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Health Effects of Subchronic Exposure to Diesel–Water Emulsion Emission

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The U.S. Environmental Protection Agency (EPA) National Ambient Air Quality Standards for ozone and particulate matter are requiring urban nonattainment areas to implement pollution-reduction strategies for anthropogenic source emissions. A type of fuel shown to decrease combustion emissions components versus traditional diesel fuels is the diesel–water emulsion. The Lubrizol Corporation in conjunction with Lovelace Respiratory Research Institute and several

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subcontracting laboratories recently conducted a rodent health assessment of inhaled combustion emissions of PuriNO$_x$ diesel fuel emulsion. Combustion emissions from either of two 2001 model Cummins 5.9-L ISB engines were diluted with charcoal-filtered air to exposure concentrations of 100, 200, and 400 μg total particulate matter/m^3. The engines were operated on a continuously repeating, heavy-duty certification cycle (U.S. Code of Federal Regulations, Title 40, Chapter I) using Rotella-T 15W-40 engine oil. Nitrogen oxide and particulate matter were reduced when engines were operated on PuriNO$_x$ versus California Air Resources Board diesel fuel under these conditions. Male and female F344 rats were housed in Hazleton H2000 exposure chambers and exposed to exhaust atmospheres 6 h/day, 5 days/wk for the first 11 wk and 7 days/wk threafter. Exposures ranged from 58 to 70 days, depending on the treatment group. Indicators of general toxicity (body weight, organ weight, clinical pathology, and histopathology), neurotoxicity (glial fibrillary acidic protein assay), genotoxicity (Ames assay, micronucleus, sister chromatid exchange), and reproduction and development were measured. Overall, effects observed were mild. Emulsion combustion emissions were not associated with neurotoxicity, reproductive/developmental toxicity, or in vivo genotoxicity. Small decreases in serum cholesterol and small increases in platelet values in some groups of exposed animals were observed. Particulate matter accumulation within alveolar macrophages was evident in all exposure groups. These findings are consistent with normal physiological responses to particle inhalation. Other statistically significant effects were present in some measured parameters of other exposed groups but were not clearly attributed to emissions exposure. Positive mutagenic responses in several strains of *Salmonella typhimurium* were observed subsequent to treatment with emulsion emissions subfractions. Based on the cholesterol and platelet results, it can be concluded that the 100-μg/m^3 exposure level was the no-observed-effect level. In general, biological findings in diesel emulsion emission-exposed animals and bacteria were consistent with exposure to petroleum diesel exhaust in the F344 rat and Ames assays.

Variations in ambient concentrations of ozone and particulate matter (PM) in urban areas have been associated statistically with concurrent variations in adverse respiratory and cardiovascular health outcomes in populations (Dockery et al., 1993; Pope et al., 1995, 2004; Samet et al., 2000; Dominici et al., 2002; U.S. EPA, 1996, 2004a). To address this problem, the U.S. Environmental Protection Agency (EPA) has tightened the National Ambient Air Quality Standards for ozone and PM (U.S. EPA, 1996, 2004a). Although many sources of air contaminants contribute to ozone and PM levels, mobile source emissions are significant contributors in most areas. As part of a strategy to reduce all on-road and off-road mobile source emissions, the U.S. EPA and California have set increasingly stringent standards for diesel emissions (CA EPA, 1998; U.S. EPA, 2002, 2004b, 2004c, 2004d). In response, diesel engine manufacturers have implemented numerous technological advances in engine design and exhaust after-treatment, and sulfur has been reduced in diesel fuel. These changes have led to 80% reductions in emissions of PM and 80%–90% reductions in emissions of nitrogen oxides (a key precursor for ozone formation) from new diesel vehicles during the past decade (Langer et al., 2000; Liedtke, 2004).

Many older diesel-powered vehicles are still in use, due to the long service life of diesel engines; thus, reducing emissions from the entire diesel fleet requires technologies that can readily be applied to older vehicles. One solution is for national, state, and local governments to require mobile on-road and off-road vehicles to employ "retrofit" emission reduction technologies (U.S. EPA, 2004b, 2004c, 2004d). Most of these technologies are designed to treat emissions downstream of the combustion chamber. Although after-treatment devices such as PM traps have been shown to decrease adverse health indices in animal studies, these and other mechanical technologies require the user to modify the existing vehicle/exhaust system (Langer et al., 2000; McDonald et al., 2004a). An alternative approach is to burn alternative diesel fuels that, by design, reduce emissions versus petroleum diesel (Park et al., 2001; Langer et al., 2000; Barnes et al., 2000). One type of fuel shown to reduce NO_x and PM emissions versus traditional diesel fuels is the diesel–water emulsion (Park et al., 2001; Langer et al., 2000; Barnes et al., 2000). The presence of water in these fuel blends (1) decreases PM precursors in the premix burn phase and (2) lowers the peak combustion temperature in the engine to decrease the formation of NO_x (Langer et al., 2000).

PM and NO_x are clearly reduced when vehicles are operated on these fuels (Barnes et al., 2000). However, given that the emulsion formulation alters combustion and requires the presence of emulsifiers and other chemicals components, there is a need to confirm that the new fuel does not present new health hazards (U.S. EPA, 1999).

Currently, there is a void of toxicity data on emissions from combustion of diesel–water emulsion in comparison to 100% petroleum diesel fuels. As interest in the use of diesel–water fuel blends builds within the United States and abroad, there is a need for a broader health assessment of the emission hazards of this technology. This study was undertaken to fulfill the U.S. EPA Tier II requirements for registration of alternative diesel fuels under Section 211(b) of the Clean Air Act as amended in 1999, and conducted in concordance with the guidelines outlined in

TABLE 1
Experimental design

Treatment group[a]	Males (subgroup)[d]						Females (subgroup)[d]					
	GST	GSTR	SH/NTX	DART	MN/SCE/GFAP	Total	GST	GSTR	SH/NTX	DART	MN/SCE/GFAP	Total
Clean air control	10	10	5	10	5	40	10	10	5	30	5	60
Low	10	—	5	10	5	30	10	—	5	30	5	50
Mid	10	—	5	10	5	30	10	—	5	30	5	50
High	10	10	5	10	5	40	10	10	5	30	5	60
CP[b]	—	—	—	—	10	10	—	—	—	—	10	10
GFAP PC[c]	—	—	—	—	5	5	—	—	—	—	5	5
Total	40	20	20	40	35	155	40	20	20	120	35	235

Note. GST, general subchronic toxicity subgroup; GSTR, general subchronic toxicity recovery subgroup (30 days post exposure); SH/NTX, special histopathology/neurotoxicity subgroup; DART, developmental and reproductive toxicity subgroup; MN/SCE/GFAP, micronucleus/sister chromatid exchange/glial fibrillary acid protein subgroup.

[a] Treatment group denotes clean-air control, low-, mid-, or high-concentration level, or positive control treatments.

[b] Cyclophosphamide treatment by ip injection to provide positive controls for MN and SCE assays.

[c] Positive control trimethyltin hudroxide-10.

[d] An additional 22 rats (11/sex) served as sentinel animals for microbiological screening.

the U.S. Code of Federal Regulations (CFR) 79 F. The emissions tested are from a commercially available diesel-emulsion fuel blend (PuriNOx Summer Fuel Blend) developed by the The Lubrizol Corporation. This study was conducted as a requirement for registration of the fuel in the United States.

MATERIALS AND METHODS

GLP Guidelines

All work was conducted and completed under the U.S. EPA Good Laboratory Practice (GLP) regulations consistent with those described in U.S. EPA 79.60, Good Laboratory Practices Standards for Inhalation Exposure Health Effects Testing.

Experimental Subgroups

Animals were assigned to each of four blocks that corresponded to specific health assays (Table 1), special histology and neurotoxicity (SH/NTX), micronucleus, sister chromatid exchange, and glial fibrilary acidic protein (MN/SCE/GFAP) assays, general subchronic toxicity and general subchronic toxicity and recovery (GST/GSTR) assessments, and development and reproductive toxicity (DART) assays. Blocks started exposure on a staggered schedule over approximately 3 wk to facilitate health assessment. Specific health assays measured within each block are presented in Table 2.

Animals and Housing

Four hundred and twelve 6- to 8-wk-old CDF (F344)/CrlBR rats (Charles River Laboratories, Inc., Raleigh, NC) were received, quarantined, and conditioned prior to study start. Rats were conditioned while quarantined in wire cages in stainless-steel inhalation chamber basket units (Hazleton H2000, Lab Products, Maywood, NJ). Animals were housed in chambers throughout the study except those animals assigned to the GSTR subgroup (during recovery), DART breeding pairs (daily after the 6-h exposure during mating), and DART females during gestation days 15–20. The latter subgroups of animals were housed in polycarbonate shoebox cages during the specified times.

TABLE 2
Health evaluations

- GST/GSTR (general subchronic toxicity/general subchronic toxicity recovery)
  - Ophthalmology
  - Hematology and clinical chemistry
    - Preexposure
    - After 30 days of exposure
    - Study termination
  - Gross necropsy
  - General histopathology
  - Postexposure recovery
- DART (development and reproductive toxicity)
  - Fertility, reproductive toxicity, teratology
- MN/SCE/GFAP
  - Micronucleus (MN)
  - Glial fibrillary protein (GFAP)
  - Sister chromatid exchange (SCE)
- SH/NTX (special histology/neurotoxicity)
  - Histology of brain and peripheral nerve
  - Histology of reproductive organs
  - Histology of lungs and lesions
  - Particle distribution in lungs

Chambers were serviced daily and washed weekly, and chamber baskets were rotated one position weekly to facilitate equivalent exposure among all groups of animals. Chamber temperature, humidity, flow, pressure, and oxygen were monitored throughout exposure. Room lighting was on a 12-h/day cycle from approximately 6 a.m. to 6 p.m. Unlimited tap water was available ad libitum. Rats were fed (Teklad certified rodent diet (W), Harlan Teklad, Madison, WI) ad libitum except during the daily 6-h exposure period and during the periods of fasting prior to necropsy (GST/GSTR).

