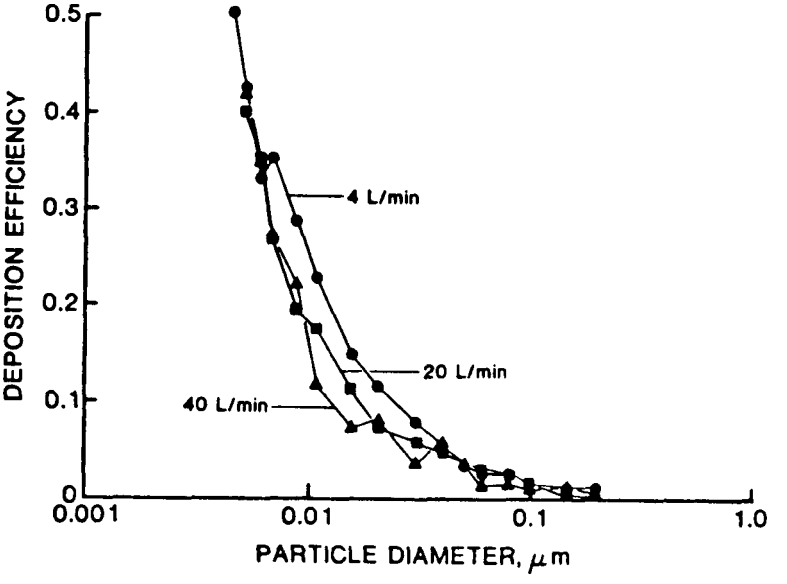


Figure 2. Oral deposition efficiency as a function of particle diameter and flow rate for inspiratory flow.

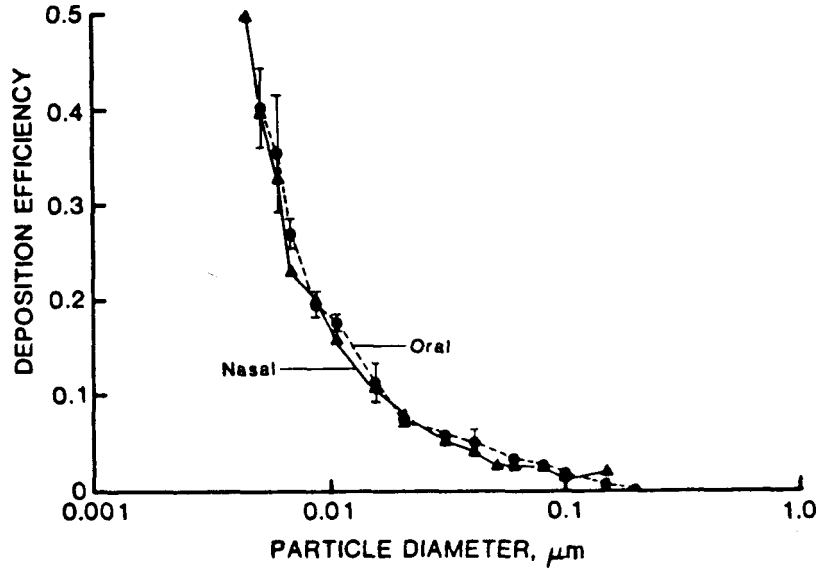


Figure 3. Comparison of nasal and oral deposition efficiency at 20 L/min inspiratory flow.

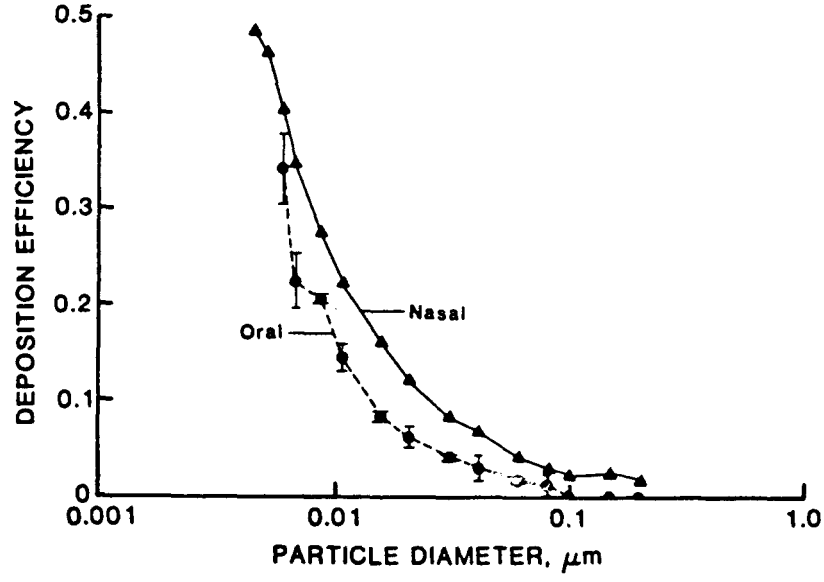


Figure 4. Comparison of nasal and oral deposition efficiency at 20 L/min expiratory flow.

S E S S I O N I I

EFFECTS ON CELLULAR SYSTEMS AND DIFFERENTIATION

SUMMARY OF SESSION II

EFFECTS ON CELLULAR SYSTEMS AND DIFFERENTIATION

By Dr. Eliezer Huberman
Argonne National Laboratory

Chemicals that are byproducts of energy-generating processes have the potential to cause health problems. These health effects can result from chemically induced cytotoxicity, immune response suppression, modulation of normal cell differentiation processes, somatic and heritable mutations, malignant transformation, or other abnormal physiological changes. The studies in Session II, "Effects on Cellular Systems and Differentiation," deal with such changes and attempt to delineate the underlying mechanisms.

To initiate their biological activity, chemicals must interact with specific target molecules, including proteins and nucleic acids. The type of interaction may vary from a ligand-receptor-type interaction to covalent binding of the chemical with its target molecule. For such interactions (especially covalent binding) to occur, the agents must be chemically reactive. Because many of the byproducts of energy generating processes are chemically inert, they must be activated by appropriate cellular enzymes to exert their biological action. The first report in this section, by Hankinson *et al.* (UCLA), dealt with the control of the expression and inducibility of such an enzyme, P450IA1 oxygenase. As a tool for their studies, these investigators developed a series of mutants from a mouse hepatoma cell line -- mutants altered in either the structure of the enzyme, the *Ah* receptor that regulates enzyme induction, or the repressor that affects the transcription of the enzyme. With the help of these mutants, Hankinson *et al.* are determining the structure, role, and mode of action of the *Ah* receptor and the repressor on the expression of the enzyme.

Two other studies dealt with the subsequent step, namely, the type and structure of adducts formed after the interaction of a reactive chemical mutagen/carcinogen with DNA.

Hettich and Buchanan (ORNL) used laser ionization Fourier transform mass spectrometry for the analysis of adducts formed after DNA was reacted with the alkylating carcinogen/mutagen, methylnitrosourea, whereas Broyde (NYU) used a combination of theory and experimentation to develop energy-minimized modules

for a 2-aminofluorene modified DNA duplex 11-mer. Broyde's models suggest that the carcinogen 2-aminofluorene is situated in the minor groove of an overall B DNA-type structure in an orientation that simultaneously maximizes both base-base and carcinogen-base stacking.

Brooks and Griffith (ITRI) are studying the binding and removal of benzo(a)pyrene diol epoxide (BPDE), [the reactive metabolite of the carcinogenic polycyclic aromatic hydrocarbon, benzo(a)pyrene] from chromosomes of Chinese hamster ovary (CHO) cells. Treatment of the CHO cell with radiolabeled BPDE did not reveal "hot spots" of bound BPDE along the various chromosomes. However, the study raised the possibility of a differential removal of the radiolabel between chromatids during the G-2 and S phase of the cell cycles.

Four studies dealt with the genetic consequences of the interaction between DNA, bacterial cells, or mammalian cells with mutagens/carcinogens. Weiss (U. Chicago) observed large deletions and illegitimate (nonhomologous) recombinations (following transfection into E. coli) in the progeny of M13 viral RF DNA containing an oligomer that harbors in each of the duplex strands single but closely spaced BPDE adducts or TT pyrimidine dimers. Transfection of E. coli with M13 vector containing either undamaged constructs or constructs having only one BPDE adduct or one TT pyrimidine dimer showed no such alteration. On the basis of these experiments, Weiss suggests that it is not the specific lesion that activates the recombinant machinery but rather the rare distortion of the DNA structure created by closely spaced adducts. Fritz and Pelroy (PNL), using strains of S. typhimurium containing a defined DNA target involving the lac operon, showed that polycyclic aromatic hydrocarbons, such as 6-aminochrysene,2-aminoanthracene and benzo(a)pyrene after appropriate activation caused an induction of mutations in or near the DNA targets. Their results also indicate that the sensitivity of their DNA targets was affected not only by their primary DNA sequences but also by the strength of the promotor for expression of the lac operon. Thilly and co-workers (MIT) are studying means to determine whether genetic changes caused by chemicals are prevalent in human populations. Using a series of selective drugs as genetic markers in a human lymphoid cell line, they have shown that a number of chemical mutagens have a defined mutational spectra. At present, they are devising new strategies and developing the necessary methodologies to study chemically induced point mutations in blood cells; these strategies involve the use of denaturing gradient gel electrophoresis

and high-fidelity DNA amplification by using polymerase chain reaction. The study of Albertini *et al.* (U. Vermont) involves the characterization of the spectrum of *hprt* mutations in peripheral blood T-lymphocytes. T-cell receptor gene rearrangement patterns were included to provide independent markers for the isolated T-cell clones and serve as a marker for their *in vivo* stage of development in order to determine in what stage the mutation occurred. Their results indicate that, (1) among the mutant colonies there are frequent replicates with some outgrowing others, which suggests that the *hprt* mutations occur in dividing T-cells; (2) *hprt* mutations in the adults arise in post-thymic T-cells; (3) T-cells mutants in newborns are found at a frequency that is approximately 10-fold lower than that in adults and a significant fraction of these mutations arise during pre- or intra-thymic events; and (4) *hprt* mutant frequency values in individuals receiving total body irradiation were higher than those for controls and the mutation spectrum in those individuals differs from that seen in either control adults or control newborns.

In addition to chemicals that bind covalently to DNA or proteins, others that do not bind to these molecules and are devoid of mutagenic activity may still potentially harm humans by abnormal modulation of physiological events, including cell differentiation. Huberman *et al.* (ANL) are investigating these types of chemicals, specifically tumor-promoting phorbol diesters, of which phorbol 12-myristate 13-acetate (PMA) is a prototype. They have shown that after PMA binds to its receptor (protein kinase C), it causes the induction of cell differentiation in a variety of human cell types, including HL-60 leukemia cells. Using HL-60 cells that are either susceptible or resistant to PMA-induced differentiation, they have found that maturation in these cells requires the activation of protein kinase C, which in turn causes a reduction in topoisomerase II activity. This reduced activity may then cause a change in DNA topology and with it the expression of genes associated with a mature phenotype. In another study, Huberman *et al.* have cloned the human and rodent cDNA of inosine 5'-monophosphate dehydrogenase (IMPDH), an enzyme that regulates guanine nucleotide biosynthesis. Using these IMPDH DNA probes, they demonstrated an association between the degree of IMPDH expression and the degree of growth of normal and malignant human cells, with the enzyme being more effective in tumor cells than in their normal counterparts. Huberman *et al.* suggest that their results further support previous notions that enhanced IMPDH is associated with tumor growth progression.

Two studies make use of gene transfer technologies to investigate the acquisition of malignancy in human and rodent cells. The study of McCormick and Maher (Michigan State U.) has shown that transfection of H-, N- or v-K-ras genes into nontumorigenic diploid cell strains with an infinite life span resulted in the development of cell foci that caused progressively growing and invasive fibroblastic tumors when they were inoculated into athymic mice. No such tumorigenic cells were obtained after transfection of these oncogenes into fibroblasts that have a limited life span. McCormick and Maher suggest that human fibroblasts with finite life span have too few potential doublings to acquire the needed malignant characteristics. Hays et al. (UCLA) are exploring methods for introducing genes into mouse bone marrow. More specifically, they are using retroviruses as a vehicle to transfer the human T-cell leukemia virus II transactivating gene (tax/vex), the hemopoietic growth factor gene (GM-CSF), and the bacterial gene that conveys neomycin resistance. Their initial results indicate that they are capable of obtaining neomycin-resistant colony-forming cells from the spleens of treated mice. They hope that their studies will lead to an effective means for gene transfer in leukemia development studies.

A study by Tang et al. (Johns Hopkins) involves the use of microfluorometry and in situ hybridization to study the production of beta-interferon at various stages of the cell cycle in cells treated with the inducer poly I:poly C. Their results show that the beta-interferon gene is expressed throughout the cell cycle and is not restricted to any specific stage. The same is true for the production of the beta-interferon protein. Tang et al. suggest that the combination of microfluorometry and in situ hybridization offer the unique advantage of measuring DNA content and mRNA expression in the same cell. When used in conjunction with a specific monoclonal antibody, these techniques provide a valuable tool to investigate specific gene expression related to ploidy or cell-cycle stage in an unsynchronized population.

GENETIC AND MOLECULAR ANALYSIS OF CYTOCHROME P-450IA1 INDUCTION

O. Hankinson, R. M. Bannister, B. A. Brooks,
N. Carramanzana, F.-F. Chu, J. Nanthur, E. C. Hoffman
H. Reyes, F. Sander, and A. J. Watson

University of California
Los Angeles

Certain polycyclic aromatic hydrocarbons (PAHs), such as benzo(a)pyrene (BP), and certain halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are important environmental pollutants. These compounds also bind the soluble Ah receptor and are thereby capable of inducing cytochrome P-450IA1. Cytochrome P-450IA1-dependent aryl hydrocarbon hydroxylase (AHH) activity in turn plays a central role in metabolism of PAHs to their ultimate carcinogenic derivatives. Pathogenesis by HAHs also depends upon action of the Ah receptor. However, in the case of these compounds, the HAHs themselves, rather than metabolites, are the pathogenic agents, and P-450IA1 activity is not involved (Reviewed in Ref. 1).

Cytochrome P-450IA1 and associated AHH activity are highly inducible by PAHs and HAHs in the mouse hepatoma cell line, Hepa-1. We previously isolated mutants of Hepa-1 that are no longer inducible for P-450IA1 and lack AHH activity. The majority of the mutants are recessive, while a few are dominant. The recessive mutants were assigned to four complementation groups (i.e. genes) (2,3). Gene A is the P-450IA1 structural gene (4,5). Mutants in gene B have much reduced levels of the Ah receptor; mutants in gene C are defective in nuclear translocation of the Ah receptor-ligand complex; the D^- mutant has diminished levels of the Ah receptor, which is also reduced in its ability to translocate to the nucleus (3,6). Thus, mutations in three complementation groups can affect functioning of the Ah receptor. This is in marked contrast to the situation with the apparently similar glucorticoid receptor where mutants in only one gene have been isolated (7).

The dominant mutants synthesize a repressor of P-450IA1 transcription. We have shown that at least one site of repressor action lies within 1186 bp upstream of the cap site of the rat P-450IA1 gene (Chu, F.-F., Fujii-Kuriyama, Y. and Hankinson, O., unpublished). We are performing additional experiments directed towards identifying the exact site(s) and mode of interaction of the dominant repressor with the P-450IA1 gene, using

transient transfection assays, and gel retardation and DNA footprinting techniques. One possible explanation for the origin of the dominant mutants is that they arose by the activation of a gene that normally regulates tissue and/or developmental expression of P-450IA1 that is not normally expressed in hepatocytes.

We developed a "reverse selection procedure" which can be used to select for P-450IA1-inducible cells growing in the presence of a vastly greater number of non-inducible cells (8). We have used this procedure to isolate transfectants of each recessive class of AHH-deficient mutant, using human DNA as donor material. A fragment of the C gene has been cloned from a secondary transfectant of the C mutant by screening a genomic library of the secondary with a human repetitive Alu sequence. A genomic fragment was used to isolate a cDNA for the C gene, and this in turn was used to detect two messenger RNAs, of 2.5 kb and 4 kb, in liver cells (unpublished). We have also successfully transfected the dominant gene into Hepa-1 cells (9) and propose to clone this gene by a marker rescue strategy.

Success in cloning the B, C, D and dominant genes should lead to important insights into the structure, role and mechanism of action of the Ah receptor; the mechanism of interaction of the Ah receptor and dominant repressor with the P450IA1 gene; the possible role of the Ah receptor in cell division and differentiation; and the possible involvement of one or more of the genes in determining individual differences in susceptibility to cigarette-induced cancer in the human population. (Research supported by the U.S. Department of Energy under Contract No. DE-FC03-87-ER60615 and PHS Grant CA 28868 awarded by the National Cancer Institute.)

REFERENCES

1. Nebert, D.W., and Gonzalez, F.J., Annual Rev. Biochem. 56:945-993 (1987).

2. Hankinson, O., Somat. Cell Genet. 9:497-514 (1983).

3. Karenlampi, S.O., Legraverend, C., Gudas, J.M., Carramanzana, N. and Hankinson, O., J. Biol. Chem. 263:10111-10117 (1988).

4. Montisano, D.F. and Hankinson, O. Mol. Cell Biol. 5:698-704 (1985)

5. Kimura, S., Smith, H.H., Hankinson, O. and Nebert, D.W., EMBO J. 6:1929-1934 (1987).

6. Legraverend, C., Hannah, R., Eisen, H., Owens, O., Nebert, D. and Hankinson, O., J. Biol. Chem. 257:6502-6507 (1982).

7. Gehring, U., Yamamoto, K.R., and Tomkins, G.M., Res. Steriods, 7:43-48 (1977)

8. Van Gurp, J.R. and Hankinson, O., Cancer Res. 43:6031-6038 (1983).

9. Watson, A.J. and Hankinson, O., Carcinogenesis, 9:1581-1586 (1988)

STRUCTURAL CHARACTERIZATION OF DNA ADDUCTS USING FOURIER TRANSFORM MASS SPECTROMETRY

Robert L. Hettich and Michelle V. Buchanan

Oak Ridge National Laboratory
Oak Ridge, TN 37831-6120

The structural characterization of DNA adducts is vital to understanding the nature of DNA modification by carcinogenic and mutagenic agents. Since these DNA adducts occur at low concentrations in a matrix dominated by normal nucleic acid constituents, any instrumental technique used to characterize these adducts must be able to obtain structural information on a small amount of material. In our laboratory, we have been examining the applicability of laser ionization Fourier transform mass spectrometry (FTMS) for the investigation of DNA adducts.

