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JET FUEL KEROSENE IS NOT IMMUNOSUPPRESSIVE IN MICE OR RATS FOLLOWING INHALATION FOR 28 DAYS

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Previous reports indicated that inhalation of JP-8 aviation turbine fuel is immunosuppressive. However, in some of those studies, the exposure concentrations were underestimated, and percent of test article as vapor or aerosol was not determined. Furthermore, it is unknown whether the observed effects are attributable to the base hydrocarbon fuel (jet fuel kerosene) or to the various fuel additives in jet fuels. The present studies were conducted, in compliance with Good Laboratory Practice (GLP) regulations, to evaluate the effects of jet fuel kerosene on the immune system, in conjunction with an accurate, quantitative characterization of the aerosol and vapor exposure concentrations. Two female rodent species (B6C3F1 mice and Crl:CD rats) were exposed by nose-only inhalation to jet fuel kerosene at targeted concentrations of 0, 500, 1000, or 2000 mg/m^3 for 6 h daily for 28 d. Humoral, cell-mediated, and innate immune functions were subsequently evaluated. No marked effects were observed in either species on body weights, spleen or thymus weights, the T-dependent antibody-forming cell response (plaque assay), or the delayed-type hypersensitivity (DTH) response. With a few exceptions, spleen cell numbers and phenotypes were also unaffected. Natural killer (NK) cell activity in mice was unaffected, while the NK assessment in rats was not usable due to an unusually low response in all groups. These studies demonstrate that inhalation of jet fuel kerosene for 28 d at levels up to 2000 mg/m^3 did not adversely affect the functional immune responses of female mice and rats.

In 1979, the U.S. Air Force (USAF) began transitioning from the use of jet propulsion fuel-4 (JP-4) to jet propulsion fuel-8 (JP-8) in all of its aircraft, due to a number of performance and safety advantages. The conversion was completed in 1994, and JP-8, which is composed primarily (>99%) of jet fuel kerosene (Ritchie et al., 2003), is now the primary jet fuel used by the U.S. military (Marchant-Borna et al., 2012; Mattie and Sterner, 2011). In 2010, approximately 22 billion gallons of kerosene-based jet fuels were consumed in the United States (U.S. EIA, 2013).

The generic term "kerosene" is used to describe the fraction of crude oil that boils approximately in the range of 302 to 554°F (150 to 290°C) and consists of hydrocarbons approximately in the range of C9–C16 (Lam et al., 2012). The refinery process streams used to blend kerosene-based products are complex substances listed on the U.S. Toxic Substance Control Act (TSCA) Chemical Inventory as

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UVCB substances, that is, Chemical Substances of Unknown or Variable Composition, Complex Reaction Products, and Biological Materials (API, 2010; U.S. EPA, 1995). The predominant use of kerosene in the United States is aviation turbine fuel for civilian (using Jet A or Jet A-1) and military (using JP-8 or JP-5) aircraft. Kerosenes are also used as diesel fuel (no. 1), domestic heating fuel (fuel oil no. 1), and illuminating kerosene (no. 1-K) (Lam et al., 2012). Kerosene-based fuels differ from each other in performance specifications (primarily freezing point or sulfur concentration) and in the minor amounts of performance additives that may be added (generally less than 0.1% v/v) (ASTM, 2001a, 2001b, 2002).

The chemical compositions of kerosene-based jet fuels are not fixed by their specifications; however, they are bounded by specification requirements such as aromatics, density, boiling range, and freezing point. A world survey of aviation turbine fuels reported an average composition (volume percent) of 54% paraffins (normal + iso), 26% cycloparaffins, 14% alkylbenzenes, and 4.8% indans and tetralins (Hadaller and Johnson, 2006). The concentration of individual chemical constituents like benzene and naphthalene were below the limits of detection (LOD).

Early toxicity studies evaluating JP-8 showed no evidence of treatment-related effects on the primary immune organs, that is, the spleen and the thymus, in either mice or rats exposed to JP-8 by continuous inhalation for 90 d (Mattie et al., 1991). In addition, studies conducted in rats and dogs exposed to deodorized kerosene vapors at doses up to 100 mg/m^3 for 13 wk (6 h/d, 5 d/wk) also demonstrated no histopathological effects on the spleen (Carpenter et al., 1976). However, a series of later studies by Harris et al. (1997, 2000a, 2000b, 2002) reported significant effects on cell-mediated immunity (CMI) in C57BL/6 mice exposed (1 h/d for 7 d) to JP-8 aerosol at concentrations as low as 100 mg/m^3. Further, Harris et al. (2007, 2008) also reported that JP-8 jet fuel inhalation resulted in increased B16F10 melanoma tumor burden and a decreased resistance to influenza A virus in exposed animals.

Concerns about the exposure levels in the Harris et al. studies were expressed by the National Research Council (NRC) Committee on Toxicology (National Research Council, 2003). Indeed, a subsequent report from the Harris lab indicated that the exposure levels in the earlier studies were approximately eight-fold higher than vapor/aerosol levels obtained using different equipment and quantified using more accurate and reliable systems (Hilgaertner et al., 2011). Further, the NRC Committee on Toxicology also recommended that additional inhalation studies be conducted in conjunction with an accurate characterization of the exposure atmosphere, utilizing standardized, interlab validated protocols (Luster et al., 1988, 1992), to assess the immunotoxicity of JP-8 and some of its primary constituents, such as the base hydrocarbon fuel (National Research Council, 2003).

The present studies were conducted in female B6C3F1 mice and female Crl:CD rats to evaluate the effects of jet fuel kerosene inhalation on various components of the immune system. The studies were conducted in conjunction with an accurate and quantitative characterization of the aerosol and vapor exposure concentrations, with targeted exposure levels of 0, 500, 1000, and 2000 mg/m^3. Assessments of the effects on humoral, cell-mediated, and innate immunity were conducted following a 28-d nose-only exposure protocol.

MATERIALS AND METHODS

Compliance

This study was conducted in compliance with the following guidelines and regulations:

1. U.S. Environmental Protection Agency (EPA) Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards (GLP), Final Rule, 40 CFR Part 792 (1989).
2. Test Conditions sections of Organization for Economic Cooperation and Development (OECD), Section 4 (Part 412): Repeated

Dose Inhalation Toxicity: 28-Day or 14-Day Study, *Guideline for the Testing of Chemicals* (1981).
3. European Economic Community (EEC), Method B.8 Directive 92/69/EEC: Repeated Dose (28 Days) Toxicity (Inhalation), *Methods for the Determination of Toxicity* (1992).
4. U.S. EPA, OPPTS 870.7800: Immunotoxicity, *Health Effects Test Guidelines* (1998).

In addition, this study was conducted in compliance with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR) as well as the NRC Guide for the Care and Use of Laboratory Animals (1996).

Test Substance

Unadditized jet fuel kerosene (CAS no. 8008-20-6) was provided by the U.S. Air Force Research Laboratory (Wright-Patterson AFB, Dayton, OH). The test article was a "Composite Jet A Blend" created in 2004 (and designated as POSF-4658) by blending five separate unadditized Jet A samples from different manufacturers. Part of the blended test substance was transferred to EPL Archives, Inc., and designated as sample 107796. It was then shipped to DuPont Haskell and designated as test article 27294.

Animals

The in-life phase of this study was conducted at DuPont Haskell in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International under a protocol reviewed by the Haskell Animal Welfare Committee. Female B6C3F1 mice and female Crl:CD rats were obtained from Charles River Laboratories (Raleigh, NC). Animals were quarantined upon arrival and examined for gross signs of disease or injury and for adequate body weight gain prior to being placed on study. Mice were 8-10 wk of age and weighed between 19 and 26.1 g at the beginning of the study, while rats were approximately 7-9 wk of age and weighed between 176.3 and 253.1 g.

