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DNA Adduct Formation and T Lymphocyte Mutation Induction in F344 Rats Implanted with Tumorigenic Doses of 1,6-Dinitropyrene

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI supports research on all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate materials) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 120 projects at institutions in North America and Europe. Consistent with its mission to serve as an independent source of information on the health effects of motor vehicle pollutants, the Institute also engages in special review and evaluation activities.

Typically, HEI receives half its funds from the U.S. Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the United States. Occasionally, revenues from other public or private organizations either support special projects or provide resources for a portion of an HEI study. However, in all cases HEI exercises complete autonomy in setting its research priorities and in disbursing its funds. An independent Board of Directors governs the Institute. The Research Committee and the Review Committee serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded research and evaluations have been used in public and private decision-making.

Statement

Synopsis of Report Number 72

DNA Mutations in Rats Treated with a Carcinogen Present in Diesel Exhaust

BACKGROUND

Diesel engine exhaust contains carbon particles and many chemicals that cause cancer in laboratory animals and mutations in cells in culture. Inhaling high doses of diesel exhaust for most of their lives produces lung cancer in rats. Moreover, some epidemiologic studies suggest that workers exposed to diesel exhaust have an increased risk of lung cancer. Although recent evidence indicates that the carbon particles themselves are largely responsible for the induction of lung cancer in rats, the chemicals adsorbed onto the carbon particles may still have an important role in the possible induction of lung cancer in humans by diesel exhaust.

When the mutagenic chemicals present in diesel exhaust are inhaled, the body metabolizes them into activated substances that damage DNA by reacting with it to form adducts. If this damage is not repaired by the cell before the DNA is replicated during cell division, mutations may be introduced that persist through future generations of cells. If it were possible to measure the numbers, kinds, and locations (or patterns) of mutations induced by these chemicals, scientists may then be able to develop a biomarker assay to assess environmental exposure to the mutagens in diesel exhaust.

Dinitropyrenes are one class of chemicals in diesel engine exhaust that are good candidates to use as biomarkers because they are highly potent mutagens and carcinogens in rats. HEI supported the study described here to develop an assay to measure mutations induced by dinitropyrenes in rats. Analyzing the patterns of mutations in rat spleen T lymphocytes might indicate eventual tumor development in other organs, and could be compared with other measurements of DNA damage, such as levels of DNA adducts formed in other tissues. If successful, the rat assay would be an important first step in developing an applicable biomarker assay for humans.

APPROACH

Dr. Beland and his associates analyzed the mutations in a selected gene in spleen T lymphocytes from rats treated with 1,6-dinitropyrene under conditions that induced lung tumors at the highest dose tested. They also examined DNA adduct levels in lung and liver tissues and in spleen lymphocytes and white blood cells.

RESULTS AND IMPLICATIONS

The investigators successfully developed an assay to measure mutations in spleen T lymphocytes from rats treated with 1,6-dinitropyrene. They also demonstrated, for the first time in any species, that 1,6-dinitropyrene is mutagenic in rats. The number of mutations in lymphocytes was related to the number of adducts in lung DNA and the incidence of lung tumors. Future studies need to determine whether the assay is sufficiently sensitive to respond to concentrations of 1,6-dinitropyrene found in the air, and to identify the kinds and locations or pattern of mutations. This pattern may be specific for each chemical and, thus, could lead to a useful biomarker of human exposure to diesel engine exhaust. This rat assay establishes the basis for a similar assay in humans if it can be adapted to tissues that are more readily obtainable from humans, such as lymphocytes from blood.

This Statement, prepared by the Health Effects Institute and approved by the Board of Directors, is a summary of a research project sponsored by HEI from 1990 to 1993. This study was conducted by Dr. Frederick A. Beland of the Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR. The following Research Report contains both the detailed Investigator's Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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DNA Adduct Formation and T Lymphocyte Mutation Induction in F344 Rats Implanted with Tumorigenic Doses of 1,6-Dinitropyrene

Frederick A. Beland

ABSTRACT

Diesel emissions are known to induce tumors in laboratory animals and are suspected of being carcinogenic in humans. Of the compounds associated with diesel exhaust, 1,6-dinitropyrene is a particularly potent mutagen and carcinogen; thus, monitoring the toxic effects of 1,6-dinitropyrene may provide a means for assessing the carcinogenic risk associated with exposure to diesel emissions. In these experiments, 1,6-dinitropyrene was implanted into the lungs of rats according to a protocol known to induce lung tumors; the DNA adducts were characterized and quantified in target (lung) and surrogate (liver, white blood cells, and spleen lymphocytes) tissues. In addition, mutation induction was assayed at the hypoxanthine-guanine phosphoribosyltransferase (*hprt*)* locus in spleen T lymphocytes, and the relation between adduct concentration and mutation induction was elucidated .

Rats were administered 30 μg or 100 μg of [ring-^{3}H]1,6-dinitropyrene and adduct levels were quantified for up to one month after treatment. In lung tissue, white blood cells, liver tissue, and spleen lymphocytes, one major DNA adduct, *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene, was detected. In each tissue, the levels of this adduct reached a maximal level one to seven days after treatment and decreased to approximately 25% of the peak values by 28 days after treatment. DNA adduct formation in the lung was approximately tenfold higher than that observed in the other tissues. A dose-response relation was not observed in the lung, white blood cells, or liver; but in the spleen lymphocytes, a threefold increase in dose resulted in approximately a twofold increase in adduct formation.

After a similar treatment with 1,6-dinitropyrene, mutations were assayed at the *hprt* locus in spleen T lymphocytes for up to 51 weeks. Compared with control animals treated with solvent, 1,6-dinitropyrene induced a significant increase in mutant frequency with the 100-μg dose, typically producing twofold more mutants than the 30-μg dose. With both doses, the mutant frequency increased until 21 weeks after treatment with 1,6-dinitropyrene, remained constant until week 40, and then began to decrease. Nonetheless, nearly one year after treatment, the mutant frequency in rats treated with 1,6-dinitropyrene was greater than that observed in control rats.

In a subsequent experiment, rats were administered 0, 0.3, 1, 3, 10, 30, 100, or 150 μg of 1,6-dinitropyrene and the extent of adduct formation was determined seven days after treatment. In the lung nuclei, liver nuclei, and spleen lymphocyte DNA, a significant dose-response relation was observed, with the extent of adduct formation increasing significantly at a dose of 10 μg. A twofold increase in dose resulted in a twofold increase in adduct formation up to the 30-μg dose in lung nuclei DNA, and up to the 10-μg dose in liver nuclei DNA. At higher doses, the extent of adduct formation still increased but the rate of increase was much lower than that occurring at lower doses.

To assess the mutation frequency as a function of dose, additional rats were treated with 0, 0.3, 1, 3, 10, 30, 100, or 150 μg of 1,6-dinitropyrene and mutations were assayed at the *hprt* locus in spleen T lymphocytes 21 weeks after treatment. In this experiment, a significant dose-response relation was observed, with the increase in mutants becoming significant at 1 μg and higher doses of 1,6-dinitropyrene.

These data indicate that 1,6-dinitropyrene, a constituent of diesel emissions, is metabolically activated by nitroreduction to produce DNA adducts in target and surrogate tissues. They further suggest that T lymphocyte mutations may be a more sensitive and longer-lived biomarker than DNA adducts for assessing previous exposures to genotoxic agents, such as nitro-polynuclear aromatic hydrocarbons.

* A list of abbreviations appears at the end of the Investigator's Report.

This Investigator's Report is one part of Health Effects Institute Research Report Number 72, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigator's Report may be addressed to Dr. Frederick A. Beland, Division of Biochemical Toxicology, National Center for Toxicological Research, HFT-100, Jefferson, AR 72079.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Agreement 816285 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement should be inferred. The contents of this document also have not been reviewed by private party institutions including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

INTRODUCTION

Epidemiologic data have suggested an association between exposure to diesel exhaust and the induction of lung and bladder cancer in humans (International Agency for Research on Cancer 1989; Mauderly 1992; Silverman et al. 1992). For example, in a large retrospective cohort study involving more than 50,000 railroad workers, Garshick and associates (1988) found a slight, but statistically significant, increase in lung tumors that appeared to be related to diesel exposure. Likewise, Boffetta and associates (1988) reported significant increases in lung cancer for miners and heavy equipment operators who reported exposure to diesel emissions, and Hayes and associates (1989) found an elevated incidence of bladder cancer in truck drivers and railroad workers with more than twenty years of exposure to diesel exhaust.

In addition to being a suspected human carcinogen, diesel exhaust induces lung tumors in some animal models, an effect attributed to the particulate phase (International Agency for Research on Cancer 1989; Mauderly 1992), which contains a variety of compounds, including polynuclear aromatic hydrocarbons (PAHs). Certain of these PAHs, as exemplified by benzo[*a*]pyrene, are clearly carcinogenic in laboratory animals and may contribute to the increased incidence of lung and bladder cancer observed in individuals exposed to diesel exhaust. Another class of chemicals detected in diesel particulates is the nitro-PAHs (International Agency for Research on Cancer 1989). These compounds, which result from the reaction of nitrogen oxides with PAHs during the combustion process, are typically found at much lower concentrations than PAHs. Pyrene, for example, occurs at approximately 5000 μg/g of particulate extract; in comparison, its mononitration and dinitration products, 1-nitropyrene and 1,6-dinitropyrene, are detected at about 75 and 0.40 μg/g of particulate extract, respectively. Although nitro-PAHs are found at much lower concentrations than PAHs, certain members of this class are powerful mutagens and carcinogens. 1,6-Dinitropyrene, for instance, is one of the most mutagenic compounds ever tested in the Ames *Salmonella* reversion assay and induces tumors at a number of sites in a variety of laboratory animals (Takayama et al. 1985; Maeda et al. 1986; Wislocki et al. 1986; Ishizaka et al. 1987; Iwagawa et al. 1989; Imaida et al. 1991). Because nitro-PAHs are interrelated through metabolism to aromatic amines (Beland and Kadlubar 1990; Beland and Marques 1994), a class of carcinogens known to cause bladder cancer in humans (Parkes and Evans 1984; Silverman et al. 1992), they may contribute to the increased incidence of both lung and bladder tumors in individuals exposed to diesel exhaust.

Recently there has been considerable effort to develop biomarkers that detect exposures to carcinogens, such as those in diesel emissions (Perera 1990). Two markers that have received considerable attention are DNA adducts (Phillips 1990; Poirier and Weston 1991) and protein adducts (Skipper and Tannenbaum 1990); a number of reports have documented correlations between carcinogen exposure and adduct concentrations (Bartsch et al. 1988; Garner et al. 1991; Poirier and Beland 1992; Beland and Poirier 1993). Although valuable, DNA and protein adduct measurements suffer from the limitation that, as a result of DNA repair and protein and cell turnover, only relatively recent exposures can be assessed. A technique having the potential to indicate past carcinogen exposures could be particularly important for assessing exposure to diesel emissions. One such approach is to measure the biological effects resulting from DNA adducts, in particular the induction of T lymphocyte mutations (Albertini and Robison 1991). An advantage of this technique is that if mutations are induced in a stem cell population, they may be detected long after the initial carcinogen insult. In addition, molecular analysis of the mutants may reveal patterns of DNA sequence alterations indicative of the specific carcinogen inducing the mutation, which could be particularly useful in assessing the extent of diesel exposures, as compared with other environmental pollutants.

In research previously sponsored by the Health Effects Institute (Beland 1991), my colleagues and I focused on 1-nitropyrene as a prototype for nitro-PAHs found in diesel emissions. Our results indicated that 1-nitropyrene was not metabolized very efficiently to DNA-binding derivatives and suggested that dinitropyrene-DNA adducts might be a more useful biomarker for diesel exhaust exposure. Based upon this premise, our current project addressed three questions: First, what is the relation between the administered dose of 1,6-dinitropyrene and the DNA adduct concentration in a target tissue (one in which 1,6-dinitropyrene is known to induce tumors)? Second, what is the relation between the administered dose of 1,6-dinitropyrene and the DNA adduct concentration in circulating white blood cells and spleen lymphocytes? Third, what is the relation between the administered dose of 1,6-dinitropyrene and the number and types of mutations induced in spleen T lymphocytes? The animal model used to address these questions was derived from Maeda and associates (1986), who demonstrated that the direct administration of a single dose of 1,6-dinitropyrene to the lungs of male F344 rats resulted in a high incidence of squamous cell carcinoma. More recently, this same research group conducted a dose-response relation study (Iwagawa et al. 1989) and observed a relatively linear relation between the

administered dose, especially at low doses of 1,6-dinitropyrene, and lung tumor incidence. Thus, by using this animal model it should be possible to relate adduct and mutation levels to tumor incidence.

SPECIFIC AIMS

1. To determine the relation between the administered dose of 1,6-dinitropyrene and the DNA adduct concentration in a target tissue.
2. To determine the relation between the administered dose of 1,6-dinitropyrene and the DNA adduct concentration in peripheral white blood cells and in spleen lymphocytes.
3. To determine the relation between the administered dose of 1,6-dinitropyrene and the number and types of mutations induced in spleen T lymphocytes.

MATERIALS AND METHODS

ANIMALS

These experiments were conducted in 12-week-old, male F344 rats obtained from the breeding colony at the National Center for Toxicological Research, Jefferson, AR. Before treatment, the animals were housed two or three per cage in polycarbonate cages with hardwood-chip bedding; after treatment, they were housed one per cage. The animal room received 10 to 15 air changes per hour, and had an average temperature of 22.6°C, a mean relative humidity of 51.1%, and a 12-hour light-dark cycle. The rats received NIH-31 feed pellets and Millipore-filtered water ad libitum.

CHEMICALS

[4,5,9,10-^{3}H]1,6-Dinitropyrene (21.5 Ci/mmol; lot number MRI-83-201-10-11) was obtained from Chemsyn Science Laboratories (Lenexa, KS), and was diluted with 1,6-dinitropyrene (99% pure; lot number MRI-83-201-11-6, Chemsyn) to a specific activity of 98 to 1257 mCi/mmol, as determined by ultraviolet (UV) spectrophotometry, using an extinction coefficient of 17.2 mM^{-1} at 415 nm, and liquid scintillation counting. The radiolabeled dinitropyrene was dissolved in a 1:1 (v:v) mixture of beeswax and tricaprylin, both of which were obtained from Sigma Chemical Company (St. Louis, MO), at a concentration of 0 to 150 µg/50 µL.

1-Acetylamino-6-nitropyrene and 1-amino-6-nitropyrene were synthesized as described in Fifer and associates (1986).

[7,8-^{3}H]Benzo[*a*]pyrene (5.87 Ci/mmol; lot number CLS-87-157-33-26, Chemsyn) was purified on silica gel by elution with benzene and diluted with benzo[*a*]pyrene (98%, lot number 03705LX, Aldrich Chemical Co., Milwaukee, WI) to a specific activity of 187 mCi/mmol, as determined by UV spectrophotometry, using an extinction coefficient of 22.3 mM^{-1} at 385 nm, and liquid scintillation counting. The radiolabeled benzo[*a*]pyrene was dissolved in beeswax and tricaprylin at a concentration of 300 µg/50 µL.

SPECIFIC AIM 1

Binding of 1,6-Dinitropyrene to Lung DNA as a Function of Time (Experiment 1)

Male F344 rats were anesthetized with 100 µL of an 87:13 (w:w) mixture of ketamine and xylazine (Van Pelt 1977) and subjected to a left lateral thoracotomy. [4,5,9,10-^{3}H]1,6-Dinitropyrene (0, 30, or 100 µg; 98 to 1059 mCi/mmol) in 50 µL of beeswax and tricaprylin was then administered using the lung implantation method of Stanton and associates (1972), as described by Iwagawa and coworkers (1989). In order to keep the beeswax and tricaprylin from solidifying, the mixture was kept at 76°C in a bath of boiling ethanol. The solution was injected into the lung using a 100-µL Hamilton syringe that was fitted with a custom-made 6-mm, 20-gauge needle. The animals typically recovered from the anesthesia within three hours and were placed on 250 mg chlortetracycline per liter of drinking water for ten days after surgery.

At intervals of 1, 3, 7, 14, and 28 days after treatment, three to five animals were exposed to carbon dioxide and decapitated; blood was collected in heparinized tubes, and the lungs, livers, and spleens were quickly excised. Lung nuclei were prepared as described in Poirier and associates (1990) and DNA was extracted from the nuclei by slight modifications of the method reported in Beland and associates (1984). The DNA was quantified by UV spectrophotometry, using an extinction coefficient of 6.6 mM^{-1} at 260 nm, and the extent of adduct formation was determined by liquid scintillation counting.

DNA Adduct Analyses

To analyze DNA adducts by high-pressure liquid chromatography (HPLC), the DNA samples were adjusted to approximately 1 mg/mL in 5 mM Bis-Tris-HCl buffer, 0.1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.1), and then treated with 10 µL/mL of 1 M magnesium chloride and 100 µg/mL of DNase I (Sigma). After incubating for three hours at 37°C, 40 mU/mL of snake venom phosphodiesterase (Sigma) and 1 U/mL of bacterial alkaline phos-

phatase (Sigma) were added and the incubations were continued overnight. Further hydrolyses were conducted in which 70 μg mL of nuclease P_1 (Sigma) either replaced or was added in addition to the snake venom phosphodiesterase. The samples were extracted two times with an equal volume of water-saturated *n*-butanol, and the *n*-butanol was washed one time with an equal volume of *n*-butanol–saturated water. After the *n*-butanol was evaporated under reduced pressure, the residue was dissolved in 100 μL of 50% methanol for HPLC analysis.

High-pressure liquid chromatography was performed with a Waters Associates (Milford, MA) system consisting of two 510 pumps, a 680 automated gradient controller, a U6K injector, and a Hewlett-Packard 1040A diode array spectrophotometric detector. The adducts were separated with a 10-μm, 3.9-mm × 300-mm μBondapak C_{18} column that was eluted with a 30-minute nonlinear gradient (Waters #2) of 20% to 56% methanol at a flow rate of 2 mL/minute. The absorbance was monitored at 280 nm and one-minute fractions were collected for radioactivity measurements by liquid scintillation counting. The adduct marker *N*-(deoxyguanosin-8-yl)-2-aminofluorene (Beland et al. 1980), which was added as an internal standard, eluted at 24.5 minutes.

In addition to enzymatic hydrolyses, DNA also was hydrolyzed by the trifluoroacetic acid method of Tang and Lieberman (1983). Specifically, 1.5 mg of DNA was dried under vacuum and then treated with 1 mL of anhydrous trifluoroacetic acid (Aldrich Chemical Co., Milwaukee, WI) for 30 minutes at 70°C. The sample was evaporated under a stream of nitrogen and further dried under a vacuum. The black residue was suspended in 1 mL of water and extracted three times with 1 mL of water-saturated *n*-butanol. After evaporation of the *n*-butanol under reduced pressure, the residue was dissolved in 100 μL of methanol for HPLC analysis, using the conditions described above.

^{32}P-Postlabeling of DNA adducts was conducted with 10 μg of DNA, using slight modifications of the *n*-butanol enhancement procedure of Gupta (1985). DNA adducts then were separated by the contact-transfer method of Lu and associates (1986), with the solvent conditions described in Smith and associates (1990). Specifically, a 70% fraction of the kinase reaction was applied 1.5 cm from the bottom of a 10-cm × 20-cm Machery-Nagel polyethyleneimine-cellulose plate, containing a 20-cm Whatman 1 wick, and eluted with 900 mM sodium phosphate, pH 6.8 (D1). The origins were excised, attached to individual 10-cm × 10-cm Merck polyethyleneimine-cellulose plates, and eluted with 3.6 M lithium formate–8.5 M urea, pH 3.5 (D2). The plates then were turned 90° and chromatography was continued with 1.2 M lithium chloride, 500 mM Tris-HCl buffer–8 M urea, pH 8.0 (D3), followed by a final elution (D4) in the direction of D3, with 900 mM sodium phosphate, pH 6.8, onto a 3-cm Whatman 1 wick.