Prior to exposure, animals were weighed and randomly assigned to a group by a computerized data acquisition system (Path/Tox; Xybion, Cedar Knolls, NJ) and identified by tail tattoo. Blood from sentinel animals was drawn for serological analysis prior to study start, at the end of the exposure period, and at the end of the recovery period. The serum was analyzed for the presence of antigens to common rodent pathogens. These included cilia-associated respiratory bacillus, Kilham rat virus (KRV), H-1 virus (H-1), lymphocytic choriomeningitis virus (LCM), *Mycoplasm pulmonis* (*M. pul.*), parvovirus (KRV, RPV), pneumonia virus of mice (PVM), rat coronavirus/sialodacryoadenitis virus (RCV/SDA), reovirus (Reo 1, 2, 3), and Sendai virus (Sendai). Tested rats were negative for all pathogens.

Exposures

Animals were exposed to diesel emulsion emissions (DEE) 6 h/day, 5 days/wk except during the mating and gestation period, when all animals were exposed 7 days/wk. No more than 2 sequential nonexposure days (e.g., weekends) occurred during the study, and no exposure week consisted of fewer than 4 exposure days. Due to the staggered-start study, combined with the in-study implementation of 7-day/wk exposures, groups were exposed for variable times. Study day "1" was considered to be the start of DEE exposure for each subgroup. Total exposure days for each subgroup were as follows:

	SH/NTX	MN/SCE/ GFAP	GST/ GSTR	DART
Total number of exposure days	66/Sex	68/Sex	68/Sex	Males 70; Females 58–69

The exposures included four treatment groups: three dilutions of engine exhaust and clean air (control). Target DEE exposures were set based on PM at 100 (low), 200 (mid), and 400 (high) μg PM/m^3. These PM concentrations were spaced evenly to facilitate exposure response assessment. The high exposure level was chosen as the maximal achievable concentration based on the maximal allowable chamber temperature for animals.

Generation of Emissions

DEE were generated alternately from two 2001 model 5.9-L Cummins ISB turbo diesel engines using diesel–water emulsion fuel (PuriNO$_x$ Summer Fuel Blend, The Lubrizol Corporation, Wickliffe, OH) and 15W-40 lubrication oil (Rotella T, Shell, Houston, TX). Crankcase oil and filters (Fleetguard LF3349, Cummins, Columbus, IN) were changed after every 200 h of engine operation. The intake was from the clean air source used for control exposure chambers. The engines were mated to eddy current dynamometers (Alpha-240, Kiel, FRG) and operated on repeated, slightly modified, transient engine cycles based on the U.S. EPA engine dynamometer schedule for heavy-duty diesel engines (40 CFR, Chapter I). The two engines/dynamometers were rotated on an approximately monthly basis so that the total exposure hours were roughly equivalent for both engines. Further details on the exposure system design and the duty cycle are described elsewhere (Reed et al., 2004; McDonald et al., 2001, 2004a).

PuriNO$_x$ fuel was supplied in four 550-gall stainless-steel totes as a water-blend diesel fuel comprised of 77% w/w California Air Recources Board (CARB) diesel fuel (Cal. Air, 2003; U.S. DOE, 2004) + 3% w/w proprietary emulsifying package (PNOX 1121A, The Lubrizol Corporation, Wickliffe, OH) + 20% w/w deionized water. Specifications for the CARB diesel fuel used in the blend were consistent with standard specifications (API Gravity [35], sulfur percent mass [0.006], cetane number [53.8], aromatic hydrocarbon percent mass [19], and distillation temperature amount recovered, 50% [564°F], 90% [627°F]). The fuel was characterized in detail for emulsion characteristics and was found to be acceptable for product specification for specific gravity (15.6°C American Society for Testing and Materials [ASTM] D4052 method), kinematic viscosity (40°C ASTM D445 method), and percent water (ASTM D6304 method).

Characterization of Exposure

PM mass was the key concentration parameter by which exposure chamber DEE concentrations were monitored and controlled. PM was measured gravimetrically each day by filter measurement and was reported as the aerosol concentrations. A real-time PM monitor (DUSTTRAK, TSI, St. Paul, MN) was used to assist operational adjustments of exposure atmospheres to target values.

Table 3 summarizes the daily and periodic measurements that were conducted, including the frequencies of measurements and calibrations, measurement techniques, methods of calibration, and sampling intervals. Daily measurements were collected from each chamber for PM, NO_x, sulfur dioxide (SO_2), total hydrocarbon (THC), carbon monoxide (CO), and carbon dioxide (CO_2). Specific methods of analyses have been described elsewhere (McDonald et al., 2004a; Reed et al., 2004). PM was measured once during the study during nonexposure hours to ensure soot was not reentrained and passed to exposure chambers.

TABLE 3
Exposure atmosphere characterization measurements, techniques, and sample frequency

Measurement	Technique	Measurement frequency	Sampling interval
Filter particle mass	Filter (Pallflex, 47 mm diameter)	Daily	Duration of exposure
Real-time particle mass	Dust-Trak nephelometer	Daily	Duration of exposure
Oxides of nitrogen	Chemiluminescence	Daily	20 min alternating[a]
Carbon monoxide	Photoacoustic gas analyzer	Daily	1, 20-min sample/level
Carbon dioxide	Photoacoustic gas analyzer	Daily	1, 20-min sample/level
Total hydrocarbon	Flame ionization detector photoacoustic gas analyzer[b]	Daily	1, 20-min sample/level
Sulfur dioxide	Photoacoustic gas analyzer[c]	Daily	1, 20-min sample/level
Particle size	Impactor,[d] SMPS[e]	4 times during study[f]	1 prestudy, 3 during study

[a] Samples alternated every 20 min between chambers.

[b] The photoacoustic gas analyzer was used as a backup in the case of flame ionization detector failure.

[c] The photoacoustic gas analyzer was subject to interferences from other analytes when measuring sulfur dioxide. Samples were collected twice per month at each exposure level to measure sulfur dioxide directly by wet chemical techniques.

[d] Cascade impactor.

[e] SMPS, scanning mobility particle sizer.

[f] Measurements were made once prior to study start and three times during study duration (beginning, middle, and end of study).

The homogeneity of test atmosphere distribution was determined during the prestudy test and early in the exposure period. The size distribution of the particles was measured in the high-level exposure chamber in the prestudy phase and three times during the study, at approximately the beginning, middle, and end of the exposure period, to establish the stability and consistency of particle size distribution. Size was measured by two methods, a micro-orifice uniform deposit impactor (MOUDI, MSP Corp., Minneapolis, MN) and a scanning mobility particle sizer (SMPS, TSI, Inc., Minneapolis, MN). This combination was necessary because the MOUDI was used to measure the material from 50 nm to 10 μm in diameter, and the SMPS was used to measure material in the smaller size range from $\sim$15 to 500 nm.

Following the completion of animal exposures, exhaust PM and semivolatile organic compounds (SVOCs) were collected on 8 inches by 10 inches Teflon-impregnated glass fiber filters (Pall-Gelman, East Hills, NY) followed by 2 (in series) PUF/XAD-4 PUF cartridges using a custom-built in-line aluminum filter holder (Tisch Environmental, Inc., Village of Cleves, OH). Material was collected at the same dilution as the high exposure level with no exposure chamber in place (filter holder replaced the chamber). The flow through the samples was approximately 300 L/m for a 6- to 8-h duration. The organic fraction of the PM and SVOC samples was extracted separately with dichloromethane for 24 h in a Soxhlet extractor. Extracts were concentrated to approximately 4 ml by rotary evaporation (vacuum distillation) followed by a gentle stream of nitrogen. Each extract concentration was determined by spiking 3 separate 10-μl aliquots onto a microbalance and allowing the dichloromethane to evaporate at room temperature for several minutes. After the mass of the aliquot stabilized, the residual mass was weighed, giving the concentration of the extract. Equivalent mass concentrations of PM and SVOC collected from each engine were combined. Extracts were then evaporated under nitrogen and exchanged into dimethyl sulfoxide for mutagenicity tests. The PM + SVOC extract and a filter (PM) and sorbent (SVOC) blank extract (processed same way as sample) were analyzed at BioReliance (Rockville, MD) for mutagenicity.

PM and SVOC samples were subjected to a bacterial reverse mutation assay (Ames assay) as described in 40 CFR 79.68. Briefly, the samples were tested individually using standard procedures both with and without S9 metabolic activation in five tester strains: TA1535, TA1537, TA98, TA100, and TA98NR. TA98NR is an acetylase-deficient strain, thus rendering the strain less susceptible to nitroaromatics in the assay (gift of Dr. H. Rosenkranz, University of Pittsburgh; Dr. L. Claxton, U.S. EPA). Thus, when used in parallel with TA98, the presence of nitroaromatics in the exhaust emissions could be identified by a profile of positive mutagenicity results in TA98 and attenuated results in TA98NR. Negative (solvent) and strain-specific positive controls were included for PM and SVOC. The potential of these subfractions extracts to cause point mutations in these tester strains in a dose-related manner was measured relative to the solvent groups. Appropriate positive-control groups served as intra-assay controls; their sole purpose was to demonstrate the reliability of the test procedures employed.

Dose levels tested for PM were 15, 50, 150, 500, 1500, and 5000 μg/plate for TA1535, TA1537, TA98, and TA100, and 10, 30, 100, 300, 1000, 2000, 3000, and 5000 μg/plate for TA98NR.

For SVOC, dose levels tested for TA98, TA100, and TA1537 were 100, 333, 667, 1000, 1200, and 3600 μg/plate and 33, 100, 333, 667, 750, and 2100 μg/plate in the presence and absence of S9 activation, respectively. The dose levels tested for TA1535 in the presence of S9 activation were 333, 667, 1000, 1200, 3600, and 5000 μg/plate and 33, 100, 333, 667, 750, and 2100 μg/plate in the absence of S9. The dose levels tested for TA98NR were 10, 30, 100, 300, 1000, 2000, 3000, and 5000 μg/plate in the presence and absence of S9 activation.