All animals were housed individually in solid bottom cages with bedding (mice) or stainless-steel wire-mesh cages (rats) and maintained on Certified Rodent LabDiet 5002 (PMI Nutrition International) and tap water ad libitum, except during exposures. Animal rooms were maintained at a temperature of 18–26°C and relative humidity of 30–70% on a 12-h light/dark cycle.

Experimental Design

The 28-d inhalation study was divided into three parts: Parts 1, 2, and 3. Animals randomly assigned to Part 1 were immunized with sheep erythrocytes (sRBC) 4 d prior to euthanasia, in order to assess the T-dependent antibody-forming cell (AFC) response. Animals randomly assigned to Part 2 were not immunized and were evaluated for effects on spleen and thymus weights, spleen cell numbers, spleen cell phenotypes, anti-CD3 antibody-mediated T cell proliferation, and natural killer cell activity. Animals randomly assigned to Part 3 were sensitized with formalin-fixed *Candida albicans* and evaluated for effects on the delayed-type hypersensitivity (DTH) response.

In each part, three jet fuel kerosene exposure groups (targeted atmospheric concentrations of jet fuel kerosene: 500, 1000, and 2000 mg/m^3) and a nose cone–air only control group (i.e., "control"; 0 mg/m^3 jet fuel kerosene) were utilized. Each exposure group consisted of 10 animals. In addition, positive control groups of 10 animals each were utilized for each immunological assay as detailed below. In Part 3, a challenge-only group of 10 untreated animals was also included. Finally, all mouse studies also included an unrestrained group of mice ("control unrestrained") that were not placed into nose cone holders. These animals were exposed to ambient room air (0 mg/m^3 jet fuel kerosene). These mice were utilized to determine if the stress related to confinement in the nose-only inhalation chambers affected the various immune parameters.

Inhalation Exposures

Animals in the 0, 500, 1000, and 2000 mg/m^3 jet fuel kerosene exposure groups were exposed by nose-only inhalation for 6 h/d, 7 d/wk, for 4 wk, resulting in a total of 28 exposures. The exposure chambers were constructed of stainless steel and glass (NYU style) with a nominal internal volume of 150 L. A stainless-steel baffle at the chamber inlet promoted uniform chamber distribution of the test atmosphere. During exposure, animals were individually restrained in perforated stainless-steel cylinders with conical nosepieces. The restrainers were inserted into a polymethylmethacrylate faceplate attached to the exposure chamber so that the nose of each animal extended into the exposure chamber. Before the start of the exposure phase, animals were placed in restrainers on 2 separate days for approximately 15 min each day to acclimate the animals to the nose-only restrainers.

The atmospheric concentration, chamber distribution, and atmosphere stability of jet fuel kerosene were determined and previously validated by gravimetric analysis and gas chromatography for the aerosol and vapor components, respectively (DeLorme et al., n.d.). Atmosphere for the low exposure chamber was generated by flash evaporation of the liquid test substance in a round-bottom, flash evaporation flask covered with a heating mantle heated to 175°C. The test substance was metered into the evaporation flask with a Harvard Apparatus syringe infusion pump. High pressure generation air was passed over the test substance and the resulting vapor traveled into a glass tube filled with glass wool, which was wrapped with heat tape, heated to 95°C prior to entering the chamber turret, where it was mixed with dilution air. The purpose of the glass wool was to trap most of the aerosol phase of test substance and facilitate its evaporation.

Atmospheres for the intermediate and high exposure chambers were generated by aerosolization of test substance in air with a Spraying Systems nebulizer. The test substance was metered into the nebulizer with a Harvard Apparatus model 22 syringe infusion pump. High-pressure air was metered into the nebulizer by a Brooks mass flow controller. The nebulizers sprayed directly into the top of the chamber turret. Dilutional air was metered to a side entry port of the chamber turret with a Brooks mass flow controller.

Known volumes of chamber atmosphere were drawn from the breathing zone of the animals through a sampling train consisting of a gravimetric filter apparatus containing a preweighed glass-fiber filter followed by a glass fritted midget impinger containing acetone as the trapping media. Following the air sampling procedure, the filters were removed from the filter apparatus and weighed on a Cahn microbalance. The aerosol concentration of the exposure atmosphere was determined by measuring the difference between the pre- and postsampling filter weights divided by the volume of chamber atmosphere sampled. Aliquots of collection medium in the impinger were injected into an Agilent Technologies model 6890N gas chromatograph equipped with a flame ionization detector. The atmospheric concentration of the vapor components was determined from a standard curve derived from liquid jet fuel kerosene standards. Standards were prepared by weighing known volumes of the liquid test substance and quantitatively diluting the test substance in acetone. Because jet fuel kerosene is a complex substance with hundreds of individual components, all component peak areas were summed to produce a total area count for quantifying the amount of jet fuel kerosene in the chamber samples and standard solutions. On a weekly basis, samples were injected a second time to determine the area percentages of 11 components (octane, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, and naphthalene) using the same gas chromatography (GC) conditions but different integration parameters to determine area percentage instead of total concentration. The identification of the peaks from the 11 components was compared to retention times determined by injecting individual samples of the components

purchased from Sigma-Aldrich. The total exposure concentrations were calculated by adding the aerosol and vapor concentrations.

Samples to determine the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were taken from the 1000 and 2000 mg/m^3 target concentration atmospheres with a Sierra series 210 cyclone preseparator/cascade impactor and Sierra series 110 constant flow air sampler.

Positive Controls

In each part, animals in a group of 10 were treated with cyclophosphamide (CPS; 50 mg/kg for mice; 25 mg/kg for rats) by *ip* injection once daily for 4 d prior to euthanasia by CO_2 overexposure. A second positive control group was utilized in Part 2 for natural killer (NK) cell activity and NK cell phenotyping. These animals were administered anti-asialo GM1 antibody (AAGM1), diluted 1:10 in 0.9% saline, in a single iv injection (0.2 ml for mice; 1 ml for rats) approximately 24 h prior to euthanasia by CO_2 overexposure.

Clinical Observations and Body Weights

Body weights were obtained at least once each week, before the daily exposure. Immediately following every exposure and every weighing, all animals were individually examined for abnormal behavior and appearance.

Splenocyte Preparation

Spleens from animals in Parts 1 and 2 were collected aseptically, placed into 3 ml (mice) or 6 ml (rats) of Earle's balanced salt solution (EBSS) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and gentamicin, packed on wet ice, and shipped overnight to ImmunoTox®, Inc. The delayed immunotoxicological assessment of lymphoid tissues for up to 24 h has been shown to produce results comparable to those of studies conducted on freshly harvested tissues (Jovanovic et al., 1999; Woolhiser and McCay, 1999). Upon receipt, spleens were prepared into single-cell suspensions. For mice, spleens were pressed between the frosted ends of two microscope slides, placed into 12 × 75-mm snap-cap tubes, centrifuged for 10 min at 300 × g, and resuspended in assay-specific media. For rats, spleen cell suspensions were prepared by mashing spleens using a Stomacher 80 Lab Blender. Cells were placed into 17 × 100-mm snap-cap tubes, centrifuged for 10 min at 300 × g, and resuspended in assay-specific media. The medium used for Part 1 samples was EBSS with HEPES, while the medium used for Part 2 samples was Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS). Spleen cell numbers were determined using a Z1 Coulter counter in the presence of ZAP-OGLOBIN II lytic reagent (Coulter Corporation, Miami, FL). Viability testing was conducted on spleen cell preparations on a Coulter Epics XL-MCL flow cytometer, following staining with propidium iodide (PI).