Adducts were located by autoradiography as described by Gupta and associates (1982). Adducts were characterized through comparison with a DNA adduct standard prepared by reacting *N*-hydroxy-1-amino-6-nitropyrene with DNA as reported in Djurić and associates (1988) to produce DNA modified with *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene at a level of 15 pmol/μg DNA. Initially, DNA adducts were quantified by liquid scintillation counting using this standard. Subsequently, the levels of adducts were quantified using DNA isolated from the lungs and livers of rats treated with [4,5,9,10-^{3}H]1,6-dinitropyrene, which produced levels of adducts between 0.2 and 2.5 fmol/μg DNA. These DNA standards were included in every ^{32}P-postlabeling assay.

DNA adducts from rats treated with benzo[*a*]pyrene were assayed by ^{32}P-postlabeling using the nuclease P_1 enhancement procedure of Reddy and Randerath (1986), as described in Culp and Beland (1994). A standard prepared by reacting *anti*-benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide with DNA (Culp and Beland 1994) to produce an adduct level of 2.2 fmol/μg DNA was included in every assay.

Binding of 1,6-Dinitropyrene to Lung DNA as a Function of Dose (Experiment 2)

Male F344 rats were treated as described in Specific Aim 1, Experiment 1, with 0, 0.3, 1, 3, 10, 30, 100, or 150 μg [4,5,9,10-^{3}H]1,6-dinitropyrene (1257 mCi/mmol). Seven days after treatment, three to five animals from each treatment group were killed, tissues were isolated, and lung nuclei and DNA were prepared as described for Specific Aim 1, Experiment 1.

SPECIFIC AIM 2

Blood Levels of 1,6-Dinitropyrene, and Binding of 1,6-Dinitropyrene to White Blood Cell, Spleen Lymphocyte, and Liver Nuclei DNA as a Function of Time (Experiment 1)

The cells and tissues for this experiment were obtained from animals treated in Specific Aim 1, Experiment 1. White blood cells were prepared from heparinized whole blood by the procedure of Ciulla and associates (1988), and lymphocytes were isolated from the spleen by the technique of Aidoo and associates (1989). DNA was extracted from these cells, quantified, and the extent of adduct formation was determined as indicated in Specific Aim 1, Experiment 1. The amount of white blood cell DNA was

insufficient to measure adduct formation by direct analysis of the ^{3}H content; the level of adduct formation in these cells was determined by ^{32}P-postlabeling analyses.

DNA adduct formation also was quantified in another nontarget tissue, the liver. Nuclei were prepared from liver by the method of Poirier and associates (1990), using liver obtained in Specific Aim 1, Experiment 1. DNA was extracted from the nuclei, quantified, and the extent of adduct formation was determined as indicated in Specific Aim 1, Experiment 1.

The blood levels of [4,5,9,10-^{3}H]1,6-dinitropyrene, or its metabolites, or both, were assayed in lysed red blood cells after separation from the white blood cells by centrifugation. The hemoglobin content was quantified using Drabkin's reagent (Sigma), following the directions provided by the supplier, and the extent of radioactivity was measured by liquid scintillation counting after treatment with perchloric acid and hydrogen peroxide as described by Mahin and Lofberg (1966). Specifically, 500 µL of lysed blood cells was treated with 50 µL of 70% perchloric acid and 100 µL of 30% hydrogen peroxide at 70°C for one hour. After adding the scintillation cocktail, the samples were stored at 4°C overnight before counting.

Organic-soluble metabolites of [4,5,9,10-^{3}H] 1,6-dinitropyrene in the blood were assayed by extracting 5 mL of lysed red blood cells with a 4-mL mixture of chloroform and methanol (1:1), followed by two additional extractions with 2 mL of chloroform. The combined organic phases were washed one time with water, the solvent was evaporated under reduced pressure, and the residue was dissolved in methanol for analysis by HPLC. Similar extractions were conducted with ethyl acetate instead of chloroform and methanol. Metabolites were separated with a 10-µm, 3.9-mm × 300-mm µBondapak C_{18} column that was eluted with a 25-minute linear gradient of 25% to 80% acetonitrile in 100 mM ammonium acetate, pH 6.7, at a flow rate of 2 mL/minute. One-minute fractions were collected for analysis of radioactivity, and metabolites were identified by coelution with synthetic standards.

Hemoglobin adducts were assayed by adding 250 µL of 10 N sodium hydroxide to 5 mL of lysed red blood cells and heating the mixture at 75°C for 3 hours. The mixture then was evaporated *in vacuo*, the residue was suspended in 3 mL of ethyl acetate, and 15 µL of triethylamine and 25 µL of acetyl chloride or acetic anhydride were added. After heating at 75°C for 30 minutes, the reaction mixture was washed three times with an equal volume of water, the ethyl acetate was evaporated *in vacuo*, and the residue was dissolved in methanol for analysis of radioactivity.

Binding of 1,6-Dinitropyrene to Spleen Lymphocyte and Liver Nuclei DNA as a Function of Dose (Experiment 2)

The spleen lymphocytes and liver nuclei used in this experiment were obtained from animals treated in Specific Aim 1, Experiment 2. These cells were prepared and DNA was isolated and quantified as indicated in Specific Aim 2, Experiment 1.

SPECIFIC AIM 3

Mutation Induction at the *hprt* Locus of Spleen T Lymphocytes as a Function of Time (Experiment 1)

Male F344 rats were treated as described in Specific Aim 1, Experiment 1, with 0, 30, or 100 µg [4,5,9,10-^{3}H]1,6-dinitropyrene [575 and 829 mCi/mmol, respectively) or injected intraperitoneally with 40 mg ethylnitrosourea (ENU) in 2.5 mL calcium- and magnesium-free, phosphate-buffered saline. The latter group served as positive controls in the mutagenesis assays. At intervals of 1, 3, 6, 9, 12, 15, 21, 27, 40, and 51 weeks after treatment, two control rats treated with solvent, two rats from each dose group treated with 1,6-dinitropyrene, and one rat treated with ENU (except for week 51) were killed by exposure to carbon dioxide. Their spleens were removed aseptically, lymphocytes were isolated and pooled by treatment group, and the number of T lymphocytes with mutations at the *hprt* locus, as evidenced by growth of the T lymphocytes in the presence of the guanine analog 6-thioguanine, was determined by a limiting dilution clonal assay developed by Aidoo and associates (1991), modified by using conditioned medium as a source of T cell growth factor (Aidoo et al. 1993)(Figure 1). Specifically, lymphocytes were primed for 40 hours in the presence of phytohemagglutinin (Burroughs Wellcome, Research Triangle Park, NC) (µg/mL culture medium). After this period of stimulation, two sets of microtiter plates were prepared for each group of cells. Cloning efficiency plates contained four primed target cells, 5×10^{3} irradiated TK6 feeder cells (an *hprt* deletion mutant obtained from Dr. W.G. Thilly, Massachusetts Institute of Technology, Cambridge, MA), and 5×10^{4} primed, irradiated, autologous cells per well. Selection plates contained 5×10^{3} irradiated TK6 cells and 5×10^{4} primed target cells per well. Each well contained 200 µL of growth medium, except that interleukin-2 was replaced by conditioned medium at a concentration of 20%, and 2-mercaptoethanol (Sigma) was included at a concentration of 50 µM. Conditioned medium was the filtered supernatant from a 36-hour mass culture of rat spleen lymphocytes, inoculated at 2×10^{6} cells per mL in growth medium that was supplemented, per mL, with 500 ng phytohemagglutinin, 20 µg lipopolysaccharide

(from *Escherichia coli* serotype 055:B5; Sigma), and 5 μg concanavalin A (Sigma). Selection plates contained, in addition, 2.5 μg 6-thioguanine/mL medium. The number of positive clones present in both sets of plates was determined 11 to14 days after cloning. Cloning efficiencies and frequencies of 6-thioguanine–resistant (TG^r) lymphocytes for each treatment were calculated as described in Aidoo and associates (1991). In additional experiments, rats were treated in an identical manner with 0, 30, or 100 μg [4,5,9,10-^{3}H]1,6-dinitropyrene (98 to 854 mCi/mmol) and the animals were killed 3, 5, 6, 9, 12, 15, 21, or 27 weeks after treatment. In some of these latter experiments, lymphocytes from individual animals were processed individually instead of being pooled by treatment group.

Mutant Sequence Analysis

6-Thioguanine–resistant T lymphocyte clones (200 μL; approximately 50,000 to 150,000 cells) were transferred into 500 μL phosphate-buffered saline and centrifuged at 500 × *g* for 10 minutes. The supernatant was removed by aspiration and cell pellets were frozen in a dry ice–ethanol bath and stored at 70°C until analysis.

RNA was isolated from the pellets with an AutoGen 540 automated nucleic acid extractor (Integrated Separation Systems, Natick, MA) according to a protocol specified by the manufacturer. The purified RNA was reverse-transcribed into complementary DNA (cDNA) using SuperScript reverse transcriptase (GIBCO-BRL, Grand Island, NY), an oligo dT primer (GIBCO-BRL), and the directions provided with GeneAmp RNA polymerase chain reaction (PCR) kits (Perkin-Elmer Cetus, Norwalk, CT). The *hprt* cDNA (Figure 2) was amplified by two rounds of PCR, which were conducted with either *Taq* (Perkin-Elmer Cetus) or *Pfu* (Stratagene, La Jolla, CA) DNA polymerase. The first round consisted of 30 amplification cycles (94°C, 1 minute; 50°C, 2 minutes; 72°C, 3 minutes, with the last extension step being 10 minutes), and used primers Zee-1 and R11 (Figure 2). The products of this reaction, which usually were not visible by agarose gel electrophoresis, were diluted 1:5000 into a second PCR mix containing primers 210R and R11 (Figure 2) and were subjected to 30 more cycles of amplification (94°C, 1 minute; 55°C, 1 minute; 72°C, 2 minutes, with the final extension step being 9 minutes). The PCR amplification products were purified

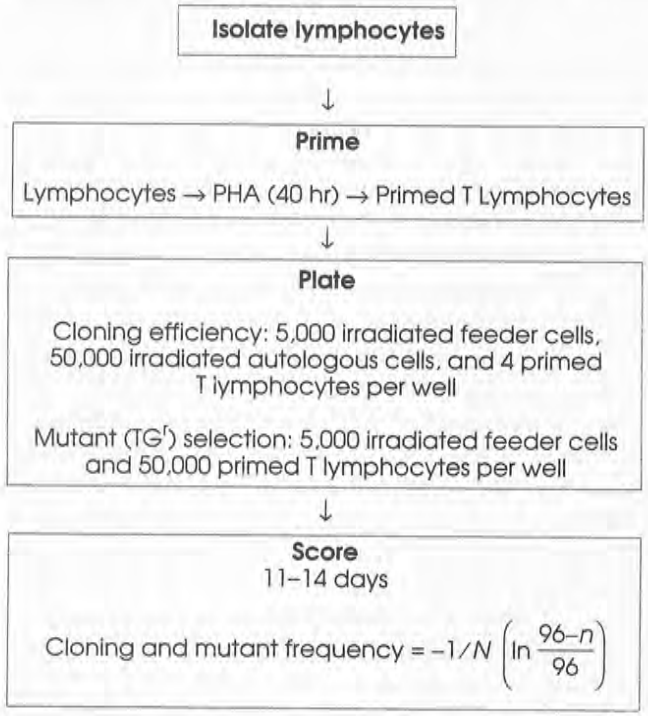

Figure 1. Assay for the induction of mutations at the *hprt* locus of spleen T lymphocytes. PHA = phytohemagglutinin; *N* = primed lymphocytes per well; *n* = number of positive wells per plate.

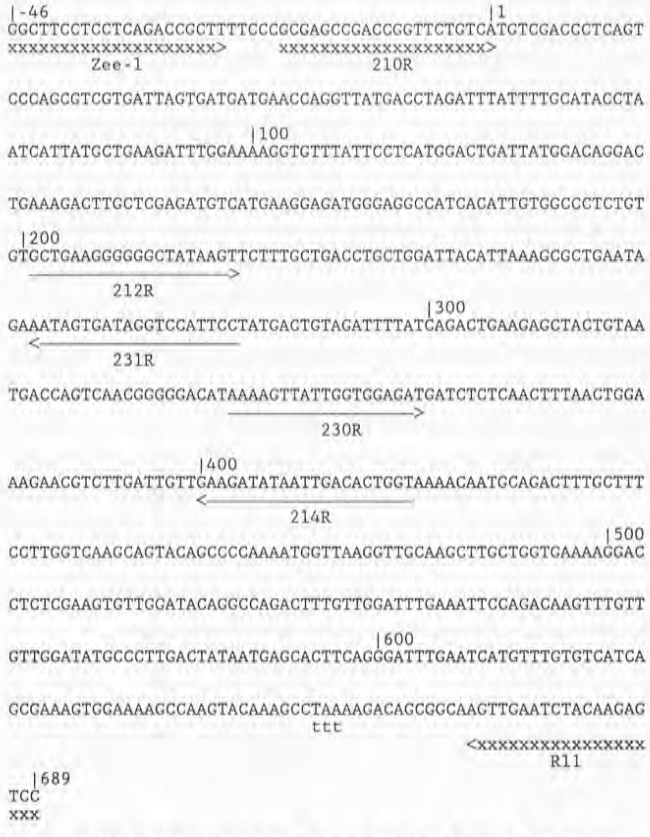

Figure 2. Rat *hprt* cDNA sequence showing location of PCR and sequencing primers. The protein coding sequence starts with base 1 (noted above the rows of letters) and terminates with nonsense codon marked with "ttt". The location of the primers used for both polymerase chain reactions and sequencing are marked as xxxx>, and the location of primers used only for sequencing are marked as →. Direction of the reation is indicated with an arrow.

by ultrafiltration through Centricon 100 microconcentrators (Amicon, Beverly, MA) and 20 to 50 ng (1 to 2 μL) was used as a template for cycle sequencing reactions, which were conducted using the protocol provided with *Taq* DyeDeoxy Terminator Cycle Sequencing kits (Applied Biosystems, Foster City, CA). Excess dye terminators were removed by extraction of the completed sequencing reactions with a mixture of phenol and chloroform, and the resultant *hprt* gene reaction products were analyzed on an Applied Biosystems 373A DNA Sequencer. Six sequencing primers (210R, R11, 212R, 214R, 231R, and 230R; Figure 2) were used to sequence the entire *hprt* protein-coding sequence in both directions. Only complete sequence changes were considered to be mutations (i.e., mixtures of wild-type and mutant sequences were excluded). All mutations were confirmed by sequencing both strands.

Mutation Induction at the *hprt* Locus of Spleen T Lymphocytes as a Function of Dose (Experiment 2)

Male F344 rats were treated as described in Specific Aim 1, Experiment 1, with 0 (solvent-treated control animals), 0.3, 1, 3, 10, 30, 100, or 150 μg 1,6-dinitropyrene or 40 mg ENU. At intervals of 15 and 27 weeks after treatment, two control rats treated with solvent, two rats treated with 30 μg 1,6-dinitropyrene, and two rats treated with 100 μg 1,6-dinitropyrene were killed and processed for the induction of mutations as described in Specific Aim 3, Experiment 1, except that each treated rat was assayed individually. Twenty-one weeks after treatment, two control rats treated with solvent, two rats from each of the 1,6-dinitropyrene treatment groups, and one rat treated with ENU were analyzed in a similar manner.

STATISTICAL ANALYSES

Dose-response relation trends in adduct levels and mutant frequency were assessed by a linear regression trend test. Comparisons between doses were made using a modified Bonferroni procedure (Hochberg 1988) to account for multiple comparisons. The degrees of freedom were calculated by Satterthwaite's formula.

RESULTS

SPECIFIC AIM 1

Binding of 1,6-Dinitropyrene to Lung DNA as a Function of Time (Experiment 1)

Initially, twelve-week-old male F344 rats were anesthetized with an intramuscular injection of ketamine (Iwagawa et al. 1989). This proved to be an unsatisfactory agent for surgical anesthesia and therefore was replaced by a mixture of ketamine and xylazine (Van Pelt 1977). Using this combination of sedative and muscle relaxant, the surgical mortality was approximately 20%. No other post-surgery complications were noted in any of the experiments.

In an initial experiment, 24 rats were subjected to a left lateral thoracotomy, and 30 μg [4,5,9,10-^{3}H]1,6-dinitropyrene in a 50-μL mixture of beeswax and tricaprylin was administered into their lungs. Twenty-five rats were treated likewise with 100 μg [4,5,9,10-^{3}H]1,6-dinitropyrene. At intervals of 1, 3, 7, 14, and 28 days after treatment, three to five animals from each group were killed and their lungs were dissected. In nearly every rat, a pellet of beeswax was clearly evident within the lung. To determine the retention of the 1,6-dinitropyrene, homogenates were made of the lungs from animals treated with 30 μg of 1,6-dinitropyrene and killed after 1, 3, 7, or 14 days. The results (Table 1) indicate that the compound rapidly dissipated from the pellet and was distributed systemically.

DNA was isolated from the lungs using enzymatic digestions and solvent extractions. The yield, as determined by UV spectrophotometry, was 3.3 ± 1.0 mg per animal (mean ± SD; $n = 40$). Aliquots were assayed by liquid scintillation counting to determine the extent as an index of adduct formation. As shown in Figure 3, maximal adduct formation was observed three to seven days after treatment, and the binding slowly decreased thereafter. In addition, a dose-response relation was detected, with the 100-μg dose producing approximately twice the binding found with the 30-μg dose.

Table 1. Levels of [4,5,9,10-^{3}H]1,6-Dinitropyrene and Its Metabolites in the Lungs of F344 Rats[a]

Days After Treatment	Percent Remaining in Lung
1	9 ± 3
3	17 ± 5
7	8 ± 2
14	5 ± 3

[a] Male F344 rats were anesthetized with ketamine and xylazine, subjected to a left lateral thoracotomy, and 30 μg [4,5,9,10-^{3}H]1,6-dinitropyrene (103 mCi/mmol) was implanted directly into the lungs. At the times indicated, three or four animals were exposed to carbon dioxide, decapitated, and the lungs were excised. Homogenates were made and digested with proteinase K (Beland et al. 1984); 50-μL aliquots then were assayed by liquid scintillation counting. The data are presented as the means ± SE of the amounts initially administered; $n = 3$ for day 1, and $n = 4$ for days 3, 7, and 14.

Additional aliquots of lung DNA were hydrolyzed enzymatically, extracted with *n*-butanol, and the *n*-butanol extracts were analyzed by HPLC. The hydrolysis efficiency was approximately 10%, as determined by measuring the radioactivity extracted with *n*-butanol. When the radioactivity soluble in *n*-butanol was analyzed by HPLC, one major peak with chromatographic characteristics consistent with *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene was observed at 29 to 30 minutes (Figure 4). There was also a small amount of late-eluting radioactivity. Similar results were obtained when different enzymatic hydrolysis conditions were used, and when the DNA was hydrolyzed chemically with trifluoroacetic acid (see Materials and Methods section).