CARB versus Emulsion Diesel Emissions Characterization

Fuel specific emission rates from the engines were determined by operating the engines on CARB diesel fuel and the PuriNO_x fuel under identical test conditions (tests conducted after the conclusion of the inhalation exposure health studies). Engines were operated as described above for 2 consecutive 20-min cycles. Emissions collections followed the general guidelines specified in 40 CFR 86. Emissions were diluted ~10:1 to reduce the exhaust temperature to <48°C at the PM filter face (actual temperature at collection = ~30°C). PM was collected on a Teflon-coated glass fiber filter (Pallflex) that was equilibrated at 25°C and 45% relative humidity for at least 24 h before weights were obtained. NO_x, THC, and CO were measured according to the measurement methods shown in Table 3 (calibration procedures described elsewhere; McDonald et al., 2004a). NO_x and THC were collected every second of the engine cycle, and the values were averaged. CO was measured from an integrated sample that was collected in a Tedlar gas sampling bag. Carbonyls were collected on dinitrophenylhydrazine-impregnated silica cartridges and analyzed as described elsewhere (McDonald et al., 2004a). Three replicates of each test were conducted. Measurements were converted to emission rates by:

$$\text{Emission rate(g/bhp-h)} = \frac{\text{Concentration(g/m}^3) \times \text{DR} \times \text{Vexh(m}^3)}{\text{(bhp-h)}}$$

where DR is the dilution rate, Vexh the engine exhaust volume, and bhp-h brake horsepower-hour. Vexh was determined indirectly by measuring the dilution rate and dilution tunnel flow and solving this equation:

$$\text{DR} = \frac{\text{Vexh} + \text{Dilution tunnel flow}}{\text{Vexh}}$$

Health Evaluations

Body Weight, Clinical Examinations, and Feed Consumption. All animals were weighed individually using the Path/Tox data acquisition system on study day −7 (to randomly assign rats to groups by weight), at day −1 or −2, then twice weekly thereafter throughout the course of the study except for gestating rats. Body weights for gestating female rats were recorded on gestational days 0, 3, 6, 9, 12, 15, 18, and 20 to track gestation. Body weights were also recorded at the time of necropsy.

A detailed clinical examination was performed when body weights were measured, and any clinical abnormalities were recorded using Path/Tox software. These observations included, but were not limited to, the following: reactivity to general stimuli; description and severity of any convulsions, tremors, or abnormal motor movements (including posture or gait abnormalities); and description of any abnormal behaviors, emaciation, dehydration, masses, lesions, etc.

Food consumption data were obtained daily for each chamber basket unit holding rats at each exposure level. The food consumed per basket unit (housing up to 24 rats) was obtained by subtracting the morning feeder weight (feeder plus feed) from the full feeder weight (feeder plus feed) recorded the prior night. Food consumption data were obtained from exposure days 14 through 50. This period consisted of the only consecutive study days where food consumption data could be effectively compared between control and exposure levels. Prior to exposure days 14 through 50 and subsequent to this period, the nominal rat count per basket unit changed due to staggered starts for different study subgroups, breeding (no food consumption data were obtained during this period), and staggered sacrifices for study groups. For each basket unit, the amount of food consumed was converted to grams of food consumed per gram of rat in each basket (total weight). These numbers were based on body weight obtained at the weekly weigh sessions (e.g., data were converted at only those dates corresponding to weigh sessions). Food consumption data were statistically analyzed for only a select group of chamber basket units among all exposure levels. For males, basket 1 (of 6 total) at each exposure level was analyzed. This basket contained male animals from each representative subgroup (same number of animals from each group at each exposure level). For females, basket 4 (of 6 total) in each chamber was analyzed comparatively. All baskets analyzed contained 24 animals per basket with a full compliment of body weights.

Ophthalmology. All rats assigned to the GST/GSTR subgroups were examined within 1 wk prior to the initiation of exposures. The consulting board-certified veterinary ophthalmologist, based on the prestudy examination, recommended rats to exclude from placement on the study. Only rats designated for the terminal sacrifice subsequent to exposure (GST) were examined again within 5 days of the conclusion of the exposures.

Examinations were performed following pupillary dilation with tropicamide eye drops. Examinations were performed using an indirect ophthalmoscope and included observation of the external globe and adnexa as well as the optical media and retina.

Development and Reproductive Toxicity (DART). The DART subgroup involved an additional 10 male and 30 female rats per treatment group as shown in Tables 1 and 2. Beginning at 8 wk of age (i.e., 2 wk before exposures started), approximately 144 female rats received daily vaginal cytology smear examinations. A saline vaginal lavage was performed on each animal and examined for cell types present. Acyclic rats were

culled and not placed on study. Rats having a normal estrus cycle were randomly assigned to exposure groups.

After 7 wk of exposure, daily vaginal cytology examinations of the 30 females per exposure group in the DART subgroup resumed. These evaluations were conducted immediately following the daily exposure, or at approximately that same time on nonexposure weekend days. At 9 wk of exposure, each of the 30 females was mated to a male rat from the same treatment group. Males from the DART, GST/GSTR, SH/NTX, and MN/SCE/GFAP subgroups were used for mating ($n = 30$ males per exposure group). The mating pairs were removed from the chambers after each day's exposure, or at approximately that same time of day on nonexposure weekend days. The mating pairs were housed in shoebox-type cages under similar environmental conditions as the exposure chambers. Three days prior to the start of the mating period, males were removed from the chambers after the exposure period and placed in shoebox cages designated with their animal number. During this period, no bedding was changed. These males were returned to their normal chamber spaces prior to exposure on each of these 3 days. Throughout the mating period, the males were placed into their designated shoebox cages, and the appropriate females were introduced into the cage several minutes later. During the mating period, the shoebox cages were not washed, and the frequency of bedding changes was minimized (1 change/wk). In the morning, before exposures began, each of the paired rats was returned to the normal individual housing location within the exposure chambers. Daily overnight mating continued for 2 wk or until daily vaginal cytological examinations of the mated females revealed a plug and the presence of sperm in the vaginal lavage cell smear. This day was defined as gestation day 0. Vaginal smears for the detection of mating evidence were made prior to daily exposures during the mating periods. Any female that did not become pregnant after mating with the initial male was placed with a proven male from the same treatment group. Females with evidence of mating were exposed every day from gestation day 0 through gestation day 15 (inclusive). Any female that aborted was sacrificed at the first evidence of abortion.

On gestation day 20, all surviving rats were euthanized by CO_2 inhalation. The abdominal, thoracic, and pelvic cavities were opened by a ventral midline incision, and the ovaries and uterus were removed from the body; pregnancy status (gravid or nongravid) and gross necropsy finding were recorded. The uterus was weighed and examined. For gravid females, the number of corpora lutea on each ovary was recorded. The uterus was opened and the development of the fetuses was classified using the following criteria: (a) live fetus: a fetus that responds to touch; (b) dead fetus: a fetus that did not respond to touch and was not breathing; (c) early resorption: an implantation for which it was not grossly evident that organogenesis had occurred; (d) late resorption: an implantation for which it was grossly evident that organogenesis had occurred; and (e) a fetus with autolysis was considered a late resorption.

The number and location of implantation sites, live and dead fetuses, and early and late resorptions were recorded. Uteri with no macroscopic evidence of implantations were opened and placed in 10% aqueous ammonium sulfide solution for detection of implantation sites.

External examinations of fetuses included the eyes, palate, head shape, trunk, and extremities. Each live fetus was then weighed, externally sexed, and euthanized by an intrathoracic injection of sodium pentobarbital. Dead fetuses were examined, but not weighed or sexed. One half of the fetuses of each litter had visceral contents removed and fixed in Bouin's solution for visceral evaluation using the Wilson free-hand slicing technique (Wilson, 1965). The remaining one-half of the live fetuses were fixed in 95% isopropyl alcohol for skeletal examination. These fetuses were subsequently macerated in 2% potassium hydroxide, stained with alizarin red S, and cleared with increasing concentrations of 25% and 100% glycerin. The fetuses were then examined for skeletal formation and ossification.

The incidences or the means and standard deviations of maternal and fetal observations were calculated. Calculations and statistical analysis of Cesarean section and fetal parameters were performed using the litter as the unit of analysis. The number of corpora lutea, implantations, live and dead fetuses, early and late resorptions, and gravid uterine weights were calculated as the total number for each exposure group divided by the number of litters evaluated. The following parameters were calculated by determining the percent loss for each litter followed by group mean calculations.

$$\text{Preimplantation loss } \% = \frac{\text{Number of Corpora lutea} - \text{number of implants}}{\text{Number of corpora lutea}} \times 100$$

$$\text{Postimplantation loss } \% = \frac{\text{Number of implants} - \text{number of viable fetuses}}{\text{Number of implants}} \times 100$$

General Histopathology. Rats in the GST and GSTR subgroups were sacrificed by overdose of barbiturate and received a full necropsy. Fresh weights were obtained for the liver, kidneys, lungs, adrenal glands, brain, vagina, uterus, ovaries, testes, epididymides, seminal vesicles with coagulating gland, and prostate. Pituitary weights were obtained after fixation. All organs were preserved in 10% neutral buffered formalin (NBF), except for the testes, epididymides, and eyes, which were preserved in Bouin's solution. In addition to the listed organs, the nasopharynx, pituitary, thyroid/parathyroid, thymus, trachea, heart, sternum with marrow, salivary gland, spleen, pancreas, aorta, skin, gastrointestinal tract, urinary bladder, lymph node, mammary gland, thigh muscle, femur, spinal cord, exorbital lachrymal gland, and zymbal gland were examined microscopically. Microscopic evaluations were limited to the control and high-exposure-level groups with the exception of the lungs, which were examined in all exposure groups. Tissues

were trimmed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Histopathologic evaluations included determining the presence, nature, and severity of morphologic abnormalities. A scale from 0 to 4 was used to score the severity of morphologic abnormalities.