Spleen IgM AFC Response to SRBC

All animals assigned to Part 1 of the study were immunized (iv) with sRBC 4 d prior to euthanasia. Mice received 7.5×10^7 sRBC in 0.2 ml EBSS with HEPES, and rats received 2×10^8 sRBC in 0.5 ml EBSS with HEPES. On the day of euthanasia, spleens were removed, weighed, and shipped to ImmunoTox®, Inc., on wet ice for next-day evaluation. On the day samples were received, spleen-cell suspensions were prepared as described earlier, and the primary immunoglobulin M (IgM) AFC response to the T-dependent antigen sRBC was evaluated using the hemolytic plaque assay of Jerne et al. (1963) with modifications described by White et al. (2010). Results were expressed in terms of specific activity (AFC/10^6 Spleen cells) and total Spleen activity (AFC/Spleen).

Splenocyte Phenotyping

Splenocytes from animals assigned to Part 2 of the study were analyzed by flow cytometry in order to determine percentages and

absolute numbers of various spleen cell populations. The populations evaluated were B cells, total T cells, T helper (T_H) cells, T cytotoxic (T_{CTL}) cells, NK cells, and macrophages. For mice, the procedure was conducted using 96-well plates (5 wells per sample). Each well contained 100 μl of cells (1×10^7 cells/ml), and 100 μl of appropriate antibodies (diluted 1:80) to identify the desired populations: IgG^+ (B cells), $CD3^+$ (total T cells), $CD3^+CD4^+$ (T_H cells), $CD3^+CD8^+$ (T_{CTL} cells), $NK1.1^+CD8^-$ (NK cells), and Mac-3^+ (macrophages). For rats, the procedure was conducted using 12 × 75-mm tubes as previously described (Mann et al., 2008), and the following populations were identified: $CD45RA^+$ (B cells), $CD5^+$ (total T cells), $CD4^+CD5^+$ (T_H cells), $CD8^+CD5^+$ (T_{CTL} cells), $NKRP1A^+CD8^+$ (NK cells), and Mac^+ (clone His36; macrophages). All antibodies were obtained from BD Biosciences. Samples were analyzed using a Coulter® Epics® XL-MCL™ flow cytometer.

AntiCD3 Antibody-Mediated T-Cell Proliferation

One day prior to assay, flat-bottomed 96-well microtiter plates were treated overnight at 2–8°C with 100 μl/well of either phosphate-buffered saline (PBS) or a 1:500 dilution of anti-CD3 antibody (purified anti-mouse CD3e or purified anti-rat CD3; BD Pharmingen) in PBS. On the day of the assay, plates were washed with PBS (200 μl/well). Spleen cells were adjusted to 2×10^6 cells/ml (mice) or 5×10^6 cells/ml (rats) in complete RPMI 1640 media and were added in a volume of 100 μl/well in quadruplicate to both PBS-treated and anti-CD3-treated wells. An additional 100 μl media was added to each well. Plates were incubated for 3 d at 36–38°C and 4–7% CO_2. Approximately 18–24 h prior to harvest, all wells were pulsed with 1 μCi [^{3}H]thymidine/well. Cells were harvested onto filter mats using a Tomtec harvester and counted on a Wallac 1450 Microbeta Trilux liquid scintillation and luminescence counter (Turku, Finland). The incorporation of [^{3}H]thymidine into proliferating cells was used as the endpoint of the assay, and results are presented as counts per minute (CPM)/(2×10^5 splenocytes) for mice and as CPM/(5×10^5 splenocytes) for rats.

Delayed-Type Hypersensitivity Response to *Candida albicans*

Animals assigned to Part 3 of the study were evaluated using a modification of the *Candida albicans* DTH assay developed by Nghiem et al. (2002). Formalin-fixed *C. albicans* and chitosan antigen were obtained from AlerChek, Inc. (Springvale, ME). For mice, the assay was conducted as previously described (Smith and White, 2010; White et al., 2012). For rats, the assay was conducted using the time course, sensitization dose, and challenge level previously optimized in our lab. Specifically, on exposure d 21, rats were sensitized by sc injection of 0.2 ml formalin-fixed *C. albicans* organisms (1×10^8 organisms/ml) in the right flank. On d 29, that is, 1 d after the last inhalation exposure, rats were challenged in the right footpad with chitosan antigen (approximately 1 mg/ml) in a volume of 100 μl. Footpad swelling was measured prior to challenge and again 24 ± 2 h after challenge. All results were presented in terms of footpad swelling with units of mm × 100. Animals assigned to the challenge only groups in Part 3 were not sensitized with *C. albicans* on d 21. However, these animals were challenged with chitosan antigen on d 29 to control for nonspecific inflammation resulting from the chitosan challenge.

Natural Killer Cell Activity

The cytotoxic activity of NK cells was evaluated using the method of Reynolds and Herberman (1981) in a 4-h ^{51}Cr release assay, with modifications as described previously (Mann et al., 2008). The target cells utilized were YAC-1 cells, which had been labeled with ^{51}Cr, and six effector-to-target (E:T) ratios were evaluated for each spleen cell suspension. The release of ^{51}Cr into the supernatant was used as the endpoint of the assay, and data were expressed in terms of percent cytotoxicity,

which was calculated as the difference between experimental and spontaneous releases divided by the difference between maximum and spontaneous releases.

Statistical Analysis

All results are presented in terms of mean ± standard error (SE) for 9 or 10 animals per group. Bartlett's test for homogeneity was utilized to determine the appropriate analysis to be conducted. Homogeneous data were evaluated using a one-way analysis of variance (ANOVA), and nonhomogeneous data were analyzed using a non-parametric ANOVA. When significant differences occurred, exposure groups were compared to the control group using Dunnett's test (homogeneous data) or the Wilcoxon rank test (nonhomogeneous data). All positive control groups and the challenge-only group in the DTH assay were compared individually to the control group using the Student's *t*-test. Jonckheere's test was used for trend analysis. In all evaluations, $p \leq .05$ indicated statistically significant differences.

RESULTS

Exposure Atmosphere Characterization

There was no detectable jet fuel kerosene in the control chambers during either the mouse or rat studies. For the mouse exposures, the overall mean concentration of aerosol in the 0 mg/m^3 (control nose cone) chamber was 0.23 ± 0.16 mg/m^3, which represented background aerosol levels. The overall mean jet fuel kerosene concentration in the low-exposure chamber was 510 ± 5.7 mg/m^3 vapor phase and 0.89 ± 0.032 mg/m^3 aerosol phase, which represented a total exposure concentration of 510 mg/m^3 (rounded to two significant digits). The overall mean jet fuel kerosene concentration in the intermediate-exposure chamber was 880 ± 7.3 mg/m^3 vapor phase and 130 ± 2 mg/m^3 aerosol phase, which represented a total exposure concentration of 1000 mg/m^3. The aerosol was characterized by an MMAD (GSD) of 2.7 μm (2) ($n = 6$). The overall mean jet fuel kerosene concentration in the high-exposure chamber was 1500 ± 13 mg/m^3 vapor phase and 530 ± 11 mg/m^3 aerosol phase, which represented a total exposure concentration of 2000 mg/m^3. The aerosol was characterized by an MMAD (GSD) of 3.2 μm (2) ($n = 6$). The area percentages of the 11 selected components (octane, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, and naphthalene) in all 3 exposure chambers remained steady over the course of the study and no test material instability was indicated (Table 1).

For the rat exposures, the overall mean concentration of aerosol in the control chamber was 0.027 ± 0.1 mg/m^3. The overall mean jet fuel kerosene concentration in the low-exposure chamber was 510 ± 11 mg/m^3 vapor phase and 2.5 mg/m^3 ± 0.31 mg/m^3 aerosol phase, which represents a total exposure concentration of 510 mg/m^3. The overall mean jet fuel kerosene concentration in the intermediate-exposure chamber was 880 ± 10 mg/m^3 vapor phase and 120 mg/m^3 ± 4 mg/m^3 aerosol phase, which represents a total exposure concentration of 1000 mg/m^3. The aerosol was characterized by a MMAD (GSD) of 2.4 μm (2.4) ($n = 5$). The overall mean jet fuel kerosene concentration in the high-exposure chamber was 1400 ± 14 mg/m^3 vapor phase and 590 ± 16 mg/m^3 aerosol phase, which represents a total exposure concentration of 2000 mg/m^3. The aerosol was characterized by a MMAD of 2.8 μm (2.4) ($n = 6$). The area percentages of the 11 selected components in all 3 exposure chambers remained steady over the course of the study and no test substance instability was indicated (Table 2).