Laser ionization Fourier transform mass spectrometry is well-suited for the examination of small quantities (picomole) of nonvolatile, thermally labile compounds such as nucleosides and nucleotides. Laser desorption provides a relatively soft ionization method, although the laser energy can be varied to influence the amount of fragmentation. Since this ionization method does not require a solvent such as glycerol, the spectra obtained by laser ionization are usually cleaner than spectra obtained by ionization methods such as fast atom bombardment (FAB). Once the ions have been created by laser desorption, they can be examined by the experimental sequences available with FTMS. Fragmentation can then be induced in these ions by interaction with photons (photodissociation) or atoms (either collision-induced dissociation or selected ion-molecule reactions). These techniques often provide the information which is required to unambiguously identify the ions.

Reaction of DNA with alkylating agents such as methyl-nitroso urea (MNU) generates a variety of methyl adducts. The most abundant modification in this specific case is alkylation of the guanine residues. Structural characterization of these adducts requires identification and differentiation at the isomeric level. Laser ionization of methyl guanosine isomers generates negative ions corresponding to $(M-H)^-$ and to the nucleic base (elimination of the sugar ring). Cleavage of the sugar ring indicates whether the methyl substitution was on the sugar ring or the nucleic base. Laser ionization of isomers with methyl substitution on the nucleic base

gave negative ions at m/z 164. The negative ion spectrum of 3'-O-methyl guanosine indicated an ion at m/z 150 but not at m/z 164, verifying that the methyl group was on the sugar ring for this compound. The ions at m/z 164 for the methylated guanosines could be isolated and collisionally dissociated to give daughter ions which were sufficient to differentiate each isomer, as shown in the following Table. The ions from both 1-methyl guanosine and N^2-methyl guanosine fragment primarily by loss of CH_3NH_2, whereas the ions from 7-methyl guanosine and O^6-methyl deoxyguanosine lose CH_3. Other fragment ions are also present for each of these isomers and provide the information necessary to resolve each of the four isomers. Loss of CH_3OH is exclusive to the O^6-methyl isomer and is diagnostic for methyl substitution of the O^6-oxygen. Accurate mass measurement and sequential collision-induced dissociation were used to investigate the fragmentation pathways.

These same structural techniques can be used to investigate other alkylated nucleosides. Isomeric differentiation of methylated thymidines can also be obtained by these techniques, providing a method to resolve the isomer of biological interest, O^4-methyl thymidine, from the O^2-methyl and 3-methyl thymidine isomers. In addition to nucleosides, small intact oligonucleotides can be investigated for modifications. For example, laser ionization of an oligomer hexamer containing all four normal nucleic bases of DNA as well as one methylated adenine d(N^6meA-T-G-C-A-T) generated negative ions which could be used to identify the specific nucleic bases present in the oligomer. Even though sequence information was not obtained for this compound, the identities and modifications of each nucleic base in the oligomer could be determined. Isolation and collisional dissociation of the ion corresponding to the methylated adenine provided the information necessary to determine the position of methyl substitution (which was N^6 in this case).

In summary, laser ionization FTMS is useful for the characterization of modified nucleic acid constituents. Specifically, laser ionization of alkylated nucleosides provides negative ions which can be used to determine the molecular weight and fragmentation mechanisms for the modified components. These negative ions can be collisionally dissociated to investigate fragmentation pathways which give the structural information necessary to identify these species at the isomeric level. (Research sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract No. DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.)

DIFFERENTIATION OF METHYL GUANOSINE ISOMERS
BY COLLISIONAL DISSOCIATION

ION	NEUTRAL LOST								
	CH_3	CH_3OH	NH_3	HNCO	CH_3NH_2	C_2H_4NO	$C_4H_6N_2O$	$C_5H_6N_4$	$C_5H_6N_4O$
1-Methyl Guanine (m/z 164)					X	X	X	X	X
N^2-Methyl Guanine (m/z 164)				X	X	X	X	X	X
7-Methyl Guanine (m/z 164)	X		X	X		X		X	X
O^6-Methyl Guanine	X	X				X	X		X

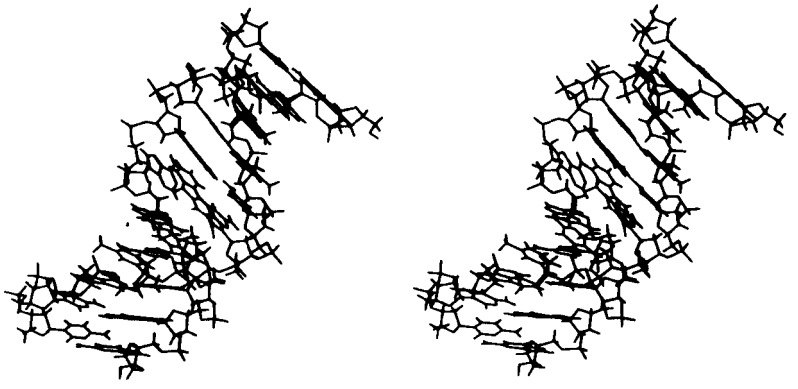
STRUCTURE OF A G-A MISMATCH MUTATION INDUCED BY 2-AMINOFLUORENE

Suse Broyde
New York University
New York, NY 10003

Brian E. Hingerty
Oak Ridge National Laboratory
Oak Ridge, TN 37831

David Norman
Columbia University College of Physicians and Surgeons
New York, NY 10032

We have produced energy minimized models of a 2-aminofluorene (AF) modified DNA duplex 11-mer, whose central trimer sequence is d(C-(AF-G)-C).d (G-A-G), by a novel combination of theory and experiment. The minimized potential energy calculations were first carried out independently, in searches of the modified duplex trimer conformation space, with experimental guidance on the following points: (1) glycosidic torsion angles of modified guanine (syn) and its opposite adenine (anti); (2) possible hydrogen bonding schemes at the modification site; (3) overall B DNA structure. Experimental distance constraints were applied to the favored trimers at this state. Constrained minimizations were further employed, together with several building strategies, to locate duplex 11-mers that incorporated the constrained trimer features. In the terminal minimization all constraints were released. A related pair of 11-mers was found, one protonated at N1 of A17 and one unprotonated, in which all previously constrained distances persisted within NOE range. In these structures the AF is situated in the minor groove of an overall B DNA type structure in an orientation which simultaneously maximizes both base-base stacking and carcinogen-base stacking. This is achieved by a kink of about 40 degrees in the helix axis. (Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC02-91ER60015.)



AF Modified Duplex 11-Mer
In Stereo View

RETENTION AND DISTRIBUTION OF BENZO(A)PYRENE-DIOL-EPOXIDE ON CHROMOSOMES OF CHO CELLS

Antone L. Brooks and William C. Griffith

Lovelace Inhalation Toxicology Research Institute
Albuquerque, NM 87185

Extensive research has been conducted to determine what relationships exist between DNA adducts and their biological effects (Yang et al., 1982). The concept of "chemical dose" has been advanced by Wogan and Gorelick (1985). This concept has been evaluated by determining the number of DNA adducts formed, the chemical nature of the adducts and the repair or removal of the adducts from DNA. Evidence has accumulated that chemical dose is not uniform in DNA. It has been determined that there is a nonrandom binding of ± anti benzo(a)pyrene-7,8-diol-9,10 epoxide (BPDE) in the nuclei of epithelial cells (Poirier et al., 1982) as well as in linker DNA regions (Jack and Brookes, 1982). Nonuniform distribution of chemical dose can also be produced by DNA repair. There is preferential repair in regions of the DNA that are transcriptionally active (Mellon et al., 1986; Hanawalt, 1987).

Our research was carried out to determine if nonuniform distribution and repair of BPDE adducts could be detected at the chromosome level. We conducted studies to measure the binding and removal of BPDE from chromosomes of Chinese hamster ovary (CHO) cells. The study consisted of three parts. First, cell cycle kinetics were determined using ^{3}H-thymidine. Second, the distribution and clearance of ^{3}H-BPDE was measured as a function of chromosome location. Finally, the distribution of silver grains from ^{3}H-BPDE or ^{3}H-thymidine between the sister chromatids was scored as an index of difference in repair rates. Differential repair was measured both with and without the addition of 5-bromodeoxyuridine to label the chromatid containing both strands of newly synthesized DNA.

To determine the uniformity of carcinogen distribution, four marker chromosomes were divided into segments of approximately equal lengths and the frequency of silver grains recorded in each segment. No significant "hot spots" or areas with high chemical doses were observed along the chromosomes at any harvest time (data not shown). However, the sensitivity of the method used to detect nonuniform distribution of label was limited because of the rather large areas of chromosome being evaluated and the difficulty in defining the exact size and location of the silver grains in each area.

To define cell cycle kinetics, we determined the frequency of metaphase cells with label in zero, one or two of the chromatids as a function of time after ^{3}H-thymidine labeling. This study made it possible to determine the stage of cell cycle where repair or loss of the BPDE occurred. By 9 hours after labeling, all cells had incorporated ^{3}H-thymidine into both chromatids and by 24 hours, 80% of the cells had label in a single chromatid. This suggests that most of the cells had gone through a single S phase by 9 hours and two S phase periods by 24 hours. Labeling with ^{3}H-thymidine and harvest at 9 and 24 hours were then used to determine the distribution of silver grains between the different chromatids and served as positive and negative controls for the study on the random distribution of BPDE label. For cells harvested at 9 hours after ^{3}H-thymidine labeling each chromatid should be equally labeled, while cells harvested at 24 hours should have all of the label on the light staining chromatid.

Clearance of label from the interphase nuclei was rapid over the first 9 hours with only 60% of the label retained. The labeling over the interphase nucleus was constant between 9 and 24 hours after exposure.

The distribution of label between chromatids after exposure to BPDE appeared to be nonrandom, with some areas of label on one chromatid where there was little label observed on the sister chromatid. A distribution index (D.I.) was calculated for cells from each treatment. The D.I. of 0.5 would indicate that the silver grains are randomly distributed between the two chromatids, while numbers larger than 0.5 provide an indication that one chromatid has more label than the other. The D.I. and the standard error for the null hypothesis of D.I. = 0.5 is $D.I. = N(d)/[N(d) + N(a)] \pm \sqrt{\frac{1}{4}[N(d) + N(a)]}$, where N(d) is the number of silver grains which have their nearest neighbor located on the same chromatid and N(a) is the number of silver grains where the nearest neighbor is on the opposite sister chromatid. ^{3}H-Thymidine-labeled cells harvested at 9 hours had the label randomly distributed (D.I. = 0.48 ± 0.03); cells harvested at 24 hours after ^{3}H-thymidine had the silver grains nonrandomly distributed (D.I. = 0.80 ± 0.04). Results of the distribution index for the ^{3}H-BPDE were 0.52 ± 0.03, 0.59 ± 0.03 and 0.60 ± 0.04 for cells harvested at 0, 3 and 6 hours after the end of a three hour exposure. These data demonstrate at a 95% level of confidence that silver grains associated with BPDE were not randomly distributed between the chromatids 3 and 6 hours after exposure.

Cells were labeled with BrdU prior to the treatment with BPDE. Double labeling made it possible to determine the potential magnitude of the error associated with scoring silver grains on chromatids. The double label also made it possible to determine if nonrandom distribution of silver grains between the chromatids is associated with unifilarly and bifilarly substituted DNA. The frequency of silver grains over the chromatid with uni- and bifilarly substituted DNA (light and dark staining chromatid) was measured at 5 1/2 hours after the end of a 1/2 hour exposure to BPDE. Cells exposed to ^{3}H-thymidine and harvested at 9 hours have a uniform distribution of silver grains between chromatids while at 24 hours 72% of the grains are on the light staining chromatid. Since the predicted distribution of grains would be 100% on the light staining chromatid at 24 hours, the observed value provides an estimate of the degree of error associated with assigning silver grains to a given chromatid. For cells exposed to BPDE 52% of the label was classified as being over the light chromatid; only 37% of the grains were scored over the dark chromatid.

Cell cycle data reported here suggest that, at the time when cells are in G-2 and S stages of the cell cycle, differential repair between the two chromatids is possible. Our data also indicate that during repair, when BPDE adducts were removed from a chromatid, the probability that repair would occur on the same chromatid was higher than that for an adduct being lost from the opposite chromatid. We have additional data that suggests the DNA rate of repair rate may be higher in unifilarly substituted chromatids than in the bifilarly substituted chromatids. This difference in repair rate may account for the difference in the distribution index for chromatids exposed to BPDE.

Additional studies on the time course of BPDE removal from chromatids are necessary to understand these observations. It will be important to determine if the observed nonrandom repair process is related to the carcinogen studied and to the loss of adduct from linker DNA. Finally, it is important to evaluate if the nonrandom repair process is related to the observed nonrandom distribution of genetic damage. (Research sponsored by the U. S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.)

REFERENCES

1. Hanawalt P.C. Preferential DNA repair in expressed genes. Environ. Health Perspect. 76: 9-14; 1987.

2. Jack P.L.; Brookes P. Mechanism for the loss of preferential benzo(a)pyrene binding to the linker DNA of chromatin. Carcinogenesis 3: 341-344; 1982.

3. Mellon I.; Bohr V.A.; Smith C.A.; Hanawalt P.C. Preferential DNA repair of an active gene in human cells. Proc. Natl. Acad. Sci. (USA) 83: 8878-8882; 1986.

4. Poirier M.C.; Stanley J.R.; Beckwith J.B.; Weinstein I.B.; Yuspa S.H. Indirect immunofluorescent localization of benzo(a)pyrene adducted to nucleic acids in cultured mouse keratinocyte nuclei. Carcinogenesis 3: 345-348; 1982.

5. Wogan G.N.; Goerlick N.J. Chemical and biochemical dosimetry of exposure to genotoxic chemicals. Environ. Health Perspect. 62: 5-18; 1985.

6. Yang L.L.; Maher V.M.; McCormick J.J. Relationship between excision repair and the cytotoxic and mutagenic effect of the 'anti' 7,8-diol-9,10-epoxide of benzo(a)pyrene in human cells. Mutat. Res. 94: 435-447; 1982.

INDUCTION OF ILLEGITIMATE RECOMBINANTS IN ESCHERICHIA COLI BY BENZO[a]PYRENE DIOL EPOXIDE AND ULTRAVIOLET LIGHT

Samuel B. Weiss

The University of Chicago
Chicago, Illinois 60637

Certain chemical and physical agents such as polycyclic aromatic hydrocarbons (PAH) and ultraviolet light are known to be mutagenic and carcinogenic in living systems. It is widely believed that damage of DNA by these agents, which cause alterations in DNA sequence arrangement, is primarily responsible for the transformation of normal cells to a malignant phenotype.

We have demonstrated that short DNA oligomers, employed as targets for alkylation by benzo[a]pyrene diol epoxide (BPDE), can be used to explore DNA mutations induced by this activated PAH metabolite (1). The alkylated oligomer is inserted into M13 viral RF DNA and, after transfection of recipient Escherichia coli cells, progeny viral DNAs are examined for modifications in nucleotide arrangement by DNA sequence analysis at the site of construct insertion.

Recently, we reported that duplex constructs (32 bp) containing a single BPDE adduct in each of the two duplex strands and closely spaced, induced large deletions and illegitimate (nonhomologous) recombinations, both of which removed the damaged construct insert (2). These sequence alterations occurred in both recA$^+$ and recA$^-$ host cells suggesting the noninvolvement of SOS processing. Transfection of E. coli with the M13 vector containing undamaged constructs and constructs having only one BPDE adduct, showed no alteration of sequences in progeny viral DNAs. To explain the above findings, we proposed that, in our system, it is not the specific lesion that is recognized for repair, but rather the rare distortion of DNA structure created by the closely spaced adducts that activates the recombinant machinery. If this hypothesis is correct, agents other than polycyclic aromatic hydrocarbons that damage and distort DNA in the same manner might also be expected to induce deletions and illegitimate recombinations.

To test this idea, we have used ultraviolet irradiation to prepare duplex constructs that contain a single TT pyrimidine dimer in each of the

two complementary DNA strands and the dimers were closely spaced. Insertion of these UV-damaged constructs into M13 RF DNA, followed by transfection of E. coli, resulted in the detection of large deletions and illegitimate recombinants in progeny viral DNAs. Although the frequency of these events is less with UV damaged constructs than with BPDE alkylated constructs, the results obtained tend to support the hypothesis that, in our system, distortion of DNA structure by DNA damaging agents can induce illegitimate recombination. Continuing studies using UV irradiated constructs are in progress. (Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-FG02-86ER60394.)

REFERENCES

1. Wei, S.-J.C., Desai, S.M., Harvey, R.G. and Weiss, S.B. (1984) Proc. Natl. Acad. Sci. USA 81, 5936-5940.

2. Kokontis, J.M., Vaughan, J., Harvey, R.G. and Weiss, S.B. (1988) Proc. Natl. Acad. Sci. USA 85, 1043-1046.

USE OF SYNTHETIC DNA TARGETS FOR INDUCED MUTAGENESIS BY BULKY ADDUCTS

L. K. Fritz and R. A. Pelroy

Pacific Northwest Laboratories
Richland, WA

RESULTS:

Synthetic oligonucleotides (DNA-targets) specifying (+1) or (+2) frameshift mutations were cloned into a BamHl restriction site near the N-terminal end of the lacZ gene in the Escherichia coli lactose operon. The design of the targets was as follows: BamHl sites were located at the 5-prime and 3-prime ends of the target. Six base pair (bp) "core" sequences of alternating guanine (G)/cytosine (C), adenine (A)/thymine (T), or runs of G or A, were placed near the middle of the DNA targets. Such repeated sequences are common in genetic hot spots in bacterial and mammalian cells (Foster et al., 1982; Calos and Miller, 1981; Miller, 1985; Gentil et al., 1986; Refolo et al., 1987; Koffel-Schwartz et al., 1984). Stop codons were placed to bracket the core sequences to try and confine reversing mutational events within the synthetic target sequence. The lac operon, plus a DNA-target, was cloned into the transformable plasmids, PDM and PLPP. Plasmid PLPP is distinguished by possession of a high-expression constitutive synthetic promoter used to drive expression of the lac operon. PDM was derived from PLPP by selection of a "down" mutation near the translational start codon of the synthetic promoter that lowered expression of lacZ gene (beta-galactosidase production).