In addition to quantifying the ^{3}H content of the lung DNA, aliquots from 1, 3, and 7 days after treatment were assayed by ^{32}P-postlabeling. A single major adduct was detected (Figure 5B) that had the same elution characteristics as the adduct obtained from reacting [4,5,9,10-^{3}H]*N*-hydroxy-1-amino-6-nitropyrene with DNA (Figure 5A). This adduct has been characterized as *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene (Djurić et al. 1988).

The adduct levels in the lung DNA samples were quantified against DNA standards that were included in every postlabeling assay. The results (Table 2) show poor agreement between the adduct levels determined by measuring the ^{3}H associated with the lung DNA and those determined by ^{32}P-postlabeling. However, the low adduct levels determined by ^{32}P-postlabeling were consistent with the low extent of radioactivity that partitioned into *n*-butanol when the DNA was hydrolyzed for HPLC analysis.

To reconcile the differences between the adduct levels determined by the measurement of ^{3}H and those indicated by ^{32}P-postlabeling, a second experiment was conducted in which rats were treated with 30 or 100 μg [4,5,9,10-^{3}H]1,6-dinitropyrene in a manner similar to the first experiment. At death, the lungs were dissected; however, instead of isolating DNA from the whole lung, nuclei were prepared. DNA then was isolated from the nuclei and the extent of binding was quantified by liquid scintillation counting. As shown in Figure 6, the extent of adducts in the DNA prepared from the nuclei was approximately 10-fold lower than when DNA was isolated from the whole lung, which is consistent with the previous ^{32}P-postlabeling data (Table 1). These results indicate a good correlation between the amount of adduction based upon ^{3}H incorporation and ^{32}P-postlabeling when lung nuclei DNA was assessed; thus, most of the ^{3}H associated with the DNA isolated from whole lungs was not due to DNA adducts but must be due to material that copurifies with the DNA. In light of these data, all subsequent adduct analyses from lungs were conducted with DNA purified from lung nuclei.

At each sampling point, similar adduct levels were found with both doses. With the 100-μg treatment, maximal binding levels occurred seven days after treatment; but with the 30-μg dose, the highest level of binding was found one day after treatment. With both treatments, the adduct levels slowly decreased so that by 28 days after treatment, it had decreased to 25% to 50% of the peak values. In subsequent studies associated with the analysis of mutant induction in spleen T lymphocytes (Specific Aim 3, Experiment 1), the extent of binding to lung nuclei DNA was determined at times up to 12 weeks after treatment. The adduct levels were similar to those detected at four weeks (data not shown).

Aliquots of lung nuclei DNA from rats killed three days after treatment were hydrolyzed enzymatically to nucleosides, and the adducts were partitioned into *n*-butanol and

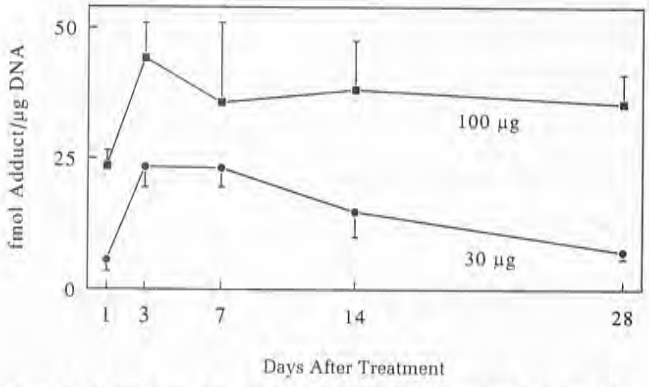

Figure 3. Adduct formation of [4,5,9,10-^{3}H]1,6-dinitropyrene with lung DNA of male F344 rats as a function of time. 1,6-Dinitropyrene (30 μg or 100 μg) was administered directly to the lungs of the rats, animals were killed at the times indicated, DNA was purified from whole lungs, and the extent of adduct formation was measured by liquid scintillation counting. The data are presented as means ± SE; $n = 4$, except for the data point for 30 μg at day 1, for which $n = 3$, and the data point for 100 μg at day 28, for which $n = 5$.

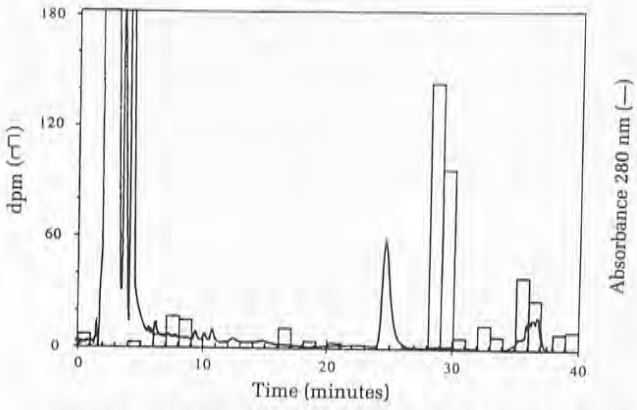

Figure 4. High-pressure liquid chromatography profile of lung DNA from a male F344 rat treated with [4,5,9,10-^{3}H]1,6-dinitropyrene. Lung DNA was enzymatically hydrolyzed to nucleosides, and adducts were partitioned into *n*-butanol for analysis by reversed-phase HPLC. The adduct marker *N*-(deoxyguanosin-8-yl)-2-aminofluorene, which was added as an internal standard, eluted at 24.5 minutes.

separated by HPLC. With these samples, 80% of the radioactivity associated with the DNA partitioned into the *n*-butanol, and when analyzed by HPLC, a major peak eluted in a region identical to the 29- to 30-minute histogram peak shown in Figure 4. These results confirm that the ^{3}H associated with lung nuclei DNA is indeed due to *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene.

Binding of 1,6-Dinitropyrene to Lung DNA as a Function of Dose (Experiment 2)

The extent of adduct formation, as a function of dose, was determined by implanting 0, 0.3, 1, 3, 10, 30, 100, or 150 μg [4,5,9,10-^{3}H]1,6-dinitropyrene in the lungs of male F344 rats. Seven days after treatment, the rats were killed, lung nuclei were prepared, DNA was isolated, and the levels of adducts were determined by liquid scintillation counting. A significant ($p < 0.0005$) dose-response relation was observed (Figure 7), with the extent of adduct formation increasing significantly at a dose of 10 μg. From 0 through 30 μg 1,6-dinitropyrene, the amount of binding to lung DNA increased with dose such that a twofold increase in the amount of 1,6-dinitropyrene resulted in a 1.8-fold increase in binding ($r = 0.96$; $p < 0.0005$). At doses above 30 μg of 1,6-dinitropyrene, the amount of binding still increased with dose, but the rate of increase was much less than that observed at the lower doses.

SPECIFIC AIM 2

Blood Levels of 1,6-Dinitropyrene, and Binding of 1,6-Dinitropyrene to White Blood Cell, Spleen Lymphocyte, and Liver Nuclei DNA as a Function of Time (Experiment 1)

The amount of radioactivity in the lysed red blood cells was assessed in rats treated in Specific Aim 1. The maximal amount of radioactivity occurred at the initial sampling point (Figure 8), which was one day after treatment, and by 28 days it had returned to background levels. Although a 3.3-fold difference in dose was administered, the maximal difference in radioactivity in the blood was only 1.3-fold. The supernatant containing the lysed blood cells was extracted with a mixture of chloroform and methanol or with ethyl acetate. Only 8% ± 2% (mean ± SD; $n = 6$) of the radioactivity partitioned into the organic phase. When this organic-soluble material was analyzed by HPLC, small amounts of radioactivity coeluted with 1-acetylamino-6-nitropyrene, 1-amino-6-nitropyrene, and 1,6-dinitropyrene (results not shown).

Hemoglobin adducts were assayed in red blood cells from rats administered [4,5,9,10-^{3}H]1,6-dinitropyrene and killed one day later. Only 7% ± 5% (mean ± SD; $n = 4$) of the radioactivity became soluble in methanol upon alkali treatment and derivatization (see the Methods section). Because this did not differ appreciably from what was found when extracting the blood samples directly with organic solvents, this procedure did not appear to release the covalently bound radioactivity; thus, the identity of this material remains unknown.

The amount of DNA (118 ± 53 μg, mean ± SD; $n = 32$) from the white blood cells was insufficient to determine the adduct levels directly by scintillation counting. These samples were, therefore, assayed by ^{32}P-postlabeling. In each instance, a single adduct was detected that had thin-layer chromatography (TLC) elution characteristics similar to *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene (results not shown). The concentration of this adduct in the white blood cell DNA (Figure 9) was considerably less than that found in the lung nuclei DNA (Figure 6). As with lung nuclei DNA, however, maximal adduct formation occurred seven days after treat-

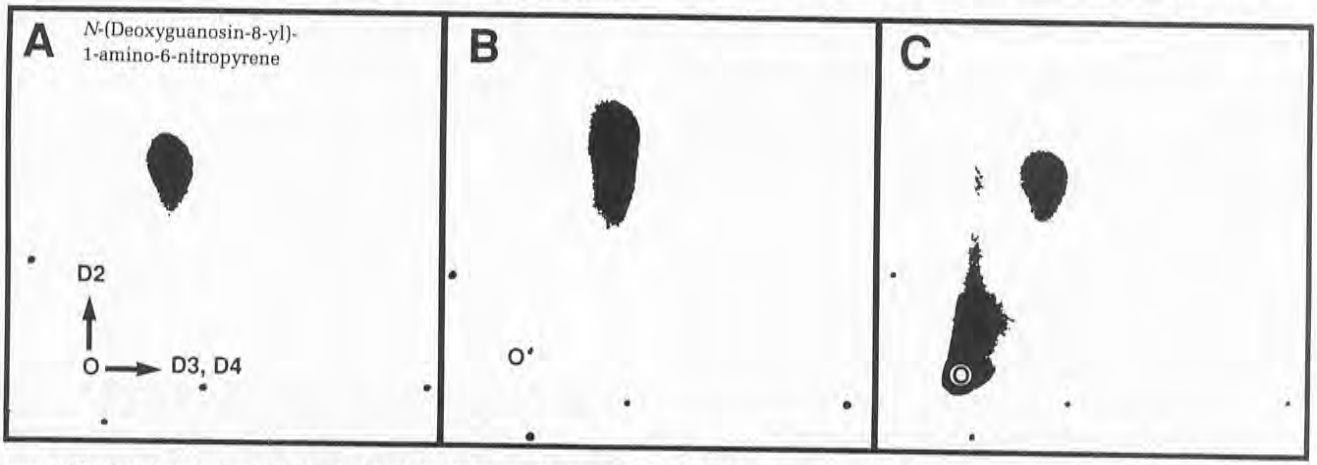

Figure 5. ^{32}P-Postlabeling autoradiographs of (A) the DNA standard modified with N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene, and (B) lung DNA and (C) spleen lymphocyte DNA of a male F344 rat treated with [4,5,6,9,10-^{3}H]1,6-dinitropyrene. The origin of the chromatogram is located at position "O". The direction of development of the chromatogram is designated by the arrows, and the composition of the solvent systems and the sequence in which they were used is signified by D2, D3, and D4 (see Materials and Methods section).

Table 2. Binding of [4,5,9,10-^{3}H]1,6-Dinitropyrene to Lung DNA of F344 Rats as Determined by Measuring ^{3}H and by ^{32}P-Postlabeling[a]

Dose (μg)	Days After Treatment	Determination Method (fmol adduct/μg DNA)	
		^{3}H Counting	^{32}P-Postlabeling
30	1	5.7 ± 2.2	2.5 ± 0.2
	3	23.4 ± 3.9	2.6 ± 0.5
	7	23.2 ± 3.6	11.8 ± 3.2
100	1	23.6 ± 2.9	5.3 ± 0.9
	3	44.2 ± 6.7	2.6 ± 0.3
	7	35.7 ± 15.3	13.2 ± 2.9

[a] Male F344 rats were anesthetized with ketamine and xylazine, subjected to a left lateral thoracotomy, and 30 μg or 100 μg [4,5,9,10-^{3}H]1,6-dinitropyrene (103 or 98 mCi/mmol, respectively) was implanted directly into the lungs. At the times indicated, three or four animals were exposed to carbon dioxide, decapitated, and the lungs were excised. DNA was isolated from the whole lungs and the extent of adduct formation was determined by assaying the ^{3}H content using liquid scintillation counting. Additional aliquots were assayed by ^{32}P-postlabeling. The data are presented as means ± SE; n = 4, except for 30 μg at day 1, for which n = 3.

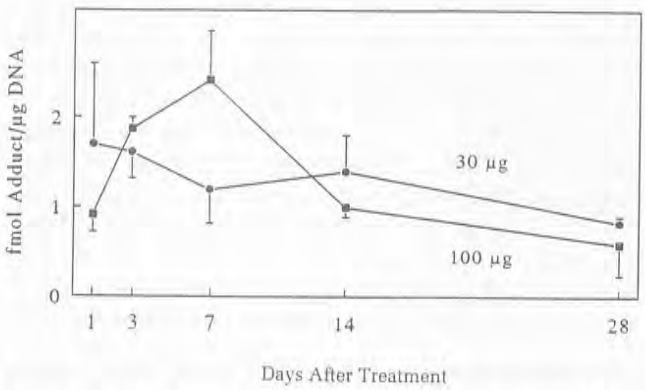

Figure 6. Adduct formation of [4,5,9,10-^{3}H]1,6-dinitropyrene with lung nuclei DNA of male F344 rats as a function of time. 1,6-Dinitropyrene (30 μg or 100 μg) was administered directly to the lungs of the rats, animals were killed at the times indicated, lung nuclei were prepared, DNA was purified, and the extent of adduct formation was measured by liquid scintillation counting. The data are presented as means ± SE from three rats, except for the 28-day data points, which had only two rats per dose.

ment with the 100-μg dose (Figure 9). Likewise, there was not a significant difference in adduct formation in the white blood cell DNA between the 30-μg and 100-μg doses, and by 28 days after treatment the adduct levels from both doses had decreased to approximately 20% of the peak values.

The yield of DNA from the spleen lymphocytes was 457 ± 115 μg per animal (mean ± SD; n = 25). The extent of binding by [4,5,9,10-^{3}H]1,6-dinitropyrene in the spleen lymphocyte DNA (Figure 10), as quantified by liquid scintillation counting, was similar to that observed in the white blood cell DNA (Figure 9). As with the white blood cell DNA, adduct formation with spleen lymphocyte DNA reached a maximal level at seven days after treatment with the 100-μg dose and three days after treatment with the 30-μg dose. In contrast to both the white blood cell and lung nuclei DNA, however, a significant dose-response relation was noted in the adduct levels with spleen lymphocyte DNA, with the 100-μg dose producing 1.5- to 2.7-fold more adducts than the 30-μg dose at each time point. Twenty-eight days after treatment, the adduct levels had decreased to approximately 30% of the peak values.

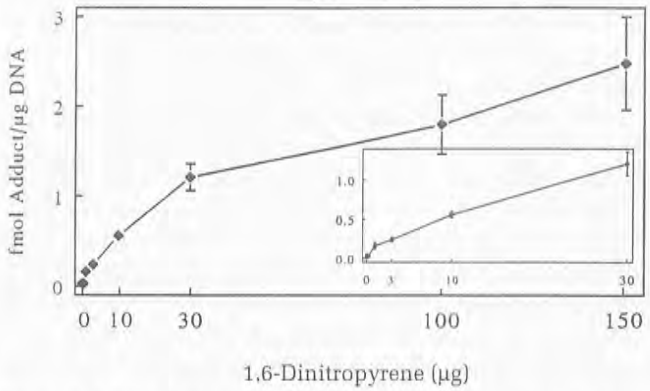

Figure 7. Adduct formation of [4,5,9,10-^{3}H]1,6-dinitropyrene with lung nuclei DNA of male F344 rats as a function of dose. 1,6-Dinitropyrene (0, 0.3, 1, 3, 10, 30, 100, or 150 μg) was administered directly to the lungs of the rats, animals were killed seven days after treatment, lung nuclei were prepared, DNA was purified, and the extent of adduct formation was measured by liquid scintillation counting. The data are presented as means ± SE; n = 2 for 0 μg; n = 3 for 30, 100, and 150 μg; n = 4 for 1 and 3 μg; and n = 5 for 0.3 and 10 μg. The inset shows the dose-response relation for 0 μg to 30 μg of 1,6-dinitropyrene.

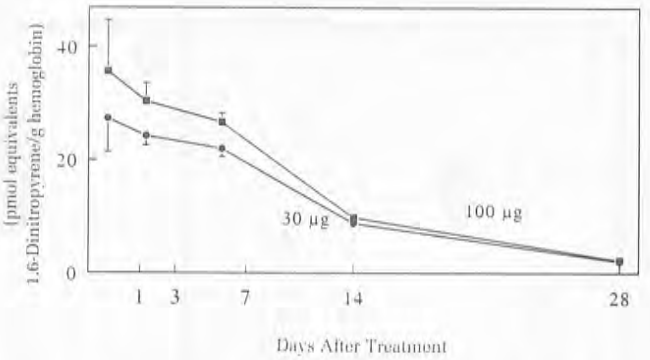

Figure 8. Blood levels of [4,5,9,10-^{3}H]1,6-dinitropyrene, or its metabolites, or both. 1,6-Dinitropyrene (30 μg or 100 μg) was administered directly to the lungs of the rats, animals were killed at the times indicated, blood was collected in heparinized tubes, red blood cells were lysed, and white blood cells were isolated by centrifugation. The hemoglobin content of the lysed red blood cells was quantified and the extent of radioactivity was measured by scintillation counting. The data are presented as means ± SE from three rats.

Aliquots of the spleen lymphocyte DNA were assayed by ^{32}P-postlabeling (Figure 5C). In each instance, a single major adduct was detected that had the same elution characteristics as *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene.

DNA was isolated from liver nuclei as described by Poirier and associates (1990) and quantified by UV spectrophotometry; the yield was 11.7 ± 2.8 mg per animal (mean ± SD; $n = 40$). The extent of binding (Figure 11), as assayed by liquid scintillation counting, was similar to that observed with white blood cell (Figure 9) and spleen lymphocyte (Figure 10) DNA. No significant difference in adduct formation was apparent between the two doses, and with both doses, maximal adduct formation occurred three days after treatment and by 28 days the adduct levels had decreased to 13% to 30% of the peak values. ^{32}P-Postlabeling analyses of the liver nuclei DNA indicated a major adduct with chromatographic characteristics identical to *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene (results not shown).

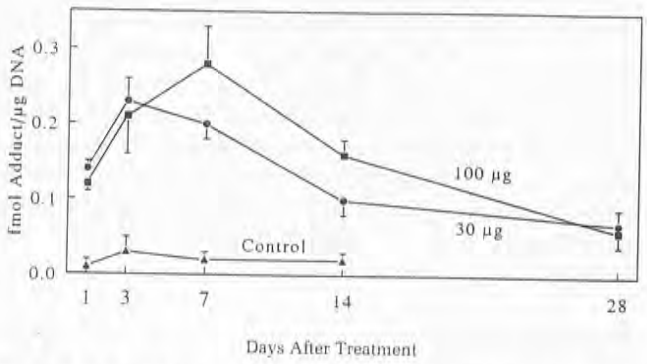

Figure 9. Adduct formation of [4,5,9,10-^{3}H]1,6-dinitropyrene with white blood cell DNA of male F344 rats as a function of time. 1,6-Dinitropyrene (0, 30, or 100 μg) was administered directly to the lungs of the rats, animals were killed at the times indicated, blood was collected in heparinized tubes, red blood cells were lysed, and white blood cells were isolated by centrifugation. DNA was purified from the white blood cells and the extent of adduct formation was measured by ^{32}P-postlabeling. The data are presented as means ± SE from three rats, except for the control rats treated with solvent, for which only one or two rats were analyzed at each time point.