General Toxicology

No statistically significant effects were observed in terminal body weights and either absolute or relative weights of the spleen and thymus, between control and jet fuel kerosene-exposed animals for either species

TABLE 1. Eleven Component Vapor Phase Analysis for the Mouse Exposure Atmospheres

	Area Percent for:					
Exposure	Octane	Nonane	Decane	Undecane	Dodecane	Tridecane
500 mg/m^3	0.94 ± 0.056	3.5 ± 0.18	6.8 ± 0.25	8.4 ± 0.21	5.7 ± 0.22	3.2 ± 0.20
1000 mg/m^3	1.1 ± 0.033	3.7 ± 0.66	7.2 ± 0.13	8.9 ± 0.25	5.8 ± 0.18	2.9 ± 0.073
2000 mg/m^3	1.5 ± 0.15	5.1 ± 0.55	8.5 ± 0.38	8.4 ± 0.32	4.1 ± 0.39	1.6 ± 0.22
	Area Percent for:					
Exposure	Tetradecane	Pentadecane	Hexadecane	Heptadecane	Naphthalene	
500 mg/m^3	1.1 ± 0.12	0.36 ± 0.16	<MDL ± NA	<MDL ± NA	<MDL ± NA	
1000 mg/m^3	0.83 ± 0.31	0.15 ± 0.050	<MDL ± NA	<MDL ± NA	<MDL ± NA	
2000 mg/m^3	0.35 ± 0.18	0.050 ± NA	<MDL ± NA	<MDL ± NA	<MDL ± NA	

Results represent the mean ± SD, n = 6. <MDL-below minimum detection level, NA-not applicable.

TABLE 2. Eleven Component Vapor Phase Analysis for the Rat Exposure Atmospheres

	Area Percent for:					
Exposure	Octane	Nonane	Decane	Undecane	Dodecane	Tridecane
500 mg/m^3	0.94 ± 0.16	3.7 ± 0.49	6.7 ± 0.32	7.7 ± 0.35	5.4 ± 0.44	3.3 ± 0.22
1000 mg/m^3	1.1 ± 0.034	3.8 ± 0.11	7.2 ± 0.16	8.7 ± 0.17	5.4 ± 0.17	2.6 ± 0.083
2000 mg/m^3	1.1 ± 0.079	4.0 ± 0.22	7.3 ± 0.22	8.4 ± 0.11	4.9 ± 0.24	2.3 ± 0.17
	Area Percent for:					
Exposure	Tetradecane	Pentadecane	hexadecane	Heptadecane	Napthalene	
500 mg/m^3	1.3 ± 0.13	0.23 ± 0.050	0.051 ± NA	<MDL ± NA	<MDL ± NA	
1000 mg/m^3	0.56 ± 0.11	0.15 ± NA	<MDL ± NA	<MDL ± NA	<MDL ± NA	
2000 mg/m^3	0.68 ± 0.12	0.15 ± NA	<MDL ± NA	<MDL ± NA	<MDL ± NA	

Results represent the mean ± SD, n = 5–6. <MDL-below minimum detection level, NA-not applicable.

(Table 3). As anticipated, the positive control (CPS) produced significant decreases in the absolute and relative weights of both the spleen and the thymus in both species. In addition, some statistically significant differences between control (i.e., nose cone) and control unrestrained groups of mice were observed. Specifically, when compared to the unrestrained control animals, the nose cone control animals demonstrated reduced terminal body weights (7%), decreased absolute and relative thymus weights (26 and 22%, respectively), and increased relative spleen weights (14%), while absolute spleen weights were unaffected (Table 3). Previous studies conducted by Pruett and colleagues (2000) demonstrated the immunosuppressive effects of restraint on various immune parameters, including decreased spleen and thymus cell numbers and suppression of anti-keyhole limpet hemocyanin (KLH) antibody production (Pruett and Fan, 2001). While one might speculate that the observed differences in immune organ weights and selected immune functions in the present report (as discussed later) between the nose cone control and control unrestrained groups were the result of restraint stress, extensive well-designed studies focused on this question would be required before a definitive conclusion could be reached.

Immunology

Spleen Cell Phenotyping The results of the spleen cell phenotypic analysis conducted in mice exposed to jet fuel kerosene are given in Table 4. While increases in spleen cell numbers were observed in all jet fuel kerosene exposure groups, as compared to control, these did not reach the level of statistical significance. No significant differences were observed in the absolute or percentage values of any

TABLE 3. Terminal body weights, spleen weights, and thymus weights of female B6C3F1 mice and female Crl:CD® rats exposed to Jet Fuel Kerosene by inhalation for 28 days

Parameter	Control Unrestrained	Control Nose Cone Air Only	Jet Fuel Kerosene (mg/m^3) 500	1000	2000	CPS 50 mg/kg (mice) 25 mg/kg (rats)	H/NH	Trend Analysis
Mice								
Body Wgt (g)	24.6 ± 0.3*	23.0 ± 0.4	23.6 ± 0.3	23.3 ± 0.3	23.5 ± 0.3	22.5 ± 0.5	H	NS
Spleen (mg)	116 ± 5	124 ± 4	139 ± 10	136 ± 12	126 ± 6	93 ± 6*	NH	NS
% Body Wgt	0.471 ± 0.016*	0.539 ± 0.016	0.588 ± 0.041	0.583 ± 0.053	0.536 ± 0.024	0.414 ± 0.026*	NH	NS
Thymus (mg)	55.1 ± 2.3*	40.9 ± 2.1	42.1 ± 3.2	44.3 ± 4.1	44.5 ± 3.3	27.0 ± 2.3*	H	NS
% Body Wgt	0.226 ± 0.009*	0.177 ± 0.009	0.177 ± 0.013	0.189 ± 0.017	0.189 ± 0.013	0.121 ± 0.011*	H	NS
Rats								
Body Wgt (g)	NA	254.1 ± 4.5	252.3 ± 7.6	240.4 ± 6.6	239.1 ± 5.7	251 ± 9.0	H	$p \leq 0.05$
Spleen (mg)	NA	556 ± 21	544 ± 33	489 ± 21	529 ± 25	310 ± 19*	H	NS
% Body Wgt	NA	0.22 ± 0.01	0.22 ± 0.01	0.20 ± 0.01	0.22 ± 0.01	0.12 ± 0.01*	NH	NS
Thymus (mg)	NA	390 ± 19	390 ± 26	415 ± 29	328 ± 24	159 ± 19*	H	NS
% Body Wgt	NA	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.14 ± 0.01	0.06 ± 0.01*	H	NS

Values represent the mean ± SE derived from 9 or 10 animals per group; *$p \leq 0.05$ compared to the control nose cone air only group; H = homogeneous data; NH = non-homogeneous data; NS = not significant; NA = not applicable.