Insertion of the DNA-targets into lacZ essentially abolished lacZ gene expression on mineral base medium for both DNA-targets in both PLPP and PDM, and prevented transformed Salmonella typhimurium from growing on lactose as a carbon source. Spontaneous rates of reverse mutation for DNA-targets in PDM was on the order of 10 minus 8 to 10 minus 7; rates comparable to spontaneous mutations in naturally occurring DNA sequences. Spontaneous rates for DNA-targets in PLPP strains were low (10 minus 8 to 10 minus 7) for DNA-targets with GC and AT cores. Intermediate spontaneous rates of between 10 minus 6 to 10 minus 5, were obtained for the DNA-target with a G-6-core in PLPP (G-6-PLPP). The DNA-target with an alternating AT-core sequence had a very high spontaneous background in PLPP (A/T-3-PLPP).

Exposure of strains of S. typhimurium containing the DNA-targets in PDM or PLPP to several polycyclic hydrocarbons resulted in the induction of mutations in or near the DNA-targets. Mutational events were detected by selection for growth on lactose. Specifically, 6-aminochrysene (6AC), 2 aminoanthracene (2AA) and benzo(a)pyrene (BaP), in the presence of metabolic activation, resulted in a significant elevation in the frequency of induced mutations for DNA-targets with alternating GC core sequences in PDM and for G core sequences in PLPP. The DNA-targets in PDM with alternating A/T or A core sequences showed little or no revertability by the polycyclic compounds. One of the mutationally active DNA-targets consisted doubly of inserted synthetic oligonucleotides, each with stop codons and GC-core sequences. This DNA-target is designated 35/21-PDM was the most active of the PDM targets in terms of revertability Target 35/21-PDM detected 6AC and 2AA but not BaP. Deletions covering all of the inserted DNA of 35/21 appear to be the favored mutational event induced in this DNA-target by these aromatic amines. A DNA-target in PLPP with a 6 bp G core sequence (G6-PLPP) was highly sensitive to induced mutations by 6AC, 2AA and BaP. Mutability of this DNA-target was dependent on the use of the strong synthetic promoter and was not observed for the same target in plasmid PDM. The profile of mutation frequency vs mutagen appears to be different for 35-21-PDM and G-6-PLPP. The other DNA-targets were inactive (G-6-PDM, A/T-3-PDM, GC-PLPP), or failed to confine the mutational events to the DNA-target (G/C-3-PLPP, A-6-PLPP) region, or had unacceptably high spontaneous mutations rates (A/T-3-PLPP).

DISCUSSION:

Detection of mutagens by DNA-targets appears to be possible using alternating G/C or repeated runs of G as their core sequences. Levels of sensitivity are comparable to those obtained with conventional mutagen/genotoxin bioassays. But the use of the plasmid constructions allows analysis of the molecular changes that accompany induced mutations more easily than with the standard systems. In particular, DNA-targets can be used for mechanistic studies by correlating the activity and selectivity of primary sequence with changes in DNA-sequence that accompany induced mutations. Use of the DNA-targets in the plasmid constructions have features advantageous for mechanistic work in molecular genetics. First, it

is possible to control sequence of the DNA-target and the surrounding flanking regions in a way not easily available in other molecular systems based on forward mutations (e.g. lacI, or tetracycline gene; see Miller, 1985; Koffel-Schwartz et al., 1984). Second, the data for 35/21-PDM suggests that stop codons can be used to confine mutational events to the selected sequences of the DNA-targets. This is an important consideration in trying to related genetic activity of frameshift mutations to designated DNA sequences, since reversing mutations can occur at a second site some distance from the initial frameshift (Refolo et al., 1987). It is noteworthy that 35/21-PDM is a doubly inserted DNA-target composed of tandem insertions of the GC-3-PDM target. It therefore contains two stop codons in the reading frame of lacZ (in place of one for GC-3-PDM) and is a (+2) rather than a (+1) frameshift mutation (as for all of the other DNA-targets). This did not appear to be the case in the GC-3-PDM DNA-targets, which appeared to be unchanged in frameshifted lactose revertants. The explanation for this may lie in the occurrence of both direct repeats and palindromic sequences that could promote secondary stem/loop structures in mRNA. Such secondary structures appear capable of allowing synthesis of lacZ past the stop codons that are masked within secondary structures of the mRNA (Albertini and Miller, 1983). The next generation of DNA-targets are being designed without the extensive palindromic and/or directly repeated sequences found in the DNA-targets reported in this study to minimize the possibilities for secondary structure in mRNA. Third, the sequencing of mutations is simplified over the procedures used for molecular analysis of mutations in the lacI repressor gene of E. coli or the structural tetracycline gene of pBR322 (Miller et al., 1985; Koffel-Schwartz et al., 1984), or for comparable molecular studies of the hisD gene in S. typhimurium (Fuscoe et al., 1988). The results obtained in this study show that the sensitivity of the DNA-targets is affected both by their primary DNA-sequences (as expected) and by the strength of the promoter for expression of the lactose operon.

REFERENCES

1. Foster et al., 1983, Proc. Natl. Acad. Sci, USA 80:2695-2698.
2. Gentil, A. et al., 1986, Proc. Natl. Acad. Sci, USA, 83:9556-9560.
3. Calos, M.P. and J.H. Miller, 1981, J. Mol. Biol. 153:39-66.
4. Koffel-Schwartz,N. et al., 1984, J. Mol. Biol., 177:33-51.
5. Miller, J.H., 1985, J. Mol. Biol., 182:45-68.
6. Miller, J.H. and A.M. Albertini, 1983, J. Mol. Biol., 164:59-71.
7. Refolo, L.M. et al., 1987, J. Mol. Biol. 193:609-636.
8. Fuscoe et al., 1988, Mutation Res., in press.

MUTATIONAL SPECTRA IN HUMAN CELLS

William G. Thilly, Neal Cariello, and Phoothone Keohavong

Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Human beings suffer from a wide variety of diseases which begin with genetic changes in germ line or somatic cells. These include point mutations, chromosome rearrangements, large deletions and aneuploidy. Chemicals found in the environment can cause these types of genetic changes in human cells in tissue culture. It is a primary goal of genetic toxicology to discover if there is a casual link between environmental mutagens and human mutations. If such a link is established, it will then become the responsibility of genetic toxicologists to discover which environmental factors are significant contributors to human genetic change in order that appropriate measures to protect public health may be adopted.

We have previously proposed that the phenomenon of mutational specificity discovered by Seymour Benzer (1961) can be used to diagnose the actual causes of human genetic changes (6). Mutagens show remarkable specificity with regard to the nature and locations of the mutations, thus the pattern of mutation produced, called mutational spectra, may be diagnostic for exposure to a particular mutagen. Here we report progress in our attempts to obtain human mutational spectra by adopting, improving and combining several of the techniques of molecular analysis of DNA.

In 1961, Seymour Benzer [1] showed that mutagens used to treat T4 bacteriophage gave a unique and reproducible distribution of mutations within the rII region of the bacteriophage genome. In particular, these sets of induced mutations were different from the set of spontaneous mutations occurring in the same region. Similar observations of specific mutagen-induced mutational spectra were reported for the lacI gene of *E. coli* by Miller and co-workers [2,3,4,5]. Based on this concept, we [6] have developed an approach to determine whether genetic change caused by chemicals is prevalent in human population. Dr. Phaik Mooi Leong (Ph.D. thesis, MIT, Cambridge, 1985) examined the ability of different mutagens to induce resistance to the drugs 6-thioguanine (6-TG), ouabain (OUA), podophyllotoxin (PPT) and 5,6 dichlororibofuranosyl benzymidazole (DRB). For OUA, DRB and PPT, only restricted kinds of missense mutations were

expected because these toxins binds to essential proteins, thus the functionality of these proteins is necessary for cell survival. However, in the case of 6TG resistance, since the HPRT gene product is not essential for cell survival, a wide variety of mutations were expected at this locus. From data of the frequencies of spontaneous mutations and mutations induced by ethyl methane sulfonate (EMS), N-methyl-N'-nitro-N-nitrosoquanidine (MNNG) and nitroquinoline oxide (NQO), she showed the existence of reproducible mutational spectra in human cells. The radio of $DRUG^r/TG^r$ was found to vary with different mutagens, and it was possible to discriminate among the mutagens based on the OUA^r/TG^r, DRB^r/TG^r and PPT^r/TG^r ratios.

An important limitation of this technology was that the low frequency of specific missense mutations obligated one to use large number of human cells. About 3×10^8 cells were necessary for this approach. These are easily obtained in human lymphoblastoid tissue culture but would represent about 300 ml of human blood were one to seek the same information from human $6TG^r$ T-cells. However, Leong made it clear that mutational spectra of marked specificity did exist in human cell populations. Seeing in mutational spectra a sufficient and perhaps unique means toward technology to obtain spectra from human blood samples and other tissues.

At this time, two techniques have been significantly improved and used in tandem to create what is believed to be a major advance in the study of point mutations: denaturing gradient gel electrophoresis (DGGE) and high fidelity DNA amplification (HFDA).

REFERENCES

1. Benzer, S. (1961), On the topography of the genetic fine structure, Proc. Natl. Acad. Sci., USA, 47:403-415.

2. Miller, J.H., Ganem, D., Lu, P. & Schmitz, A. (1977), Genomic study of the Lac repressor: I. Correlation of mutational sites with specific amino acid residues: Construction of colinear gene-protein map, J. Mol. Biol., 109:275-298.

3. Coulondre, C. & Miller, J.H. (1977a), Genetic study of the Lac repressor: III. Additional correlation of mutational sites with amino acid residues, J. Mol. Biol., 117:525-575.

4. Coulondre, C. & Miller, J.H. (1977b), Genetic study of the Lac repressor: IV. Mutagenic specificity in the LacI of E.coli. J. Mol. Biol., 117:577-606.

5. Calos, M.P. & Miller, J.H. (1981), Genetic sequence analysis of frameshift mutations induced by ICR-191, J. Mol. Biol., 153:39-64.

6. Thilly, W.G., Leong, P.M. & Skopek, T.R. (1982), Potential of mutational spectra for diagnosing the cause of genetic change in human cell populations. In: Banbury report 13. Indicators of Genotoxic Exposure in Man and Animals. Bridges, B.A., Butterworth, B.E. & Weinstein, I.B. (eds), Cold Spring Habor Laboratory, Cold Spring Habor, N.Y., pp 453-465.

MOLECULAR ANALYSIS OF hprt MUTATIONS ARISING in vivo AND in vitro IN HUMAN T-LYMPHOCYTES

R. J. Albertini, M. J. McGinniss, J. A. Nicklas, J. P. O'Neill and T. R. Skopek

University of Vermont
Burlington, VT 05401

CIIT
Research Triangle Park, NC 27709

Analysis of 326 in vivo-derived hprt mutants and 94 in vivo-derived wild type T-cell clones, isolated over a period of 1 1/2 years from three normal young adult males, has recently been completed. Between 10 and 16% of the mutant clones showed gross structural alterations involving the hprt gene. Gross structural alterations are defined as those large enough to be detected by Southern blot analysis. These deletions and complex alterations have been characterized as to location of breakpoints, which have occurred along the length of the entire hprt gene at a frequency of approximately 0.77 breaks per kb. T-cell receptor (TCR) gene rearrangement patterns were also studied in these mutant and wild type clones. TCR patterns provide independent markers of in vivo independence of the isolated T-cell colonies and serve as markers for the developmental stage in vivo where hprt mutation occurred. As so defined, all of the isolated wild type colonies in the current study were independent. However, there were frequent replicates among the hprt mutants, suggesting that mutation in vivo preferentially occurs in dividing T-cells. Simultaneous analysis of hprt and TCR patterns showed that hprt mutations in the adult arose in post-thymic T-cells. However, one interesting mutation was noted in several isolates which may have had an intra-thymic origin.

These recent results in normal adults are added to our earlier data base obtained in adults and in human newborns. T-cell hprt mutants are found at a frequency in newborns that is approximately ten-fold lower than in adults. Also, the background newborn hprt mutational spectrum was found to be different in the newborn, with approximately 85% of the mutants showing gross structural alterations as revealed by Southern blot analysis. In addition, exon 2-3 deletions/changes predominated in the hprt mutants recovered from newborns. Finally, the pattern of clusters of mutants showing the same hprt structural alteration, but different TCR gene

rearrangement patterns, occurred frequently enough in newborns to suggest that, in contrast to adults, these hprt mutations arose in pre- or intra-thymic events.

These mutational spectra for background hprt mutations in the normal adults and newborns serve as a basis for evaluating induced hprt mutations in humans. Blood samples from patients receiving radioimmunoglobulin therapy (RIT) at the Johns Hopkins Cancer Center have been studied. Hprt mutant frequency values in this group of individuals receiving total body irradiation were grossly elevated compared to normals. At the molecular level, the hprt mutational spectrum in these "radiation-induced" mutants was clearly different from the spectrum seen for normal adults or newborns, with 30% of the mutant clones from irradiated patients showing gross structural alterations involving hprt. However, similar to background mutations in the adult, breaks within the hprt gene in "irradiation mutants" occurred as a function of gene length, but with a higher frequency per kb.

For further comparisons, the molecular mutational spectrum at hprt resulting from in vitro ionizing radiation of human T-lymphocytes was characterized. Normal human T-lymphocytes were given gamma irradiation in vitro. In this instance, gross structural alterations were observed in 66% of the independent hprt mutant clones isolated. Again, however, the pattern of breaks was the same as seen for normal and in vivo irradiated adults, with linearity over the length of the gene. Analysis of TCR rearrangement patterns allowed isolation of independent mutants despite the fact that some mutants grossly "outgrew" others during phenotypic expression.

We have thus identified adult and fetal molecular patterns at the level of Southern blots for "spontaneous" hprt mutations arising in vivo in human T-cells. The adult pattern has persisted following ionizing irradiation exposure in vivo, but with a higher frequency of breaks per kb. In vitro studies of gamma irradiation-induced hprt mutations gave results that were similar to the in vivo irradiation results, but with a still higher frequency of breaks per kb of hprt gene.

As noted, the majority of hprt mutant T-cell clones isolated from adults had molecular lesions smaller than those detectable at the level of Southern blot analysis. We, therefore, have begun studies utilizing the polymerase chain reaction (PCR) amplification of cDNA copied of mRNA templates derived from mutant clones to allow direct sequencing of in vivo-derived mutant genes. Our preliminary studies with this technique

using cultured T-cells from members of a French-Canadian Lesch-Nyhan family have identified a new heritable mutation at *hprt* which we named $HPRT_{Montreal}$. In analyzing mutations at the somatic level, our first studies of *in vivo*-derived *hprt* mutant T-cell clones isolated from a normal highway worker showed a splice-site mutation involving exon 2 in two clones. We are now analyzing the remaining 84-90% of mutant clones not showing gross structural alterations on Southern blots obtained from the three normal males described above in an effort to "fill in" the "spontaneous" mutational spectrum for *in vivo*-arising *hprt* mutations in humans. This is a necessary first step in launching a search for *in vivo* and *in vitro*-induced mutational spectra that may allow the diagnosis of human exposures to specific mutagens or classes of mutagen.

THE CONTROL OF GROWTH AND DIFFERENTIATION IN CULTURED CELLS BY AGENTS THAT PROMOTE TUMOR FORMATION

E. Huberman, Y. Homma, S. I. Murao,
A. Constantinou, and F. R. Collart

Argonne National Laboratory
Argonne, IL 60439-4833

Our research addresses the cellular and molecular events that govern tumor promotion and progression. Because cancer cells are marked by uncontrolled replication and blocked differentiation, our studies focused mainly on factors that affect these events. Current studies involve the following: Tumor promoters such as phorbol 12-myristate 13-acetate (PMA), which are potent inducers of differentiation in various cultured human cell types including the human promyelocytic HL-60 cell line, are tested for their ability to initiate early biochemical events that control differentiation processes. Cell maturation in these cells begins after PMA binds to its receptor, which is a calcium- and phospholipid-dependent kinase, protein kinase C. To study the role of this kinase in cell maturation, we developed HL-60 cell variants that are either susceptible or resistant to PMA-induced differentiation. Treatment of the maturation-susceptible HL-205 cells with PMA results within minutes in a different protein phosphorylation pattern than that of treated maturation-resistant HL-525 cells. Because this new pattern may result from differences in protein kinase C activity between the two cell types, we examined the characteristics of this activity from both HL-205 and HL-525 cells. Using partially purified enzymes, we established that the activities of protein kinase C from the two cells types differed in a number of characteristics, including requirements for the enzyme cofactors calcium and phospholipids. We also found that the addition of exogenous proteins and peptides to the two different enzyme preparations resulted in different protein phosphorylation profiles. On the basis of these results, we suggest that differences in the protein kinase C activities in the two cell types, and the resulting new substrate specificities, may be responsible for the different phosphorylation patterns and susceptibilities of the two cells types to the induction of maturation by PMA and related agents.

Characterization of specific proteins (especially nuclear proteins) that are phosphorylated in one cell type but not the other can lead to the

identification of molecules that are critical in initiating cell differentiation in HL-60 and other related cell types. One or more of these proteins may be topoisomerases because these enzymes are localized in the nucleus and alteration in their activities affects the transcription of specific genes. We found that the topoisomerase II inhibitor novobiocin functioned in the same fashion as PMA, causing the HL-205 cells (but not the HL-525 cells) to acquire maturation markers. Furthermore, novobiocin and PMA were able, in the HL-205 cells but not the HL-525 cells, to reduce the activity of topoisomerase II within 30 min after treatment. This reduction in topoisomerase II activity and acquired cell differentiation was suppressed by H-7, an inhibitor of protein kinases, including protein kinase C. On the basis of these results, we suggest that induction of differentiation in HL-60 and other related cell types by some tumor-promoting agents requires the activation of protein kinase C, which causes a reduction in topoisomerase II activity and consequent alterations in the DNA topology, resulting in specific expression of genes that are associated with the mature phenotype.