Special Histopathology. Five males and five females per exposure groups were designated for special neuropathology, reproductive-system pathology, and pulmonary distribution of particles. Rats were weighed, heparinized, euthanized by overdose of barbiturate, their hearts were canulated, and their tissues were fixed by whole-body perfusion of buffered 4% paraformaldehyde. The lungs were fixed in situ by intratracheal instillation of 4% paraformaldehyde via gravity feed at a pressure of approximately 25 mm Hg.

After perfusion fixation, the pelts were removed from the dorsal head, neck, shoulders, and both hind legs. The bones of the cranium and cervical vertebral column were exposed. The nasal cavity was gently flushed with 4% buffered paraformaldehyde. The lungs were carefully removed and tied off. Other organs and tissues (see General Histopathology section earlier), including reproductive organs, but not the brain, spinal cord, peripheral nerve, or nasal tissue, were removed and placed in 10% NBF, except for the testes, epididymides, and eyes, which were preserved in Bouin's solution. The carcasses were placed in a tissue bag with 4% buffered paraformaldehyde and held at 4°C overnight. The following day, the cranium and cervical vertebral column were removed. The brain (cerebrum, cerebellum with pons, and medulla) was removed, weighed, and the length and width of the brain were recorded. The brain, spinal cord at the cervical swelling, the left and right sciatic nerves at the mid-thigh and sciatic notch, the left tibial nerve at the knee, and the nasal cavity were removed and placed in 10% NBF. All of the tissues were held in 10% NBF for at least 48 h before further processing.

After at least 48 h of formalin fixation, the sciatic or tibial nerve(s) and crosssections of the forebrain, center of the cerebrum, midbrain cerebellum, and medulla oblongata were processed for embedding and histopathology. The spinal cord at the cervical swelling (C3–C6) and proximal right sciatic nerve (mid-thigh and sciatic notch) were placed in labeled cassettes. A longitudinal section of the left sciatic nerve was also placed in a cassette with the cross sections of the right sciatic nerve. The lungs and other organs were trimmed and processed and stained with hematoxylin and eosin. Neural tissues from only the clean-air control and high-level treatment groups were processed and examined. The testes, epididymides, seminal vesicles, and ovaries were examined microscopically from all SH/NTX subgroup animals (all exposure groups). The lungs and trachea of all whole-body perfusion-fixed test animals were examined for inhaled particle distribution. These assessments were qualitative, noting regional distribution of particles or particle-containing macrophages and intralumenal versus interstitial particle location.

Clinical Chemistry and Hematology. Hematology and clinical chemistry were evaluated on all rats assigned to the GST/GSTR subgroup after 1 mo of exposure, at the end of exposure, and at the end of the recovery period (GSTR only). In addition, at the 1-mo bleed, the SH/NTX rats group served as clinical chemistry and hematology backup animals for those GST/GSTR rats where adequate samples were not obtained. Prior to blood collection, rats were fasted overnight. At the end of exposure and recovery, the rats were anesthetized with halothane (1 mo) or overdose of barbiturate for necropsy. Blood was collected via the retro-orbital sinus at the 1-mo bleed (prior to exposure) and by cardiac puncture during necropsy at the end of exposure and after the recovery period.

For hematology analyses, approximately 0.5–1.0 ml of whole blood was collected from each rat into tubes containing ethylenediamine tetraacetic acid (EDTA) as an anticoagulant. For clinical pathology analyses, approximately 1 ml of whole blood was collected from each rat into microtube serum separator tubes for centrifugation and separation into cellular and serum fractions. Clinical chemistry parameters included the following (Monarch 2000, Instrumentation Lab., Lexington, MA): alanine aminotransferase (ALT), albumin, albumin/globulin ratio, alkaline phosphatase (ALP), aspartate aminotransferase (AST), bile acids, blood urea nitrogen (BUN), BUN/creatinine (CRE) ratio, serum calcium, serum chloride, cholesterol, creatinine, gamma-glutamyl transpeptidase (GGT), glucose, inorganic phosphorus, potassium, sodium, sorbitol dehydrogenase (SDH), total bilirubin, total globulin, and total protein. Hematology parameters were assayed by automated analyzer (Advia 120, Bayer Corp., Tarrytown, NY) and included the following: differential white blood cell count (lymphocytes, neutrophils, eosinophils, monocytes), hematocrit, hemoglobin, platelet count, red blood cell count, white blood cell count, and methemoglobin (IL 682 CO-oximeter, Instrumentation Lab., Lexington, MA).

Glial Fibrillary Acidic Protein. Subsequent to blood collection for the SCE assay, the skull was opened and the brain was removed for the glial fibrillary acidic protein (GFAP) assay from all MN/SCE/GFAP rats plus rats dosed with 8.0 mg/kg, intraperitoneally (ip), of the known hippocampal neurotoxicant trimethyltin (TMT). Brains were dissected into the following six regions: cerebellum, cerebral cortex, hippocampus, striatum, thalamus/hypothalamus, and remaining brain, as specified in the U.S. EPA Guidelines (40 CFR 79.67). Each brain region was placed into individually labeled tubes, frozen over dry ice, and stored at -70°C until processing for protein analysis. GFAP analyses were performed on each brain region by sandwiched enzyme-linked immunosorbent assay (ELISA) versus known GFAP standards as described previously (O'Callaghan, 2002).

Sister Chromatid Exchange. On the day following the last day of exposure, MN/SCE/GFAP animals were sacrificed by overdose of barbiturate. The chest was opened, and heparinized peripheral blood samples (2–4 ml) were obtained from the abdominal aorta of all exposed rats in this subgroup plus the

appropriate cyclophosphamide (CP)-treated, positive-control animals (5 males and 5 females exposed to clean air throughout the exposure period and dosed with 5 mg/kg CP ip 24 h prior to sacrifice). Blood samples were analyzed by BioReliance Corporation. The frequency of SCEs was determined by culturing whole-blood lymphocytes in the presence of 5-bromo-2′-deoxyuridine (BrdU) for two cell cycles. Briefly, cultures were initiated by adding 0.5 ml of whole blood per 5.0 ml of complete culture medium [RPMI 1640 culture medium supplemented with 10% fetal bovine serum, antibiotics (penicillin G, 100 μg/ml, and streptomycin sulfate, 0.1 mg/ml), 25 m*M* HEPES buffer, 20 μg/ml phytohemagglutinin, and an additional 2 m*M* L-glutamine] in T-25 sterile disposable tissue culture flasks and incubated at 37°C. Approximately 21 h after initiation of the cultures, the cells were exposed to 5.0 μg/ml of BrdU. Dividing cells were arrested in metaphase and harvested for microscopic evaluation of SCEs. At approximately 68 h, colcemid at 0.2 μg/ml was added to each flask and incubated for approximately 3 h. The cells were swollen by hypotonic treatment (0.075 *M* potassium chloride, KCl), fixed, and washed with methanol:acetic acid (3:1), capped, and stored overnight or longer at 2–8°C. To prepare slides, the cells were collected by centrifugation and resuspended in fresh fixative. One to two drops of fixed cells were dropped onto a glass microscope slide and air-dried. The slides were stained using a modified fluorescence-plus-Giemsa technique (Perry & Wolff, 1974). Slides were stained in Hoechst 33258, 5 μg/ml, for 10 min; mounted in phosphate buffer, pH 6.8; exposed to an ultraviolet lamp at approximately 60°C for 4–6 min; and then stained in 5% Giemsa for 6–10 min. The potential of the test substance to increase sister chromatid exchanges (SCEs) in an exposure-responsive manner was measured relative to the clean-air control group. The CP-treated group served as a positive control to demonstrate the reliability of the test system.

Slides were coded. Metaphase cells were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 25 second-division metaphases per animal was scored for SCEs. At least 100 consecutive metaphase cells were scored for the number of cells in first-, second-, or third-division metaphase for each animal as an indicator of toxicity (cell cycle delay). At least 1000 cells were scored for mitotic index per animal.

Micronucleus. Subsequent to blood removal for the SCE assay, for MN/SCE/GFAP animals and CP-treated, positive-control animals (5 males and 5 females exposed to clean air throughout the exposure period and dosed with 40 mg/kg CP ip 24 h prior to sacrifice), both femurs were removed to provide marrow cells for the micronucleus (MN) assay. Bone-marrow smears for this endpoint were prepared and scored for micronuclei. Briefly, femur proximal heads were removed and the contents of each femur were eluted in fetal bovine serum by aspiration through a needle fitted to a plastic syringe. The resulting cell suspension was centrifuged to isolate cells. The final cell pellets were resuspended in a small volume of fetal calf serum to facilitate smearing on glass microscope slides. Two smears were prepared from each animal. The slides were air-dried and shipped to BioReliance for analyses. Slides were coded, fixed by dipping in methanol, stained with the nucleic acid-specific stain acridine orange, and scored. Using medium magnification, an area of acceptable quality was selected such that the cells were well spread and stained. Using oil immersion, 2000 polychromatic erythrocytes were scored per animal for the presence of micronuclei. The number of micronucleated normocytes in the field of 2000 polychromatic erythrocytes was also enumerated. The proportion of polychromatic erythrocytes to total erythrocytes was recorded per 1000 erythrocytes. The proportion of polychromatic erythrocytes to total erythrocytes in test substance-treated animals was never less than 20% of the control value. The potential of the test substance to increase MNs in an exposure-responsive manner was measured relative to the clean-air control group. The CP-treated group served as a positive control to demonstrate the reliability of the test system.

Statistical Analyses. For all analyses, multivariate and univariate, the criterion for statistical significance was set at $p \leq .05$.

Body weight and organ weight data were tested for homogeneity with Bartlett's test. For homogenous data, Dunnett's *t*-test was performed, and for nonhomogenous data, the modified *t*-test was performed to determine statistical difference from control values. For food consumption, least-squares regression lines with a common slope and different y intercepts were fit to the data from each exposure group for both males and females for gram of food consumed per gram of bodyweight values over the period for which data was analyzed. A *p* value was assigned to deviation from the control group line.