TABLE 4. Spleen cell phenotypes in female B6C3F1 mice exposed to Jet Fuel Kerosene by inhalation for 28 days

Exposure	Spleen Cells	Ig^{+a}	$CD3^{+b}$	$CD3^{+}CD4^{+c}$	$CD3^{+}CD8^{+d}$	$NK1.1^{+}CD8^{-e}$	$Mac\text{-}3^{+f}$
Absolute Values ($\times 10^6$)							
Control (Unrestrained)	124.3 ± 7.7*	38.03 ± 3.41*	20.26 ± 2.16*	12.18 ± 1.26*	6.09 ± 0.76*	2.63 ± 0.24*	4.71 ± 0.54
Control (Nose Cone)	94.0 ± 9.7	21.98 ± 1.92	12.54 ± 1.08	7.04 ± 0.56	3.80 ± 0.33	1.60 ± 0.17	4.10 ± 0.73
Jet Fuel Kerosene							
500 mg/m^3	118.5 ± 9.5	24.95 ± 0.86	13.35 ± 0.67	7.40 ± 0.34	4.22 ± 0.22	2.16 ± 0.15*	5.55 ± 0.67
1000 mg/m^3	123.0 ± 4.7	24.19 ± 1.82	12.18 ± 1.08	6.99 ± 0.66	3.78 ± 0.31	2.11 ± 0.10*	5.22 ± 0.32
2000 mg/m^3	106.1 ± 3.8	20.50 ± 0.78	11.39 ± 0.55	5.94 ± 0.30	3.64 ± 0.20	1.98 ± 0.09	5.20 ± 0.35
CPS (50 mg/kg)	49.9 ± 2.9*	8.08 ± 0.68*	11.02 ± 1.12	6.29 ± 0.63	3.76 ± 0.41	NA	2.34 ± 0.16*
AAGM1 (1:10)	127.2 ± 2.8*	NA	NA	NA	NA	1.31 ± 0.08	NA
H/NH	NH	NH	H	H	H	NH	NH
Trend Analysis	NS	NS	NS	$p \leq 0.05$	NS	NS	NS
Percent Values							
Control (Unrestrained)		30.27 ± 1.25*	16.02 ± 0.95	9.68 ± 0.59	4.79 ± 0.37	2.11 ± 0.12*	3.76 ± 0.40
Control (Nose Cone)		23.99 ± 1.27	13.99 ± 1.09	7.90 ± 0.62	4.22 ± 0.31	1.70 ± 0.09	4.23 ± 0.46
Jet Fuel Kerosene							
500 mg/m^3		21.96 ± 1.66	11.72 ± 0.96	6.46 ± 0.47	3.68 ± 0.28	1.87 ± 0.13	4.63 ± 0.28
1000 mg/m^3		20.07 ± 1.89	10.17 ± 1.14	5.84 ± 0.69*	3.15 ± 0.33	1.75 ± 0.12	4.29 ± 0.31
2000 mg/m^3		19.52 ± 0.96	10.90 ± 0.76	5.70 ± 0.43*	3.48 ± 0.25	1.88 ± 0.10	4.88 ± 0.24
CPS (50 mg/kg)		16.04 ± 0.74*	21.89 ± 1.61*	12.51 ± 0.93*	7.43 ± 0.59*	NA	4.76 ± 0.28
AAGM1 (1:10)		NA	NA	NA	NA	1.03 ± 0.06*	NA
H/NH		H	H	H	H	H	H
Trend Analysis		$p \leq 0.05$	$p \leq 0.05$	$p \leq 0.05$	$p \leq 0.05$	NS	NS

[a]B-lymphocyte; [b]T-lymphocyte; [c]Helper T-lymphocyte; [d]Cytotoxic T-lymphocyte; [e]Natural Killer cell; [f]Macrophage; Values represent the mean ± SE derived from 9 or 10 animals per group; *$p \leq 0.05$ compared to the control nose cone air only group H = homogeneous data; NH = non-homogeneous data; NS = not significant; NA = not applicable.

of the spleen cell phenotypes evaluated, with the exception of absolute NK cells, which were significantly elevated (35 and 32% at 500 and 1000 mg/m^3, respectively) compared to control, and %T_H cells, which were significantly decreased (10 and 12% at 1000 and 2000 mg/m^3, respectively) compared to control (Table 4). Of note in Table 4 are the significant differences between control (i.e., nose cone–air only) and control unrestrained animals. The nose cone control animals had significantly lower spleen cell numbers (24%), percent of B cells (21%), and percent of NK cells (19%), as well as significantly reduced absolute numbers of B cells (42%), T cells (38%), T_H cells (42%), T_{CTL} cells (38%), and NK cells (39%) compared to unrestrained controls (Table 4).

In the rat studies, spleen cell numbers were decreased at the two highest exposure levels, however only the reduction at the middle dose (18%) reached the level of statistical significance (Table 5). No significant effects were observed on the absolute numbers of T cells, T_H cells, T_{CTL} cells, NK cells, or macrophages. A significant decrease (21%) was observed in the absolute number of splenic B cells in rats exposed to 1000 mg/m^3 jet fuel kerosene. However, this effect was not observed at the higher dose of 2000 mg/m^3. No marked effects were observed when the data were analyzed in terms of percentage values. The positive controls (CPS and AAGM1) produced the anticipated alterations in spleen cell numbers and phenotypes in both species.

Spleen IgM AFC Response to SRBC Figure 1 shows the AFC response to sRBC for the mouse inhalation studies. The left panel gives the results in terms of specific activity (AFC/10^6 Spleen cells), and the right panel gives the results as total Spleen activity (AFC/Spleen). No marked effects were observed between the control and the jet fuel kerosene-exposed groups. When the control unrestrained group was compared to control, the AFC response was significantly decreased when evaluated both as specific activity (46% decrease) and as total spleen activity (39% decrease).

Figure 2 depicts the results of the AFC study conducted in rats exposed to jet fuel by inhalation. Again, the left panels show the specific activity results and the right panels show the total spleen activity. The top two panels show the mean + SE for each of the groups, while the bottom two panels present the results of each individual animal as a scatter plot, showing the variability in the responses of these outbred rats. Similar to mouse studies, no significant differences in the AFC response were observed between control rats and those exposed to jet fuel kerosene. In both species, treatment with the positive control (CPS) produced the anticipated decreases in the AFC response.

Anti-CD3 Mediated T-Cell Proliferation Figure 3 shows the effects of jet fuel inhalation on T cell proliferation stimulated by anti-CD3 antibodies. The left panel gives the results for the B6C3F1 mice, and the right panel shows the results for the Crl:CD rats. No marked effects were observed on either the unstimulated or the anti-CD3 stimulated proliferative responses in the mouse study. In the rat, however, significant increases, as compared to control, were noted in both the unstimulated and anti-CD3 stimulated proliferative responses at all jet fuel kerosene exposure levels, except for unstimulated proliferation at the 500-mg/m^3 exposure level. However, the biological significance of these results is questionable, as the response of the control animals was low when compared to other anti-CD3 proliferation studies conducted with female Crl:CD® rats in this lab. CPS, the positive control, suppressed the T cell proliferative responses in both species.

DTH Response to *C. albicans* Results of the DTH studies are shown in Figure 4, with the top panel giving the results for mice and the bottom panel for rats. No significant effects were observed in either species between control and jet fuel kerosene-exposed groups, while the positive control (CPS) produced the expected decreases in the footpad swelling. In the mouse study, the response of the control group (i.e., nose cone control) was significantly reduced (48%) compared to the control unrestrained animals.