To determine the possible involvement in tumor progression of inosine 5'-monophosphate dehydrogenase (IMPDH), a key enzyme that regulates quanine nucleotide biosynthesis, we analyzed its expression in normal and malignant human cells. To obtain an appropriate IMPDH probe for such studies, we purified the enzyme to homogeneity and used a polyclonal antibody directed against the purified protein to isolate human and Chinese hamster IMPDH cDNA clones. These clones were sequenced and found to contain an open reading frame of a protein containing 514 amino acids. A sequence of 35 amino acids obtained by analysis of the purified protein was found to be identical to a segment of the protein sequence deduced from the IMPDH cDNA. Comparison of the protein sequences deduced from the human and Chinese hamster cDNA clones indicates only eight amino acid differences, suggesting that IMP dehydrogenase is a highly conserved protein. Using the IMPDH probe and the Northern blotting technique, we demonstrated an association between IMPDH expression and the degree of growth of normal and malignant human cells. Furthermore, our studies indicated an increase in IMPDH expression in the tumor cells relative to their normal counterparts. In both the normal and the tumor cells, the level of IMPDH mRNA expression paralleled the amount of cellular IMPDH enzyme and activity. These results indicate an association of IMPDH expression with cell growth and support a possible link of enhanced enzyme expression in the growth progression of tumor cells. These results

also suggest that agents that modulate IMPDH activity may be useful for studies on tumor progression and may perhaps help in controlling the growth of some tumors. We anticipate that the work on the development and use of the various assays that incorporate cultured human cells will lead to the development of rapid and reliable methods by which a broad spectrum of environmental contaminants can be tested for their potential tumor-promoting and -progressing activity. (Research supported by the U.S. Department of Energy under Contract No. W-31-109-ENG-38.)

MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS BY TRANSFECTION OF ONCOGENES

J. J. McCormick and V. M. Maher

Michigan State University
East Lansing, MI 48824

Human fibroblasts in vitro have not been successfully transformed to malignancy by carcinogen exposure. This is surprising since there is excellent data that indicates that carcinogen exposure is the cause of most human tumors. To understand why human fibroblasts are so refractory, we have transfected them with oncogenes active in transformed rodent fibroblasts or human tumorigenic fibroblasts derived from fibrosarcomas. We have found that activated H- or N-ras genes flanked by suitable enhancer and promoter sequences were able to cause normal human fibroblasts to express many transformed phenotypes. However, such cells senescenced after the expected in vitro lifespan and did not form tumors in athymic mice. When we transfected H-, or N-ras genes in the same constructions used above or the v-K-ras gene into MSU-1.1 cells, an infinite lifespan near diploid non-tumorigenic cell strain we have developed, foci formed. Cells from these foci formed progressively growing, invasive fibroblastic tumors in athymic mice. The premalignant, as well as the tumor-derived cells, transfected with the H- and N-ras gene overexpress the p21 ras protein. The p21 viral K-ras p21 ras protein was not overexpressed. However, unlike the H- and N-ras genes used, the K-ras was a double mutant with changes in codon 12 and 60. The H-ras gene in the same construction used above caused malignant transformation of two other infinite lifespan cell lines of the KMST-6 cells of Namba and GM637 cells, which was transformed with SV40. This suggests that the infinite lifespan phenotype is required for malignant transformation. At least in vitro, finite lifespan cells such as human fibroblasts appear to have too few potential doublings to acquire the needed transformed characteristics and then form a life-threatening tumor.

Studies on human fibrosarcoma-derived cells show that 6 out of 6 cell lines examined did not show overexpression of the p21 ras protein. Studies are underway to determine if expression of a normal level of the mutant H- or N-ras is sufficient for malignant transformation of MSU-1.1 cells or whether the activation of some other gene(s) in addition to ras is required to complement the infinite lifespan phenotype. Since the oncogenes used in

these studies are homologous to the human proto-oncogenes, we propose to replace transfection of such oncogenes with carcinogen-induced activation of the proto-oncogene as the appropriate selection techniques are understood. By this method, it should be possible to come to a quantitative mechanistic understanding of malignant transformation. (Research supported by the U.S. Department of Energy Grant 60524, DHHS Grants CA21289 from NCI and ES65152 from NIEHS.)

GENE TRANSFER WITH RETROVIRAL VECTORS

E. Hays, J. Gasson, J. Dipersio, I. Chen

University of California
Los Angeles, CA 90024

In the course of development of leukemia as the result of chemical carcinogens, ionizing radiation retroviruses or as yet unknown environmental factors, normal cellular genes are amplified or rearranged. Gene products from them are increased or altered so that aspects of the complex functions that regulate cell division cannot operate and uncontrolled growth occurs.

We are exploring methods for introducing genes into bone marrow stem cells to study this process. This system of gene transfer provides a new approach to the developmental biology of replicating differentiating cells and offers promise for understanding altered gene expression in the generation of human malignancies. We are currently exploring the use of retroviruses (defective RNA viruses) to transfer the tax/rex, (the transactivating gene of Human T cell leukemia virus II, HTLV II), GM-CSF and other hemopoietic growth factor genes to marrow cells as models to investigate effects of expression of these genes by marrow stem cells on hematopoiesis and development of leukemia. Primary efforts are directed to consideration of vector design, the specificity of internal promoters, optimal infection protocols for marrow cells, and long term expression following transplantation of infected marrow to irradiated hosts.

We report in these studies the preliminary results of gene transfer of tax/rex and GM-CSF genes into murine hemopoietic stem cells with these vectors. In addition, we have infected a slow growing sarcoma cell line with the GM-CSF-containing retroviral vector and describe <u>in vitro</u> and <u>in vivo</u> studies using these cells.

HTLV II is a retrovirus that is associated with a T cell leukemia in man. This virus encodes a protein responsible for transcriptional activation of the HTLV II long terminal repeat (LTR). The virus gene responsible for this activity is called tax/rex. Tax/rex is the putative transforming gene for HTLV II. GM-CSF is a factor which stimulates growth and differentiation progenitors of granulocytes and macrophages and produces enhanced function of mature granulocytes. Its disregulation has been postulated, but not proven, to be an etiological factor in the development of leukemia.

To carry out these experiments, the genes of interest are placed in expression vectors with a CMV promoter and the Neo-R gene (to confer resistance to neomycin for the purpose of cell selection). These vectors are transfected into fibroblast cell lines which can package the vector into retroviruses. These viruses are replication-defective but can infect cells co-cultured with the fibroblasts and become integrated into the DNA of the cell of interest. Retrovirus vectors containing the tax/rex gene and the Murine GM-CSF genes have been used in protocols to transfer them to hemopoietic stem cells by co-culturing bone marrow from mice treated with 5-florouracil (to enrich for stem cells), with the fibroblasts containing the retroviral vector. After infection, cells are selected with the antibiotic G-418 (neomycin) and the resistant cells are tested in a standard colony assay. The majority of the cultured G-418 resistant cells are injected into 900 rad-treated syngeneic mice. The spleen colonies (12 day) of the radiation chimeras are tested for neomycin resistant colony forming cells. The DNA from these spleens is evaluated for the presence of tax/rex and GM-CSF genes.

Results to date show that we are able to detect neomycin resistant colony forming cells from the spleens of treated mice. Analysis of the DNA for tax/rex and GM-CSF genes from four spleens by polymerase chain reaction was positive in one animal. Eight mice have been transplanted with tax/rex bone marrow cells. Two animals died with visible spleen colonies and remarkable wasting in the second week post transplant. Two animals were sacrificed when moribund the third week post transplant. One had profound anemia and neutropenia and marrow aplasia, the other had a cellular marrow with a predominance of mature granulocytes. Both showed loss of body fat and thymic atrophy. The GM-CSF chimeras (6 survivors) and the 4 surviving tax/rex mice show no evidence of disease two months past transplant.

In a second approach the retroviral vector has been used to infect a slow growing murine sarcoma line. This infected line produces high levels of GM-CSF *in vitro*. These cells were transplanted subcutaneously ($5x10^5$) to a single mouse. At 4 weeks after transplant the recipient had a 1 x 1.5 cm tumor at the site of injection. GM-CSF activity was present in the serum. The white blood cell count was 260,000/cu mm with a marked shift to the left. At autopsy splenomegaly with predominance of granulopoiesis and a bone marrow replaced by immature granulocytes were the remarkable findings. Selected clones from this cell line have been studied. All mice with tumors

have had similar findings to those described above. The peripheral blood granulocytes have ranged from 250,000 to 750,000/cu mm. High serum GM-CSF levels are present.

The goal of these studies is to develop an effective means for gene transfer to use these models to determine if tax/rex and GM-CSF gene expression by hemopoietic stem cells alters hemopoiesis or is associated with leukemogenesis. In the case of the GM-CSF gene, we have described an additional mechanism to study the in vivo effect of the continued production of high levels of this growth factor on hematopoiesis and leukemogenesis. Mice pretreated with ionizing radiation or xenobiotic agents also will be studied for leukemia development in the presence of high levels of GM-CSF produced by this GM-CSF-producing tumor. (Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-FC03-87-ER60615.)

POLY I·POLY C INDUCTION OF BETA INTERFERON DURING THE CELL CYCLE - A STUDY AT THE SINGLE CELL LEVEL

K. F. J. Tang, P. O. P. Ts'o and S. A. Lesko

The Johns Hopkins University
Baltimore, MD 21205

Human beta-interferon is produced in fibroblasts by treatment with double-stranded RNA. Production of beta-interferon by poly I·poly C (polyinosinic and polycytidylic acids) is a transient phenomenon; after poly I·poly C induction, there is a short lag period (about 1/2-2 hrs) followed by a burst of beta-interferon synthesis which persists for a short period, and sharply decreases thereafter. Studies on the relationship between levels of beta-interferon protein and mRNA indicates a very close correlation between production of beta-interferon protein and relative amount of beta-interferon mRNA in the cells.

We have investigated the production of beta-interferon at various stages of cell cycle in synchronized and unsynchronized cell populations induced by poly I·poly C. The beta-interferon appeared 1/2 hr post-induction, and maximal activity was reached approximately at 1-2 hrs post-induction in our human fibroblasts, HF926 cells and JHU-1 cells. Human fibroblasts were synchronized with mitotic detachment and, at different stages of cell cycle, poly I·poly C was added for induction of beta-interferon. One hour after induction, cell-free medium was collected and assayed for secreted beta-interferon. The cells were then fixed and stained with a DNA specific fluorochrome, 4',6-diamidino-2-phenylindole (DAPI), for cell cycle analysis by microfluorometry. The data indicated that beta-interferon was produced in every stage of cell cycle examined, and there was no significant elevation in beta-interferon activity at any particular stage. In an unsynchronized experiment, the level of intracellular beta-interferon was quantitatively measured in single cells using a specific antibody followed by a FITC conjugated secondary antibody. In the same individual cell, DAPI fluorescence intensity was measured for determination of cell cycle position. The results show that beta-interferon protein can be detected throughout the cell cycle and is not restricted to any specific stage of the cell cycle.

In addition, we also investigated the expression of beta-interferon mRNA by in situ hybridization. In situ mRNA hybridization has become one of

the most important tools to study gene expression in individual cells during the past few years. We have extended this technique in conjunction with microfluorometry to simultaneously detect beta-interferon mRNA and DNA content in the same individual cells. Uninduced and poly I·poly C induced HF926 cells were in situ hybridized with a ^{3}H-beta-interferon probe. After autoradiographic detection, the number of silver grains was counted. The data indicate that about 50% of cells are positive for beta-interferon mRNA expression; similar results were observed at various stages of cell cycle. We also found that beta-interferon mRNA was expressed in all phases of the cell cycle in an unsynchronized population.

This method described above was also applied to a cell line (HT1080) which consisted of diploid and tetraploid cells. The level of beta-interferon mRNA detected by in situ hybridization upon induction with poly I·poly C was found to be 2-3 times higher in tetraploid compared to diploid HT1080 cells and correlated with beta-interferon activity in that a subclone of tetraploid HT1080 cells secreted 2-5 fold more beta-interferon than a subclone of diploid HT1080 cells. Both subclones appeared to exhibit the same kinetics of beta-interferon production. Interestingly, there were beta-interferon related transcripts detected during S phase in uninduced tetraploid HT1080 cells.

The combination of microfluorometry and in situ hybridization offer the unique feature of measuring DNA content and mRNA expression in the same cell. When used in conjunction with a specific monoclonal antibody these techniques provide a valuable tool to investigate specific gene expression related to ploidy or cell-cycle stage in an unsynchronized population. (Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-FG02-88ER60636.)

S E S S I O N I I I

CHEMICAL INTERACTIONS WITH DNA

SUMMARY OF SESSION III

CHEMICAL INTERACTIONS WITH DNA AND OTHER BIOMACROMOLECULES

By Dr. Roger M. Bean
Pacific Northwest Laboratory

Organic chemicals are known to interact with DNA and other macromolecules in a variety of ways. The interaction can be purely physical, arising through intramolecular attraction, clathration, or intercalation. Highly reactive chemicals, such as nitrosamines, can directly react with DNA upon exposure, to produce substituted products. In the case of many organics, specific metabolites are formed that produce either macromolecular associations or covalent reaction products. The papers presented in this session are the results of research to determine the amount and types of interactions produced from exposure to chemicals, to understand effects of DNA-organic interactions on genetic structure and function, and to shed light on the relationship between these interactions and carcinogenic response.

There continues to be a lively research interest in methods for the characterization and quantification of chemicals that are adducted to DNA and other biomolecules. Analysis of adducts to DNA or other macromolecules may be a way of obtaining a measure of individual exposure to carcinogenic chemicals. Bean *et al*. (PNL) reported on recent developments in research toward the direct analysis of DNA adducts using combinations of separation and detection methods. Capillary zone electrophoresis/laser fluorimetry has been used to successfully separate and detect 100 attomoles of adducted nucleoside. Gas chromatography/mass spectrometry has also been used to detect adducts at the 1 picogram level. In addition, Bean *et al*. also reported the formation of adducted species to DNA that are substantially more polar than "classical" adducts. Characterization of adducts formed from the reaction of benzo[a]pyrene (BaP) diol epoxide with hemoglobin was reported by Haugen and Reilly, Jr. (ANL). Although at least 6 adducts were isolated from the reaction, they did not involve reaction with the hemoglobin sulfhydryl groups, and 50% of the adducts could be liberated with 0.1 N HCl. However, when the adduct preparation was done biologically,

using a combination of BaP, human erythrocytes, and hamster embryo cells, no detectable acid hydrolysis of adducts occurred. Therefore, hemoglobin adduction by BaP does not likely occur through a diol epoxide route. The characterization of the form of the BaP-hemoglobin adducts is being investigated by Haugen (ANL). After modification of unadducted hemoglobin sulfhydryl groups with an ionic reagent, he has been able to separate unadducted hemoglobin from adducted hemoglobin with ion chromatography over DEAE-cellulose. Another approach to the dosimetry of cancer-causing agents has been developed by Lesco et al. (Johns Hopkins). To assay for cyclobutyl-dithymidine dimers formed from exposure of skin to sunlight (a known carcinogen), a quantitative competitive ELISA immunofluorescence assay was developed that could determine the photodimerization level in individual cells. The method has been used to determine the number of dimers induced at germicidal doses of radiation.

Although DNA adducts have long been associated with cellular carcinogenic response, demonstration of direct causal relationships between adducts and cancer have proven to be very difficult. Hence, correlations between adducts and carcinogenic response continue to be actively pursued. The relationship between adduct formation from exposure to inhaled diesel exhaust and subsequent carcinogenic response of the pulmonary system has been studied by Mauderly et al. (ITRI). Diesel exhaust was found to produce increased adduct levels only in nose and peripheral lung (locations of increased cancer) of exposed rats. Adduct formation, as determined by postlabeling analysis, was found to increase with exposure level. Rats exposed to carbon black particulates formed no increased adduct levels in tissues examined, although tissue inflammation levels were similar to those observed with diesel exhaust. Studies comparing the tumorigenic response to these two carbonaceous insults are continuing. Another approach to correlation of exposure dose with carcinogenic effect is being developed by Bond, Gubin and Johnson (ITRI). These workers are attempting to determine if DNA repair activity has an effect on tissue response to exposure to cancer-causing chemicals. An assay for the activity of O^6-alkylguanine-DNA alkyltransferase (AGT), an enzyme that repairs DNA methylated by alkylating carcinogens such as nitrosamines, was developed and applied to a number of respiratory tissues in rats. The results indicate that AGT activity was not uniformly distributed. These studies indicate that different regions of the respiratory tract can effectively repair methylated DNA, with lung >

ethmoturbinates > maxilloturbinates ≅ alveolar type II cells > trachea ≅ bronchi. Experiments relating AGT activity and response to administered alkylating agents are in progress. Okinaka and Strniste (LANL) are taking another approach to relate repair activity with carcinogenicity. The DNA of mutant Chinese hamster cells were treated with either N-acetoxy- or N-hydroxy-acetylaminofluorine to produce cells deficient in hypoxanthene-guanine phosphoribosyl transferase. The DNA of these mutants was studied using digestion, Southern blotting, and probing techniques. It was found that the DNA banding pattern of all mutants studied were identical to the original cell line, which led to the conclusion that mutations were not caused by gross deletions/rearrangements, but rather by point mutations or small deletions. Research into the exact nature of these small changes is continuing. Another study of the effects of DNA misrepair on DNA function is being undertaken by Giometti (ANL), who has isolated 5 liver protein variants from offspring of N-ethyl-N-nitrosourea-treated mice. The proteins are being characterized in order to provide a direct link between carcinogen-induced DNA damage and resulting protein structural changes. Using two-dimensional electrophoresis, three proteins out of the five variants have been found to be associated with the mitochondrial proteins, while another is exclusively found in the microsomal fraction. One of the mitochondrial proteins has been found to co-migrate with ornithine aminotransferase (OAT), and reacted with anti-OAT antiserum. The structural differences between normal and mutant OAT are being elucidated, and characterization of the other mutant proteins continues.