For DART data, the number of corpora lutea, implantation sites, live fetuses, gravid uterus weights, and fetal body weights by litter and by sex were analyzed by one-way analysis of variance (ANOVA). If a significant *F* ratio was obtained ($p < .05$), Dunnett's test was used for pairwise comparisons to the control group. The percent preimplantation loss, percent postimplantation loss, number of dead fetuses, early and late resorptions, and the percent of male and female fetuses were compared using the Kruskal–Wallis test. If a significant effect occurred ($p < .05$), the Mann–Whitney U-test was used for pairwise comparisons of each treated group to the control group. The incidences of malformation and variations were compared using the chi-square test with the litter as the experimental unit. The total numbers of litters with external, visceral, and skeletal malformations as well as the total number of litters with malformations and variations were also statistically compared. If a significant effect occurred ($p < .05$), Fisher's exact test was used for pairwise comparisons to the control group.

Hematology and clinical chemistry were evaluated using the Kruskal–Wallis test (Hollander & Wolfe, 1973). If significant evidence of a treatment effect ($p < .05$) was found, individual comparisons of exposed groups against controls were performed using Dunn's (1964) multiple comparison procedure.

TABLE 4
Summary of particle mass and gas analyte concentration data

	Treatment group			
	Clean air control	Low	Mid	High
CO, ppm	0.3 ± 0.2	10.9 ± 2.9	17.3 ± 2.9	28.6 ± 5.6
CO_2, ppm	1921.2 ± 676.9	2652.4 ± 571.6	3286.0 ± 523.6	4654.6 ± 1105.4
SO_2, ppb	ND	4.0 ± 6	8.0 ± 15.0	15.0 ± 4.0
Total hydrocarbon, ppm	0.3 ± 0.2	1.1 ± 0.4	1.7 ± 0.5	2.7 ± 0.7
NO_x, ppm	0.3 ± 0.6	7.4 ± 1.5	11.9 ± 2.2	20.4 ± 4.0
NO, ppm	0.1 ± 0.4	6.0 ± 1.8	9.2 ± 3.6	15.9 ± 6.1
NO_2, ppm	0.2 ± 0.3	1.4 ± 1.0	2.7 ± 2.7	4.5 ± 5.1
Particle mass, $\mu g/m^3$	6.4 ± 4.2	102.5 ± 7.8	203.3 ± 12.4	401.5 ± 21.8

Note. Values are mean plus or minus one standard deviation. ND, not detected.

For the SCE assay, Dunnett's *t*-test was used to test statistical significance from control and treated groups. For the MN assay, micronucleated polychromatic erythrocytes were tested in control vs. treated groups by Kastenbaum–Bowman's test. GFAP data were subjected to analysis of variance followed by post hoc comparison of treatment means (JMP, SAS).

RESULTS

Exposures and Characterization

Daily average chamber temperatures (∼21°C), exposure room temperature (∼20°C), chamber relative humidity (∼10–30%), oxygen concentrations (∼21%), and chamber exhaust flows (∼600 L/min) were acceptable throughout the study at all exposure levels.

Summary exposure concentrations of PM, NO_x, nitrogen oxide (NO), NO_2, CO, CO_2, THC, and SO_2 are shown in Table 4. PM concentrations were within 15% of target on 90% of the exposure days, with only 4 days in the low-exposure level and 2 days in the mid-exposure level within 20% of target. Control background PM averaged 6.4 $\mu g/m^3$. NO_x, CO, CO_2, SO_2, and THC scaled with dilution at all exposure levels. NO_x was composed of approximately 80% NO and 20% NO_2, and the concentration of NO_x at the highest exposure level was approximately 20 ppm. SO_2 values averaged 15 ppb at the highest exposure level. Small but measurable background amounts of NO_x, CO, CO_2, and THC were present in the control chamber. The reported summary of NO_x values for the control chamber (∼0.3 ppm) was low relative to the three exposure levels. However, control NO_x values were higher than expected (identified as an artifact due to a gas analyzer problem). The median diameter of PM was between 0.1 and 0.2 μm, and the median number distribution was less than 100 nm (Table 5).

DEE PM and SVOC tested positive in the bacterial reverse mutation assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537, and/or TA98NR. For PM, positive responses were observed with tester strains TA98, TA100, TA1535, TA1537, and TA98NR in the presence of S9 activation and with tester strains TA98, TA100, TA1537, and TA98NR in the absence of S9. Toxicity was observed in the absence of S9 at 1000 μg/plate with tester strain TA98NR and at 5000 μg/plate for all other strains. No precipitate was observed.

For SVOC, positive responses were observed with tester strains TA98, TA100, TA1535, and TA1537 in the presence of S9 activation and with tester strains TA98, TA100, and TA1537 in the absence of S9. Toxicity was observed in the absence of S9 beginning at 750, 1000, or 2100 μg/plate. Toxicity was observed in the presence of S9 activation beginning at 3600 or at 5000 μg/plate for tester strains TA1535, TA1537, and TA98NR. No precipitate was observed.

TABLE 5
Monthly summary of particle mass and number size distribution (high exposure level)

	Particle mass distribution		
	Mass median diameter (μm)	Percent < 1 μm	Percent 1–10 μm
Month 1	0.12	75	25
Month 2	0.1	76	24
Month 3	0.18	88	12
	Particle number distribution		
	Median size (nm)	GSD[a]	
Month 1	33	1.6	
Month 2	34	1.5	
Month 3	34	1.5	

[a]GSD, geometric standard deviation.

TABLE 6
Emissions characterization of California Air Recourses Board diesel and PuriNO$_x$ diesel emulsion fuels

Analyte	Unit[b]	CARB	PuriNo$_x$	Percent change
PM	g/bhp-h	0.045	0.022	−49.8
CO	g/bhp-h	1.532	1.918	25.2
NO_x	g/bhp-h	2.414	2.150	−10.9
HC[a]	g/bhp-h	0.138	0.299	117.2
Formaldehyde	mg/bhp-h	18.57	25.27	36.1
Acetaldyde	mg/bhp-h	6.19	7.10	14.7

[a]HC concentrations converted from ppm to $\mu g/m^3$ using a molecular weight of 44, the molecular weight of propane.
[b]Grams per brake horsepower-hour.

Emissions Reductions Characteristics of PuriNO$_x$ versus CARB Diesel

In the fuel emissions comparison portion of this study (conducted after the conclusion of the inhalation exposure health studies), PuriNO$_x$ decreased NO_x (∼11%) and PM (∼50%) emissions versus CARB diesel when operated on the Cummins engines used in the current study (Table 6). CO emissions were increased slightly for the emulsion (∼25%). Measured aldehydes were slightly increased for PuriNO$_x$ versus CARB fuel (∼14–36%).

HEALTH RESPONSES

Survival, Body Weight, Clinical Signs, Feed Consumption

There were no exposure-related effects on mortality, morbidity, or clinical signs.

No consistent exposure-related body weight effects were noted in the GST/GSTR, SH/NTX, DART, and MN/SCE/GFAP subgroups (data not shown). Some statistically significant decreases from control were seen at some measurement intervals in both sexes in high-level SH/NTX and GST/GSTR subgroups and in high-level DART males. No difference in body weight was apparent in the GSTR subgroup subsequent to exposure.

Food consumption per gram of body weight for both males and females in all exposure groups decreased over time as the study progressed (data not shown). However, the amount of food consumed (grams) per gram of rat weight for exposed groups was greater than for controls (Table 7). No exposure-response relationship was observed.

Ophthalmologic Examinations

All animals assigned to the study had congenital corneal dystrophy (CDD) at study onset. Examination of all 80 animals assigned to the GST group postexposure showed no CDD progression during the study or other signs of ocular pathology.

TABLE 7
Summary food consumption analyses

Gender	Chamber	Mean ± SEM of deviations from fitted regression line (mg food/g rat)	p value vs. control
Male	Clean air Control[a]	0.0 ± 1.5	—
	Low	5.4 ± 1.1	.004
	Mid	5.8 ± 1.1	.002
	High	4.8 ± 1.3	.009
Female	Clean air Control[a]	0.0 ± 1.2	—
	Low	4.0 ± 1.4	.035
	Mid	6.4 ± 1.1	.001
	High	6.6 ± 1.4	.001

[a]Control value set at 0 for the purpose of reporting regression analyses.

Clinical Chemistry and Hematology

Statistically significant exposure effects on clinical chemistry values of rats are summarized in Table 8. The magnitude of most effects was modest, and there was little consistent evidence for effects across genders and exposure times. Most statistically significant effects were observed after 3 mo of exposure. Most effects disappeared after 30 days of recovery in clean air exposure. Cholesterol was reduced in females after 1 mo of exposure (maximum 46%) and both females (maximum 11%) and males (maximum 36%) at 3 mo. The serum enzyme sorbitol dehydrogenase (SDH) was decreased in males (maximum 37%) at 1 mo of exposure, but was increased in both females and males at 3 mo (maximum 73% and 63%, respectively). Other effects on serum enzymes (ALT, ALP, AST, GGT) were variable among sexes and exposure times and relatively modest (maximum change from control [increase] 34%). Electrolyte values (chloride [Cl], sodium [Na], calcium [Ca], K) were decreased slightly but significantly in single sexes (male, K, maximum 14%, and female, Ca, maximum 3%) and both genders (maximum 6% Na, Cl) at 3 mo of exposure. These effects contrast with those observed at 30 days in male Cl (increase 2% maximum), in male and female Na (increase 1% maximum), and in male phosphorus (P) (increase maximum 11%) at recovery. Albumin and total protein were slightly decreased in females (maximum 4%) after 30 days, and both total protein (maximum 11%) and albumin (maximum 6%) were decreased slightly in both sexes at 3 mo. The calculated globulin and albumin/globulin ratios decreased (maximum 19%) and increased (maximum 13%) respectively after 3 mo of exposure. Decreases in BUN and BUN/CRE (maximum both 18%) were present after exposure and recovery.