Binding of 1,6-Dinitropyrene to Spleen Lymphocyte and Liver Nuclei DNA as a Function of Dose (Experiment 2)

The extent of adduct formation, as a function of dose, in spleen lymphocyte and liver nuclei DNA was determined seven days after implanting 0, 0.3, 1, 3, 10, 30, 100, or 150 μg [4,5,9,10-^{3}H]1,6-dinitropyrene in the lungs of male F344 rats. When treated with 150 μg of 1,6-dinitropyrene, adduct formation in spleen lymphocyte DNA, as determined by liquid scintillation counting, was 0.13 ± 0.02 fmol adduct/μg DNA (mean ± SE; $n = 3$). The comparable values for 30 and 100 μg of 1,6-dinitropyrene were 0.07 ± 0.01 and 0.14 ± 0.05 fmol adduct/μg DNA, respectively. The yield of DNA from the spleen lymphocytes was insufficient (518 ± 225 μg, mean ± SD; $n = 29$) to measure the extent of binding based upon the amount of ^{3}H incorporated at doses lower than 30 μg of 1,6-dinitropyrene.

Additional aliquots of the spleen lymphocyte DNA were assayed by ^{32}P-postlabeling. A significant dose-response relation was observed ($p < 0.0005$), with the values becom-

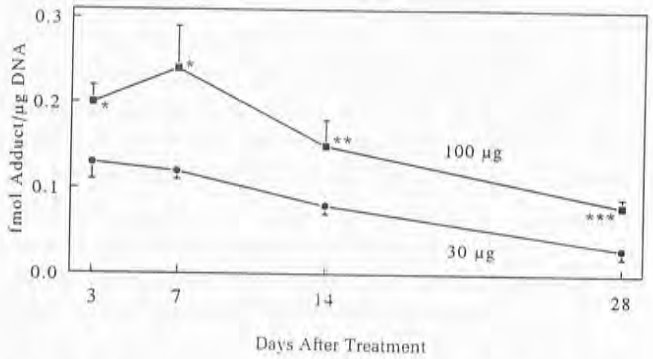

Figure 10. Adduct formation of [4,5,9,10-^{3}H]1,6-dinitropyrene with spleen lymphocyte DNA of male F344 rats as a function of time. 1,6-Dinitropyrene (30 μg or 100 μg) was administered directly to the lungs of the rats, animals were killed at the times indicated, spleen lymphocytes were isolated, DNA was purified, and the extent of adduct formation was measured by liquid scintillation counting. The data are presented as means ± SE from three rats, except for the 28-day point, which had only two rats per dose. The asterisks indicate that DNA adduct formation in the 100-μg treatment group was significantly greater than in the 30-μg treatment group, with p values of <0.1(*), <0.05(**), and , <0.01 (***).

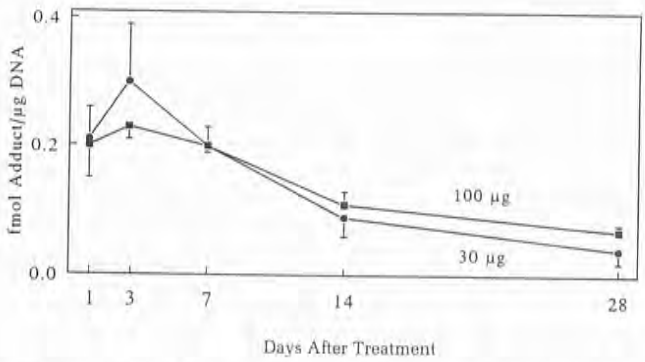

Figure 11. Adduct formation of [4,5,9,10-^{3}H]1,6-dinitropyrene with liver nuclei DNA of male F344 rats as a function of time. 1,6-Dinitropyrene (30 μg or 100 μg) was administered directly to the lungs of the rats, animals were killed at the times indicated, liver nuclei were prepared, DNA was purified, and the extent of adduct formation was measured by liquid scintillation counting. The data are presented as means ± SE; $n = 4$, except for the data point for 30 μg at day 1, for which $n = 3$, and the data point for 100 μg at day 28, for which $n = 5$.

ing significantly greater than control values at a dose of 10 μg 1,6-dinitropyrene (Figure 12). Furthermore, the results obtained were quite similar to those found by direct measurement of ^{3}H incorporation. Due to the low extent of adduct formation in the spleen lymphocyte DNA and the relatively limited quantity of DNA that could be assayed by ^{32}P-postlabeling (i.e., 20 μg), an increase in adduct formation could not be demonstrated at doses below 10 μg of 1,6-dinitropyrene.

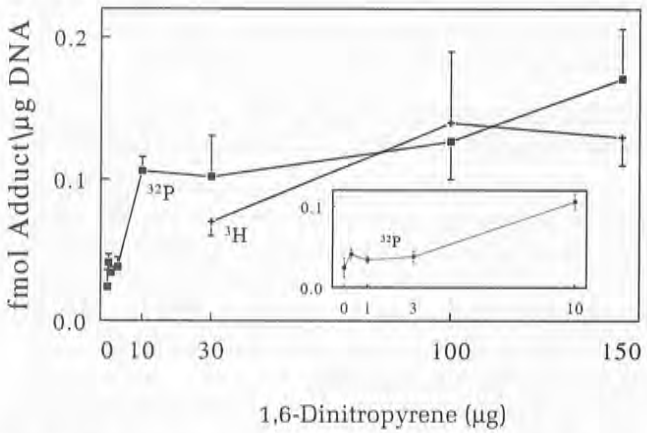

Figure 12. Adduct formation of [4,5,9,10-^{3}H]1,6-dinitropyrene with spleen lymphocyte DNA of male F344 rats as a function of dose. 1,6-Dinitropyrene (0, 0.3, 1, 3, 10, 30, 100, or 150 μg) was administered directly to the lungs of the rats, animals were killed seven days after treatment, spleen lymphocytes were isolated, DNA was purified, and the extent of adduct formation was measured by liquid scintillation counting and ^{32}P-postlabeling. The data are presented as means ± SE; $n = 2$ for 0 μg; $n = 3$ for 30, 100, and 150 μg; $n = 4$ for 1 and 3 μg; and $n = 5$ for 0.3 and 10 μg. The inset shows the dose-response relation for 0 to 10 μg of 1,6-dinitropyrene as determined by ^{32}P-postlabeling.

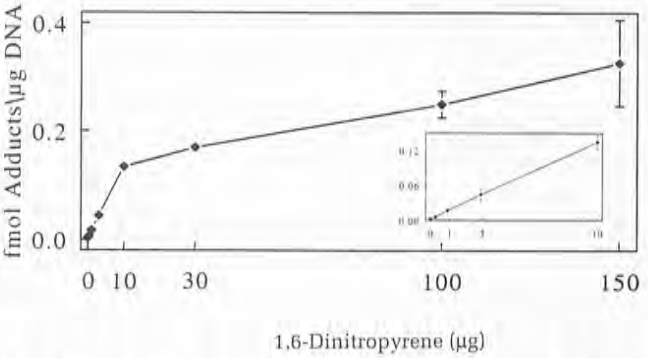

Figure 13. Adduct formation of [4,5,9,10-^{3}H]1,6-dinitropyrene with liver nuclei DNA of male F344 rats as a function of dose. 1,6-Dinitropyrene (0, 0.3, 1, 3, 10, 30, 100, or 150 μg) was administered directly to the lungs of the rats, animals were killed seven days after treatment, liver nuclei were prepared, DNA was purified, and the extent of adduct formation was measured by liquid scintillation counting. The data are presented as means ± SE; $n = 2$ for 0 μg; $n = 3$ for 30, 100, and 150 μg; $n = 4$ for 1 and 3 μg; and $n = 5$ for 0.3 and 10 μg. The inset shows the dose-response relation for 0 to 10 μg of 1,6-dinitropyrene.

The total extent of adduct formation in the liver nuclei was measured by liquid scintillation counting. A significant ($p < 0.0005$) dose-response relation was observed (Figure 13), with the values being approximately 10% of those observed in the lung (Figure 7). As with the lung DNA, the extent of adduct formation as a function of dose was described by a biphasic curve. From 0 μg through 10 μg of 1,6-dinitropyrene, the increase was linear ($r = 0.97$; $p < 0.0005$), with a twofold increase in dose resulting in a two fold increase in adduct formation. Above 10 μg of 1,6-dinitropyrene, the increase in adduct concentration was still linear, but the rate was much lower than occurred at lower doses. ^{32}P-Postlabeling analyses of liver DNA indicated the presence of one major adduct with the same TLC elution characteristics as that detected in the time course study described in Specific Aim 1, Experiment 1 (data not shown).

SPECIFIC AIM 3

Mutation Induction at the *hprt* Locus of Spleen T Lymphocytes as a Function of Time (Experiment 1)

Male F344 rats were administered solvent, or 30 μg or 100 μg [4,5,9,10-^{3}H]1,6-dinitropyrene as described in the previous specific aims. Additional rats were treated intraperitoneally with 40 mg ENU to serve as positive controls. At intervals of 1, 3, 6, 9, 12, 15, 21, 27, 40, and 51 weeks after treatment, two rats from each of the groups treated with solvent, or 30 μg or 100 μg 1,6-dinitropyrene, and one rat treated with ENU were killed to determine the number of spleen T lymphocytes with mutations at the *hprt* locus (Figure 1). In other experiments, rats were treated with 0, 30, or 100 μg [4,5,9,10-^{3}H]1,6-dinitropyrene in an identical manner; the animals were killed to assess the mutation frequency at 3, 5, 6, 9, 12, 15, 21, or 27 weeks after treatment.

The cloning efficiencies of the phytohemagglutinin-stimulated spleen T lymphocytes from these experiments, as a function of time, are presented in Table 3 and Figure 14. With all treatments, the cloning efficiencies were relatively constant through week 27, whereupon they decreased. At most time points, a higher cloning efficiency was observed in T lymphocytes from control rats or rats treated with 30 μg 1,6-dinitropyrene compared with those administered 100 μg 1,6-dinitropyrene or ENU.

The mutant frequency, as a function of time, at the *hprt* locus of spleen T lymphocytes is presented in Table 3 and Figure 15. With 30 μg and 100 μg 1,6-dinitropyrene, the

Table 3. Cloning Efficiency and TG^r Mutant Frequency, as a Function of Time, in Spleen T Lymphocytes from F344 Rats Treated with Solvent, 1,6-Dinitropyrene, or Ethylnitrosourea

Treatment	Weeks After Treatment	Number of Assays	Cloning Efficiency[a] (%)	TG^r T Lymphocytes per 10^6 Cells[a]
Solvent	1	1	8.5	3
	3	2	9.5 ± 0.5	8 ± 3
	5	4	8.2 ± 0.5	4 ± 1
	6	2	7.0 ± 0.4	9 ± 1
	9	2	8.1 ± 0.9	7 ± 4
	12	3	9.7 ± 0.2	10 ± 5
	15	3	6.8 ± 0.1	9 ± 5
	21	5	8.1 ± 0.3	6 ± 1
	27	3	6.4 ± 0.9	5 ± 1
	40	1	4.1	7
	51	1	4.8	7
1,6-Dinitro-pyrene (30 µg)	1	1	9.1	7
	3	1	8.4	20
	5	4	7.9 ± 0.4	27 ± 2[b]
	6	1	6.4	31
	9	1	8.9	20
	12	2	10.0 ± 1.9	28 ± 5[b]
	15	3	6.7 ± 0.3	36 ± 2[b]
	21	3	8.1 ± 0.3	48 ± 2[b]
	27	3	6.5 ± 0.4	53 ± 2[b]
	40	ND[c]	ND	ND
	51	1	4.8	26
1,6-Dinitro-pyrene (100 µg)	1	1	7.9	9
	3	2	7.8 ± 0.1	37 ± 5[b]
	5	4	6.2 ± 0.3	39 ± 2[b,d]
	6	2	5.7 ± 0.3	48 ± 7
	9	2	7.5 ± 1.1	46 ± 6[b]
	12	3	7.8 ± 0.6	50 ± 12[b]
	15	3	6.0 ± 0.2	68 ± 2[b,d]
	21	3	7.3 ± 0.2	99 ± 7[b,d]
	27	3	5.3 ± 0.6	89 ± 7[b,d]
	40	1	3.0	91
	51	1	4.0	49
Ethylnitro-sourea (40 mg)	1	1	7.7	67
	3	2	6.3 ± 1.1	178 ± 11[b]
	6	2	5.2 ± 0.3	244 ± 39
	5	ND	ND	ND
	9	2	6.2 ± 0.9	228 ± 40

(Table continues next column)

Table 3. Cloning Efficiency and TG^r Mutant Frequency, as a Function of Time, in Spleen T Lymphocytes from F344 Rats Treated with Solvent, 1,6-Dinitropyrene, or Ethylnitrosourea (*Continued*)

Treatment	Weeks After Treatment	Number of Assays	Cloning Efficiency[a] (%)	TG^r T Lymphocytes per 10^6 Cells[a]
	12	2	6.6 ± 0.5	252 ± 136
	15	1	6.4	226
	21	2	6.7 ± 0.1	207 ± 7[b]
	27	1	5.5	181
	40	1	2.3	218
	51	ND	ND	ND

[a] Data are presented as means ± SE. Each assay consisted of three 96-well microtiter plates; for cloning efficiency, target cells = 4/well; for TG^r lymphocytes, total target cells = 14.4×10^6.

[b] Significantly ($p < 0.05$) different from control group at the same time point.

[c] ND = not determined. An insufficient number of the rats treated with 30 µg 1,6-dinitropyrene survived surgery to allow analysis at the 40-week time point. Animals were not treated with ENU in the five-week experiment. The rat injected with ENU and assigned to the 51-week sampling point died from an intestinal tumor 50 weeks after treatment.

[d] Significantly ($p < 0.05$) different from the group treated with 30 µg 1,6-dinitropyrene at the same time point.

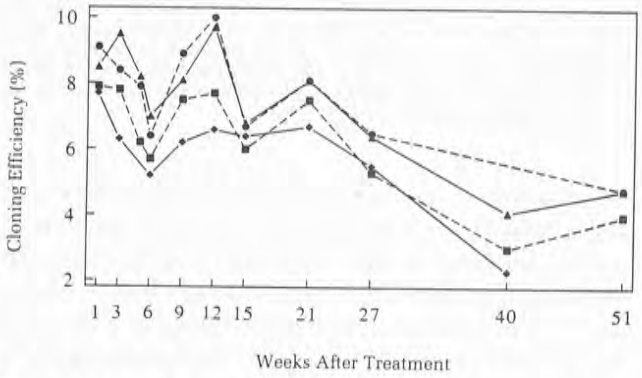

Figure 14. Cloning efficiency of spleen T lymphocytes as a function of time. 1,6-Dinitropyrene (0 (▲), 30 (●), or 100 (■) µg) was administered directly to the lungs of the rats, animals were euthanized at the times indicated, spleen T lymphocytes were isolated, and the cloning efficiency was determined. The data are presented as means of one to five assays (see Table 3), each consisting of three 96-well microtiter plates. Additional rats were analyzed in an identical manner after being administered 40 mg ENU (◆) intraperitoneally.

extent of mutation induction increased until week 3, and then remained relatively level until week 12, whereupon it increased until week 21. The mutation frequency then was constant once again until week 40 and then started to decrease. After the initial week 1 sampling, the mutation frequency induced with the 100-µg dose of 1,6-dinitropyrene was significantly greater than that observed in the

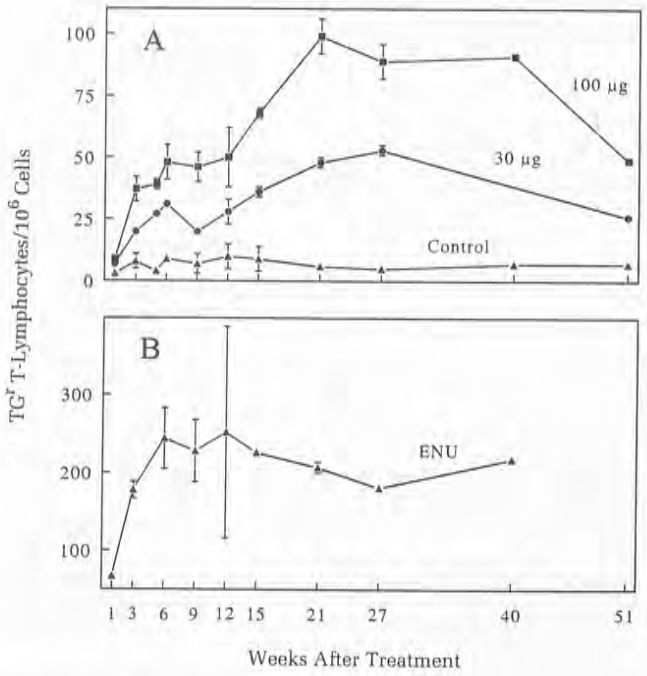

Figure 15. Mutation induction in spleen T lymphocytes as a function of time. A: 1,6-Dinitropyrene (0, 30, or 100 µg) was administered directly to the lungs of the rats, animals were killed at the times indicated, spleen T lymphocytes were isolated, and mutation induction at the *hprt* locus was determined by assaying for TG^r T lymphocytes. The data are presented as means of one to five assays (see Table 3), each consisting of three 96-well microtiter plates. B: Additional rats were analyzed in an identical manner after being administered 40 mg ENA intraperitoneally.

solvent-treated control group. Likewise, the extent of mutation induction was usually significantly greater with the 100-µg dose compared with the 30-µg dose of 1,6-dinitropyrene, with the difference typically being 1.8-fold. In animals treated with ENU, the mutation frequency increased rapidly until week 6, whereupon it remained relatively constant for the remainder of the experiment.

Mutation Induction at the *hprt* Locus of Spleen T Lymphocytes as a Function of Dose (Experiment 2)

Male F344 rats were implanted with 0, 0.3, 1, 3, 10, 30, 100, or 150 mg [4,5,9,10-^{3}H]1,6-dinitropyrene. As shown in Table 4 and Figure 16A, with the exception of the 150-mg dose, the cloning efficiencies of the spleen T lymphocytes were relatively constant when measured 21 weeks after treatment. The mutant frequencies as a function of dose are presented in Table 4 and Figure 16B. A significant dose-response relation was observed ($p < 0.005$), with the increase in mutants becoming significant at 1 µg of 1,6-dinitropyrene. As with the DNA binding (Figures 7 and 13), the mutation frequencies tended to increase with dose; however, the response was not linear and actually decreased at the highest dose of 150 µg of 1,6-dinitropyrene.

Additional rats were implanted with 0, 30, or 100 µg of [4,5,9,10-^{3}H]1,6-dinitropyrene, and the cloning efficiencies and mutation frequencies were determined after 15 and 27 weeks to ensure that the responses detected in the dose-response experiment (Specific Aim 3, Experiment 2) were consistent with the data obtained in the time-course experiment (Specific Aim 3, Experiment 1). The results (Table 4) indicate that a plateau in the mutagenic response had been obtained, and that the magnitude of the mutation induction was similar in both experiments. As an additional control, one rat was treated intraperitoneally with 40 mg ENU and the spleen T lymphocyte mutation frequency was assayed after 21 weeks. The results from this rat (Table 4) also were similar to those found in the time-course study (Table 3).