In addition to research that attempts to directly relate changes in chemical structure to carcinogenesis and mutagenesis, other laboratories are studying the effects of chemical exposure and DNA-adduction on the morphology of cellular biostructure. For example, Geacintov *et al*. (NYU) has used an assay based on linear dichroism to study the effects of diol epoxide metabolites of BaP on the unwinding of supercoiled DNA. Intercalation of the diol epoxide causes substantial DNA unwinding, which can be kinetically followed by linear dichroism. A significant finding is that the (+) enantiomer of the diol epoxide forms a DNA adduct that causes substantially more unwinding than the (-) enantiomer, even though the (+) compound forms an adduct that lies outside the DNA helix, and the (-) compound is primarily contained within the helical structure. Large differences in the mobilities on agarose gels between supercoiled DNA and

unwound, highly adducted DNA are observed. Differences in electrophoretic resolution between adducted and nonadducted DNA are a function of extent of adduction. Springer, Mann and Stiegler (PNL) are also exploring the effects of BaP adduction of the physical arrangement of genetic material. Their research is designed to determine the effects of binding on the arrangement of nucleosomes surrounding the DNA, and the effects of nucleosome position on the site of DNA adduction. To this end, they have been developing a method to determine the site-frequency of adduction by BaP diol epoxide on a specific 120-base-pair gene, the 5S rRNA gene, where the position of the nucleosome is well defined. Using a combination of restriction enzyme cleavage, radiolabeled phosphorylation, and polymerase digestion, DNA fragments are isolated that have radiolabeled phosphate on one end and the adducted base on the other end. Gel electrophoresis is then used to determine the distribution of base-pairs, and thus the distribution of adducts along the DNA chain. Preliminary evidence indicates that there are preferred sites of adduction which may either dictate, or be dictated by, nucleosome position.

ANALYSIS OF CLASSICAL AND NONCLASSICAL ADDUCTS

R. M. Bean, B. L. Thomas, D. A. Dankovic, D. B. Mann,
G. A. Ross, and D. L. Springer

Pacific Northwest Laboratory
Richland, WA 99352

Metabolites of carcinogenic organic compounds have the ability to bond with deoxyribonucleic acids (DNA) for form DNA adducts. These species are retained for relatively long periods of time in the body, and are thought to be associated with the formation of cancer. Analysis of DNA for adducts may therefore provide an estimate of individual exposure to carcinogens. Methods currently being used for analysis of DNA adducts have been reviewed by Wogan and Gorelick (1). In general, the currently available methods suffer either from a lack of sufficient sensitivity for environmental screening or from a lack of qualitative specificity. Although data on humans is sparse, we may expect, allowing for initial rapid decay after environmental exposure, adduct levels of 0.1 to 0.01 nanograms per milligram of DNA. Research at PNL is directed toward developing methods for the analysis of DNA adducts that will permit identification and quantitation of adducted polycyclic aromatic hydrocarbon (PAH) metabolites at environmental levels. Included within this objective is the preparation and characterization of DNA-adducts to be used as analytical standards.

Several approaches to the analysis of DNA adducts are being undertaken. A primary analytical approach is to isolate the bound metabolites by cleaving the covalent bonds that attach them to the purine or pyrimidine bases of the DNA, and then to treat the isolated metabolites with reagents that will increase their separability and their sensitivity to gas chromatography/mass spectrometry (GC/MS) detection. However, analysis of the metabolites while still bound to the nucleic acid base (as adducted nucleosides or nucleotides) would afford more specific information about the nature of the adduction process and its relation to carcinogenesis. Thus, separation and detection methods that do not require cleavage of the metabolite from the DNA bases are also being explored. These methods involve liquid chromatography and capillary electrophoresis, using fluorescence, chemiluminescence, or electrospray mass spectrometry detection.

Preparation of Analytical Standards - One of the limitations inherent in the direct analysis of adducts has been the lack of analytical standards. In initial experiments, we prepared nucleotide adducts of benzo[a]pyrene (BaP) in nanogram quantities with calf thymus DNA, using microsomal preparations to afford the adduction process. The microsomal method produced experimental artifacts, and did not produce adducts in the microgram quantities required for analytical study. A method using intact rat hepatocytes, developed in our Biology and Chemistry Department, appears to be a satisfactory method for obtaining the required quantities of a variety of PAH adducts (2). The products from this in vitro method seem to very closely approximate the adduct distributions found from in vivo studies of adducts. The significance of this result is that for the first time, research quantities of many different adducts can be prepared from adducting hydrocarbons and pure DNA. At the present time, about 5 μg adducted BaP, 1.5 μg adducted fluoranthene, 200 ng dibenz[a,h]anthracene, and 110 ng 7,12-dimethylbenz[a]anthracene have been made available for study. The rate of metabolism of each hydrocarbon by the hepatocyte system is different; hence, the incubation conditions require preliminary experiments before microgram-scale experiments can be successfully conducted.

GC/MS of Derivatized Metabolites - Upon treatment with hydrochloric acid, the adducting moiety from PAH adducts is liberated as a tetrahydro-tetrahydroxy compound (tetrol) that is subject to derivatization by a number of agents prior to analysis by GC/MS. BaP tetrol forms a tetramethyl ether as well as a tetraacetate that is detectable by selected ion monitoring (SIM) mass spectrometry at the picogram level (3). A principal research problem is to prepare these derivatives reliably, in good yield and at the concentrations required with background noise sufficiently low for unambiguous identification. Although we have extended to sensitivity of the permethylation method to 10 pg of tetrol (3), we have not yet been able to do so reliably and without unacceptable accompanying chemical noise. During a study of the mass spectral properties of derivatives of BaP metabolites (4), we found that the methyl ether derivatives of several PAH tetrols produce a unique spectrum resulting from a reverse Diels-Alder (RDA) cleavage. This cleavage is not observed with PAH acetate or trifluoro-acetate derivatives, which fragment by other mechanisms. We have recently found that the trimethylsily derivative of BaP tetrol also undergoes the RDA cleavage to yield a fragment ion of 404 amu that has very little interfer

ence in the single ion chromatogram. The derivative is more readily and reliably prepared than the corresponding methyl ethers, and unoptimized detection levels are approaching the 1 pg level.

Detection of Intact Adduct by Chemiluminescence - Chemiluminescence is a powerful and specific detection method that has been recently applied to the analysis of a number of PAH compounds (5). It has been shown recently that amino-PAH compounds are particularly sensitive to this detection technique, with detection limits 1 to 2 orders of magnitude lower than fluorescence detection (6). The structures of DNA adducts formed from PAH compounds comprise both a fluorescent hydrocarbon moiety and an aromatic amine linkage. Thus, preliminary studies were conducted to determine if these compounds are amenable to specific detection by chemiluminescence. The chemiluminescence response we are investigating depends on electronic excitation of the analyte by the reaction between an aryl oxalate and hydrogen peroxide. A variety of oxalates can be used for the reaction, each giving different light intensities and durations that are pH dependent. Although in principle the reaction is simple, from a practical standpoint the process is more difficult because of need for compatibility between separation column eluent and post-column reagents and solvents, the mechanical arrangements for reagent metering, and the short time required between post-column mixing and detection. For these experiments, bis-(2,4,6-trichlorophenyl) oxalate was used with hydrogen peroxide to initiate chemiluminescence response from a synthetic BaP-guanosine adduct and the response compared with fluorescence detection. The experiments were conducted without a separation column. The results of these preliminary experiments indicate that the adduct does exhibit a strong chemiluminescence response under the conditions used that is comparable in intensity with fluorescence. It is very likely that the chemiluminescence response can be increased substantially by increasing oxalate concentration, choosing a more responsive oxalate, and optimizing solvent, pH, and post-column mixing conditions. Further chemiluminescence studies are planned.

Separation of Adducts by Capillary Zone Electrophoresis (CZE) - Electrophoretic separation of high molecular weight compounds in silica capillaries filled with buffer solutions has several advantages that lend themselves well to the analysis of adducts. CZE separation efficiencies can be high for molecules with higher molecular weights since mobility depends mainly on charge. PAH-adducted nucleotides and nucleosides are sufficiently

polar to give good electrophoretic mobility with excellent separation efficiency. Good separations can also be obtained at both high and low pH. In our studies, detection was by laser fluorescence. Although detection wavelength was not optimized for these experiments, it appears that about 100 attomoles can be detected under the conditions used. We have found that the use of a mixed solvent/buffer system as the mobile phase is critical to good performance. If water alone is used, several peaks appear, presumably because of hydrolysis under the strongly acid or basic conditions of the separation. A principal problem with CZE is the small sample capacity. The column must be very small in diameter to avoid overheating the column. Only about 10 nL of sample can be applied to the column, giving an overall detection level of 10 pg/mL using fluorescence detection.

The next step in this technology will be to interface the CZE system with a mass spectrometer. Normal mass spectrometric analysis is not sensitive enough to detect femtogram quantities of analyte. An electrospray interface, pioneered at PNL (7) and winner of an R&D award (8), has been demonstrated to be capable of detecting femtogram quantities of certain polypeptide analytes. Research is being conducted to interface CZE with a new triple-quadrupole atmospheric-inlet mass spectrometer recently acquired for this and other biochemical studies. Another refinement of the CZE approach to adduct analysis will be an investigation of the CZE and mass spectrometric properties of the BaP-nucleotide adduct (containing a phosphate moiety).

Investigation of Nonclassical DNA Adducts - We are engaged in an Exploratory Research Project to identify forms of hydrocarbon adducts that do not exhibit the behavior of "classical" adducts formed through normal diolepoxide addition to DNA bases. A number of researchers, for example Shen et al. (9) and Ashurst and Cohen (10), have reported that when radiolabeled BaP adducts are isolated by the conventional method, 1/3 to 1/2 of the radiolabeled nucleoside material is not recovered as the classical adduct. We have examined this "nonclassical" adduct material by treating it with 0.12 N HCI to release it from the residual DNA structure. This process was followed by solid reverse-phase adsorption of the hydrolyzed product. Reverse phase chromatography of this material revealed that in addition to tetrols, from 10% to 33% of the nonclassical material had retention times consistent with BaP-3,6-quinone and BaP-6,12-quinone. We have confirmed the presence of these quinones with mass spectrometry. Quinones are not found

in the nonclassical fraction unless the acid hydrolysis step is performed, leading us to believe that the appearance of quinones after acid treatment results from cleavage of covalent bonds to the DNA structure. Formation of nonclassical structures may be a consequence of a competing adduct-formation mechanism, as suggested by Cavalieri (11). The project will continue through FY 1989 to confirm these results and to investigate the nature of the quinone-precursor structure. (Research supported by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC06-76RLO 1830.)

REFERENCES

1. Wogan, G.N., and Gorelick, N.J., 1985, Environ. Health Perspec. 62:5-18.

2. Dankovic, D.A., Springer, D.L., Thomas, B.L., Mann, D.B. and Bean, R.M., 1987, Proceedings, DOE Contractor Conference on Chemical Research, Monterey, CA, June 24-26, 1987.

3. Bean, R.M., Chess, E.K., Thomas, B.L., Springer, D.L., Mann, D.B., and Hendren, D.J., 1987, Abstracts, Eleventh International Symposium on Polynuclear Aromatic Hydrocarbons, Gaithersburg, MD, September, 23-25, 1987.

4. Chess, E.K., Thomas, B.L., Hendren, D.J., and Bean, R.M., 1988, Biomed. Environ. Mass Spectrometry 15:485-493.

5. Sigvardson, K.W., and Birks, J.W., 1983, Anal. Chem., 55:432-435.

6. Sigvardson, K.W., Kennish, J.M., and Birks, J.W., 1984, Anal. Chem., 56:1096-1102.

7. Olivares, J.A., Nguyen, N.T., Yonker, C.R., and Smith, R.D., 1987, Anal. Chem., 59: 1230-1232.

8. Special Report of the R&D 100 Winners, 1988, R&D 100, Des Plaines, IL, p7.

9. Shen, A.L., Fahl, W.E., and Jefcoate, C.R., 1980, Arch. Biochem. Biophys. 204:511-523.

10. Ashurst, S.W., and Cohen, G.M., 1982, Carcinogenesis 3:267-273.

11. Cavielieri, E.L., and Rogan, E.G., 1985, "Polycyclic Hydrocarbons and Carcinogenesis," ACS Symposium Series 283, R.G. Harvey, Ed., American Chemical Society, Washington, D.C., pp 289-305.

ISOLATION AND CHARACTERIZATION OF ADDUCTS FORMED BETWEEN BENZO[a]PYRENE METABOLITES AND HUMAN HEMOGLOBIN IN CELL-FREE AND INTACT CELL SYSTEMS

David A. Haugen and Christopher A. Reilly, Jr.

Argonne National Laboratory
Argonne, IL 60439-4833

One of the objectives of our studies is to identify hemoglobin-carcinogen adducts and to develop techniques for their analysis as a measure of human exposure to carcinogens, and the associated risks for carcinogenesis. An accompanying report presents the rationale for analysis of hemoglobin-carcinogen adducts as a means of molecular dosimetry. In this report, we describe results obtained during the characterization of adducts between human hemoglobin (Hb) and metabolites of benzo[a]pyrene (BP), including development of a system for exposure of intact erythrocytes to labile BP metabolites.

Initial studies in the characterization of Hb-BP adducts involved the in vitro treatment of dialyzed lysates of human erythrocytes with [^{3}H](+)anti-BP-7,8-diol-9,10-epoxide ([^{3}H]BPDE). Hb and BPDE concentrations ranged from 0.1 to 4 mM and 0.3 to 13 μM, respectively. The formation of Hb-BPDE adducts was demonstrated by precipitation and extraction of hemoglobin or globin with acetone or HCl in acetone, respectively. The maximal level of BPDE incorporation was 1-2 pmole per nmol Hb (i.e., 1-2 per 10^3).

Reaction of BPDE and Hb occurred according to pseudo-first-order kinetics, with the kinetics primarily governed by the hydrolysis of BPDE. Anion-exchange chromatography (DEAE-cellulose, pH 7.7) of the BPDE-treated Hb showed that two principal classes of adducts were formed, characterized as either more or less acidic than native hemoglobin. Reversed-phase high-performance liquid chromatography of the heme-free globins revealed the presence of at least six adducts.

Although the identity of the adducts has not been determined, the following evidence indicates that the adducts were not formed via interaction with the reactive β93 cysteine sulfhydryl group: (a) variation of the pH from 6 to 8 had little effect on the extent of adduct formation, (b) the level of adduct formation was similar for oxy- and deoxyhemoglobin, and (c) treatment of BPDE-treated hemoglobin with iodoacetamidosalicylic acid

(ISAL), and ionic sulfhydryl reagent (see accompanying abstract), similarly altered the ion-chromatographic properties of native hemoglobin and the radioactive Hb-BPDE adducts. The formation of two principal classes of Hb-BPDE adducts is consistent with our observation that approximately 50% of the adducts were subject to hydrolysis in 0.1 N HCl or NaOH. Together, the present data suggest that some of the adducts may have formed via reaction with protein carboxyl groups, yielding the corresponding esters, analogous to reaction of BPDE with acetate and with organic and inorganic phosphate.

Furthermore, the data indicated that Hb-BP adducts (and related adducts) may potentially be isolated by ion-exchange chromatography. Thus, to explore this potential and to work toward identification of human Hb-BP adducts formed *in vivo*, we developed a second model system in which human erythrocytes (0.5 to 4 x 10^9 cells per 75-cm^2 flask) and confluent Syrian hamster embryo (SHE) cells are co-cultivated for 24 h in medium supplemented with [^{3}H]BP (2 μM). The level of Hb-BP adduct formation was routinely detected by treatment of erythrocyte lysates with HCl-acetone, and confirmed by other techniques. The total amount of Hb-BP adducts depended on the erythrocyte density in the cultures, exhibiting saturation kinetics with half-maximal levels occurring at 1.3 x 10^9 cells per flask. The specific content of the adducts ranged from 0.08 to 0.2 pmole BP/nmol Hb (i.e., 0.8-2 adducts per 10^4 hemoglobin molecules).

In control cultures where the hamster cells were omitted, the apparent level of adduct formation was only 2-5% of that in the complete cultures. Negligible adduct formation occurred in erythrocytes incubated in medium removed from flasks incubated only with HE cells and BP, indicating that biosynthetic incorporation of tritium or tritium exchange was not responsible for the incorporation of radioactivity. The presence of Hb-BP adducts (rather than other HCl-acetone-precipitable products) in the lysates of BP-treated erythrocytes was confirmed by the alteration of the apparent molecular weight (gel filtration) of the adducts upon addition of hepatoglobin, a hemoglobin-binding serum protein.

Although as presented above, treatment of Hb-BPDE adducts with 0.1 N HCl or NaOH released about 50% of the radioactivity (ethyl acetate extraction), identical treatment of Hb-BP adducts and no effect, suggesting that BPDE is not a significant intermediate in the formation of Hb-BP adducts in the model system. This conclusion is in agreement with that of Wallin *et al*. [Cancer Lett., *35*, 139 (1987)] who observed that in BP-treated

mice Hb-BPDE adducts were only a small portion of total level of radiometrically detected Hb-BP adducts.

Anion-exchange chromatography (DEAE-cellulose, pH 7.7) of lysates from BP-treated erythrocytes showed that, as for Hb-BPDE adducts, two classes of adducts were resolved from native hemoglobin, these adducts being either more or less acidic than native hemoglobin. These results suggest that fractions enriched in hemoglobin-PAH adducts can be isolated by preparative chromatography. Chemical characterization of the hemoglobin-BP adducts is in progress. (Research supported by the U.S. Department of Energy, Office of Health and Environmental Research, under Contract No. W-31-109-ENG-38.)

A NEW ION-CHROMATOGRAPHIC APPROACH FOR ISOLATION OF ADDUCTS FORMED BY REACTION OF CARCINOGENS WITH THE β93 CYSTEINE SULFHYDRYL GROUPS OF HUMAN HEMOGLOBIN

David A. Haugen

Argonne National Laboratory
Argonne, IL 60439-4833

Covalent reaction with DNA is believed to be essential for the action of most chemical mutagens and carcinogens. In addition to this reaction, chemical mutagens and carcinogens react with nucleophilic sites in proteins, including hemoglobin. Studies with animals have shown that (a) similar dose-response relationships occur for covalent binding of carcinogens to DNA and hemoglobin; (b) hemoglobin-carcinogen adducts are long lived in the circulatory system, with disappearance rates similar to the naturally occurring rates for erythrocytes; (c) chronic, low-level exposure leads to accumulation of hemoglobin-carcinogen adducts, providing for an integral assay of exposure; and (d) several types of carcinogens and mutagens (e.g., aryl amines, nitrosamines, and alkyl oxides) or metabolites thereof react with the β93 cysteine sulfhydryl group of hemoglobin.