Statistically significant exposure effects on hematology values of rats are summarized in Table 9. The magnitude of most

TABLE 8
Significant effects of exposure on clinical chemistry parameters of rats

Parameter	Interim	Terminal	Recovery
Cholesterol	↓Female, M, H (46%)	↓Female, H (11%)	—
	—	↓Male, M, H (36%)	—
Sorbitol dehydrogenase	—	↑Female, M (73%)	—
	↓Male, H (37%)	↑Male, M (63%)	—
Gamma glutamyl transpeptidase	—	—	↑Female, H (37%)
	—	—	—
Alanine aminotransferase	—	↓Female, H (16%)	—
	—	↑Male, M (24%)	—
Alkaline phosphatase	—	—	—
	↑Male, L, M, H (5%)	—	—
Aspartate aminotransferase	—	↓Female, H (34%)	—
	—	—	—
Blood urea nitrogen (BUN)	—	↓Female, M, H (18%)	↓Female, H (14%)
	↓Male, L, M, H (13%)	—	↓Male, H (8%)
BUN/creatinine	—	↓Female, M, H (18%)	—
	—	—	—
Chloride	—	↓Female, H (2%)	—
	↑Male, M (2%)	↓Male, H (6%)	—
Sodium	↑Female, L (1%)	↓Female, M, H (3%)	—
	↑Male, M (1%), H	↓Male, H (5%)	—
Potassium	—	—	—
	—	↓Male, H (14%)	—
Calcium	—	↓Female, H (3%)	—
	—	—	—
Phosphorus	—	—	—
	—	—	↑Male, H (11%)
Glucose	—	↓Female,M (24%)	—
	—	—	↓Male, H (12%)
Total protein	↓Female, M (3%)	↓Female, H (11%)	—
	—	↓Male, H (5%)	—
Albumin	↓Female, M, H (4%)	↓Female, H (6%)	—
	—	↓Male, H (5%)	—
Globulin (total protein − albumin)	—	↓Female, H (19%)	—
	—	—	—
Albumin/globulin ration	—	↑Female, H (13%)	—
	—	—	—

Note. Arrows indicate increases (↑) or decreases (↓) from control ($p < .05$). Letters indicate the exposure groups differing significantly from controls (L = low, M = mid, H = high). Values in parentheses are the largest percent differences between exposed and control group means and immediately follow the indicator for the group having the greatest difference.

effects was modest, there was little coherent evidence for effects across exposure times, and many effects lacked an exposure-response relationship. Platelet values were increased in males (mid and high, maximum 34%) at 3 mo and the effect persisted to recovery (high, maximum 10%). Statistical changes in white blood cell (WBC) counts (male, mid, maximum 55% terminal sac; and high, 40% recovery) were variable among sexes and exposure times and lacked an exposure response, although increases in the monocyte and lymphocyte counts at 3 mo and recovery were statistically significant. In addition, female moncyte differential values were increased (maximum 44%) in the mid- and high-exposure-level groups at the terminal sacrifice, and male values were increased at recovery (maximum high 49%). Eosinophil changes were also variable in males at 3 mo

TABLE 9
Significant effects of exposure on hematology parameters of rats

Parameter	Interim	Terminal	Recovery
Platelets	—	—	—
	—	↑Male, M, H (34%)	↑Male, H (10%)
Hematocrit	—	↑Female, M, H (9%)	—
	↑Male, L, M (3%)	↑Male, M (4%)	—
Red blood cell count	—	↑Female, M (8%), H	—
	—	↑Male, M (8%)	—
Hemoglobin	—	↑Female, H (8%)	—
	—	↑Male, M (8%)	—
Met-hemoglobin	—	—	↓Female, H (34%)
	—	—	—
White blood cell count	—	↑Female, L (31%)	—
	—	↑Male, M (55%)	↑Male, H (40%)
Monocytes	—	↑Female, M, H (44%)	—
	↓Male, L (27%)	↑Male, M (112%)	↑Male, H (49%)
Lymphocytes	—	↑Female, L (33%)	—
	—	↑Male, L, M (141%)	↑Male, H (43%)
Eosinophils	—	—	—
	—	↓Male, H (37%)	↑Male, H (55%)

Note. Arrows indicate increases (↑) or decreases (↓) from control ($p < .05$). Letters indicate the exposure groups differing significantly from controls (L = low, M = mid, H = high). Values in parentheses are the largest percent differences between exposed and control group means and immediately follow the indicator for the group having the greatest difference from control.

and recovery. Changes in red blood cell parameters (hematocrit, red blood cell [RBC] count, hemoglobin, met-hemoglobin) were generally slight and lacked an exposure-response relationship in males. However, mid-exposure level values were statistically significantly increased in all parameters (maximum 9%) in both sexes at 3 mo, while hematocrit (maximum 3%) was increased in males at 1 mo.

Necropsy and Pathology Gross Observations

Gross observations were generally unremarkable in all study groups at all sacrifice times. Discolored lungs, consistent with inhalation of PM, were fairly prevalent in the high-level-exposure group for males and females.

Organ Weights

Organ weight changes were minimal. Absolute epididymis weights were slightly lower (5.5%) than control in the high-level-exposure group at the end of exposure. No differences existed in epididymis weights as a percentage of brain weight or body weight. Liver weights (absolute, percent brain weight) were statistically significantly decreased in the high-exposure-level males (10%) and in the mid-exposure-level females (7%) (absolute and relative). No changes in liver weights in the recovery animals were noted. High-exposure-level female adrenals (16%), brain (6%), and kidney (7%) organ to body weight ratios were increased in comparison to control. No changes were evident in absolute or organ to brain weight ratios. There were no differences in these organ weights when recovery animals were compared to the untreated control group. There were no changes in male adrenal weights at the end of exposure. However, a small decrease in male absolute adrenal weight was observed in high-exposure-level recovery animals (6%). Mid-exposure-level female lung values were slightly increased as a percentage of body weight (7%). No change was detected in absolute values or percentage of brain weight values, and no changes were observed in recovery animals. Male, high-exposure-level values for lung (5%), pituitary gland (40% total difference, actual values 0.003 vs. 0.005), and seminal vesicle weights (24% total difference, actual values 0.347 vs. 0.465) were increased as a percentage of body weight. Seminal vesicle weights were also increased as a percentage of brain weight (19% total difference, 60.78 vs. 72.85). Seminal-vesicle weight changes were not observed in absolute organ weight.

Microscopic Pathology

Exposure-related findings were limited to the lungs and due to DEE PM in GST/GSTR animals (Figure 1). Observations included accumulation of black/brown PM within the cytoplasm of alveolar macrophages and minimal alveolar macrophage hyperplasia. PM accumulation was exposure related and correlated with the presence of gross necropsy observations of discolored

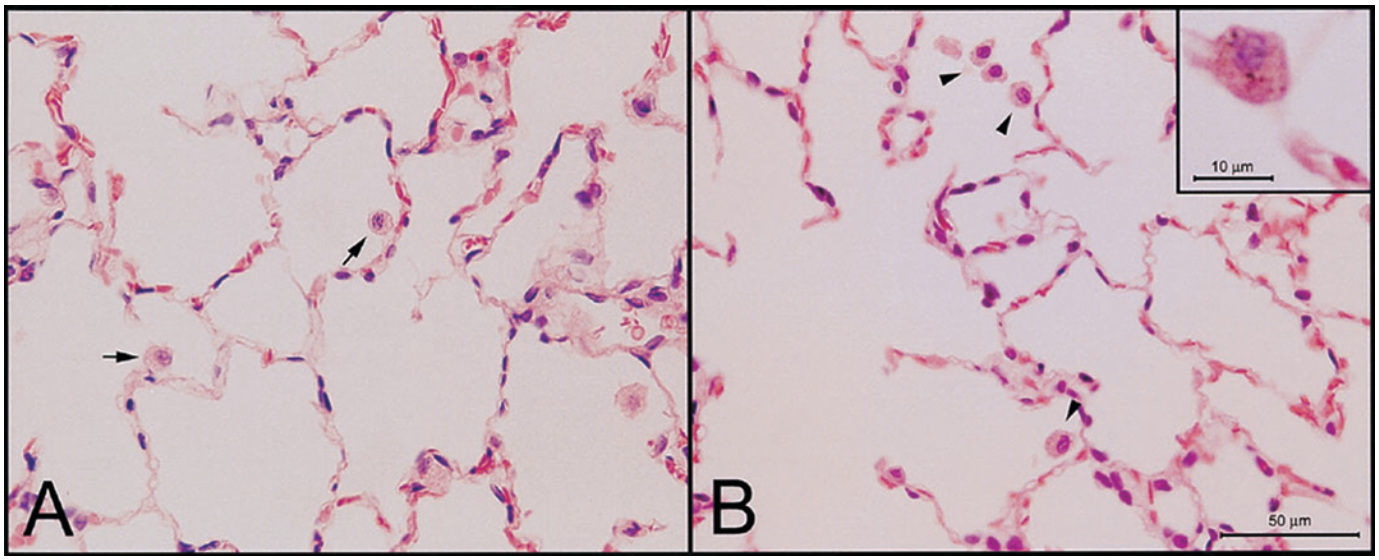

FIG. 1. Photomicrographs illustrating pulmonary histology. (A) Lung from a control male rat demonstrating normal alveolar macrophages with lightly eosinophilic, vacuolated cytoplasm (arrows). (B) Lung from high-exposure-level group (400 μg PM/m^3) male rat demonstrating macrophages with more dense, faintly brown-tinged cytoplasm with indistinct particulate material (arrowheads). Higher magnification, inset, demonstrates an alveolar macrophage with more discrete particles.

lungs. Essentially no particles were evident in the cytoplasm of any alveolar macrophages of control animals. Some resolution of PM accumulation and macrophage hyperplasia was evident after the 30-day recovery period for the GSTR animals.

Microscopic findings in other organs were minimal and similar between controls and high-exposure level animals and incidental to DEE exposure. Findings included the following: minor foci of chronic inflammation within the liver and/or myocardium; alveolar histiocytosis; minimal renal tubular concretions/mineralization; scattered foci of inflammation or mineralization in other organs/tissues; and cystic anomalies, foci of hemorrhage or fibrosis, and hepatodiaphragmatic nodules.