TABLE 5. Spleen cell phenotypes in female Crl:CD® rats exposed to Jet Fuel Kerosene by inhalation for 28 days

Exposure	Spleen Cells	CD45RA^{+a}	CD5^{+b}	CD4+CD5^{+c}	CD8+CD5^{+d}	NKRP1A+CD8^{-e}	His36^{+f}
Absolute Values ($\times 10^6$)							
Control	561.8 ± 21.1	382.1 ± 19.1	121.4 ± 10.1	77.7 ± 5.4	50.7 ± 5.3	31.1 ± 1.8	60.1 ± 6.7
Jet Fuel Kerosene							
500 mg/m^3	534.4 ± 34.8	376.2 ± 29.2	111.2 ± 11.0	74.8 ± 6.8	44.0 ± 5.9	29.9 ± 2.6	62.1 ± 5.7
1000 mg/m^3	461.9 ± 21.1*	300.8 ± 17.6*	117.3 ± 7.3	74.9 ± 5.5	47.3 ± 3.1	32.9 ± 3.3	69.1 ± 9.0
2000 mg/m^3	479.9 ± 21.5	323.3 ± 17.5	109.1 ± 9.3	74.9 ± 5.6	41.1 ± 3.7	27.4 ± 3.4	64.5 ± 6.7
CPS (25 mg/kg)	144.5 ± 11.3*	12.2 ± 2.4*	105.1 ± 9.1	56.8 ± 5.3*	54.3 ± 5.3	NA	9.8 ± 3.2*
AAGM1 (1:10)	517.0 ± 27.0	NA	NA	NA	NA	5.1 ± 0.7*	NA
H/NH	H	H	H	H	H	H	H
Trend Analysis	$p \leq 0.01$	$p \leq 0.01$	NS	NS	NS	NS	NS
Percent Values							
Control		68.04 ± 2.24	21.61 ± 1.63	13.86 ± 0.88	8.97 ± 0.82	5.61 ± 0.44	10.73 ± 1.18
Jet Fuel Kerosene							
500 mg/m^3		70.05 ± 1.82	20.75 ± 1.36	14.13 ± 1.01	8.06 ± 0.73	5.70 ± 0.52	12.25 ± 1.61
1000 mg/m^3		64.83 ± 1.41	25.38 ± 1.07	16.23 ± 0.89	10.28 ± 0.58	7.31 ± 0.87	14.83 ± 1.66
2000 mg/m^3		67.40 ± 2.13	22.71 ± 1.73	15.69 ± 1.13	8.62 ± 0.77	5.69 ± 0.69	13.37 ± 1.21
CPS (25 mg/kg)		8.34 ± 1.63*	72.55 ± 2.29*	39.02 ± 1.48*	37.51 ± 2.08*	NA	6.43 ± 1.63*
AAGM1 (1:10)		NA	NA	NA	NA	0.98 ± 0.11**	NA
H/NH		H	H	H	H	H	H
Trend Analysis		NS	NS	NS	NS	NS	$p \leq 0.05$

[a]B-lymphocyte; [b]T-lymphocyte; [c]Helper T-lymphocyte; [d]Cytotoxic T-lymphocyte; [e]Natural Killer cell; [f]Macrophage; Values represent the mean ± SE derived from 9 or 10 animals per group; *$p \leq 0.05$ compared to the control nose cone air only group H = homogeneous data; NH = non-homogeneous data; NS = not significant; NA = not applicable.

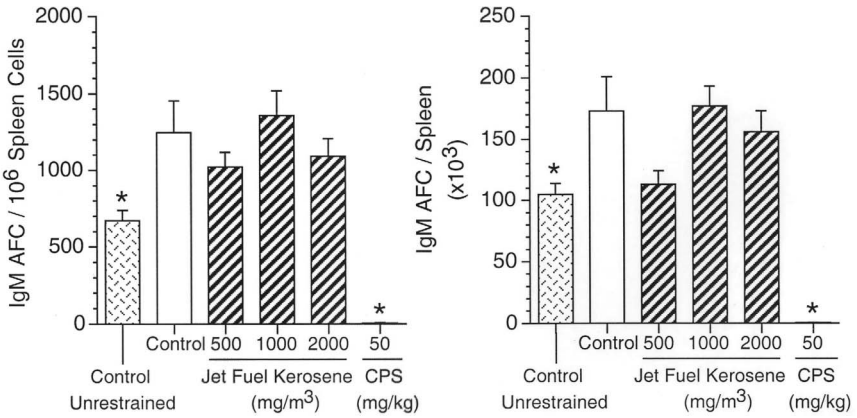

FIGURE 1. AFC response to sRBC in female B6C3F1 mice exposed to jet fuel kerosene for 28 d. On d 25 of the study, mice were immunized with 7.5×10^7 sRBC by iv injection. Positive control mice received 50 mg/kg CPS daily by ip injection for 4 d prior to study termination on d 29. Left panel: AFC specific activity (IgM AFC/10^6 splenocytes); right panel: AFC total Spleen activity (IgM AFC/Spleen). Asterisk indicates statistically significant differences from control: $^*p \leq .05$.

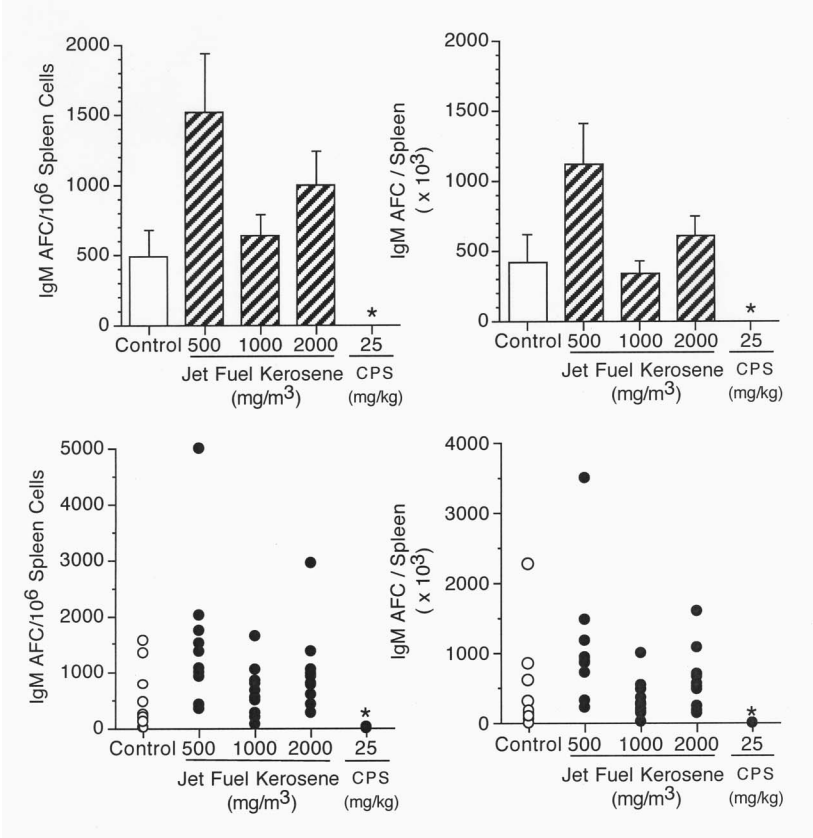

FIGURE 2. AFC response to sRBC in female Crl:CD rats exposed to jet fuel kerosene for 28 d. On d 25 of the study, rats were immunized with 2×10^8 sRBC by iv injection. Positive control animals received 25 mg/kg CPS daily by ip injection for 4 d prior to study termination on d 29. The top two panels present the mean + SE for each group, while the bottom two panels present the individual responses of each animal. Left panels: AFC specific activity (IgM AFC/10^6 splenocytes); right panels: AFC total Spleen activity (IgM AFC/Spleen). Asterisk indicates statistically significant differences from control: $^*p \leq .05$.