In agreement with the results of animals, limited studies of human populations and of human erythrocytes *in vitro* have demonstrated (a) dose-response relationships for formation of hemoglobin-carcinogen adducts *in vivo*, (b) similar kinetics of disappearance of adducts and erythrocytes *in vivo*, and (c) the β93 cysteine sulfhydryl groups as targets for reaction with electrophilic carcinogens or their metabolites. These observations led us to develop new approaches for the analysis of hemoglobin-carcinogen adducts in humans as a measure of carcinogen exposure and risk for carcinogenesis.

We report development of a method for the site-specific isolation of hemoglobin-carcinogen adducts formed by reaction of the β93 cysteine sulfhydryl group. As a model for development of the analytical approach, we used the sulfinimide adduct formed by reaction of hemoglobin with 4-nitrosobiphenyl (a metabolite of the carcinogen 4-aminobiphenyl). These adducts occur in the human population, especially in smokers.

Hemoglobin-nitrosobiphenyl adducts (Hb-Ph_2) were formed by the *in vitro* reaction (30°C, 180 min) of dialyzed lysates of human erythrocytes (0.5 mM

Hb) with 4-nitrosobiphenyl (0.4 mM) at pH 8.3. The reaction was terminated by addition of dithiothreitol (10 mM), and hemoglobin was isolated by gel filtration. Sulfhydryl analysis and spectrophotometric measurements showed that about 60% of the hemoglobin was converted to Hb-Ph_2. The model mixture of Hb and Hb-Ph_2 (1 mM total) was then treated (pH 7.2, 10°C, 2 h) with 20 mM 4-iodoacetamidosalicylic acid (ISAL), an ionic sulfhydryl reagent. After addition of dithiothreitol to react with excess ISAL, hemoglobin (Hb-SAL + Hb-Ph_2) was isolated by gel filtration.

On the basis of the results of preliminary kinetics studies (chemical measurement of SH disappearance and iodide formation, and direct spectrophotometric detection of the incorporated salicylate moiety), we estimated the conditions of reaction with ISAL to achieve 99.9% alkylation of the free sulfhydryl groups. Because the β93 cysteine sulfhydryl groups were unavailable for reaction in Hb-Ph_2 adducts, ISAL reacted only with native Hb, thus significantly modifying its charge, while having no effect on Hb-Ph_2.

The ISAL-treated mixture (Hb-SAL + Hb-Ph_2) and a parallel control mixture (Hb + Hb-Ph_2) not treated with ISAL were applied to respective DEAE-cellulose columns (pH 7.7; 20 mg protein each column) and eluted with a linear NaCl gradient. Figure 1A represents the elution of the major form of human hemoglobin prior to treatment with either 4-nitrosobiphenyl or ISAL. Figure 1B shows that native Hb and Hb-Ph_2 were partially resolved without prior treatment with ISAL presumably because local perturbations of electrostatic interactions near the site of adduct formation. Figure 1C shows that treatment with ISAL had no effect on the elution profile of Hb-Ph_2 but substantially increased the NaCl concentration required for elution of Hb-SAL (derived from the native Hb in the model mixture).

Current efforts are directed toward validating the charge-shift strategy for other carcinogens, as well as recovering and analyzing adduct-enriched fractions at the levels expected in typical samples of human blood. (Research supported by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. W-31-109-ENG-38.)

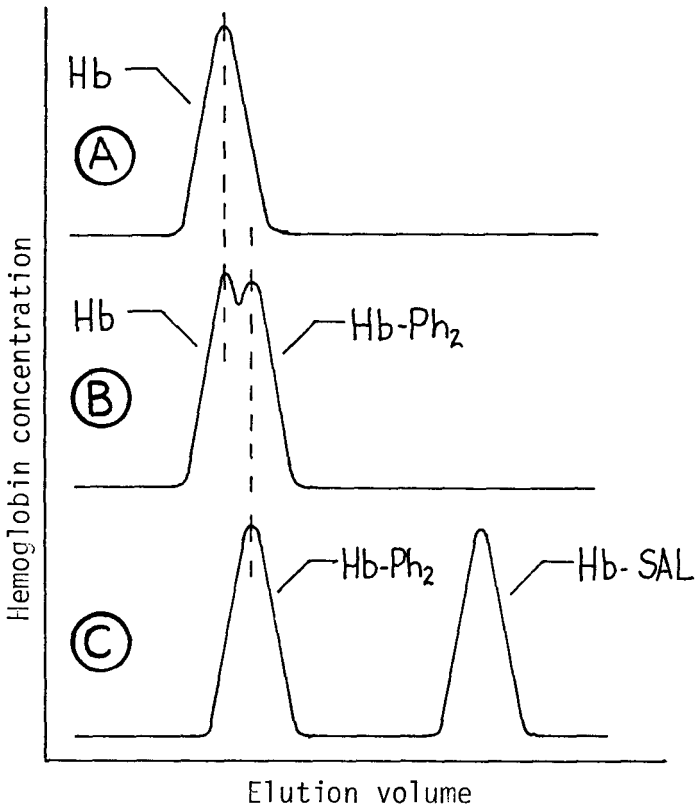


FIGURE 1. Chromatography of Hemoglobin

QUANTITATIVE IMMUNOFLUORESCENCE ASSAY FOR CYCLOBUTYLDITHYMIDINE DIMERS IN INDIVIDUAL MAMMALIAN CELLS

S. A. Lesko, D. E. Callahan, D. S. Kaplan, N. Ni, W. Li, W. R. Midden, P. T. Strickland, and G. Zheng

The John Hopkins University
Baltimore, MD 21205

Photochemical damage to DNA is generally thought to be an important factor in the development of skin cancer. The availability of sensitive and specific methods for the direct measurement of individual DNA adducts at the single cell level would greatly facilitate the study of the biological significance of these lesions.

In this investigation we have used Syrian hamster embryo cells treated with germicidal irradiation, to optimize a quantitative immunostaining procedure. With appropriate processing of the cells, T<>T were rendered accessible to binding by specific antibody, thereby generating a fluorescence signal whose intensity accurately described the T<>T content per cell. Quantitation of T<>T in single immunostained hamster cells was accomplished by means of computer-assisted microfluorometry (CAM) in conjunction with a competitive enzyme-linked immunosorbent assay (ELISA). A relationship was established between normalized fluorescence intensity and the number of cyclobutyl dithymidine dimers (T<>T) per cell. This method can be extended to other DNA adducts when the appropriate antibodies are available, and will be especially useful when only a small sample of cells or tissue is available, thus precluding DNA isolation. The monitoring of persons who are occupationally or environmentally exposed to genotoxic agents present such a scenario. Immunofluorescence assays also allow for a direct study of the formation and persistence of adducts in the different cell types of a tissue, where a particular cell type may have enhanced proclivity to undergo neoplastic transformation.

Calibration of CAM System

The first priority in conducting a quantitative immunofluorescence study is to establish a method for comparing daily fluorescence measurements. Our microfluorometry system was calibrated using a uranyl oxide-impregnated glass slide as a standard. Data was obtained with this

standard using all combinations of high voltage and amplifier gain. The parameters were adjusted to prevent PMT saturation while maintaining distinguishable intensity levels. A standard curve was obtained which showed a linear relationship between log intensity and log high voltage. Fluorescence intensity data from any experiment can be compared by extrapolation to a standard condition (high voltage - 376; gain - 1) using regression line parameters (slope and intercept from standard curve). The fluorescence of the uranyl glass slide (0.43 to 0.54) measured at this standard condition was then used to normalize fluorescence intensity data obtained with cell nuclei.

Immunocytochemical Measurement of T<>T

T<>T and DNA fluorescence was measured in the same individual cell using FITC and propidium iodide, respectively. FITC fluorescence intensity per nucleus as a percentage of the fluorescence intensity of the uranyl glass slide at the standard condition and normalized to propidium iodide fluorescence per nucleus was determined over a dose range of 0-120 J/m^2 for early passage, diploid fibroblasts (FC13) and a line of immortalized fibroblasts (21F). The data show that both cell types exhibited very similar responses with respect to T<>T fluorescence per unit DNA fluorescence in relation to dose of germicidal irradiation.

Competitive ELISA for Quantitation of T<>T

In order to quantitate T<>T formation in hamster cells with respect to UV dose, a competitive ELISA was performed with DNA isolated from FC13 and 21F fibroblasts that received identical doses of UV irradiation under the same conditions as cells used for immunocytochemical measurements. The ELISA was calibrated using serial dilutions of denatured UV-irradiated calf thymus DNAs (one T<>T per 20 and 2500 base pairs, HPLC analysis) as standards. The T<>T content of the various competitor DNAs, which were isolated from UV-irradiated cells, was determined by comparing the amount of DNA required to reduce by 40% antibody binding to the immobilized DNA to the amount of standard DNA required to reduce antibody binding by 40%.

Quantitation of T<>T in Irradiated Syrian Hamster Embryo Cells

The relationship between T<>T formation and fluorescence intensity measurements was compared, with respect to dose of germicidal irradiation,

in early passage FC13 cells and immortal 21F cells. Germicidal irradiation (10 J/m^2) produced about 9 x 10^5 T<>T cell (1 per 7450 bases of DNA) in F13 cells with a normalized fluorescence intensity of 0.027%. The values for 21F cells at 10 J/m^2 were 7.5 x 10^5 T<>T/cell (1 per 9750 bases) with a normalized fluorescence intensity of 0.021%. The limit of sensitivity of the immunofluorescence assay with the current anti-UV DNA antibody is about 5 J/m^2 (55-75% survival). The antibody was tritiated by reductive alkylation and used to examine the relationship between the number of T<>T/cell and the number of antibody molecules bound per cell. The data show that about 45% of the T<>T dimers in cells receiving 100 J/m^2 of germicidal irradiation were being detected with the indirect immunofluorescence assay. (Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under contract No. DE-FG02-88ER60636.)

EXPLORING THE MOLECULAR BASIS FOR THE PULMONARY CARCINOGENICITY OF DIESEL EXHAUST

J.L. Mauderly, J.A. Bond, J.R. Harkema, R.F. Henderson, and R.K. Wolff

Lovelace Inhalation Toxicology Research Institute
Albuquerque, NM 87185

Diesel exhaust (DE) has been shown by several studies to be a pulmonary carcinogen in rats exposed to high concentrations for 24 months or more. The tumor response is dose-related; the incidence of lung tumors was shown at this Institute to be significantly increased in rats exposed 7 hrs/day, 5 days/wk for 30 mo to exhaust at 3.5 and 7.0 mg soot/m^3, but not in rats exposed to 0.35 mg/m^3(1). Two findings suggest that the carcinogenicity of DE is related to soot-associated, solvent-extractable, organic compounds (OC), which include known mutagens and carcinogens. First, increased levels of lung DNA adducts were found in rats exposed to the high concentration at this Institute(2). Second, the tumor response has been shown(3) to be associated with the particulate, rather than the gas/vapor, phase of DE.

There is also evidence suggesting that DE-induced carcinogenicity in rats might be a nonspecific response of the rat lung to chronic particle loading. At carcinogenic concentrations, DE causes chronic, progressive lung inflammation, retarded particle clearance with progressive accumulation of soot in the lung, and progressive epithelial hyperplasia and metaplasia(1,4,5). Similar changes, and increased incidences of lung tumors of types similar to those caused by DE, have occurred in rats exposed chronically to high concentrations of particles having no bioavailable OC, such as quartz(6), oil shale dust(6), titanium dioxide(7).

The mechanism of the carcinogenicity of DE in rats is of considerable importance if the data are to be used to estimate dose-response relationships for man. Epidemiological evidence suggests a relationship between occupational DE exposure and pulmonary carcinogenesis in man(8). The regulation of occupational DE exposure levels and the development of criteria documents on automotive emissions are under consideration. We need to know if the response of rats to DE can be modeled as chemical carcinogenesis, or if it is a nonspecific response to lung loading under exposure conditions which will not be encountered by man.

We conducted three studies of F344 rats exposed 7 hr/day, 5 days/wk for up to 12 wks to DE or carbon black (carbonaceous particles similar to DE

soot, but having no mutagenic OC) to better understand the potential role of OC metabolite-induced lung DNA adducts in DE-induced pulmonary carcinogenesis in the rat. Adduct levels were measured by the ^{32}P-postlabeling method using the nuclease P_1 enrichment procedure. The studies were designed to answer the following questions: 1) Are DE-induced DNA adduct levels within the respiratory tract increased most in the region in which tumors were observed? 2) Are DNA adduct levels increased in proportion to DE concentration? 3) What is the time course of DNA adduct formation and repair? 4) Does exposure to OC-free carbonaceous particles also increase DNA adduct levels?

In the first study, microdissection was used to collect tissue from sites ranging from the nose to peripheral lung in rats exposed to DE at 10 mg/m^3 or sham-exposed as controls. DE exposure increased adduct levels only in the nose and peripheral lung and the greatest increase occurred in peripheral lung. This demonstrated that the greatest induction of adducts occurs in the region where DE-induced tumors occur.

In the second study, rats were exposed to DE at 0, 0.35, 3.5, 7.0, or 11 mg/m^3. Adduct levels were measured after 2, 4, 8, or 12 wks of exposure, and at 2, 4, and 8 wks after exposure ended. Adduct levels were increased similarly by DE at all concentrations, and were similar to those observed previously after 30 mo of exposure. Adduct levels were increased at the end of exposure, but had returned to control levels by 4 wks after exposure. This demonstrated that adducts were increased by a DE concentration (0.35 mg/m^3) that was not carcinogenic, and suggested that a steady-state level of adducts would be reached during chronic exposure.

In the third study, rats were exposed to DE or carbon black at 3.5 or 10 mg/m^3 for 12 wks. Lung burdens of particles were measured, and the inflammatory responses were evaluated by bronchoalveolar lavage. DE soot and carbon black accumulated in the lung similarly and caused similar inflammatory responses. DE increased adduct levels, but carbon black caused little or no increase. This demonstrated that adduct levels are related to soot-associated OC, but the inflammatory response is not.

The results of these studies suggest that DNA adducts from soot-associated OC are probably an initiating step in the pulmonary carcinogenicity of DE in the rat, but that other factors, such as epithelial proliferation and metaplasia are probably also important contributors. A study of rats exposed chronically to DE and carbon black is now being conducted to

determine the importance of the OC in the tumor response. This study will also address the cell types with increased DNA adduct levels, relationships among exposure, DNA adducts, oncogene activation, and tumors, markers of exposure, and specific soot-associated OC responsible for DNA adduct formation. (Research supported by U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.)

REFERENCES

1. Mauderly, J.L., R.K. Jones, W.C. Griffith, R.F. Henderson, and R.O. McClellan, Fundam. Appl. Toxicol. 9:1-13, 1987.

2. Wong, D., C. Mitchell, R.K. Wolff, J.L. Mauderly and A.M. Jeffrey, Carcinogenesis 7:1595-1597, 1986.

3. Heinrich, U., H. Muhle, S. Takenaka, H. Ernst, R. Fuhst, U. Mohr, F. Pott, and W. Stober, J. Appl. Toxicol. 6:383-395.

4. Henderson, R.F., J.L. Mauderly, R.K. Jones, J.D. Sun, J.M. Benson, J.L. Mauderly, and R.O. McClellan, Fundam. Appl. Toxicol. 11: (in press), 1988.

5. Wolff, R.K., R.F. Henderson, M.B. Snipes, W.C. Griffith, J.L. Mauderly, R.G. Cuddihy, and R.O. McClellan, Fundam. Appl. Toxicol. 9:208-221, 1987.

6. Goldsmith, D., D. Winn, and C. Shy, Eds., Silica, Silicosis, and Cancer, Praeger: New York, 1985.

7. Lee, K.P., H.J. Trochimowicz, and C.F. Reinhardt, Toxicol. Appl. Pharmacol. 79:179-192, 1985.

8. Garshick, E., M.B. Schenker, A. Munoz, M. Segal, T.J. Smith, S. R. Woskie, S.K. Hammond, and F.E. Speizer, Am. Rev. Respir. Dis. 137:820-825, 1988.

DNA REPAIR ACTIVITY THROUGHOUT THE RAT RESPIRATORY TRACT

James A. Bond, Jodi M. Gubin, and Neil F. Johnson

Lovelace Inhalation Toxicology Research Institute
Albuquerque, NM 87185

Some of the products formed by reaction of mutagenic and carcinogenic alkylating agents with DNA are O^6-alkylguanine, 7-alkylguanine, and 3-alkyladenine. One of the products of methylation with dimethylnitrosamine is O^6-methylguanine. It has been hypothesized that the inability of a cell to remove O^6-alkylguanine (i.e., persistence of O^6-alkylguanine in organs) and repair the DNA damage correlates with tissue susceptibility to alkylating carcinogens. O^6-alkylguanine, which is produced to a large extent by N-nitroso carcinogens, can mispair with thymine.

The protein referred to as O^6-alkylguanine-DNA alkyltransferase (AGT) removes the alkyl group from O^6-alkylguanine and transfers it to a cysteine acceptor site on the protein. AGT binds to DNA containing O^6-alkylguanine and transfers the alkyl group on the oxygen atom at the O^6 position of guanine to the sulfur atom of the amino acid cysteine in the AGT protein.

The bulk of evidence indicates that AGT is the major mechanism for removal of alkylated guanines, specifically methylated and ethylated guanines in DNA. While there have been several reports on the presence of O^6-methylguanine in tissues and cells of animals exposed to alkylating carcinogens there are very little data available on the AGT activity in tissues and cells of the respiratory tract. It is particularly important to determine which tissues and cells of the respiratory tract have low AGT activity, since these cells may be target cells for airborne alkylating agents. The purpose of our studies was to characterize AGT activity <u>in vitro</u> in tissues and cells in the respiratory tract.

Male F344/N rats (9 to 12 wk of age) were used in all studies. The rats were born and raised in the Institute's barrier-maintained colony. All rats were killed by CO_2 asphyxiation. Immediately after death, the thoracic cavity of the rat was opened, and the trachea and lungs were removed intact. Concomitantly, the head was removed from the carcass and the nasal cavity was split from the nares to the nasopharynx, exposing the right and left lateral and septal mucosal surfaces. Anatomically defined regions throughout the respiratory tract were sampled. These included two regions

of the lateral wall of the left and right nasal airway (maxilloturbinates and ethmoturbinates), the trachea, mainstem bronchi, and peripheral lung (i.e., alveolar tissue). In addition, a sample of liver was removed. Respiratory tract tissue samples used in this study contained not only the luminal mucosa, but also the underlying submucosal tissue that contains varying amounts of vasculature, glands, connective tissue elements and, in some sites, cartilage.