SH/NTX Subgroup

Special histopathology neuropathology indicators were normal. No lesions were detected in either paraffin-embedded nervous tissue or plastic-mounted, teased preparations of tibial nerves from high-exposure level or control animals.

For particle distribution, PM was contained within the cytoplasm of macrophages. Observations were essentially as described earlier for the GST/GSTR subgroup. PM was only rarely seen in tracheal sections of exposed animals. Particles were not found within epithelia or free within interstitial tissue.

No exposure-related lesions were observed in reproductive tissues from either males or females. No aberrancy in spermatogenesis or oogenesis was detected histologically.

DART

Animals placed on study cycled normally through the course of exposure. The pregnancy rate was 77% in the clean-air control exposure group, 79% in the mid-exposure-level group, and 83% in the low- and high-exposure-level groups. A minimum of 23 pregnant females with viable fetuses per study group was available for examination.

No statistically significant difference from control was observed for any parameter measured, including the number of corpora lutea, implantations, live and dead fetuses, early and late resorptions, the percent preimplantation and postimplantation loss, the percent of male and female fetuses, and the mean gravid uterus and mean fetal body weights by litter and by sex. No exposure-related differences from control malformation or variation incidence were observed. The pattern and distribution of the malformations did not indicate a teratogenic response in any exposure group. Various common developmental variations were observed at each exposure level at a similar frequency.

GFAP

The validity of the GFAP assay as an indicator of neurotoxicant-induced gliosis was verified with a positive control, TMT (Tables 10 and 11). Large increases in hippocampal GFAP were observed in both male and female positive control animals, even after a relatively short postdosing interval (5–7 days). A slight decrease in GFAP was observed in all areas of the male brains except rest of brain at the high exposure level, as shown in Table 10. Additionally, a slight decrease in GFAP was noted in the hypothalamus/thalamus of males at the mid-exposure level. Levels of GFAP were not affected in any brain region of the females. Thus, gliosis, which is reflected by an increase in GFAP, did not occur in the brain regions examined in either sex in this study.

TABLE 10
Mean GFAP levels in specific regions of male rat brains

Brain area	TMT	Clean air control	Low	Mid	High
Striatum		1.28 ± 0.05	1.23 ± 0.04	1.29 ± 0.07	1.07 ± 0.06[a]
Hippocampus	8.80 ± 1.61[a]	2.82 ± 0.03	2.66 ± 0.04	2.62 ± 0.08	2.44 ± 0.17[a]
Cortex		1.56 ± 0.08	1.42 ± 0.07	1.57 ± 0.10	1.26 ± 0.09[a]
Hypo/thalamus		2.82 ± 0.07	2.56 ± 0.05	2.34 ± 0.19[a]	2.42 ± 0.18[a]
Cerebellum		3.41 ± 0.10	3.10 ± 0.11	3.05 ± 0.12	2.85 ± 0.18[a]
Rest of brain		4.01 ± 0.16	3.73 ± 0.10	3.53 ± 0.11	3.57 ± 0.12

Note. Each value represents the mean ± SEM for the concentration of GFAP (μg/mg total protein). TMT, trimethyltin hydroxide, 8.0 mg/kg, ip, positive control.
[a]Statistically different from control, $p \leq .05$.

SCE

As shown in Table 11, no statistically significant increases in average SCE frequencies were observed in exposure animals. The positive control group (5 mg/kg CP) exhibited statistically significant increases in SCE frequencies in both sexes. No appreciable difference was observed in average generation time or in mitotic index in exposed versus control animals.

Micronucleus

Slight reductions (up to 8%) in the ratio of polychromatic erythrocytes to total erythrocytes were observed in exposed groups relative to the negative controls (data not shown). There was no exposure response. The number of micronucleated polychromatic erythrocytes was not statistically increased relative to controls at any exposure level (see Table 12). As shown in Table 13, CP induced a significant increase in micronucleated reticulocytes in both male and female rats.

DISCUSSION

DEE caused only slight to modest health outcomes in this study of subchronically exposed F344 rats. The most noteworthy effects of DEE were observed in the several statistical changes in clinical chemistry and hematology. However, most statistical hits were slight to modest in magnitude and inconsistent among exposure times and sexes. Based on the most consistent observations at 3 mo of exposure and recovery (e.g., cholesterol and platelets), a no-observed-effect level (NOEL) could be determined as the low exposure level (100 μg/m^3).

The PM exposure concentrations were within the range of very high environmental to occupational levels of petroleum diesel emissions (PDE) (U.S. EPA, 2002; Lloyd & Cackette, 2001). The DEE exposure concentrations employed in this study were also comparable to the latest subchronic health study conducted with PDE in this institute (whole exhaust diluted based on PM to 30, 100, 300, and 1000 μg PM/m^3) under very similar engine operation (2000 vs. 2001 Cummins) and atmospheric sampling conditions (Reed et al., 2004). In addition, the DEE exposures were roughly comparable to human equivalent concentrations of similar levels of PDE based solely on PM size and deposition fractions (Reed et al., 2004). However, one caveat of the Tier II program and the U.S. EPA-required testing of PuriNO$_x$ and other alternative diesel fuels is that no baseline Tier II study exists for any blend of petroleum diesel. Although the study conducted by Reed et al. (2004) was performed under similar animal environmental and exposure conditions, in most

TABLE 11
Mean GFAP levels in specific regions of female rat brains

Brain area	TMT	Clean air control	Low	Mid	High
Striatum		1.21 ± 0.04	1.14 ± 0.05	1.06 ± 0.05	1.20 ± 0.07
Hippocampus	13.16 ± 4.51[a]	2.85 ± 0.04	2.73 ± 0.08	2.57 ± 0.09	2.64 ± 0.09
Cortex		1.35 ± 0.07	1.27 ± 0.08	1.19 ± 0.03	1.29 ± 0.07
Hypo/thalamus		2.49 ± 0.09	2.33 ± 0.05	2.27 ± 0.11	2.24 ± 0.10
Cerebellum		2.85 ± 0.01	2.79 ± 0.16	2.69 ± 0.11	3.13 ± 0.23
Rest of brain		3.71 ± 0.19	3.93 ± 0.26	3.27 ± 0.02	3.44 ± 0.27

Note. Each value represents the mean ± SEM for the concentration of GFAP (μg/mg total protein). TMT, trimethyltin hydroxide, 8.0 mg/kg, ip, positive control.
[a]Significantly different from control, $p \leq .05$.

TABLE 12
Sister chromatid exchange (SCE) in exposed rats

	Female			Male		
Exposure	SCE, mean ± SE[a]	AGT	MI	SCE, mean ± SE	AGT	MI
Clean air control	5.7 ± 0.67	26	4.0	5.6 ± 0.72	26	3.7
Low	5.4 ± 0.58	25	3.3	5.5 ± 0.72	26	4.4
Mid	5.4 ± 0.67	26	4.8	5.6 ± 0.63	26	4.3
High	5.5 ± 0.67	26	4.5	5.3 ± 0.81	25	4.8
CP	24.1 ± 1.79[b]	26	8.0	23.2 ± 1.39[b]	25	6.8

Note. MI, mitotic index (number of cells in mitosis out of 1000 cells). AGT, average generation time: number of hours in BrdU × 100/(number M_1 cells × 1) + (number M_2 cells × 2) + (number M_3 cells × 3). CP, cyclophosphomide, 5.0 mg/kg, ip, positive control.

[a]The standard error was calculated using the data of all 125 metaphases scored.

[b]Statistically different from control, $p \leq .05$.

cases the fuel, the exposure times, and health assessments varied from those specified in 40 CFR 79. This makes direct comparisons of the possible health effects elicited in studies with PDE and DEE difficult at best.

However, the DEE generated in this study was similar in nature to PDE. Particle size and number counts were consistent with typical PDE and with PDE generated in other studies at this institute and elsewhere (Kittleson, 1998; Reed et al., 2004; McDonald et al., 2004a). At the same PM level (100 $\mu g/m^3$), however, PDE and DEE differed in concentration from component to component. For example, SO_2, CO, and NO_x differed from those measurements reported by Reed et al. (2004) and McDonald et al. (2004a) when similar engines (circa 2000) were operated on national certification diesel fuel. DEE contained roughly equivalent NO_x, slightly higher CO, and ∼10 ppm less SO_2/m^3 than the PDE used in the 2004 Reed study. When the current engines were operated comparatively on CARB and PuriNO$_x$ fuels (∼80% CARB fuel) under identical test conditions approximating those under which the health assessments were performed (Table 6), emission results were similar to those reported in the U.S. EPA Tier I test and those conducted in the field (Barnes et al., 2000; Langer et al., 2000; CA EPA, 2003, 2004). NO_x and PM were reduced in the DEE vs. PDE in all cases (Table 4), suggesting that the conditions under which the health assessments were conducted were representative of a valid test case for the fuel emulsion blend. Total DEE aldehydes were expected to be slightly increased vs. PDE (Barnes et al., 2000; CA EPA, 2003, 2004). The contributions of slightly different magnitudes of components between DEE and PDE to health outcome are unknown. As might be expected, both SVOC and PM components of DEE were mutagenic in several strains of bacteria. These results are similar to those of PDE and reflect the majority petroleum diesel component of the PuriNO$_x$ fuel (U.S. EPA, 2002).

TABLE 13
Percent micronucleated reticulocytes in exposed rats

Exposure	Female, number per 1000 PCEs (mean ± SD)	Male, number per 1000 PCEs (mean ± SD)
Clean air control	1.0 ± 0.32	0.5 ± 0.16
Low	1.1 ± 0.48	0.8 ± 0.30
Mid	0.3 ± 0.20	0.9 ± 0.46
High	0.8 ± 0.34	0.5 ± 0.32
CP	16.0 ± 2.90[a]	19.0 ± 2.14[a]

Note. CP, cyclophosphomide, 40.0 mg/kg, ip, positive control.

[a]Statistically different from control, $p \leq .05$.