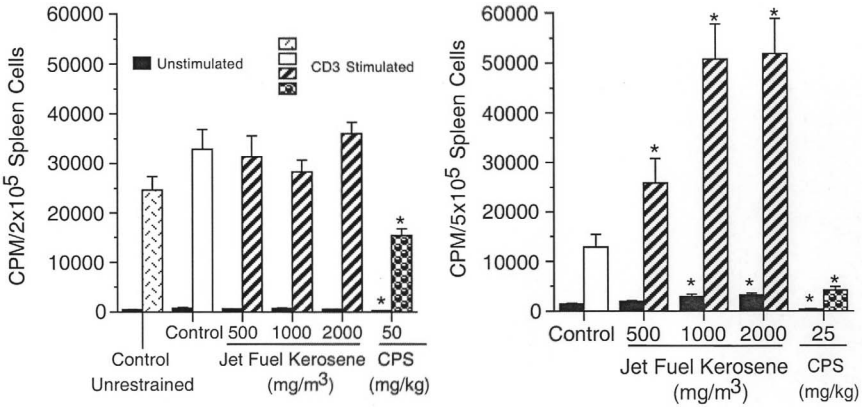

FIGURE 3. Anti-CD3 mediated proliferation of splenocytes from female B6C3F1 mice and Crl:CD rats exposed to jet fuel kerosene for 28 d. Left panel: mice. Right panel: rats. CPS, the positive control, was administered by ip injection at either 25 mg/kg (rats) or 50 mg/kg (mice) daily on d 25–28. Spleens were harvested aseptically, and spleen cells were cultured in 96-well plates (uncoated or coated with anti-CD3 antibody) for 3 d. All cultures were labeled with [^{3}H]thymidine (1 μCi/well) 18–24 h prior to harvest. Incorporation of [^{3}H]thymidine into proliferating cells was the assay endpoint. Results are expressed as CPM/2 × 10^5 splenocytes (mice) or as CPM/5 × 10^5 splenocytes (rats). Asterisk indicates statistically significant differences from control: $^*p \leq .05$.

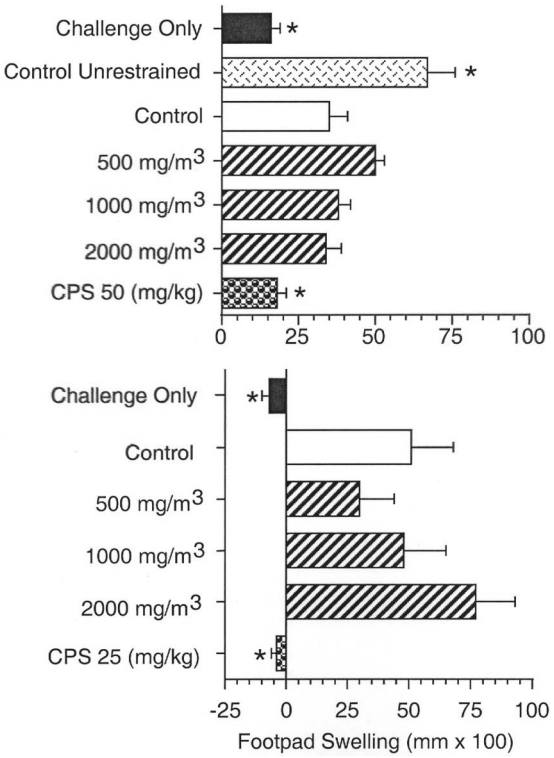

FIGURE 4. DTH response to *C. albicans* in female B6C3F1 mice and Crl:CD rats exposed to jet fuel kerosene for 28 d. Top panel: mice. Bottom panel: rats. Animals were immunized on exposure d 21 with formalin-fixed *C. albicans* organisms and challenged in the right footpad with chitosan antigen one d after the last inhalation exposure. Footpad swelling was determined 24 h postchallenge. Results are reported in terms of mm × 100. Asterisk indicates statistically significant differences from control: $^*p \leq .05$.

NK Cell Activity The results of the NK study conducted in mice are presented in Figure 5. The 200:1, 100:1, and 50:1 E:T ratios, which represented the linear response region for this study, are depicted. No significant effects were noted between control and jet fuel kerosene-exposed animals. Further, no marked differences were found between control and control unrestrained groups. As expected, the positive control (AAGM1) decreased the cytotoxic activity of the splenic NK cells.

The NK study conducted in the Crl:CD rat was conducted according to lab standard operating procedures; however, the observed responses were extremely low for each of the groups at all E:T ratios (data not shown). Typically, the response of this strain in this assay tends to be lower than other strains and species (such as the B6C3F1 mouse). However, the unusually low response observed made the results for this study not usable in the immunotoxicological evaluation of jet fuel kerosene.

DISCUSSION

A number of previous studies indicated that JP-8 is an immunotoxicant following nose-only inhalation (Harris et al., 1997, 2000a, 2000b, 2002, 2007, 2008), dermal exposure (Ramos et al., 2002, 2007; Ullrich, 1999; Ullrich and Lyons, 2000), and oral gavage (Keil et al., 2004;

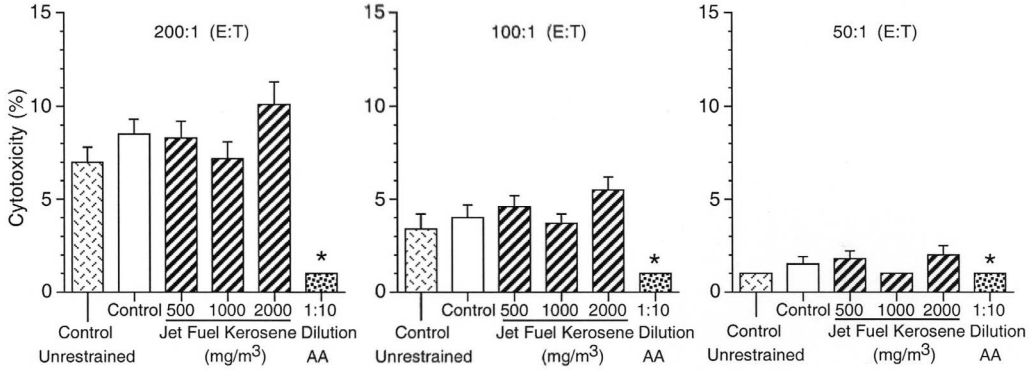

FIGURE 5. NK cell activity in female B6C3F1 mice exposed to jet fuel kerosene for 28 d. AAGM1, the positive control, was administered by iv injection 24 h prior to study termination. On d 29, spleens were harvested aseptically, and spleen cells were cultured with ^{51}Cr-labeled YAC-1 target cells for 4 h. From each culture 100 μl of supernatant was counted in a gamma counter to assess the release of ^{51}Cr from the target cells. Results are presented for three E:T ratios (200:1, 100:1, and 50:1) in terms of percent cytotoxicity, which was calculated as the difference between the experimental release and spontaneous release, divided by the difference between the maximum release and spontaneous release.

Peden-Adams et al., 2001). The inhalation and dermal studies of others implicated CMI as the primary target, while no effects on humoral immunity were observed on the T-dependent antibody response (TDAR) to KLH in the dermal studies. The inhalation studies reported by Harris et al. did not evaluate effects on humoral immunity. Results of the present studies, which demonstrated that jet fuel kerosene inhalation did not suppress innate, humoral, or cell-mediated immune functions, are in contrast with the many reports regarding the effects of JP-8 inhalation. Of note is that, in contrast to the other research studies conducted on JP-8, synthetic jet fuel (S8), and the various jet fuel additives, these inhalation studies and the dermal jet fuel kerosene study reported by Mann et al. (2008) were conducted under GLP regulations in a contract research organization (CRO) with extensive experience in conducting immunotoxicological assessments.

As indicated previously, the NRC suggested that the exposure levels in the Harris et al. studies were likely higher than reported (National Research Council, 2003), and this has since been confirmed by the Harris lab (Hilgaertner et al., 2011). The report by Hilgaertner et al. (2011) demonstrates JP-8-related immunosuppressive effects, including decreased spleen and thymus weights at exposure levels $\geq$1000 mg/m^3 and reduced IFN-γ production at doses $\geq$4000 mg/m^3. Hilgaertner et al. (2011) also indicated that these exposures were equivalent to aerosol exposures of 125 and 500 mg/m^3 in the early reports by Harris et al. (1997, 2000a, 2000b, 2002, 2007, 2008). These results, which contrast with those in the present study evaluating the base jet fuel kerosene, suggest the possibility that the additives, and not the jet fuel kerosene, are primarily responsible for the immunosuppressive effects of JP-8 jet fuel.