Primary type II alveolar cells were obtained from male F344/N rats (9-12 weeks) sacrificed by CO_2 asphyxiation. Lungs were perfused with Dulbecco's phosphate buffered saline containing calcium and magnesium. After perfusion, the lungs were lavaged with the buffered saline to remove any macrophages. Following lavage, lungs were digested with hyalurondiase and cytochalasin B for 60 min at 37°C, followed by pronase digestion for 30 min at 37°C. The lungs were then minced, filtered, and centrifuged at 10°C (1200 rpm for 15 min). The cell pellet was resuspended and lung cells were separated on a Ficoll density gradient of 1 to 2% (CelSep, Sorvall Instruments, Newtown, CT) for 90 min. Fractions were removed from the CelSep chamber and the presence of type II alveolar cells in each fraction was determined by staining with a modified Paponiculous stain. The cell preparation consisted of type II alveolar cells (85%) and alveolar macrophages (15%) as determined by electron microscopy.

Tissue and cell extracts were obtained and AGT activity in the extracts was determined as previously described[1,2]. Hydrolyzed DNA was analyzed by high-performance liquid chromatography (HPLC) using two Whatman Partisil 10-SCX columns linked in series. The bases were eluted isocratically at room temperature with 75 mM ammonium formate (pH 2.5-3.0) containing 20% methanol. The flow rate was 1.5 ml/min. Absorbance was monitored at 254 nm. Fractions of eluate were collected at 30-sec intervals; the amount of radioactivity in each fraction was determined by liquid scintillation spectroscopy. The retention times for guanine, adenine, 7-methylguanine, and O^6-methylguanine were 8.8, 13.8, 14.8, and 19.1 min, respectively. AGT activity was calculated by subtraction of the O^6-methylguanine (O^6-MG)/7-methylguanine (7-MG) ratio in test reactions from the O^6-methylguanine/7-methylguanine ratio in control reactions as follows:

$$\frac{O^6\text{-MG/7-MG}_{(\text{control})} - O^6\text{-MG/7-MG}_{(\text{sample})}}{O^6\text{-MG/7-MG}_{(\text{control})}} \times 100 = \%\ O^6\text{-MG removed.}$$

Removal of O^6-methylguanine as a function of the amount of protein added to incubation flasks was investigated in respiratory tract tissues and cells. Incubation times used for the maxilloturbinates, ethmoturbinates, trachea, bronchi, lung, type II alveolar cells, and liver were 10, 10, 30, 30, 20, 30, and 30 min, respectively. These incubation times resulted in maximal AGT activity for the protein concentration used in reaction flasks. AGT activity increased proportionally to the amount of protein added to reaction flasks. AGT activity was detected at all protein concentrations except in the maxilloturbinates, the trachea and the bronchi. For the maxilloturbinates, no AGT activity was detected using 0.5 mg protein/ml, and AGT activity in tracheal and bronchial extracts was only detected at 1.5 mg protein/ml.

AGT activity in the respiratory tract was nonuniformly distributed. The highest activity was detected in the lung (~ 75 fmole/mg protein) and a region of the nasal tissue (i.e. ethmoturbinates; ~ 45 fmole/mg protein). The lowest activity was measured in tissues of the major conducting airways, the trachea and bronchi, and in alveolar type II cells and ranged from about 10-20 fmole/mg protein. AGT activity in the maxilloturbinates was about 50% less than the AGT activity measured in the ethmoturbinates. Liver AGT activity reported here (~ 60 fmole/mg protein) was similar to AGT activity in livers of F344 and Sprague Dawley male rats (70-90 fmole/mg protein) reported elsewhere.

Analysis of the data presented in this paper on <u>in vitro</u> AGT activities in various regions of the respiratory tract leads to the conclusion that AGT activity is distributed nonuniformly throughout the respiratory tract. The highest AGT activity was detected in the lung and the lowest activity was seen in the trachea and bronchi which contained about 20% of the activity measured in the lung. It is possible that the lower AGT activities in the trachea and bronchi may be due to the higher structural connective tissue component of these tissues compared to other regions of the respiratory tract sampled. The structural connective tissue component would not be expected to contain AGT activity, but would contribute to the total protein content removed from these tissues.

Our studies indicate that different regions of the respiratory tract can effectively repair methylated DNA with lung > ethmoturbinates > maxilloturbinates ≅ alveolar type II cells > trachea ≅ bronchi. Our experiments

provide additional information on AGT activity in various regions of the rat respiratory tract (e.g., trachea, bronchi, alveolar type II cells). Additional studies are necessary to characterize AGT activity in other cells of the rat respiratory tract (e.g., Clara cells, tracheal epithelial cells). While we do not yet have enough data to allow prediction of target tissue/cell specificity following exposure to alkylating agents, the data do suggest that methylated DNA in the respiratory tract should be readily repaired, albeit to differing extents. (Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. DE-AC04-76EV01013.)

REFERENCES

1. A. E. Pegg and L. Weist, Regulation of O^6-methylguanine-DNA methyltransferase levels in rat liver and kidney, Cancer Res., 43 (1983) 972-975.

2. M. E. Dolan, K. Morimoto and A. E. Pegg, Reduction of O^6-alkylguanine-DNA alkyltransferase activity in HeLa cells treated with O^6-alkylguanines, Cancer Res., 45 (1985) 6413-6417.

AROMATIC AMINE-DNA ADDUCTS: STUDIES ON REPAIR AND CONSEQUENCES OF MISREPAIR OF THIS TYPE OF DNA DAMAGE IN MAMMALIAN CELLS

R. T. Okinaka and G. F. Strniste

Los Alamos National Laboratory
Los Alamos, NM 87545

The industrial application of aromatic amines as chemical feedstocks in the production of dyes and as antioxidants in the rubber industry led to epidemiological studies implicating exposure to these chemicals with increased tumor incidence in man [1]. Many of these compounds have since been shown to be potent promutagens and hepatocarcinogens in a variety of mammalian systems. As promutagens these compounds by themselves are relatively inert but can be enzymatically converted to reactive, mutagenic electrophiles by cellular processes termed metabolic activation [2]. Our interest in aromatic amines began with the discovery that this class of compounds is responsible for the majority of the mutagenic activity in certain synthetic petroleums [3].

Our research efforts have evolved from earlier studies on the photodynamic behavior of various polycyclic aromatic hydrocarbons and energy-related complex organic mixtures on human and rodent cell cultures [4] to the more recent characterization of the pathways by which model aromatic amines (i.e., 2-aminofluorene and 1-aminopyrene) can be converted to mutagenic photoproducts following exposure to near ultraviolet light [5]. Comparison of these photoproducts to the mutagenic derivatives resulting from the bioconversion of aromatic amines suggested a striking similarity between photo-induced and metabolically-derived N-oxidized mutagens, (e.g., N-nitro, N-nitroso, and N-hydroxyl intermediates). Current studies are focused on the molecular basis for mutations induced by these types of intermediates in DNA excision repair-proficient and repair-deficient mammalian cell culture systems.

N-acetoxy-2-acetylaminofluorene (N-OAc-AAF) and N-hydroxyl-2-acetyl-aminofluorene (N-OH-2-AAF) behave as UV-mimetic chemicals in mammalian cells [6]. Studies over the past decade indicate that there are, however, distinct differences between the recognition and removal of UV-induced lesions compared to those induced by N-OAc-AAF [7]. Model-building studies suggest that this disparity may in part be a result of differences in the

preferred conformational state of the two C-8 quanosine aminofluorene adducts nested within the DNA molecule itself. The acetylated adduct [N-(2'-deoxyguanosin-8-yl)-2-acetylaminofluorene, dG-C8-AAF], promotes the syn conformation and results in a localized distortion of the DNA helix [8]. The deacetylated adduct [N-(2'-deoxyquanosin-8-yl)-2-aminofluorene, dG-C8-AF] prefers the anti conformation and does not induce large changes in the helix structure [9].

In N-OAc-AAF-treated Chinese hamster (CHO) cell cultures the predominant DNA-adduct that can be detected biochemically is dG-C8-AF [10]. Recent data also indicates that the only adduct found in N-hydroxyl-2-aminofluorene-treated CHO cultures is the dG-C8-AF adduct [11]. These results would imply that most of the genotoxic effects observed in CHO cells result from the unacetylated C-8 guanine adduct. It is paradoxical that the lesion most likely to induce DNA excision repair effects (dG-C8-AAF) is either not detectable or not an abundant species in either human or CHO cell cultures treated with N-OAc-AAF [10,11]. Despite these findings, Carothers et al. [10] have implicated a role for mutagenesis by the acetylated (dG-C8-AAF) adduct. Their findings indicate that the metabolic processing of this minor DNA component leads to the induction of gross deletion/rearrangements at the dihydrofolate reductase (DHFR) locus in CHO cells.

In this report we describe the isolation of independent hypoxanthine-quanine phosphoribosyl transferase (HPRT) deficient mutants from N-OAc-AAF- or N-OH-AAF-treated Chinese hamster cells and the analysis of their DNA by restriction enzyme digestion/Southern blotting and probing techniques. The intent of these experiments is to determine whether failure to excise critical N-OAc-AAF induced lesions will result in elevated levels of deletion/rearrangement events at the X-linked HPRT locus. The rationale for this approach comes form our previous studies [12] in which we showed that UV-5, a DNA excision repair deficient CHO cell line [13], is extremely sensitive to the mutagenic effects of N-hydroxyl-2-acetylaminofluorene (N-OH-AAF). A crucial question is whether the elevated levels of mutations observed in UV-5 cells are the result of gross gene alteration mechanisms as proposed by Carothers et al. [10].

Genomic DNAs from these mutants were digested with the restriction enzyme Pst 1 and the digests were analyzed by Southern blotting and hybridization to the nick-translated cDNA HPRT probe, pPHT12. This enzyme creates 7 resolvable bands at 8.8, 7.8, 6.0, 5.0, 3.2, 2.6 and 0.9 kb with

the normal V79 HPRT$^+$ cell [14]. The band at 5.0 kb is a psuedogene and is not associated with the functional HPRT locus [14].

The DNA banding pattern of all the UV-5 derived mutants (27/27) isolated, thus far, from N-OAc-AAF- or N-OH-AAF-treated cultures were identical to that of the parental cell line. Since this technique cannot resolve sequence changes of 100 bp or less, it can only be concluded that these HPRT$^-$ phenotypes are the result of either point mutations, small deletions or small insertions. The average induced mutation frequency from the experiments in which each of the UV-5 derived HPRT$^-$ mutants was isolated (19 individual experiments) was approximately 25-fold higher than the average background mutation frequency. These numbers indicate that it is unlikely that any given mutant is not a result of a N-OAc-AAF-induced event. There is an obvious lack of detectable deletion events in these mutants. Such events can be observed at approximately a 10% frequency in spontaneously- or UV-irradiation-derived mutants at the HPRT locus [14]. In direct contrast are the results for cells exposed to ionizing radiation in which total or partial deletions of HPRT sequences account for 50-60% of the mutants isolated [15].

Failure to repair N-OAc-AAF induced lesions in DNA by excision repair mechanisms apparently results in the induction of point mutations or small deletions or insertions. These observations therefore suggest that gross gene alterations observed at the DHFR locus in repair competent cells [10] is probably due to an error prone repair process rather than failure to recognize specific lesions. Some of the HPRT$^-$ mutants derived from repair competent AA8-4 cells treated with N-OAc-AAF contain detectable deletions and may suggest a role for misrepair in the induction of these events. However, the data obtained to date cannot statistically eliminate the possibility of spontaneously arising mutants accounting for the deletion events witnessed in these experiments.

Experiments in progress are attempting to determine the molecular nature of the point and/or small deletions induced in UV-5 cells. A modification of the polymerase chain reaction (PCR) technique [16] is being employed to enrich mutated regions of the HPRT locus [17]. These amplified segments will allow direct sequence determination. (Research supported by the U.S. Department of Energy under Contract No. W-7405-ENG-36.)

REFERENCES

1. Scott, T.S. (1962) Carcinogenic and chronic toxic hazards of aromatic amines, In: E. Browning (ed.), Elsevier Monographs on Toxic Agents, Elsevier Publishing Company, Amsterdam and New York, pp 1-208.

2. Miller, E.C. (1978) Cancer Res., 38: 1479-1496.

3. Guerin, M.R., et al. (1980) Environ. Res., 23:42-53; Wilson, B.W., et al. (1980) Mutat. Res., 79: 193-202.

4. Strniste, G.F. and Chen, D.J. (1981) Environ. Mutat., 3:221-231; Strniste, G.F., et al. (1982) J. Natl. Cancer Inst., 69:199-203; Strinste, G.F., et al. (1982) Toxicological Letters, 13:163-167; Okinaka, R.T., et al. (1984) Photochem. Photobiol., 39:353-358.

5. Okinaka, R.T., et al. (1984) In: M. Cooke and A.J. Dennis (eds.), Polynuclear Aromatic Hydrocarbons: Mechanisms, Methods and Metabolism, Battelle, Columbus, OH, pp. 961-971; Okinaka, R.T., et al., (1984) Carcinogenesis, 5:1741-1743; Strniste, G.F., et al. (1985) Mutat. Res., 151:15-24; Okinaka, R.T., et al. (1986) Mutat. Res., 173:93-98.

6. Maher, V., Heflich, R.H. and McCormick, J.J. (1980) Natl. Cancer Inst. Monograph No. 58: Carcinogenic and mutagenic N-substituted aryl compounds, pp. 217-221; Regan, J.D., and Setlow, R.B. (1974) Cancer Res., 34: 3318-3325.

7. Ahmed, F.E. and Setlow, R.B. (1977) PNAS, 74: 1548-1552; Amacher, D.E. et al. (1977) PNAS, 74:1553-1557.

8. Fuchs, R.P.P., and Duane, M.P. (1974) Biochemistry, 13:4435-4440; Grunberger, D., and Weinstein, I.B. (1976) In: Yuhas, J.M. Tennant, R.W., and Regan, J.D. (eds), Biology of Radiation Carcinogenesis, Raven Press, New York, pp. 175-187.

9. Broyde, S., and Hingerty, B. (1983) Biopolymers, 22:2423-2441.

10. Arce, G.T., et al. (1987) Carcinogenesis, 8:515-520; Carothers, A.M., et al. (1986) PNAS, 83:6519-6523.

11. Heflich, R.H., et al. (1986) Mutagenesis, 1:201-206.

12. Okinaka, R.T., et al. (1988) Mutat. Res. (in press).

13. Thompson, L.H., et al. (1981) PNAS, 78:3734-3737.

14. Fuscoe, J.C., et al. (1983) Mol. Cell. Biol. 3:1086-1096.

15. Fuscoe, J.C., et al. (1986) Int. J. Radiat. Biol. 49:1011-1020; Thacker, J. (1986) Mutat. Res. 160:267-275.

16. Saiki, R., et al. (1988) Science, 239:487-491.

17. Vreiling, et al. (1988) Mutat. Res., 198, 107-113.

CHARACTERIZATION OF MOUSE LIVER PROTEIN VARIANTS INDUCED BY PARENTAL EXPOSURE TO N-ETHYL-N-NITROSOUREA

Carol S. Giometti

Argonne National Laboratory
Argonne, IL 60439-4833

As part of a study to detect heritable mutations as alterations in protein expression by using two-dimensional electrophoresis (2DE)(1), we have identified ENU-induced variants of five different mouse liver proteins. These protein variants give us a unique opportunity to correlate ENU-induced DNA lesions with specific changes in protein structure, thus furthering our understanding of the mechanism of ENU-induced mutagenesis. Characterization of these proteins will also provide information regarding the sorts of protein changes that can be tolerated by an organism and thus become introduced into the gene pool for succeeding generations.

Our first step in the characterization of the ENU-induced protein variants was to breed homozygous stocks. Such stocks have been successfully established for all five variants (ENU 1, ENU 2, ENU 4, ENU 6, and ENU 8), indicating that none of these genetic traits is lethal in homozygous carriers. In each case, comparable amounts of the variant and normal proteins are expressed in livers of heterozygous individuals, while homozygous carriers of either normal or variant proteins express comparable amounts of the respective proteins. These findings indicate that there is no selective expression of the normal gene product relative to the variant.

The subcellular location of the five ENU-induced variant proteins and their normal counterparts have been determined (2). ENU 2 and ENU 8 proteins are found predominantly in the mitochondrial fraction, while ENU 1 is found in the microsomal fraction, ENU 4 is found in mitochondrial, microsomal, and soluble fractions, and ENU 6 is in the soluble fraction. The significance of three out of the five variants occurring in mitochondrial proteins remains to be determined. This apparent susceptibility of mitochondrial proteins to ENU-induced alterations could be the result of selectivity inherent to 2DE analysis or could indicate that particular genes are more prone than others to survivable mutations.

The ultimate goal of the characterization of the ENU-induced protein variants is to know the identity of each protein and the primary ENU-induced alteration that caused the appearance of the variant. Thus far, one of the

five variants has been identified. The normal protein counterpart of ENU 2 was found to co-migrate with mouse liver ornithine aminotransferase (OAT). In addition, the ENU 2 protein co-purified with mouse liver OAT and reacted with anti-OAT antiserum (3). The structural differences between the normal and variant OAT is currently under study.

The subcellular location, pI and approximate molecular weight of the other four variants provide some clues to their identity. In addition, ENU 6 is a variant of a liver protein that is normally expressed predominantly in C57BL/6 mice but is barely detectable in BALB/c mice. Our approach to the identification of the remaining protein variants is to obtain partial amino acid sequences by using protein extracted from 2DE gels (4) and using those partial sequences to search existing protein databases for sequence homologies. Thus far, a 21 amino acid sequence obtained for the variant protein ENU 8 has been found to be homologous with the NH-terminal sequence of several heat shock proteins. In addition to providing presumptive identifications for the ENU-induced variant proteins, comparative partial amino acid sequencing of the variant proteins and their normal protein counterparts may also reveal amino acid substitutions related to primary ENU-induced damage. (Research supported by the U.S. Department of Energy, Office of Health and Environmental Research under Contract No. W-31-109-ENG-38.)