SEQUENCE ANALYSIS OF *hprt* MUTANTS

RNA was isolated from suspected *hprt* mutant clones and reverse-transcribed into cDNA; the cDNA was amplified via PCR with *Taq* DNA polymerase, and the *hprt* protein-coding region was sequenced. Of the 37 clones analyzed, 23 (62%) produced sufficient amounts of amplified cDNA to sequence. In the 23 reactions amplified by PCR, 19 sequence changes were detected (Table 5): ten A → G transitions, six large deletions, one insertion, and only two base-pair substitutions targeted to deoxguanosine, one being a G → T transversion and the other a G → C transition.

Taq polymerase is known to possess a relatively low fidelity, introducing errors, mainly A → G transitions at a rate of approximately 2 per 10^4 to 10^5 bases (Keohavong and Thilly 1989; Lundberg et al. 1991). Also, the Maloney murine leukemia virus reverse transcriptase used to synthesize cDNA has an error rate of about 3 per 10^5 bases (Roberts et al. 1988). In these experiments, 60 cycles of amplification by PCR were performed, which was more than sufficient to produce a visible product from just one copy of cDNA template. The finding that only 62% of the amplifications by PCR were successful indicates that each TG^r clone contained very little *hprt* messenger RNA (mRNA). Assuming that the first round of PCR started with a single copy of cDNA template, the intrinsic polymerase error rates could introduce mutations into as many as 16% of all amplified clones. This was below the frequency of base-pair substitutions that was detected (35%), but most of the identified base-pair substitutions were the type expected from *Taq* synthesis errors and not the type anticipated from a mutagen that forms adducts at G:C base pairs.

Table 4. Cloning Efficiency and TG^r Mutant Frequency, as a Function of Dose, in Spleen T Lymphocytes from F344 Rats Treated with Solvent, 1,6-Dinitropyrene, or Ethylnitrosourea

Treatment	Weeks After Treatment	Number of Assays	Cloning Efficiency[a] (%)	TG^r T Lymphocytes per 10^6 Cells[a]
Solvent	15	2	6.8 ± 0.1	4 ± 1
	21	4	7.9 ± 0.4	6 ± 1
	27	2	5.7 ± 1.1	4 ± 1
1,6-Dinitropyrene (μg)				
0.3	21	2	7.9 ± 0.5	8 ± 1
1	21	2	7.7 ± 0.2	16 ± 1
3	21	2	7.6 ± 0.3	21 ± 3
10	21	2	7.6 ± 0.5	34 ± 3
30	15	2	6.6 ± 0.4	37 ± 4
	21	2	7.9 ± 0.5	47 ± 2
	27	2	6.3 ± 0.6	52 ± 2
100	15	2	5.9 ± 0.3	69 ± 3
	21	2	7.3 ± 0.4	104 ± 8
	27	2	4.8 ± 0.4	92 ± 10
150	21	2	6.5 ± 0.4	74 ± 7
Ethylnitrosourea (40 mg)	21	1	6.6	214

[a] Data are presented as means ± SE. Each assay consisted of three 96-well microtiter plates; for cloning efficiency, target cells = 4/well; for TG^r lymphocytes, total target cells = 14.4×10^6.

To explore the possibility that polymerase infidelity was responsible for the base-pair substitutions, PCR was performed with *Pfu* DNA polymerase instead of *Taq*. *Pfu* is a thermostable DNA polymerase possessing proofreading activity and fidelity more than 10-fold higher than *Taq* (Lundberg et al. 1991). About 58% of 189 colonies amplified with *Pfu* produced a visible PCR product, and 3% of these amplified templates contained point mutations, with A → G transitions again being the major mutation detected (Table 6). The type of mutations produced by *Pfu* misincorporation has not been reported; however, the results indicate that the recovery of mutations was inversely related to the fidelity of the polymerase used for PCRs, and suggest that

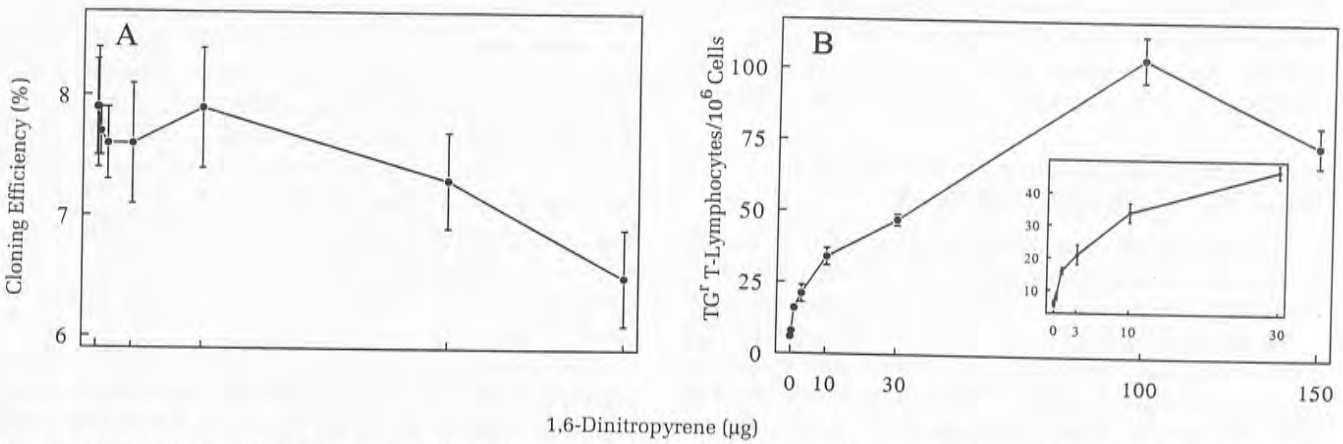

Figure 16. Cloning efficiency (A) and mutation induction (B) in spleen T lymphocytes as a function of dose. 1,6-Dinitropyrene (0, 0.3, 1, 3, 10, 30, 100, or 150 μg) was administered directly to the lungs of the rats, animals were killed 21 weeks after treatment, spleen T lymphocytes were isolated, and the cloning efficiency and induction of mutations at the *hprt* locus (as indicated by TG^r T lymphocytes) were determined. The data are presented as means of two to four assays (see Table 4), each consisting of three 96-well microtiter plates. The inset shows the dose-response relation for 0 to 30 μg of 1,6-dinitropyrene.

many of the base-pair substitution mutations found with the TG^r rat T lymphocyte clones were an artifact of the PCR procedure.

To confirm that TG^r T lymphocyte clones contain very little *hprt* mRNA, PCR was performed on cDNA transcribed from RNA extracted from several TG^r clones and from rat T lymphocytes that were freshly isolated and stimulated with phytohemagglutinin, but not cloned. With uncloned lymphocytes, 30 cycles of PCR were sufficient to produce a strong amplification product from 1×10^4 cells, although 60 cycles were required to detect an amplification product using 5 to 15×10^4 cells from TG^r clones. These results support the conclusion that cloned rat lymphocytes contain reduced levels of *hprt* mRNA.

LUNG TUMORIGENICITY OF 1,6-DINITROPYRENE

Although tumorigenesis was not an objective of this study, as part of the time-course mutagenicity experiment (Specific Aim 3, Experiment 1), one rat treated with 100 μg of 1,6-dinitropyrene developed labored breathing 32 weeks after treatment and was euthanized. This animal was diagnosed as having a large, well-differentiated squamous cell carcinoma of the lung. Two additional rats presented similar symptoms 51 weeks after treatment. Pathological analysis of one that had been administered 30 μg of 1,6-dinitropyrene indicated an undifferentiated carcinoma of the lung with no squamous differentiation present. The other, which had been treated with 100 μg of 1,6-dinitropyrene, had a very poorly differentiated carcinoma that was not associated with any organ; however, it contained caricatures of tubules that suggested it was from the lungs.

Additional rats treated with 1,6-dinitropyrene developed lung tumors during the course of this work, including 1 rat that died 96 weeks after treatment with 1 μg, 5 rats that died 51 to 98 weeks after treatment with 30 μg, 15 rats that died between 32 and 88 weeks after treatment with 100 μg, and 1 rat that died 51 weeks after treatment with 150 μg. These tumors were not subjected to histopathological analyses.

DNA ADDUCT FORMATION AND MUTATION INDUCTION WITH BENZO[*a*]PYRENE

In a preliminary experiment, 300 μg of [7,8-^{3}H]benzo[*a*]pyrene was implanted into the lungs of male F344 rats. One week after treatment, four rats were killed for DNA adduct determinations. DNA was isolated from lung and liver nuclei and the adduct levels were determined by liquid scintillation counting. The extent of binding to lung DNA was 1.48 ± 0.35 fmol adduct/μg DNA (mean ± SE; n = 4); the amount of radioactivity associated with liver nuclei DNA was too low for accurate quantification. Additional aliquots of DNA were analyzed by ^{32}P-postlabeling. Two major adducts were observed in the lung (Figure 17), both of which migrated further than the adduct standard prepared from reacting *anti*-benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide with DNA. This adduct standard has been characterized as 10β-(deoxyguanosin-N^2-yl)-7β,8α,9α-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (Culp and Beland 1994). When quantified against the DNA standard, the major adduct found in the largest amount in the lung was present at a concentration of 1.3 fmol/μg DNA, and the other adduct occurred at 0.6 fmol/μg DNA. Both of these adducts also were present in the liver, at approximately 0.05 fmol/μg DNA.

Five weeks after treatment, an additional four rats were killed to determine the mutation frequency at the *hprt* locus in spleen T lymphocytes. The cloning efficiency (6.0 ± 0.3%, mean ± SE; n = 4) was lower than that found in the control rats treated with solvent (8.2 ± 0.3%), but was similar to the cloning efficiency from rats treated in the same experiment with 100 μg of 1,6-dinitropyrene (6.2 ± 0.3%). Although the decreased cloning efficiency suggested a toxic response, the mutation frequency in the rats administered benzo[*a*]pyrene (8 ± 2 TG^r cells/10^6 T lymphocytes) did not differ significantly from the solvent-treated control group (4 ± 1 TG^r cells/10^6 T lymphocytes), and was substantially lower than that observed in rats administered 100 μg of 1,6-dinitropyrene (39 ± 2 TG^r cells/10^6 T lymphocytes) in the same experiment.

DISCUSSION

Iwagawa and associates (1989) demonstrated that the administration of 1,6-dinitropyrene, a component of diesel emissions, by the lung implantation method of Stanton and associates (1972) resulted in a dose-dependent induction of lung tumors in male F344 rats. Using their protocol, we initially implanted 30 μg of [4,5,9,10-^{3}H]1,6-dinitropyrene and monitored the loss of radioactivity from the lungs during a two-week period. Even though a beeswax pellet was clearly evident in the lungs of the rats upon euthanasia, the 1,6-dinitropyene had rapidly dissipated from the pellet so that by one day after treatment only about 10% of the initial radioactivity could be found in the lungs (Table 1). Sun and associates (1983) have noted a similar rapid clearance of 1-nitropyrene from the lungs of rats when it was coated on gallium oxide particles and administered by inhalation.

Consistent with the rapid elimination of the 1,6-dinitropyrene from the pellet were the observations that adduct formation with lung (Figures 3 and 6), white blood cell (Figure 9), spleen lymphocyte (Figure 10), and liver (Figure 11) DNA reached a maximal level one to seven days after treatment. Another indication of the rapid loss of 1,6-dini-

Table 5. Sequence Analysis of TG^r T Lymphocytes Induced by 1,6-Dinitropyrene After cDNA Was Amplified by PCR with *Taq* DNA Polymerase[a]

Colony Number	Location	Mutation	Amino Acid Change
DNP-1-2	279	A → G	None
	373	A → G	Phe → Leu
	475	A → G	Lys → Glu
DNP-1-4	318–384	Deletion of exon 4	
DNP-2-2A	111	A → G	None
DNP-2-2B	259–609	Deletion of exons 4, 5, 6, 7, and 8, and part of exon 3	
DNP-2-3	582	G → T	Asp → Glu
DNP-3-4B	163	A → G	Lys → Glu
	235/241–515/520	Deletion of exons 4, 5, and 6, and part of exons 3 and 7	
DNP-3-7	27/28	19 Base-pair insertion	(Possible splicing mutant)
DNP-4-5	318–384	Deletion of exon 4	
DNP-4-6	150	A → G	None
	284	A → G	Leu → Ser
	301	A → G	Cys → Gly
DNP-4-7	511	A → G	Ser → Arg
	143/146–229/231	Partial deletion of exon 3	
DNP-4-8B	318–384	Deletion of exon 4	
DNP-4-9	239	A → G	Phe → Ser
DNP-5-2	368	G → C	Ser → Opal

[a] RNA from TG^r T lymphocyte clones (50,000 to 150,000 cells) was isolated and reverse-transcribed into cDNA; the cDNA was amplified by PCR using *Taq* DNA polymerase, and the *hprt* protein-coding region was sequenced.

Table 6. Sequence Analysis of TG^r T Lymphocytes Induced by 1,6-Dinitropyrene After cDNA was Amplified by PCR with *Pfu* DNA Polymerase[a]

Colony Number	Location	Mutation	Amino Acid Change
DNP-12-7	319–385	Deletion of exon 4	
DNP-12-8	146	A → G	Leu → Pro
DNP-12-9	461/465	+ G	Frameshift
DNP-13-4	75	– T	Frameshift
DNP-16-6	29	A → G	Ileu → Pro
DNP-16-7	208	G → A	Gly → Arg

[a] RNA from TG^r T lymphocyte clones (50,000 to 150,000 cells) was isolated and reverse-transcribed into cDNA; the cDNA was amplified by PCR using *Pfu* DNA polymerase, and the *hprt* protein-coding region was sequenced.

tropyrene from the beeswax pellet was the observation that ^{3}H levels in the blood were highest one day after treatment and then decreased to background values by one month (Figure 8). Approximately 10% of the blood-borne radioactivity was soluble in organic solvents, and HPLC analysis of this radioactivity demonstrated the presence of 1-acetylamino-6-nitropyrene, 1-amino-6-nitropyrene, and 1,6-dinitropyrene, which indicated that 1,6-dinitropyrene was being metabolized by nitroreduction (Figure 18), a finding consistent with previous in vitro (Djurić et al. 1985, 1986, 1988; Heflich et al. 1986a,b) and in vivo (Heflich et al. 1986a) studies.

The presence of organic-soluble metabolites resulting from nitroreduction suggested that the remaining unidentified radioactivity in the blood might be due to the covalent binding of reactive metabolites formed by nitroreduction (e.g., 1-nitroso-6-nitropyrene) to hemoglobin. Similar types of metabolites from arylamine carcinogens, such as 4-aminobiphenyl (Bryant et al. 1987) and 2-acetylaminofluorene (Kinouchi et al. 1990), are known to bind readily to hemoglobin. Accordingly, blood samples were treated with base and acetylating agents in an attempt to hydrolyze and derivatize bound metabolites, but this procedure did not release additional radioactivity. Previous work has suggested that planar aromatic compounds have high noncovalent affinity for hemoglobin; for example, after base hydrolysis of adducted hemoglobin, 4-aminobiphenyl, but not 2-aminofluorene, could be extracted into organic solvents (Kinouchi et al. 1990). As with 2-aminofluorene, the extensive planar aromatic character of pyrene may have prevented the release of bound 1,6-dinitropyrene metabolites from hemoglobin upon hydrolysis with alkali.

In initial experiments, DNA was isolated from whole lungs; however, upon enzymatic hydrolysis only a small portion of the associated radioactivity appeared to be a covalently-bound DNA adduct. Likewise, poor agreement was noted between the extent of adduct formation as measured by ^{3}H incorporation into the lung DNA and that determined by ^{32}P-postlabeling (Table 2). The identity of this nonadducted radioactivity was not determined, but the interference could be removed upon preparation of lung nuclei before DNA isolation.

High-pressure liquid chromatography (Figure 4) and ^{32}P-postlabeling (Figure 5) analyses of the lung DNA indicated the formation of a major DNA adduct that had elution characteristics consistent with *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene. The identity of this adduct further supports the supposition that 1,6-dinitropyrene is metabolically activated by nitroreduction to, presumably, *N*-hydroxy-1-amino-6-nitropyene, and that this latter compound reacts with DNA, either directly or after subsequent activation (Figure 18).

As noted above, the maximum concentration of *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene in lung nuclei DNA occurred one to seven days after treatment (Figure 6). The adduct levels then decreased so that by one month after

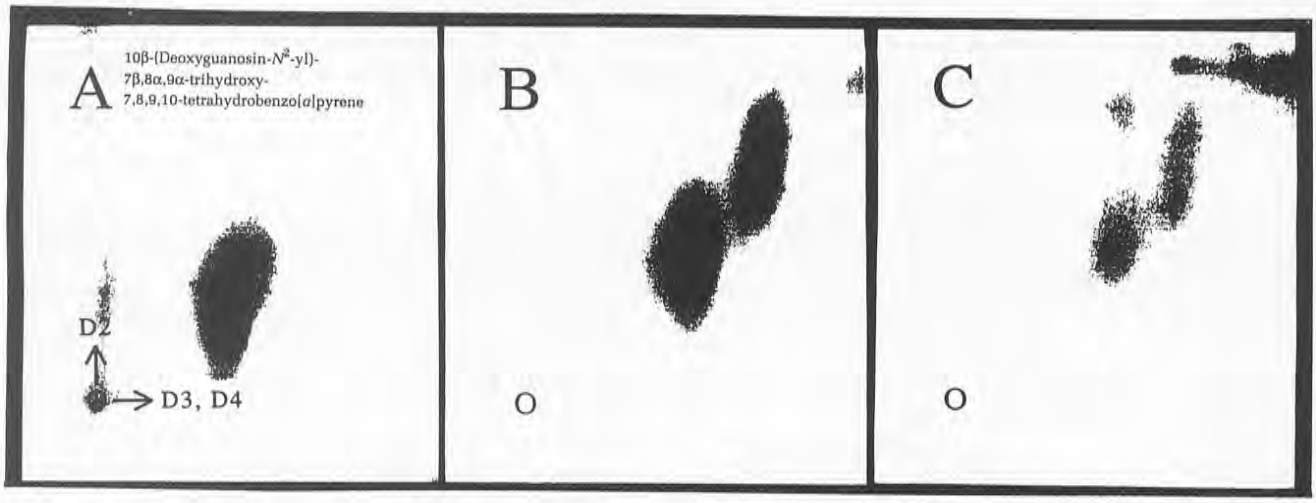

Figure 17. ^{32}P-Postlabeling autoradiographs of (A) DNA reacted with anti-benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide, (B) lung nuclei DNA, and (c) liver nuclei DNA of a male F344 rat that was treated with 300 μg (7,8-^{3}H]benzo[*a*]pyrene. The sensitivity of (A) and (B) has been attenuated five-fold compared with (C). The origin of the chromatogram is located at position "O". The direction of development of the chromatogram is designated by the arrows, and the composition of the solvent systems and the sequence in which they were used is signified by D2, D3, and D4 (see Materials and Methods section).

treatment, the values were 25% to 50% of the peak values. Although adduct measurements after this time were more limited, the adduct levels 12 weeks after treatment were similar to those detected at four weeks after treatment. This indicates that after the initial rapid removal of the majority of the DNA adducts, the remaining population is removed much more slowly. Similar adduct removal kinetics have been noted for a number of carcinogens, including benzidine (Martin et al. 1982) and benzo[*a*]pyrene (Nakayama et al. 1984), and have been studied most extensively with 2-acetylaminofluorene (Culp et al. 1993, and references cited therein). The available data indicate that certain nucleotide sequences may be resistant to DNA repair, which may explain the persistence observed for *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene in rat lung DNA.