Studies evaluating the immune effects of dermally applied jet fuels also present conflicting results. Jet A is the commercial aviation counterpart to the military's JP-8 fuel and has often been referred to as being equivalent to the kerosene base fuel of JP-8 (i.e., jet fuel kerosene), although these fuels have distinctly different performance additives (Ritchie et al., 2003). Indeed, in contrast to the reported effects of dermally applied JP-8 on CMI, Jet A was shown to exert no immunotoxic effects when administered dermally in a mineral oil vehicle (Mann et al., 2008). However, this result contrasts with another report by Ramos et al. (2002), who reported that JP-8 and Jet A both suppress cell-mediated immune functions in mice when applied directly (i.e., undiluted) to the skin. The differences between these Jet A dermal studies may be due to the use of the mineral oil vehicle, which Mann et al. (2008)

used to mitigate the irritant effects resulting from repeated exposure to undiluted jet fuel. Thus, differences in the effects of exposure to various jet fuels need to be evaluated in the context of the exact nature of the test material (Ritchie et al., 2003), as well as any irritation produced at the application site and the vehicle (if any) that is utilized for delivery.

Some studies have been conducted to assess the effects of hydrocarbon composition on JP-8-mediated immune suppression using a synthetic jet fuel (S8) for comparison. S8 is a synthetic jet fuel (synthesized by the Fischer–Tropsch process), composed primarily of aliphatic hydrocarbons and having a significantly lower aromatic hydrocarbon content than JP-8, including decreased levels of naphthalene and its derivatives (van der Westhuizen et al., 2011). Ramos et al. (2007) conducted dermal exposure studies to compare the effects of JP-8 and S8 and found that, unlike JP-8, S8 jet fuel exerted no marked effect on CMI (i.e., DTH). When Ramos et al. (2007) spiked the S8 jet fuel with a cocktail of 7 primary aromatic hydrocarbons found in JP-8, suppression of the DTH response occurred, suggesting that the aromatic component of JP-8 was responsible for the observed effects. However, the response of that S8-spiked mixture was not dose-responsive over a 10-fold increase in aromatic hydrocarbon content. Therefore, the hypothesis that the aromatic hydrocarbons are responsible for dermal JP-8-mediated immune suppression is questionable. Comparing S8 and JP-8 following nose-only inhalation (1 h/d for 7 d), Hilgaertner et al. (2011) found that S8 and JP-8 produced similar suppressive effects on the immune system, suggesting that the aromatic hydrocarbon content may not be responsible for the observed immunotoxicity. The present results are consistent with this suggestion, as no immunosuppression was observed following inhalation of the base jet fuel kerosene, which contains no performance additives.

Based on the specifications of MIL-DTL-834133, JP-8 jet fuel contains 3 to 5 performance additives, which make up less than 0.5% (w/w) of the fuel (Ritchie et al., 2003). These include an anti-icing agent (diethylene glycol monomethyl ether [DGME]), an antistatic agent (Stadis 450), and an anticorrosive agent (DCI-4A), all of which are required additives, while the addition of metal chelators and antioxidants is allowed but not required (Inman et al., 2008; Ritchie et al., 2003). According to the Air Force Logistics Management Agency, DGME is found in JP-8 at a 1:1000 dilution, while DCI-4A and Stadis 450 are present at smaller concentrations (1:66,660 and 1:666,666 dilutions, respectively) (AFLMA, 2007).

The first of these, DGME, was shown to produce no histopathological abnormalities in any of the lymphoid organs, including the spleen, thymus, lymph nodes, and bone marrow, in male and female Fischer 344 rats exposed by inhalation for 13 wk at exposures up to 1.06 mg/L (1060 mg/m^3) (Miller et al., 1985). In light of these results, any significant contribution by DGME to the reported immunosuppressive effects of JP-8 inhalation is not likely.

Stadis 450 is the only static dissipater approved for use in USAF aviation fuels (AFLMA, 2007). This additive consists of 35–60% toluene, 5–20% solvent naphtha, <5% naphthalene, <5% isopropyl alcohol, <0.06% benzene, 1–10% dodecylbenzenesulfonic acid, 1–10% dinonylnaphthylsulfonic acid, and 1–10% proprietary chemicals (Ritchie et al., 2003). Olsgard et al. (2008) reported that inhalation of a toluene/benzene mixture (8:1 ratio) for 1 h daily for 28 d produced no effects on humoral immunity with significant decreases in the DTH response to dinitrophenol (DNP)–KLH at all exposure levels tested, including the lowest dose of 0.8 ppm (i.e. 800 mg/m^3) toluene and 0.1 ppm (i.e. 100 mg/m^3) benzene. Although these results are consistent with previous reports evaluating the immune effects of JP-8, the full immunological implications of the use of Stadis 450 are unknown at this time, due in large part to the proprietary nature of as much as 10% of the formulation.

DCI-4A is the most common anti-corrosive agent found in JP-8 and consists of 20–30% xylene, 0–5% ethylbenzene, and 70–80%

proprietary chemical, according to Ritchie et al. (2003). A report prepared by the U.S. Army TARDEC Fuels and Lubricants Research Facility (Lacey and Westbrook, 1997) identifies dilinoleic acid as the primary component of DCI-4A, which suggests that dilinoleic acid constitutes, at a minimum, the majority of the proprietary chemical component of DCI-4A.

Dilinoleic acid consists of two linked units of linoleic acid, which is an unsaturated fatty acid found in a variety of food sources, including chicken fat (Nutter et al., 1943) and olive oil (Beltran et al., 2004). In vitro studies evaluating linoleic acid by inhalation showed that this fatty acid increases reactive oxygen species (ROS) production, apoptosis, and necrosis of human peripheral blood mononuclear cells (Cury-Boaventura et al., 2006). Other in vitro studies demonstrated that conjugated linoleic acid increased IL-10 production (Loscher et al., 2005) and reduced human macrophage adhesion to endothelial surfaces (Stachowska et al., 2012). Further, linoleic acid was found to suppress the mitogen-stimulated proliferative responses of human T lymphocytes and murine T and B lymphocytes (Tezabwala et al., 1995). Finally, in vivo studies in humans demonstrated that increased intake of linoleic acid elevates production of prostaglandin E2 (PGE2) (Kelley, 2001), which is consistent with the effects of dermal JP-8 exposure on ROS and PGE2 production reported by Ramos et al. (2004, 2009). Because DCI-4A is present in JP-8 at a 1:66,660 dilution (AFLMA, 2007), the concentration of dilinoleic acid in JP-8 is extremely low. However, it is not out of the realm of possibility that this compound may contribute to the previously reported immune effects of JP-8.

Toxicological assessments of a complex UVCB substance or mixture can be complicated by the interactions of the individual constituents. However, in light of the absence of immunosuppressive effects of jet fuel kerosene and the many reports of immune effects following exposure to JP-8, evidence suggests that the performance additives in JP-8 fuel are, in part, responsible for the effects observed on CMI following exposure to JP-8. Further, based upon the available scientific literature, it does not appear that any one component of the additive package is the primary immunosuppressive agent. It is therefore likely that the combination and interaction of many, or all, of the various components in JP-8 jet fuel contribute to the previously reported effects of JP-8 on the immune system.

These inhalation studies in mice and rats demonstrated that unadditized jet fuel kerosene, which constitutes more than 99% of the composition of JP-8 aviation fuel, did not suppress innate, humoral, or cell-mediated immune function when conducted under GLP. Further, these data suggest that additives that distinguish these jet fuels from the hydrocarbon base kerosene may be responsible for previously reported immunosuppression following JP-8 exposure.

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