REFERENCES

1. C.S. Giometti, M.A. Gemmell, S.L. Nance, S.L. Tollaksen, and J. Taylor, Detection of heritable mutations as quantitative changes in protein expression, J. Biol. Chem., 262:12764-12767, 1987.

2. C.S. Giometti, M.A. Gemmell, S.L. Tollaksen, and D. Grahn, Heritable protein variants induced by exposure to ethylnitrosourea; heritability, subcellular location, and tissue distribution, Mut. Res., 9-17, 1988.

3. C.S. Giometti, S.L. Tollaksen, M.A. Gemmell, J. Burcham, and C. Peraino, A heritable variant of mouse liver ornithine aminotransferase (e.c.2.6.1.13) induced by ethylnitrosourea, J. Biol. Chem., 15781-15784, 1988.

4. P. Matsudaira, Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes, J. Biol. Chem., 262:20035-20038, 1987.

UNWINDING OF SUPERCOILED DNA INDUCED BY MUTAGENIC POLYCYCLIC HYDROCARBON METABOLITES. A PROBE OF DNA DAMAGE

N. E. Geacintov, S. E. Carberry, C. E. Swenberg* and R. G. Harvey**

New York University
New York, NY 10003
*Armed Forces Radiobiology Research Institute
Bethesda, MD 20814
**Ben May Laboratory for Cancer Research
The University of Chicago
Chicago, IL 60637

Polycyclic aromatic compounds are metabolized in living systems to potent mutagens and tumorigens which bind both noncovalently and covalently to cellular DNA. The formation of the noncovalent complexes is important in determining the chemical reaction pathways of these highly reactive electrophiles with nucleic acids, while the covalent binding to one of the DNA bases is critical in the expression of their mutagenic and tumorigenic potentials.

It is well known that the noncovalent, intercalative binding of electrically charged drug molecules to supercoiled DNA induces an unwinding and a decrease in the superhelicity. This is a reversible effect since the DNA can rewind to its initial state if the intercalated drug molecules are removed. We have developed a new kinetic technique for monitoring the unwinding and rewinding of supercoiled DNA on time scales of seconds [1]. This method involves the orientation of the DNA in aqueous solution in a hydrodynamic flow gradient in a Couette cell. This cell consists of a stationary outer cylinder and a rotating inner cylinder. The aqueous solution is placed in the annular space between the cylinders, and the inner cylinder is rotated at speeds of up to 1000 rpm; the resulting hydrodynamic forces give rise to a partial orientation of the DNA. The degree of orientation of the DNA can be monitored by the linear dichroism technique; in this method, the absorbances of the DNA bases, and any polyclic aromatic mutagens or drug molecules bound to the DNA, are probed with linearly polarized light, with the directions of polarization oriented parallel or perpendicular with respect to the flow direction. As the supercoiled DNA unwinds, the physical dimensions of the DNA molecules are increased, and a larger linear dichroism signal results [1]. The conformation of the drug or polycyclic mutagen giving rise to the unwinding of the DNA can be followed simultaneously.

The interactions of the two enantiomers of trans-7,8-dihydroxy-anti-9,10-epoxy-benzo[a]pyrene (BPDE), the most mutagenic metabolites of benzo[a]pyrene, with supercoiled ΦX174 DNA (RF I) were studied. In mammalian cell systems, the enantiomer (+)BPDE is significantly more mutagenic than the (-) stereoisomer, and is also a strong tumorigen, while the (-) isomer is not. The differences in the modes of interactions of these two stereoisomers with nucleic acids, and the types of DNA damage produced, are therefore of great interest.

The diol epoxides BPDE are unstable in aqueous media, but rapidly form physical complexes with DNA (ms time scales), before any significant extent of chemical reaction can occur (time scales of minutes). The chemical reactions involve the hydrolysis of the diol epoxides to tetraols by reaction with water, and covalent binding to the DNA bases (mostly the exocyclic amino group of quanine). Hydrolysis is by far the dominant reaction pathway (80-95%, depending on the stereoisomer of BPDE). The kinetic linear dichroism technique is ideal for following these reactions and for monitoring their effects on the superhelicity of the DNA.

Immediately after the addition of the BPDE to the aqueous ΦX174 solutions, there is a sharp increase in the linear dichroism signal, indicating that the noncovalent binding of the BPDE molecules induces an unwinding of the DNA. Independent measurements have shown that the noncovalent BPDE-DNA complexes are intercalative in nature [2], and that the binding affinities are the same for (+)BPDE and for (-)BPDE [3]. In spite of the fact that both BPDE enantiomers from intercalation complexes with ΦX174 with the same affinities, the degree of unwinding induced by the (+) enantiomer is about 3 times higher than in the case of the (-) isomer. These results suggest that the modes of noncovalent intercalation of the two enantiomers into DNA are different from one another; these differences apparently lead to differences in the chemical adduct distributions [4], and their orientations at the DNA binding sites [5]. Both effects may be linked to the differences in the biological activities of these two stereoisomers [4,6].

As the chemical reactions of the noncovalently bound BPDE molecules take place, BPDE molecules are removed from their intercalation sites, and a rewinding of the DNA is observed with the same kinetics as those of the chemical reactions. The linear dichroism characteristics of the covalent adducts show that the (+)BPDE stereoisomer gives rise to covalent adducts

which are external to the DNA helix, while (-)BPDE gives rise to intercalative-type conformations [5]. The effects of these carcinogen-DNA lesions on the superhelicity were assayed by agarose gel electrophoretic techniques. Massive differences in the mobilities of the modified supercoiled DNA were observed in the case of the covalent (+)BPDE-ΦX174 DNA adducts. The fast supercoiled form becomes partially unwound at low binding ratios of $r < 0.01$ (BPDE residues/base), and a heterogeneos distribution of mobilities on the agarose gels are observed. The covalently closed, relaxed form, however, is characterized by faster mobilities. It is noteworthy that large unwinding effects are observed by the covalently bound (+)BPDE, even though the pyrene residues are not intercalated [5]. This observation suggests that DNA adduct lesions, other than intercalative ones, produce DNA damage which causes unwinding of supercoiled DNA. This is in contrast to the noncovalent drug-DNA complexes, where unwinding is caused only by intercalation complex formation.

In contrast to the (+) stereoisomer, the covalent binding of (-)BPDE to supercoiled DNA gives rise to only minor changes in the agarose gel migration patterns at similar values of the binding ratios r. Even though the pyrenyl residues are predominantly stacked with the DNA bases [5], as in intercalation complexes, there is less unwinding than in the case of the biologically highly active (+)BPDE stereoisomer. Thus, the covalent lesions derived from the covalent attachment of (-)BPDE, even though they are characterized by significant base stacking interactions, are not of the classical intercalation-type. These findings are interpreted in terms of the minimum energy forms of (+)BPDE-$(dGdC)_n$ and (-)BPDE-$(dGdC)_n$ dodecameric duplex structures calculated by minimized semi-empirical potential energy computer methods [7]. (Research sponsored by the U.S. Department of Energy, Office of Health and Environmental Research under Grants DE-FG02-88ER 60674 and DE-FG02-86ER 60405.)

REFERENCES

1. H. Yoshida, C.E. Swenberg, and N.E. Geacintov, Biochemistry (1987), 26, 1351.

2. C.J. Roche, N.E. Geacintov, V. Ibanez, and R.G. Harvey, (1988), submitted for publication.

3. M.C. MacLeod and K. Zachary, Chem. Biol. Interact, (1985) 45, 54.

4. P. Brookes and M.R. Osborne, Carcinogenesis, (1982) 3, 1223.

5. D. Zinger, N.E. Geacintov, and R.G. Harvey, Biophys. Chem., (1987), 27, 131.

6. R.G. Harvey and N.E. Geacintov, Acc. Chem. Res., (1988), 1, 66.

7. S. Broyde and B. Hingerty, to be published.

CHARACTERIZATION OF BaP ADDUCTS TO THE 5S rRNA GENE

D. L. Springer, D. B. Mann, AND G. L. Stiegler

Battelle-Pacific Northwest Laboratories
Richland, WA 99352

Although the initial event in the formation of tumors by chemical carcinogens is the formation of carcinogen-DNA adducts, the mechanism by which the cell then progresses into a tumor is not yet understood. Efforts to understand this progression must begin with an understanding of how individual genes are turned on and off. One of the indicators of the activity or inactivity of genes appears to be the structural formation of the gene's chromatin. Transcriptionally active genes have been shown to have a more open chromatin structure than those that are inactive. At a very basic level, this structure is controlled by the positioning of the nucleosomes and other control proteins within a gene. The induction of one such system was shown to be accompanied by the loss or alteration of a single nucleosome (Richard-Foy and Hager, 1987). It has been shown that for the ß-major globin gene, the nucleosomes are in a phased array on specific sites in L-cells in which the gene is inactive; however, in Friend cells where the gene is expressed, there is a 300 base pair (bp) nucleosome free region at the 5'-end of the gene (Benezra et al., 1986). Although this mechanism needs further evaluation, these results suggest that the positioning of certain nucleosomes are critical to gene expression.

The position of the nucleosomes in genes is determined to a large extent by the DNA sequence of the gene. It has been shown that certain sequences have preferred orientations with respect to the core histones, and other sequences, most notably long AT or GC tracts tend to be excluded from the nucleosome (Drew and Calladine, 1987). Moreover, benzo[a]pyrene (BaP) adduction of DNA has been shown to cause a distortion in the DNA helix favoring a transition from B to Z DNA at a point several bp from the adduct (Chen, 1985). The left-handed Z DNA is substantially stiffer than the right-handed B DNA and subsequently has a decreased ability to bind to the core histones. This implies that in addition to the direct effect of the adduct, it may also influence nucleosome positioning at some distance from the site of adduction.

Because of this we are looking at the influence of bulky chemical adducts on the positioning of nucleosomes within a gene. The system we are using is the pXP-14 plasmid which is 3250 bp in length and contains the 5S rRNA gene and the SP6 promoter. This model system is ideal for this type of study because the 5S rRNA gene, which is about 120 bp long, forms a single nucleosome that is located at a tightly fixed position along the DNA with the midpoint of the nucleosome located at the beginning (5' end) of the transcribed gene.

We have adducted the pXP-14 plasmid with (+) r-7,t-8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (anti-BPDE) at levels which result in an average of 6.7 adducts per plasmid. This corresponded to an adduction level of one BPDE per 4 copies of the 5S rRNA gene. The plasmid was then digested with restriction enzymes, dephosphorylated and 5'-end labeled with ^{32}P. Using other restriction enzymes the fragments were digested for a second time to remove the ^{32}P from one of the strands. This resulted in fragments containing either the 5S rRNA gene or the SP6 promoter with a ^{32}P on the 5'-end of either the transcribed or the non-transcribed strand. Each of these 5' end labeled fragments were digested with the T4 polymerase which contains a 3'-5' exonuclease activity in the absence of free nucleotides. This resulted in a population of fragments with a BPDE adduct on the 3' end and a ^{32}P label on the 5' end. These fragments were separated on a denaturing polyacrylamide gel. This approach provided a map of the adduct locations within each strand of the 5S rRNA gene and the SP6 promoter. Preliminary data indicated that there were preferred binding sites for the BPDE within the DNA, with certain deoxyguanosines being more heavily adducted than others. Using this approach in conjunction with footprinting techniques will allow us to determine the effects of chemical adducts on nucleosome position.

REFERENCES

1. Benezra, R., C.R. Cantor and R. Axel, 1986, Nucleosomes are phased along the mouse β-major globin gene in erythroid and nonerythroid cells, Cell, 44: 697-704.

2. Chen, F.M., 1985, Covalent binding of (+)- and (-)-trans-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene of B and Z DNAs, Biochemistry, 24:6219-6227.

3. Drew, H.R. and C.R. Calladine, 1987, Sequence-specific positioning of core histones on an 860 base-pair DNA, J. Mol. Biol., 195: 143-173.

4. Richard-Foy, H., and G.L. Hager, 1987, Sequence-specific positioning of nucleosomes over the steriod-inducible MMTV promoter, EMBO J, 6:2321-2328.

ATTENDEES OF DOE CONTRACTORS MEETING
ALBUQUERQUE, NM
on
November 17-18, 1988

Albertini, Richard J. Genetics Laboratory University of Vermont Burlington, VT 05401	FTS & Commercial (802) 863-5716
Bartley, Jack C. Cell & Molecular Biology Division Lawrence Berkeley Laboratory Berkeley, CA 94720	(415) 486-4191 FTS 451-4191
Beall, James R. Office of Health & Environmental Research Office of Energy Research U.S. Department of Energy Washington, D.C. 20545	(301) 353-4507 FTS 233-4507
Bean, Roger M. Biology & Chemistry Department Battelle-Pacific Northwest Laboratories P. O. Box 999 Richland, WA 99352	(509) 376-8834 FTS 444-8834
Bhattacharyya, Maryka H. Biological and Medical Research Division Argonne National Laboratory 9700 S. Cass Ave. Argonne, IL 60439	FTS & Commercial (312) 972-3923
Bice, David E. Lovelace Inhalation Toxicology Research Institute P. O. Box 5890 Albuquerque, NM 87185	FTS & Commercial (505) 844-1406
Bond, James A. Lovelace Inhalation Toxicology Research Institute P. O. Box 5890 Albuquerque, NM 87185	FTS & Commercial (505) 844-7989
Brooks, Antone L. Lovelace Inhalation Toxicology Research Institute P. O. Box 5890 Albuquerque, NM 87185	FTS & Commercial (505) 844-8681

Broyde, Suse B.
Department of Biology
New York University
Washington Square
New York, NY 10003
(212) 998-8231

Buchanan, Michele V.
Analytical Chemistry Division
Oak Ridge National Laboratory
P. O. Box 2008, MS 6120
Oak Ridge, TN 37830
(615) 574-4868
FTS 624-4868

Dahl, Alan R.
Lovelace Inhalation Toxicology
Research Institute
P. O. Box 5890
Albuquerque, NM 87185
FTS & Commercial
(505) 844-7990

Geacintov, Nickolas E.
Department of Chemistry
New York University
Washington Square
New York, NY 10003
(212) 998-8407

Giometti, Carol S.
Biological and Medical Research Division
Argonne National Laboratory
9700 S. Cass Ave.
Argonne, IL 60439
FTS & Commercial
(312) 972-3839

Haley, Patrick J.
Lovelace Inhalation Toxicology
Research Institute
P. O. Box 5890
Albuquerque, NM 87185
FTS & Commercial
(505) 844-6041

Hankinson, Oliver
Laboratory of Biomedical
and Environmental Science
University of California
900 Veteran Ave.
Los Angeles, CA 90024
(213) 825-2936

Harkema, Jack R.
Lovelace Inhalation Toxicology
Research Institute
P. O. Box 5890
Albuquerque, NM 87185
FTS & Commercial
(505) 844-0943

Haugen, David A.
Biological and Medical Research Division
Argonne National Laboratory
9700 S. Cass Ave.
Argonne, IL 60439
FTS & Commercial
(312) 972-3916

Hays, Esther F.
Laboratory of Biomedical
and Environmental Science
University of California
900 Veteran Ave.
Los Angeles, CA 90024
(213) 206-1921

Henderson, Rogene F.
Lovelace Inhalation Toxicology
Research Institute
P. O. Box 5890
Albuquerque, NM 87185
FTS & Commercial
(505) 844-8676

Hobbs, Charles H.
Lovelace Inhalation Toxicology
Research Institute
P. O. Box 5890
Albuquerque, NM 87185
FTS & Commercial
(505) 844-2435

Huberman, Eliezer
Director, Biological
and Medical Research Division
Argonne National Laboratory
9700 S. Cass Avenue
Argonne, IL 60439
FTS & Commercial
(312) 972-3819

Lesko, Steven
Division of Biophysics
The Johns Hopkins University
School of Hygiene and Public Health
615 N. Wolfe Street
Baltimore, MD 21205
(301) 955-3170

Mahlum, D. Dennis
Biology and Chemistry Department
Battelle-Pacific Northwest Laboratories
P. O. Box 999
Richland, WA 99352
(509) 376-3140
FTS 444-3140

Mauderly, Joe L.
Lovelace Inhalation Toxicology
Research Institute
P. O. Box 5890
Albuquerque, NM 87185
FTS & Commercial
(505) 844-1169

Mitchell, Charles E.
Lovelace Inhalation Toxicology
Research Institute
P. O. Box 5890
Albuquerque, NM 87185
FTS & Commercial
(505) 844-9729

Okinaka, Richard T.
Life Sciences Division
Los Alamos National Laboratory
Los Alamos, NM 87545
(505) 667-2743
FTS 843-2743

Oller, Adriana
Center for Environmental Health Sciences
Massachusetts Institute of Technology
E18-666
Cambridge, Massachusetts 02139
(617) 253-6220

Pelroy, Richard A.
Biology and Chemistry Department
Battelle-Pacific Northwest Laboratories
P. O. Box 999
Richland, WA 99352
(509) 375-2107
FTS 444-2107

Reilly, Jr., Christopher A.
Section Head, Toxicology
Biological and Medical Research Division
Argonne National Laboratory
9700 S. Cass Ave.
Argonne, IL 60439
FTS & Commercial
(312) 972-3879

Springer, David L.
Biology and Chemistry Department
Battelle-Pacific Northwest Laboratories
P. O. Box 999
Richland, WA 99352
(509) 376-1515
FTS 444-1515

Strniste, Gary
Life Sciences Division
Los Alamos National Laboratory
Los Alamos, NM 87545
(505) 667-2746
FTS 843-2746

Thilly, William G.
Center for Environmental Health Sciences
Massachusetts Institute of Technology
E18-666
Cambridge, Massachusetts 02139
(617) 253-6221
FAX 617-258-5424

Tischler, Allan
Cell & Molecular Biology Division
Lawrence Berkeley Laboratory
Berkeley, CA 94720
(415) 986-4193
FTS 451-4193

Yeh, Hsu-Chi
Lovelace Inhalation Toxicology
Research Institute
P. O. Box 5890
Albuquerque, NM 87185
FTS & Commercial
(505) 844-2151