Dose-response relations for the formation of *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene were examined by administering 0.3 μg to 150 μg of 1,6-dinitropyrene and measuring the adduct formation seven days after treatment. In the lung DNA (Figure 7), the extent of adduct formation increased linearly with doses up to 30 μg of 1,6-dinitropyrene. Beyond this dose, the adduct levels still increased, but the rate of increase was much lower than that observed at lower doses. Biphasic dose-response curves for adduct formation, with linear increases at low doses but proportionally smaller increases at higher doses, have been observed with other carcinogens, including *N,N*-diethylnitrosamine in male rat liver DNA (Boucheron et al. 1987), 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone in male rat lung DNA (Belinsky et al. 1990), and 4-aminobiphenyl in female mouse liver DNA (Beland et al. 1992). With these carcinogens, the decreased rates of adduct formation at higher doses have been attributed to metabolic saturation, or the induction of toxicity and cell proliferation, or both. In the lungs of rats treated with 1,6-dinitropyrene, both of these mechanisms could contribute to the biphasic response. Furthermore, if toxicity and cell proliferation occurred at higher doses, this could explain the difference in tumor incidence between 30 μg and 100 μg of 1,6-dinitropyrene even though the adduct levels with these two doses were similar. The kinetics of adduct formation also could differ among the cell types within the lung; for example, Bond and associates (1990a) have found more extensive DNA adduct formation in alveolar type II cells compared with whole lung tissue in rats administered diesel exhaust by inhalation.

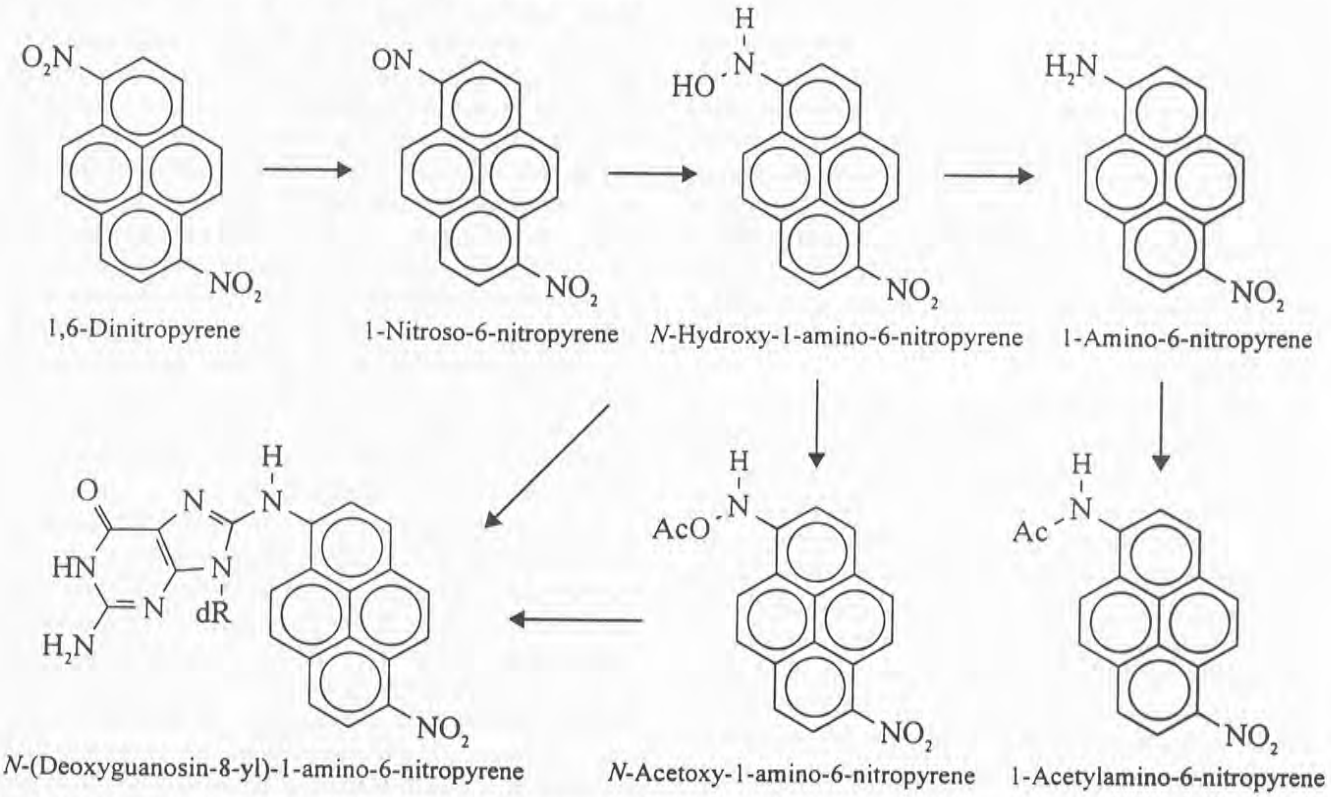

Figure 18. Metabolic pathways of 1,6-dinitropyrene. 1,6-Dinitropyrene undergoes nitroreduction to 1-nitroso-6-nitropyrene, *N*-hydroxy-1-amino-6-nitropyrene, and 1-amino-6-nitropyrene, wihch is acetylated to 1-acetyl-amino-6-nitropytrene. *N*-Hydroxy-1-amino-6-nitropyrene can react with DNA either directly or after subsequent esterification (e.g. *O*-acetylation).

The adduct formation of 1,6-dinitropyrene with DNA also was measured in nontarget (i.e., liver; Figure 11) and readily obtainable surrogate (i.e., white blood cells; Figure 9) tissues. Because mutations were assayed in spleen T lymphocytes, adduct levels were determined in spleen lymphocytes as well (Figure 10). With each of these tissues, the extent of adduct formation was approximately 10% of that found in the lung, with the same major adduct being detected. Maximal adduct formation occurred three to seven days after treatment; by one month after treatment, the extent of adduct formation had decreased to approximately 25% of the peak adduct levels.

In addition to determining the time course of adduct formation, dose-response relations were established in the liver and spleen lymphocytes. In liver, 1,6-dinitropyrene gave a biphasic dose-response, with a twofold increase in dose resulting in a twofold increase in adduct formation at doses up to 10 μg (Figure 13). At higher doses, the increase in adduct formation was still linear as a function of the dose, but the rate of increase was much lower. The biphasic response in the liver may be due to metabolic saturation or the induction of toxicity and cell proliferation, or both, although toxicity and cell proliferation are probably unlikely due to the low adduct levels detected. Clearance of the 1,6-dinitropyrene from the lungs also may be impaired at higher carcinogen doses, which would decrease the amount of compound available for forming adducts with liver DNA. In this regard, it should be noted that peak adduct levels in all tissues were typically detected at later times (i.e., seven days after treatment) with the 100-μg treatment, compared with the 30-μg treatment (Figures 6, 9, 10, and 11). Due to the relatively small amount of DNA that could be obtained from the spleen lymphocytes, coupled with the low extent of adduct formation, a complete dose-response relation curve could not be described for these cells (Figure 12).

A major goal of this project was to determine if treatment with 1,6-dinitropyrene would induce mutations in T lymphocytes. Due to the relatively small volume of blood that can be obtained from rats, plus the poor cloning ability of T lymphocytes obtained from rat peripheral blood, spleens were used as the source of the T lymphocytes. Initially, rats were treated with either 30 μg or 100 μg of 1,6-dinitropyrene and mutations were assayed at the *hprt* locus in spleen T lymphocytes for up to 51 weeks (Figure 15). Compared with control rats treated with solvent, 1,6-dinitropyrene induced a significant increase in mutation frequency, with the 100-μg dose typically producing approximately twofold more mutations than the 30-μg dose. With both doses, the mutation frequency increased until 21 weeks after treatment, remained constant until week 40, and then began to decrease; but almost one year after treatment, the mutation frequency in the rats treated with 1,6-dinitropyrene was still greater than in control animals.

In these experiments, ENU was used as a positive control. Although the maximal mutagenic response with ENU was approximately three times that observed with the highest dose of 1,6-dinitropyrene (Figure 15), it should be noted that, based on molar equivalence, approximately 1000-fold more ENU was administered. This emphasizes the potent mutagenicity of 1,6-dinitropyrene. Another difference between ENU and 1,6-dinitropyrene is that the maximal induction of TG^r T lymphocytes occurred after 6 weeks in rats administered ENU, but the maximal response with 1,6-dinitropyrene was detected 21 weeks after treatment. The reasons for this are not known but may be due to differences between the chemicals in the timing of mutation fixation or mutant clone expansion. In mice, mutations appear to occur primarily before the T lymphocytes migrate to the spleen (Jones et al. 1988; Walker and Skopek 1993). With rats (Aidoo et al. 1994) and humans (Nicklas et al. 1987; 1988; Albertini et al. 1990), however, it appears that most mutations are induced in mature T lymphocytes after they are processed in the thymus. This implies that, in this study, the DNA damage occurred in fully differentiated cells that were mainly in the G_0 phase of the cell cycle, and that the rate of cell division in this population was an important factor in the kinetics of mutation induction. With 1,6-dinitropyrene, DNA damage was relatively persistent and, therefore, new mutations may have been induced many weeks or months after the initial treatment. It also is possible that different chemical treatments may induce or inhibit the normal rate of cell division in T lymphocytes. Stimulating selected T lymphocytes to divide not only would allow the fixation of new mutations, but also might result in the expansion of preexisting mutations (Nicklas et al. 1988). Clonal expansion of *hprt* mutations has been noted with both the mouse (Skopek et al. 1992; Walker and Skopek 1993) and rat (Mittelstaedt and Heflich 1994) TG^r T lymphocyte systems.

The twofold difference in mutant frequency between 30 μg and 100 μg of 1,6-dinitropyrene correlated with the twofold difference in adduct concentration in these cells upon administration of the same doses. To investigate further dose-response relations for mutant induction, rats were treated with 0.3 μg to 150 μg of 1,6-dinitropyrene and the frequency of TG^r T lymphocytes was assayed 21 weeks after treatment. In this experiment, the induction of T lymphocyte mutants tended to increase with dose, but when compared with the increase in DNA adduct formation, the response was not linear at low doses of the car-

cinogen. The curvilinear response throughout the entire dose range may be a direct reflection of the extent of DNA adduct formation, which we have not been able to determine at low doses in spleen lymphocytes. In addition, these relations are undoubtedly complicated by the heterogeneous nature of the lymphocyte population; thus, overall adduct formation with T lymphocyte DNA may be linear, but the adduct levels or the effects of the adduct levels may be different for individual lymphocyte populations. Nonetheless, the results suggest that mutations are being induced by the 1,6-dinitropyrene treatment and that these are the direct result of the formation of *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene in the spleen lymphocyte DNA.

An interesting feature of the mutagenicity results was the apparent efficiency with which 1,6-dinitropyrene adducts induced TG^r mutations. In a previous study measuring mutations at the *hprt* locus in Chinese hamster ovary cells (Heflich et al. 1986b), 1 fmol *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene/μg DNA was associated with less than 30 mutants/10^6 cells. Several other compounds that form adducts with deoxyguanosine at the C-8 position produced a similar quantitative relation between adduct levels and mutation induction. Results from the present study, however, indicate that 1 fmol *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene/μg DNA is associated with approximately 100 TG^r cells/10^6 clonable T lymphocytes. This increased sensitivity in vivo may reflect the longer time the in vivo system had to fix and express mutations relative to the time available in an in vitro system, or it may reflect the complex cellular kinetics of rodent T lymphocytes. Whatever the cause, the data suggest that TG^r T lymphocyte mutations are extraordinarily sensitive indicators of DNA damage from 1,6-dinitropyrene.

Molecular analyses of the DNA alterations in the putative mutants were conducted to support the supposition that *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene was responsible for the mutation induction. These were not successful because the majority of the TG^r clones analyzed contained no mutations, and most of those that were detected occurred at deoxyadenosine rather than at deoxyguanosine as expected. The sequencing methodology used in these experiments was based upon reverse transcription of *hprt* mRNA, and it appeared that rat TG^r clones contained only very low levels of *hprt* mRNA. Furthermore, the *hprt* mRNA that was present seemed to be a mixture of wild-type and mutant sequences. Authentic mutant sequences were probably identified only in those clones that possessed splicing-site mutations, because these alterations would not result from polymerase errors and the PCR products from these templates would be sufficiently different in size from wild-type sequences to be isolated. Similar difficulties in analyzing mutations in TG^r clones from mouse T lymphocytes have been reported by Skopek and associates (1992). Recently, denaturing-gradient-gel electrophoresis and single-strand conformation polymorphism methods have been applied to identify the mutations induced by ENU in exon 8 of the rat *hprt* gene (Mittelstaedt and Heflich 1994). The results obtained are consistent with those previously published for mutations in the *hprt* gene of mice and monkeys (Harbach et al. 1992; Skopek et al. 1992), and suggest that this approach will be applicable for the analysis of the TG^r T lymphocytes induced by treatment with 1,6-dinitropyrene.

The doses selected for this study correspond to those used by Iwagawa and associates (1989) to induce lung tumors in male F344 rats. In their bioassay, 10 μg of 1,6-dinitropyrene induced a 13% incidence of lung tumors, and the incidence increased in a relatively linear manner to 85% at the 100-μg dose. At 150 μg of 1,6-dinitropyrene, the incidence decreased slightly, but this may reflect the fact that this treatment group contained very few animals. (Although tumorigenesis was not an objective of the project, we were able to confirm the potent lung tumorigenicity of 1,6-dinitropyrene.) Because DNA adduct levels were measured at the same doses used in the bioassay, it is possible to relate the extent of DNA adduct formation to the tumor incidence; such an analysis is shown in Figure 19A. These results indicate that a 50% incidence of lung tumors would be associated with a concentration of approximately 1.3 fmol *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene/μg DNA, a value similar to what has been observed for aflatoxin B_1 (Bechtel 1989). Interestingly, both this carcinogen and 1,6-dinitropyrene induce tumors in rats at very low concentrations. In our study, we also detected DNA adducts at dose levels (e.g., 3 μg of 1,6-dinitropyrene) at which Iwagawa and associates (1989) did not detect lung tumors. Assuming a linear relation between the DNA adduct levels and the induced tumor incidence, our data predict that 3 μg of 1,6-dinitropyrene should induce approximately a 5% incidence of lung tumors. Iwagawa and associates (1989) would have had to treat considerably more rats than the 30 animals they used to detect this level of tumor induction.

Also shown in Figure 19 is the relation between the mutant frequency at the *hprt* locus in spleen T lymphocytes and the tumor incidence (Figure 19B). As with DNA adducts (Figure 19A), the incidence of mutations was significantly elevated in the absence of a detectable increase in lung tumors, which, again, is probably a reflection of the relatively limited number of animals used in the tumorigenicity bioassay. A comparison of Figures 19A and 19B also suggests that T lymphocyte mutations may be a more sensitive marker for exposure to dinitropyrenes. This

concept is further reinforced by the fact that DNA adduct levels in surrogate tissues, such as white blood cells, were tenfold lower than those found in the target tissues, which would decrease the sensitivity of adduct measurements even further.

It has been reported that F344 rats exposed to a concentration of 7.1 mg diesel emissions/m^3 of air for 30 months (7 hours/day, 5 days/week) developed a 13% incidence of lung tumors (Mauderly et al. 1987). Assuming a respiratory rate of 210 mL/minute (Sun et al. 1983) and a concentration of 400 ng of 1,6-dinitropyrene/mg of diesel particles (International Agency for Research on Cancer 1989), the rats would have been exposed to 160 ng of 1,6-dinitropyrene during this treatment protocol. Although we detected increased levels of DNA adducts and T lymphocyte mutations when rats were administered approximately twice this amount of 1,6-dinitropyrene in a single dose, Iwagawa and associates (1989) administered 10 μg of 1,6-dinitropyrene in order to obtain a 13% tumor incidence. This suggests that 1,6-dinitropyrene is probably not solely responsible for the lung tumors induced in rats exposed to diesel emissions. Rosenkranz (1993) recently arrived at a similar conclusion.

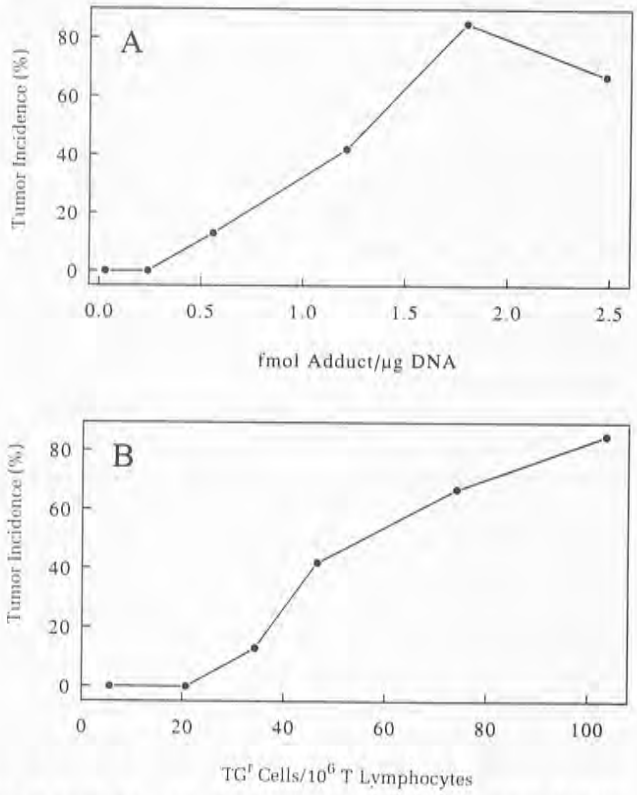

Figure 19. Lung tumor incidence in male F344 rats as a function of (A) the level of *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene in lung DNA and (B) the frequency of TG^r T lymphocytes. 1,6-Dinitropyrene (0, 3, 10, 30, 100, or 150 μg) was administered directly to the lungs of the rats. The lung tumor incidence is from Iwagawa and associates (1989) and was determined two years after treatment. In the current experiments, DNA adduct levels and the frequency of TG^r T lymphocytes were measured at seven days and 21 weeks after treatment, respectively.

Treatment-related DNA adducts have been reported in rats exposed to diesel exhaust (Wong et al. 1986; Bond et al. 1988, 1990a,b). The identities of these adducts are unknown; but other highly genotoxic compounds, such as benzo[*a*]pyrene, which are found at far greater concentrations in diesel particles than dinitropyrenes (International Agency for Research on Cancer 1989), could be responsible for the formation of these adducts. To investigate this possibility, a preliminary experiment was conducted in which rats were implanted with 300 μg of benzo[*a*]pyrene and then analyzed for DNA adducts and mutations. Two adducts were clearly evident by ^{32}P-postlabeling (Figure 17) and their levels (1.5 fmol/μg DNA) were similar to the level of *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene found after administering 100 μg 1,6-dinitropyrene. Because 100 μg of 1,6-dinitropyrene induces the same lung tumor incidence as 300 μg of benzo[*a*]pyrene (Iwagawa et al. 1989), this indicates that the DNA adducts formed by these two compounds have similar tumorigenic potency.