The generally mild effects observed in survival, clinical signs, body weight, ophthalmology, and food consumption were consistent with the relatively low concentrations of DEE used in this study and other studies of rodents exposed to PDE (Mauderly, 1999; U.S. EPA, 2002). Overt toxicity was not a necessary goal of this study. Similar gross indicators of toxicity were unaffected by up to 6 mo of PDE at similar levels (Reed et al., 2004) and in an earlier Tier II study of biodiesel emissions (Finch et al., 2002). Ophthalmology and food consumption endpoints have not been addressed thoroughly in these or other PDE exposure studies. The corneal dystrophy observed in all exposure groups, including control, is widely recognized as a congenital condition in the F344 rat strain (Losco & Troup, 1988; Bruner et al., 1992). The lack of progression and the lack of other ophthalmology indicators of toxicity indicated no effect of DEE on this parameter. Interestingly, the DEE-associated slight increase in food consumption has not been observed in other studies with PDE and biodiesel emissions (Mauderly, 1999; Finch et al., 2002). In fact, PDE exposure has been shown to decrease food consumption in cases where the food was present in the exposure chamber during exposures (Maejima & Nagase, 1989). The modest nature, the lack of a clear exposure response, and the lack of a consistent body-weight exposure effect suggest that these observations were not clinically significant and not clearly associated with DEE exposure.

The effect of PDE and other combustion emissions on developmental and reproductive parameters have not been assessed thoroughly. In the current study, there were no exposure effects on pregnancy rates or on developmental and reproductive indices. These observations reflect the results observed in mouse fertility/reproduction after exposure to 6 g PM/m^3 PDE for 5 to 10 wk (Pepelko & Periano, 1983) and those of rats exposed to biodiesel emissions (Finch et al., 2002). Although fetal masculinization has been reported when pregnant females have been exposed to high levels of PDE (5.63 mg PM/m^3) (Watanabe & Kurita, 2001), there were no such observations in the current study.

The slight to modest nature of the several observations of organ weight changes were not associated with clear histopathology nor clearly attributed to exposure to DEE. In general, slight and variable effects on organ weights were observed in F344 rats exposed to similar levels of PDE for up to 6 mo (Reed et al., 2004). Although measured by absolute, percent body weight, and percent brain weight indices, no consistent or coherent pattern of organ alterations exists in PDE/DEE exposure studies that span the environmental range of PDE or DEE (U.S. EPA, 2002; Reed et al., 2004). Outside of the lung and beyond occasional and sporadic observations unrelated to exposure, there was no histopathology present in any of the organs or tissues examined in this study.

One of the most pronounced but expected results of DEE exposures was accumulation of PM within macrophages and associated macrophage hyperplasia. These observations are comparable with PDE and other PM sources and constitute a normal response of the lung to PM (U.S. EPA, 2002; Mauderly, 1999). Consistent with the observations in this study, no overt pathology resulted from PM accumulation in macrophages from rats exposed to low-level PDE for up to 6 mo (Reed et al., 2004). In general, except in conditions of severe particle overload where normal clearance mechanisms are overcome, PM accumulation and PM associated macrophages have little pathological effect (Mauderly, 1999; Sydbom et al., 2001).

Of the numerous statistically significant observations within clinical chemistry and hematology parameters measured or calculated in this study, those most clearly related to exposure were serum cholesterol and platelet values at 3 mo of exposure and platelet values after recovery. The former observation has precedents in the PDE literature. Reed et al. (2004) observed decreases in cholesterol in male and female F344 rats exposed to PDE for 1 wk and 6 mo. Similar changes have been observed in rats exposed to levels of PDE ranging from 0.5 to 6.6 mg PM/m^3 PDE (Maejima & Nagse, 1989; Brightwell et al., 1986). This observation is certainly consistent among PDE exposures, but the mechanism of effect remains uncertain. Other effects likely associated with exposure after 3 mo were serum electrolytes (Na and Cl), SDH, and albumin–total protein. Slight decreases in Na and Cl were observed in F344 rats exposed to PDE for 6 mo (Reed et al., 2004) as well as those exposed to PDE for up to 30 mo at 0.5–3.7 mg PM/m^3 (Maejima & Hagase, 1989). Differential effects on total protein and albumin were observed in the latter studies, but all were slight and of undetermined clinical significance. BUN measurements subsequent to PDE have been variable. Maejima and Nagase (1989) observed slight increases in BUN in some groups of animals exposed to PDE, while Reed et al. (2004) observed slight decreases in females exposed to PDE for 6 mo. In the current study, the observed slight decreases subsequent to DEE exposure at various time points in both sexes are certainly consistent with PDE exposure studies but are difficult to interpret and likely of little clinical significance. SDH increases in this study may seem rather large when compared to other changes in clinical chemistry parameters, but in the context of serum liver enzymes, changes in excess of 20-fold or more are seen with overt toxicities (O'Brien et al., 2002). The lack of corroborating histopathology suggests that liver function was likely not significantly imparied. SDH was not measured in previous PDE exposure studies, although Reed et al. (2004) observed similar changes in GGT subsequent to PDE exposure. However, since effects on other serum enzymes were minimal and variable, the equivocal nature of changes in liver enzymes subsequent to PDE/DEE exposure suggests that they were not exposure related.

The persistent increase in male platelet values through 3 mo of DEE exposure and at recovery is puzzling. Reed et al. (2004) observed no changes in male or female platelet values in rats exposed to PDE for up to 6 mo. Other, higher concentration PDE studies also revealed no platelet alterations after PDE exposure (Maejima & Nagase, 1989; Brightwell et al., 1986). The differential effect between sexes in the current study is difficult to interpret as well. Nevertheless, these observations would suggest that slight platelet alterations may exist in male rats exposed to DEE. The clinical significance of this alteration is uncertain.

The minimal increases in other RBC parameters (hematocrit, RBC count, hemoglobin) and the variable changes observed in the WBC count were not observed in the latest PDE study conducted at this institute (Reed et al., 2004). Other high-exposure PDE studies observed alterations in many of these parameters, but the clinical significance was not determined (Brightwell et al., 1986). Although the statistically significant observations in RBC parameters were consistent in direction of change (increase), the lack of a clear exposure response in males and the overall minimal magnitude of change from control indicate that these observations are likely not clinically significant. Similarly, the lack of exposure response and the variable nature of the WBC indices suggest that these changes were not related to DEE exposure.

In summary, consistent with the good general health of all exposed rats, the statistically significant changes in clinical chemistry and hematology were not supported by any gross/microscopic pathology or damage to any major organ system.

The current study was not designed to evaluate the carcinogenic effects of DEE. The carcinogenicity of chronic PDE exposures has been assessed in several studies of animals (Pepelko & Peirano, 1983; Heinrich et al., 1986, 1995; Mauderly et al., 1987, 1996; Ishinishi et al., 1988; Brightwell et al., 1989) and reviewed extensively (HEI, 1995; CA EPA, 1998; Mauderly, 1999; U.S. EPA, 2002). In short, rats exposed chronically to extremely high concentrations of PDE develop lung tumors, which appear to be a somewhat nonspecific response accompanying the overloading effect of the lung with poorly soluble PM (Mauderly, 1997; Heinrich et al., 1995; Nikula et al., 1995). Lifetime exposure studies of tumorigenesis are not called for in the U.S. EPA Tier II design and were not possible in the 90-day exposure format in the current study. However, indicators of clastogenesis and chromosomal alterations (e.g., carcinogenic potential),

bone-marrow micronuclei and SCE, were unaffected by DEE in the current study. The absence of exposure effects is similar to those observed by Reed et al. (2004) in strain A/J mice exposed to PDE for 6 mo at similar exposure levels. In rats, no changes in micronucleus counts or SCE have been observed subsequent to exposure to PDE (Morimoto et al., 1986; Ishinishi et al., 1988; Pepelko & Periano, 1983) or biodiesel emissions (Finch et al., 2002). In fact, the only rodent model showing PDE-induced, exposure-related increases in MN or SCE was Syrian hamsters exposed to PDE at 6 mg PM/m^3 for 6 mo (Pepelko & Peirano, 1983).

A universal cellular reaction to damage of the central nervous system is hypertrophy of astrocytes. The defining characteristic of this response, often termed reactive gliosis, is the enhanced expression of the major intermediate filament protein of astrocytes, GFAP. Thus, an increase in the brain concentration of GFAP serves as a biochemical indicator of neurotoxicity. Central nervous system levels of GFAP have not been assessed previously in rats exposed to PDE or DEE but, nevertheless, are a requirement of the Tier II testing procedures. The decreases observed in GFAP levels in some brain regions of some exposure groups in the current study, although small (none $>19\%$), were statistically significant. However, these observations cannot be interpreted as adverse given that prior studies (and the positive controls in this study) indicate that GFAP levels increase rather than decrease in response to known neurotoxicants (e.g., TMT) (O'Callaghan, 1991; Norton et al., 1992). One speculative possibility for these effects may be associated with male corticosterone levels. Small, neuroendocrine-regulated decreases in GFAP have been reported in association with high physiological levels of corticosterone (O'Callaghan et al., 1991). Thus, it is possible that the exposed males had elevated levels of corticosterone sufficient to result in small decreases in GFAP. While enhanced expression of GFAP serves as a signature of brain injury-induced glial activation (gliosis), small decreases in GFAP, such as those observed in the present study, are likely to reflect exaggerated physiological effects with no known adverse consequences (O'Callaghan et al., 1991). Overall, the data suggest that under the exposure conditions employed, damage-induced gliosis did not occur in the brain regions examined.

Using these cumulative data to survey the comparative risks of PDE, DEE, and other alternative fuels (e.g., biodiesel emissions) is difficult given that no baseline PDE exposure study has been conducted in the Tier II program. However, the results of this, the second of three Tier II studies conducted at this institute on a novel alternative diesel fuel, were consistent with the mild biological responses observed after exposure to reasonable concentrations of biodiesel emissions (Finch et al., 2002) and a third study of DEE (an all-weather fuel blend developed by The Lubrizol Corporation; Reed et al., unpublished, in preparation). This body of evidence and that of other PDE exposure studies suggests that the risks of DEE are no greater than that of PDE and other alternative diesel fuels (Reed et al., 2004).

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