Neither of the adducts derived from benzo[*a*]pyrene appeared to be derived from the major adduct of benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide. Recently, Ross and associates (1990, 1991) have proposed that DNA adducts of benzo[*a*]pyrene detected in rat lung tissue arise from the metabolism of benzo[*a*]pyrene to 9-hydroxybenzo[*a*]pyrene. Additional studies will be necessary to determine if this metabolic pathway is responsible for the adducts we detected.

Although DNA adducts clearly were present in the lungs and livers of rats treated with benzo[*a*]pyrene, we observed only a twofold increase in TG^r T lymphocytes, which was not statistically significant compared with control animals treated with solvent. The low extent of mutation induction may be due to the failure to form DNA adducts in the T lymphocytes; however, in other work, we have detected DNA adducts and TG^r T lymphocytes with another PAH, 7,12-dimethylbenz[*a*]anthracene, albeit administered at a higher dose (Manjanatha et al. 1994). It also is possible that the kinetics of mutation induction may vary among compounds; for example, as noted above, the maximal mutagenic response with ENU occurred after six weeks, although with 1,6-dinitropyrene the maximal frequency was observed 21 weeks after treatment.

The 300-μg dose of benzo[*a*]pyrene used in this preliminary experiment approximated the total exposure to benzo[*a*]pyrene that would have occurred during a two-

year inhalation study of diesel exhaust in F334 rats (Mauderly et al. 1987). Because this dose of benzo[*a*]pyrene is clearly carcinogenic (Iwagawa et al. 1989), this suggests that the benzo[*a*]pyrene contained in the diesel emissions is much more likely to contribute to the observed lung tumorigenicity in rats than is 1,6-dinitropyrene. Nonetheless, although diesel exhaust–induced adducts that are suggestive of those derived from benzo[*a*]pyrene 7,8-dihydrodiol 9,10-epoxide have been detected in some experiments (Bond et al. 1988), this has not always been the case (Bond et al. 1990b). Moreover, in a comparative carcinogenicity study in which F344 rats were treated with either diesel exhaust or carbon black by inhalation, the tumor incidence from both types of exposures was similar (Mauderly et al. 1994). These results indicate that organic material associated with the exhaust particles is probably not solely responsible for the tumors observed in rat lungs, a conclusion also proposed by Rosenkranz (1993).

Although the benzo[*a*]pyrene and 1,6-dinitropyrene present in diesel emissions do not appear to be responsible for rat lung tumorigenicity, the situation for carcinogenesis in humans is less clear. Recently, exposure-related DNA adducts have been found in T lymphocytes from individuals with relatively high exposure to diesel exhaust (Hemminki et al. 1994), and other work has demonstrated that adduct levels in humans are many-fold higher than would be predicted from extrapolating data obtained in laboratory animals (Poirier and Beland 1992). For example, after exposure to nanogram amounts of 4-aminobiphenyl in cigarette smoke, DNA adducts derived from 4-aminobiphenyl were clearly detected in the urinary bladder, a target tissue for tobacco carcinogenesis (Talaska et al. 1991). In laboratory animals, substantially higher amounts of 4-aminobiphenyl and its DNA adducts appear to be required for bladder tumor induction (Poirier and Beland 1992). Individuals exposed to high levels of diesel emissions receive daily exposures of nanogram amounts of nitro-PAHs, such as 1,6-dinitropyrene, and microgram quantities of PAHs, such as benzo[*a*]pyrene. This suggests that it may be possible to detect DNA adducts indicative of 1,6-dinitropyrene, or benzo[*a*]pyrene, or both, as well as TG^r T lymphocytes resulting from these adducts, in exposed human populations.

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ABBREVIATIONS

cDNA	complementary DNA
dG-C8-ANP	*N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene
ENU	ethylnitrosourea
^{3}H	tritium
HPLC	high-pressure liquid chromatography
hprt	hypoxanthine-guanine phosphoribosyltransferase gene
hprt	hypoxanthine-guanine phosphoribosyltransferase enzyme
mRNA	messenger RNA
^{32}P	phosphorus-32
PAHs	polynuclear aromatic hydrocarbons
PCR	polymerase chain reaction
TG^{r}	6-thioguanine–resistant
TLC	thin-layer chromatography

INTRODUCTION

The environment contains a wide variety of pollutants generated by human activities, particularly in heavily industrialized and urbanized settings. The impact of most of these substances on human health is unknown because their toxicologic effects are not completely understood. Since 1983, the Health Effects Institute has conducted a multidisciplinary program to fund scientific research into how motor vehicle emissions, and their individual constituents, impact human health. The ultimate goal of HEI research is to provide regulators with the necessary information to conduct sound risk assessments for humans. Because a major weakness in most cancer risk assessments is a lack of information regarding human exposures to suspected and known carcinogens, a part of HEI's program has been directed toward developing better methods for assessing human exposure to pollutants and related compounds from mobile sources. The study described in this report was funded to address that need.

Among the pollutants of concern in mobile-source emissions are nitro-substituted polynuclear aromatic hydrocarbons (nitro-PAHs)*, many of which are known to be mutagenic to cells in culture, or carcinogenic to laboratory animals, or both (International Agency for Research on Cancer 1983, 1989). These compounds are ubiquitous in the environment and originate primarily from the incomplete combustion of organic materials, including gasoline, diesel fuel, and engine lubricants. Diesel engine exhaust is unique from other combustion emissions because it contains a preponderance of "direct-acting" bacterial mutagens; these are mutagens that do not require the addition of rat liver homogenate (S9) for the mutagenic process to occur (Huisingh et al. 1978; Pederson and Siak 1981). The high level of direct-acting mutagens is due primarily to the presence of nitropyrenes, foremost of which are 1,6-dinitropyrene (1,6-DNP) and 1,8-dinitropyrene (1,8-DNP) (Rosenkranz 1982; Nakagawa et al. 1983). Because diesel engine emissions contribute over two-thirds of the airborne elemental carbon particles (with diameters of less than 2 μm) in the Los Angeles area (Cass and Gray 1995), and because the dinitropyrenes are extraordinarily potent mutagens and carcinogens, these compounds may adversely affect human health (Rosenkranz and Mermelstein 1985; Tokiwa and Ohnishi 1986; International Agency for Research on Cancer 1989; Shirnamé-Moré 1995).

In the light of recent evidence that lung tumors induced in rats by diesel engine exhaust are largely attributable to the inorganic carbon particles (Mauderly et al. 1994; Heinrich et al. 1995; Nikula et al. 1995), the role for dinitropyrenes and other organic compounds in tumor induction has been questioned (Rosenkranz 1993). However, because we do not yet know how these findings in rats may relate to human health, it is premature to dismiss the role of dinitropyrenes as a possible public health problem. At the very least, information about 2the biologic effects of dinitropyrenes may lead to developing biomarkers to measure human environmental and occupational exposure to diesel emissions.

In 1988, HEI issued a general Request for Preliminary Applications, RFPA 88-2, "Health Effects of Automotive Emissions," which solicited applications for research that would use new techniques or investigate new mechanisms to study pollutants emitted from motor vehicles in urban environments.

In response to this RFPA, Dr. Frederick A. Beland of the University of Arkansas for Medical Sciences submitted an application to investigate the relation between the dose of 1,6-DNP, the DNA adducts formed in target (lung) and surrogate (lymphocytes, liver) tissues, and the types of mutations induced in T lymphocytes. His application was considered by the Health Research Committee in June 1989 and Dr. Beland was encouraged to submit a full proposal. His proposal, "Relation Between Administered Dose, DNA Adducts, and Mutation Induction in Vivo," was received in August 1989, and after minor revisions, was approved as a three-year study starting in January 1990. A six-month no-cost extension was approved by the Research Committee in December 1992 to permit the investigator to examine (a) whether other types of enzymes (polymerases) used to copy the DNA in the mutation assay were more accurate than the enzyme initially used, and (b) if the types of mutations found in spleen T lymphocytes at shorter intervals after treatment with 1,6-DNP were different from those found at longer intervals. Total expenditures for the study were $232,346. Dr. Beland's final report was received in June 1994. After discussion by the Health Review Committee, Dr. Beland revised his report, which was accepted for publication in April 1995. During the review of the Investigator's Report, the Review Committee and the investigator had an opportunity to exchange comments and to clarify issues in the Investigator's Report and in the Review Com-

* A list of abbreviations appears at the end of the Investigator's Report for your reference.

mittee's Commentary. This Commentary is intended to place the Investigator's Report in perspective as an aid to the sponsors of HEI and to the public.

REGULATORY BACKGROUND

Because of concerns about the potential health consequences of inhaling diesel engine exhaust, the U.S. Environmental Protection Agency (EPA) sets emission standards for diesel engines and vehicles under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1) of the Act directs the Administrator of the EPA to "prescribe (and from time to time revise) . . . standards applicable to the emissions of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Section 202(a)(3)(A)(i) of the Act, as amended by Section 201 of the 1990 Amendments, specifically directs the Administrator to set standards for the "emissions of hydrocarbons, carbon monoxide, oxides of nitrogen, and particulate matter from classes . . . of heavy-duty vehicles or engines."

The EPA has taken a variety of regulatory actions with respect to diesel engines and vehicles under the authority given it by Sections 202(a)(1) and 202(a)(3)(A)(i). For example, the EPA has set emission standards that were initially made applicable to all engines and vehicles produced in a given model year. Engines and vehicles of the same class that are produced in succeeding years also must comply with these existing standards unless the EPA establishes a new set of standards.

Determining appropriate emission standards for particles and hydrocarbons depends, in part, on assessing the health risks they present. That process requires a better understanding than currently exists of the levels and biologic effects of pollutants to which individuals are exposed. Because of the apparent preponderance of nitro-PAHs in diesel exhaust, measuring human exposure to nitro-PAHs may serve as a surrogate or indicator of exposure to diesel exhaust. Alternatively and preferably, a biomarker of an effect, such as actual damage to the genetic material (DNA) that could lead directly to deleterious health effects, might be measured (Harris 1991; Hemminki 1992; Lambert 1992). Dr. Beland's study was funded by HEI to develop such techniques or biomarkers that would be applicable to measuring individual exposures to and biologic effects from 1,6-DNP. Such research is essential to the informed regulatory decision-making required by the Clean Air Act.

SCIENTIFIC BACKGROUND

Nitro-PAHs are PAH derivatives that contain one or more nitro groups (NO_2) covalently bound to cyclic carbon atoms. They are present as environmental pollutants either from direct emissions from the combustion of fuels and other organic materials (International Agency for Research on Cancer 1989), or are formed in the atmosphere from the reaction of PAHs with hydroxyl radicals and nitrogen dioxide or with nitrate radicals (Pitts et al. 1978; Pitts 1987). Nitro-PAHs pose possible significant human health risks because some members of this group, such as certain dinitropyrenes, are highly mutagenic to cells in culture and carcinogenic to rodents (reviewed by Rosenkranz and Mermelstein 1985; Tokiwa and Ohnishi 1986; International Agency for Research on Cancer 1989).

OCCURRENCE OF 1,6-DINITROPYRENE AND ITS BIOLOGIC ACTIVITY

1,6-Dinitropyrene was found in ambient air particulate samples at concentrations of 0.004 to 0.1 μg/g of particles (Tokiwa et al. 1983; Gibson 1986). It occurred in extracts of particle emissions (1 to 2 μg 1,6-DNP/g particles) from a domestic kerosene heater, from manufactured gas and propane flames (Tokiwa et al. 1985), and in diesel engine emissions (0.4 to 1.2 μg/g particles) (Nakagawa et al. 1983; Paputa-Peck et al. 1983). Despite their relatively low concentrations, dinitropyrenes accounted for 20% to 50% of the total mutagenicity in extracts of diesel exhaust particles when tested in the *Salmonella* assay (Nakagawa et al. 1983; Schuetzle 1983).

Among the most potent bacterial mutagens known, 1,6- and 1,8-DNP typically are 2 to 4 orders of magnitude more mutagenic than mutagenic mononitropyrenes, such as 1-nitropyrene (Rosenkranz and Mermelstein 1985). 1,6-Dinitropyrene is highly genotoxic to rodent and human cells in culture, and induces mutations, DNA fragmentation, and chromosomal abnormalities (International Agency for Research on Cancer 1989; Sawada et al. 1991; Busby et al. 1994).

The carcinogenic activity of 1,6-DNP in rodents has been established in several studies. 1,6-Dinitropyrene induced sarcomas at the site of injection in rats and mice (Tokiwa et al. 1984; Ohgaki et al. 1985; Imaida et al. 1991). In general, these tumors developed rapidly (3 to 9 months) in animals given 1 to 5 mg of compound. Small quantities (0.003 to 0.15 mg) of 1,6-DNP implanted into the lungs of rats in a pellet dissolved in beeswax and tricaprylin caused an increase in lung tumors related to the level of dose (Iwagawa et al. 1989).

METABOLISM AND MECHANISMS OF MUTATION

A mechanism by which many chemical mutagens, such as PAHs and nitro-PAHs, damage DNA is by the activation of the compound to an electrophilic metabolite that can bind covalently with various nucleophilic sites in DNA and form adducts (Miller 1978; Williams and Weisburger 1991). The metabolic activation of 1,6-DNP occurs by nitroreduction of one of the nitro groups followed by *O*-acetylation, culminating in the formation of the adduct *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene (dG-C8-ANP) (reviewed by Beland and Marques 1994). This adduct has been detected in the liver, lung, kidney, mammary gland, and urinary bladder of rats treated with 1,6-DNP (Djurić et al. 1988).

DNA adducts contribute to carcinogenesis because they interfere with the normal replication of DNA (Harris 1991; Williams and Weisburger 1991). When DNA with adducts is replicated during cell division, mutations may be introduced as a result of a change in the nucleotide sequence of the newly synthesized DNA. When the DNA strands containing mutations are themselves replicated in further cycles of cell division, the genetic changes are made permanent and passed along to future generations of cells. Because cancer is a multistage process involving multiple mutational and nonmutational events, scientists often measure the ability of chemicals to cause mutations in cultured cells and laboratory animals as a possible indicator for predicting risk of cancer in humans (Harris 1991).

hprt MUTATION ASSAYS

Measuring mutations in vivo in laboratory animals has significant advantages over in vitro cell assays. Not only do in vivo assays take into account variations in the tissue distribution and metabolism of the chemical that occur within complex organisms, but if the assay is extended to human subjects, an individual's susceptibility to mutagens might be assessed and later correlated with the development of cancer, should it occur (Albertini et al. 1993).

Most mutation assays measure mutations only within a specified genetic locus (or gene) for which there is a simple technique that will select and quantify the mutant cells. Assays based upon measuring mutations in the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene fulfill this criterion and have been extensively employed in many laboratories (Albertini et al. 1993). The *hprt* gene contains the nucleotide sequence necessary to produce the hprt enzyme, a protein that aids the cell in utilizing biochemicals called purines. If the purine is a natural biochemical (for example, hypoxanthine or guanine), the hprt enzyme converts it into a nucleotide that can be incorporated into newly synthesized DNA when the cell divides. If the purine is a synthetic, toxic purine analogue (for example, 6-thioguanine), the hprt enzyme converts it into a nucleotide derivative that results in death or other toxic effects in the cell. This process is critical to the *hprt* mutation assay because the success of the assay lies in being able to count only the mutated cells. When mutations have destroyed the ability of the *hprt* gene to produce the hprt enzyme, the enzyme is not available in the mutated cells to convert 6-thioguanine into the toxic derivative that makes the cells unable to grow. Therefore, only the cells with *hprt* mutations, which lack the hprt enzyme, can grow and form visible colonies that can be counted in the presence of 6-thioguanine. Furthermore, because the hprt enzyme is not requred for the cell to grow in this assay, its absence in the mutated cells does not prevent them from growing and being counted.

Mutation assays can be performed with a number of different kinds of cells. However, T lymphocytes have several advantages over other cell types. First, these cells can be propagated in vitro to produce a visible colony from each mutant cell for counting purposes (Albertini et al. 1993). This clonal expansion also provides sufficient material for the mutations in each mutant cell to be identified. Second, because T lymphocytes circulate in the blood of adult animals, these cells could be exposed to mutagens present in any tissue. Thus, the mutagen does not have to be transported to a particular site within the living organism before mutations can be induced in the T lymphocytes. Third, because T lymphocytes can be readily obtained from blood samples, the need for procuring cells by biopsy or other invasive procedures is eliminated.

In vivo measurements of *hprt* mutations have been made in spleen T lymphocytes from mice and rats treated with *N*-ethyl-*N*-nitrosourea (Skopek et al. 1992; Aidoo et al. 1993) and from mice treated with ethylene oxide (Walker and Skopek 1993). In humans, increased *hprt* mutations in peripheral blood T lymphocytes have been attributed to cigarette smoking, cancer chemotherapeutic agents, ionizing radiation, PAHs, and 1,3-butadiene (Albertini et al. 1993; Perera et al. 1993; Ward et al. 1994).

Scientists can obtain additional information from mutation assays by analyzing the specific kinds of mutations that are induced. Molecular analysis of the DNA strands can be performed to determine the mutational spectrum, that is, the pattern of the locations and kinds of deviations from the normal nucleotide sequence that have occurred in the DNA

strand (Skopek et al. 1992; Albertini et al. 1993). This has potential value because the mutational spectrum may be unique for each kind or each class of chemical (Harris 1991; Lambert 1992). This knowledge may ultimately permit estimates of cancer risk to be made for individuals exposed to a number of chemicals present in complex mixtures in the environment.

RATIONALE FOR THE STUDY

In an earlier study funded by HEI, Beland (1991) demonstrated that when mice were exposed to a carcinogenic dose of 1-nitropyrene, the only DNA adducts detected in target and surrogate tissues were derived from very low levels of dinitropyrenes (including 1,6-DNP) that were present as contaminants in the batch of 1-nitropyrene used. This indicated that dinitropyrenes preferentially formed DNA adducts in mouse tissues and, therefore, might be better compounds to use as biomarkers of exposure and effect than 1-nitropyrene in other laboratory animals and humans.

Although much is known about the biologic activity and metabolism of 1,6-DNP, HEI wanted to determine whether 1,6-DNP caused functional alterations in DNA, such as the induction of mutations in vivo. The present study was undertaken to determine the relation between 1,6-DNP–DNA adducts in the rat lung (a target tissue) and adducts and mutations in rat lymphocytes, a surrogate tissue potentially applicable to human studies. In addition, HEI was interested in whether the types of mutations induced by 1,6-DNP were characterized by specific changes in the DNA sequence that might be used as a "fingerprint" to identify previous exposures to 1,6-DNP (and, therefore, to diesel exhaust) in humans. This is important because, unlike the measurement of DNA adducts, which are removed more or less quickly by various DNA repair processes following exposure, induced mutations are fixed in the cell and in its succeeding generations. The ability to measure and characterize mutations in cells would provide important information on cumulative exposures in humans.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

This study was designed to examine DNA adducts and mutations in target and surrogate tissues of rats under the same conditions in which a test chemical (1,6-DNP) had been shown to cause lung tumors in rats (Iwagawa et al. 1989). The experimental system involved injecting 1,6-DNP in a beeswax-tricaprylin solution into the lungs of male F344 rats.

Specifically, Dr. Beland had three major specific aims:

1. To determine the relations between the administered dose of 1,6-DNP (30 or 150 μg/rat) and the levels of DNA adducts in the lungs (target tissue) of rats up to 28 days after treatment;
2. To determine the relations between the dose of 1,6-DNP and the levels of DNA adducts in livers, peripheral white blood cells, and spleen lymphocytes (surrogate tissues) of rats up to 28 days after treatment; and
3. To ascertain whether 1,6-DNP treatment induced mutations at the *hprt* locus in rat spleen T lymphocytes up to 51 weeks after treatment; if mutations are found, to relate their numbers and types to the dose of 1,6-DNP, and compare these results to the DNA adduct levels found in target and surrogate tissues from experiments related to the first two objectives. Additional rats treated with the potent mutagen ethylnitrosourea (40 mg/rat administered intraperitoneally) served as positive control animals.

As amply documented in the Investigator's Report, Dr. Beland attained most of the study objectives. He demonstrated that 1,6-DNP migrated from the site of deposition in the lung, and defined the time course and the dose-response kinetics of the formation of the DNA adduct dG-C8-ANP in the lungs (Specific Aim 1), and in livers, peripheral white blood cells, and spleen lymphocytes (Specific Aim 2).

Toward Specific Aim 3, the investigator used a rat spleen T lymphocyte *hprt* assay, demonstrated that the mutagenicity of 1,6-DNP was a function of time and dose, and showed that the induced mutations were still present one year after treatment. The only portion of this objective that was not totally achieved was characterizing the induced mutations at the *hprt* locus. Part of the problem, as noted by the investigator, may have been the very low level of *hprt*-specific messenger RNA (mRNA) that was present in the spleen T lymphocytes. Furthermore, he expressed concern about the relative lack of fidelity of the *Taq* polymerase used in the polymerase chain reaction amplification step in that it may have introduced some errors into the amplified DNA. Indeed, the investigator carried out a series of experiments using a polymerase with higher fidelity (*Pfu*) to define the extent of the problem.

Other observations were made, relating to the yield of DNA adducts and the time course of mutation induction. In addition, preliminary experiments were carried out on

DNA adduct formation in the lungs and livers and on mutations induced in spleen T lymphocytes from rats implanted with a carcinogenic dose of benzo[*a*]pyrene.

METHODS AND STUDY DESIGN

This study is a logical extension of the work Dr. Beland and his associates have published on the metabolism and DNA adduct formation of dinitropyrenes. The choice of 1,6-DNP as a surrogate chemical for the nitro-PAHs present in diesel emissions is justified because it and a related isomer, 1,8-DNP, are the most mutagenic compounds present in diesel engine exhaust.

Adopting the rat lung implantation model is a major departure from the laboratory animal systems previously employed by the investigator. Studies by Iwagawa and associates (1989) with this model clearly demonstrated a linear relation between the dose of 1,6-DNP administered to the animals and the induction of lung tumors. Moreover, this intrapulmonary route of administration may be more relevant to the probable human exposure to environmental 1,6-DNP than studies that have used other routes of exposure.

Investigating the formation of DNA adducts from 1,6-DNP and measuring their persistence in target and nontarget or surrogate tissues was an appropriate approach. The metabolism of 1,6-DNP has been well characterized in a number of systems and, unlike 1-nitropyrene, 1,6-DNP appears to generate only a single DNA adduct (dG-C8-ANP). Furthermore, exquisitely sensitive methods are available to measure this adduct, including direct identification by high-pressure liquid chromatography, immunochemical analysis, and the ^{32}P-postlabeling technique if lesser amounts of adduct are present. Information on the time course of adduct formation and the level of adducts formed in response to various doses of compound could show a relation between the level of DNA adducts and the lung tumor incidence in the experiments of Iwagawa and associates (1989), as well as between the level of DNA adducts and the yield and kinetics of induced mutations. In addition, such information could help to interpret the basis of the time course by which induced mutations appear, that is, whether they arise from clonal expansion of mutations already present or from the continuing induction of mutations by persistent DNA adducts.

The technique for determining the frequency and type of mutations induced in T lymphocytes at the *hprt* locus in mice and humans has been worked out in a number of laboratories. However, because carcinogenicity studies of diesel emissions and 1,6-DNP had been performed in the rat, using the rat *hprt* mutation assay for 1,6-DNP was justified in this study.

STATISTICAL METHODS

In the following discussion, suggestions are made for a more rigorous statistical analysis of the investigator's results. They are offered because the quality of these data is high, and because the T lymphocyte mutation assay has applications to measuring mutations in humans. The suggested additional analyses would not alter the investigator's scientific conclusions.

Many of the data in this report are presented as dose-response and time-response curves. The investigator compared the effects of different doses of 1,6-DNP by performing sophisticated multiple *t* tests using the Bonferroni rule to control type I errors, and used the further refinement of the Satterthwaite formula, which suggests that the *t* tests were performed without assuming equal variance in the two groups being compared. However, any kind of *t* testing, regardless of the quality, does not extract all the information from the longitudinal nature of a dose-response or time-response curve. The investigator also employed a linear regression trend test to analyze these curves, but did not explain how this test for linear data applies to data that are essentially nonlinear. Linear regression was applied to the initial segment of each curve, but the fact that the DNA binding curves leveled out at higher doses or longer times was not analyzed quantitatively.

These data could support a more comprehensive analysis by curve-fitting approaches. Dose-response curves could be fitted by a simple binding model comprising both saturable and nonsaturable components to account for the observed shape of the curve. This process would yield interpretable parameters such as values for maximal binding and half-maximal dose. Time-course curves could be fitted by biexponential functions to give kinetic constants as parameters. Although it can be argued that curve-fitting is not necessary when the data are few and when the mechanisms underlying the binding are not well understood, these techniques still are useful. Time and rate constants are valuable even when no explicit compartmental structure is hypothesized, and curve-fitting is particularly useful when small sample sizes are involved because it pools random variability around the fitted curves and, therefore, provides more residual degrees of freedom, better precision, and more statistical power for comparing parameters across experimental groups. When curve-fitting may not be appropriate (as perhaps in Figures 14 and 15 in the Investigator's Report), then two-way analysis of variance (ANOVA), with

time and dose as the two factors, is an appropriate way to pool error and estimate relative response in the context of the whole data set.

The investigator stopped pooling preparations of lymphocytes from two animals because the quantity of lymphocytes sufficient to conduct the assay was obtainable from one rat. When possible, it is always preferable to perform statistical analyses upon samples from inidividual animals. Animals are never identical, and animal-to-animal variability should be measured and taken into account in analyses because it is likely to be a major source of variance in an experiment. It is also important to keep each source of variability separate (for example, repeated mutation assays within a time point, or replicate plates within an assay). Nested ANOVA would be more appropriate than *t* testing for these data analyses (even for comparisons within a single time point).

RESULTS AND INTERPRETATION

DNA Adducts

Preliminary experiments established that [^{3}H]1,6-DNP is lost from the lung at a rapid rate after implanting the carcinogen. Approximately 90% of the radiolabel was removed within 24 hours after treatment with little additional loss over the next 13 days. Whole blood levels of radioactivity were maximal one day after treatment (the first time point measured) and gradually declined to near zero after 28 days in rats given either 30 μg or 100 μg 1,6-DNP. No differences were noted in the blood concentrations nor the kinetics of loss of the radiolabel at these two doses.

Dr. Beland demonstrated in vivo formation of dG-C8-ANP, the adduct found in earlier in vitro and in vivo studies (Djurić et al. 1988), in time-course studies with DNA from the lungs, peripheral white blood cells, spleen lymphocytes, and livers of rats treated with 1,6-DNP. The highest adduct level was found in lung DNA, the site of eventual tumor formation, three to seven days after treatment depending upon the initial dose. Maximal adduct levels in DNA from peripheral white blood cells, spleen lymphocytes, and liver were generally similar to each other, but only about 10% to 15% of that measured in lung DNA.

Dr. Beland also studied the relation between dose of 1,6-DNP and adduct levels in tissue DNA. In lung DNA, a biphasic increase in adduct level was noted, with a relatively rapid increase during the first phase up to a dose of 30 μg [^{3}H]1,6-DNP, followed by a slower increase at higher doses. Similar kinetics were observed in liver DNA, except that the rapid first phase ended at a dose of 10 μg. DNA adducts in spleen lymphocytes increased only slightly between doses of 10 μg and 150 μg [^{3}H]1,6-DNP, but were not detected at doses lower than 10 μg. No dose studies were performed with peripheral white blood cells.

The investigator conducted an interesting preliminary experiment in which rats were implanted with a dose of the PAH benzo[*a*]pyrene (300 μg), which is known to be carcinogenic to the rat lung in this procedure. Two uncharacterized DNA adducts were measured 7 days after treatment in lung and liver DNA. Other investigators also have found uncharacterized benzo[*a*]pyrene-DNA adducts in vivo (Ross et al. 1990; Nesnow et al. 1993). Peripheral white blood cells and spleen lymphocytes were not examined for DNA adducts. When rats were treated with a dose of either benzo[*a*]pyrene or 1,6-DNP that induced the same incidence of lung tumors, similar amounts of lung DNA adducts were found in both cases.

hprt Mutation Assay

Dr. Beland and colleagues demonstrated that dose- and time-dependent increases in *hprt* mutations in rat spleen T lymphocytes reached a maximal level 21 to 27 weeks after treatment with 1,6-DNP. A detailed study of the effect of 1,6-DNP dose at a single time point (21 weeks) demonstrated that all doses above 0.3 μg produced significant numbers of mutations that ranged to levels 20 times higher than comparable control values. A significant observation made in this study was that a second wave of mutations was induced 12 weeks after treatment. This might be explained by persistent 1,6-DNP–DNA adducts, shown to be present in spleen lymphocytes, continuing to induce the mutations.

In a preliminary study, no mutations were induced in spleen T lymphocytes from animals treated with a single 300-μg dose of benzo[*a*]pyrene, an amount known to be carcinogenic to the rat lung.

In summary, the investigator established unambiguously (and probably for the first time in any species) that 1,6-DNP is a somatic mutagen in rats. Moreover, these mutations persisted for at least one year and were induced under experimental conditions in which lung tumors were known to develop.

DNA Sequence Analysis of *hprt* Mutants

The investigator conducted a sequence analysis of the *hprt*-coding region of DNA to identify the specific nature of the mutations in spleen T lymphocytes. The sequencing was performed on complementary DNA (cDNA). First, the cDNA was transcribed from *hprt* mRNA isolated from clones of the mutant (6-thioguanine–resistant) T lymphocytes; then, the cDNA was amplified by *Taq* DNA polymerase to provide sufficient amounts of identical DNA

strands for analysis. Only 60% of the clones from two experiments provided enough DNA to sequence, which was one indication that very little *hprt* mRNA was present in the mutant cells. The investigator had problems analyzing the mutations because, of the 19 different sequence changes noted using the *Taq* DNA polymerase, only 2 were base substitutions at G sites in the DNA, which is the type of mutation expected to result when dG-C8-ANP forms from 1,6-DNP. Moreover, 10 of the sequence changes were A-to-G transitions, a type of mutation known to be induced by the occasional errors introduced into the DNA by amplifying it with *Taq* DNA polymerase. There was some indication that these A-to-G mutations might well correspond to *Taq* error. The investigator stated that the DNA amplified from the mutant clones contained a mixture of both mutant and normal (wild-type) sequences. Therefore, a DNA sequence amplified from the cloned cells would correspond to either a mutant or a wild-type form of the *hprt* gene. On the other hand, if mutant sequences were erroneously produced by the *Taq* polymerase amplification, a significant fraction of the amplified DNA would contain these mutations, depending on when in the amplification cycle the error was introduced.

In an attempt to clarify this problem, the investigator performed another sequence analysis experiment using *Pfu* DNA polymerase, an enzyme that is 10 times less likely to introduce errors than the *Taq* polymerase. Only six sequence changes were found in a series of new mutant clones that were not previously characterized by *Taq*. None of these mutations were base substitutions at G sites, although several A-to-G mutations were again detected with *Pfu*. These results did not clarify whether these mutations were produced by the test chemical or by the amplification procedure because the types of errors induced by *Pfu* are not known and may well be similar to those induced by *Taq*.

One possibility to address this problem would be to use another DNA polymerase, such as Sequenase, which is known to produce mostly G-to-A transition errors. A second possibility is that genomic DNA (rather than cDNA transcribed from *hprt* mRNA) from the same clones that had previously been characterized by *Taq* to contain an A-to-G mutation could be analyzed with Sequenase to see whether the results observed with *Taq* could be reproduced. An affirmative result would indicate that the A-to-G mutations preexisted at the DNA level and were not artifacts produced by the *Taq* polymerase amplification. Identifying the source of the A-to-G mutations is germane to resolving the problem of mutant sequences in amplified DNA because it has been suggested that 1,6-DNP could induce mutations involving A sites in addition to G sites.

Finally, rather than indirectly analyzing cDNA obtained from transcribing the mutant cell mRNA, the investigator could directly analyze DNA from clones of the mutant cells to resolve the problem of characterizing mutations. The DNA could be amplified using the polymerase chain reaction, then screened for mutant alleles with other techniques, such as single-strand conformational polymorphism or denaturing gradient gel electrophoresis, before sequence analysis.

IMPLICATIONS FOR FUTURE RESEARCH

The investigator suggests that because the rat T lymphocyte *hprt* mutation assay is sensitive to 1,6-DNP, a similar assay with human T lymphocytes may hold promise as a biomarker of exposure to environmental mixtures containing 1,6-DNP. This development would be welcome for several reasons, among which is that this assay could provide an answer to the question of whether the 1,6-DNP in diesel emissions is "bioavailable" to humans and therefore capable of inducing such mutations. First, however, it would have to be established how sensitive the human assay would be relative to the rat *hprt* assay. It should also be noted that the investigator measured *hprt* mutations in spleen T lymphocytes, rather than the peripheral blood T lymphocytes, of rats because sufficient quantities of T lymphocytes could not be obtained from rat blood. Future epidemiologic monitoring studies with humans, of course, would be restricted primarily to samples of peripheral blood T lymphocytes. Therefore, it would be important to define in rat studies the relation, if any, between mutation induction in spleen and peripheral blood lymphocytes. Moreover, the demonstration of the in vivo mutagenicity of 1,6-DNP toward the *hprt* gene implies that 1,6-DNP may also have such a mutagenic effect on genes more relevant to the carcinogenic process such as proto-oncogenes or tumor suppressor genes.

In order to be able to use *hprt* mutations as a biomarker of 1,6-DNP exposure, it would also be necessary to determine whether 1,6-DNP leaves a specific "fingerprint" with respect to the location and nature of the induced mutations in the DNA strand. This would require solving some of the problems encountered by Dr. Beland in characterizing the mutational spectrum (see comments included in the Results and Interpretation section under Technical Evaluation). Knowledge of the mutational spectrum will greatly aid in evaluating the mechanisms of mutagenicity and carcinogenicity of 1,6-DNP.

It is necessary to resolve the question of whether the rat T lymphocyte *hprt* mutation assay is sufficiently sensitive to respond to environmental levels of 1,6-DNP. Dr. Beland calculated that, under the conditions of exposure to diesel engine exhaust (7.1 mg/m^3 for 7 hours per day, 5 days per week for up to 30 months) in a rat carcinogenicity study (Mauderly et al. 1987), he would have expected to see DNA adducts and induced *hprt* mutations in spleen T lymphocytes if all of the cumulative dose of 1,6-DNP was bioavailable. Although this level of exposure to diesel emissions is too high to be considered an environmental dose, it would be instructive to determine whether the rats exposed to these carcinogenic levels of diesel particles would indeed exhibit *hprt* mutations in spleen T lymphocytes. (It should be noted that under the conditions of this and similar rat carcinogenicity studies, the carbonaceous core of the inhaled particles appeared to be responsible for the rat lung tumors, and not the organic compounds adsorbed to the core [Mauderly et al. 1994; Heinrich et al. 1995; Nikula et al. 1995].) Of course, any mutations observed may or may not be due to 1,6-DNP. Indeed, Dr. Beland presented calculations indicating that there was not sufficient 1,6-DNP present in the diesel emissions to account for the number of lung tumors observed, which was the conclusion reached earlier by Rosenkranz (1993). In fact, Dr. Beland suggests that benzo[*a*]pyrene, also present in diesel emissions, is more likely than 1,6-DNP to contribute to the lung tumorigenicity observed in animals exposed to diesel exhaust.

An observation relevant to assessing the usefulness of *hprt* mutations in T lymphocytes as a biomarker of exposure to 1,6-DNP is the fact that significant numbers of mutations are induced by doses of 1,6-DNP (1 μg) that do not produce lung tumors. Whereas the carcinogenicity assay may not detect tumors in small numbers of animals, a necessary and expected feature of a biomarker assay would be the capability to detect an increase in mutations at carcinogen exposure levels below those that produce tumors and before tumors appear. When the level of DNA adducts formed in the present study was compared with the results from the carcinogenicity study performed by Iwagawa and associates (1989) under the same experimental conditions, a 50% incidence of lung tumors corresponded to an approximate level of 1.3 fmol dG-C8-ANP/μg lung DNA. This is approximately 100-fold less DNA adduct than has been found for some other carcinogens, such as 4-aminobiphenyl, and about 3-fold less than has been found for aflatoxin B_1, a known human carcinogen (Poirier and Beland 1992; Beland and Poirier 1994).

Furthermore, 1,6-DNP was more potent as an in vivo mutagen in rat spleen T lymphocytes than the positive control mutagen, ethylnitrosourea. 1,6-Dinitropyrene appeared to be three times more effective in inducing mutations in vivo (that is, less chemical produced more mutations) than has been reported by the same group of investigators in earlier studies with 1,6-DNP in vitro (Heflich et al. 1986). This finding has significant implications for the practice of deriving risk estimates from information obtained with cells in culture rather than with intact animals. Taken together, these data reinforce the unusually potent mutagenicity of 1,6-DNP that has been consistently noted since this chemical (and the related 1,8-DNP) was reported as an environmental pollutant.

CONCLUSIONS

The primary conclusion from this study is that 1,6-DNP is mutagenic in vivo in an animal tissue (rat spleen T lymphocytes) remote from the site of deposition (lung) at which this compound induced tumors. This finding supports the possibility that readily obtainable tissues (such as T lymphocytes from blood samples) could serve as a dosimeter to measure mutations from exposure to environmental pollutants, thereby avoiding the difficulties associated with sampling the internal tissues considered to be at risk of tumor development. Prior to this study, none of the highly carcinogenic dinitropyrenes had been proven to induce somatic mutations in vivo. For the *hprt* mutation assay to be a successful biomarker of human exposure to diesel exhaust, the methods used to characterize the mutational spectrum of 1,6-DNP in treated animals must be improved. In addition, a search for such mutations in exposed human populations could help establish a better estimate of the cancer risk for humans.

Dr. Beland provided further evidence for the extraordinary carcinogenic and mutagenic potency of 1,6-DNP by clearly establishing the high efficiency in which low levels of dG-C8-ANP were translated into high yields of mutations. He found that 1 fmol of dG-C8-ANP in spleen lymphocyte DNA was associated with the induction of approximately 100 mutant clones per 10^6 clonable cells, and that a similar level of dG-C8-ANP (1.3 fmol) in lung DNA was associated with a lung tumor incidence of 50%. These data complement Dr. Beland's earlier study in which extremely low concentrations (0.02% to 0.15%) of contaminating dinitropyrenes were apparently exclusively metabolized

to DNA adducts in vivo in target tissues of rats and mice treated with a carcinogenic dose of 1-nitropyrene (Smith et al. 1990; Beland 1991).

Dr. Beland's results suggest that his continued efforts to characterize the mutational spectrum of 1,6-DNP in the rat T lymphocyte *hprt* mutation assay will, if successful, provide a good foundation to extend the assay to measuring mutations in peripheral blood T lymphocytes in humans. This promises to form the basis of measuring exposure of humans to 1,6-DNP and, eventually, to other constituents of motor vehicle emissions.

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