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Immunological Function in Mice Exposed to JP-8 Jet Fuel *In Utero*

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Immunological parameters, host resistance, and thyroid hormones were evaluated in F_1 mice exposed *in utero* to jet propulsion fuel–8 (JP-8). C57BL/6 pregnant dams (mated with C3H/HeJ males) were gavaged daily on gestation days 6–15 with JP-8 in a vehicle of olive oil at 0, 1000, or 2000 mg/kg. At weaning (3 weeks of age), no significant differences were observed in body, liver, spleen, or thymus weight, splenic and thymic cellularity, splenic CD4/CD8 lymphocyte subpopulations, or T-cell proliferation. Yet, lymphocytic proliferative responses to B-cell mitogens were suppressed in the 2000 mg/kg treatment group. In addition, thymic CD4−/CD8+ cells were significantly increased. By adulthood (8 weeks of age), lymphocyte proliferative responses and the alteration in thymic CD4−/CD8+ cells had returned to normal. However, splenic weight and thymic cellularity were altered, and the IgM plaque forming cell response was suppressed by 46% and 81% in the 1000 and 2000 mg/kg treatment groups, respectively. Furthermore, a 38% decrease was detected in the total T4 serum hormone level at 2000 mg/kg. In F_1 adults, no significant alterations were observed in natural killer cell activity, T-cell lymphocyte proliferation, bone marrow cellularity and proliferative responses, complete blood counts, peritoneal and splenic cellularity, liver, kidney, or thymus weight, macrophage phagocytosis or nitric oxide production, splenic CD4/CD8 lymphocyte subpopulations, or total T3 serum hormone levels. Host resistance models in treated F_1 adults demonstrated that immunological responses were normal after challenge with *Listeria monocytogenes*, but heightened susceptibility to B16F10 tumor challenge was seen at both treatment levels. This study demonstrates that prenatal exposure to JP-8 can target the developing murine fetus and result in impaired immune function and altered T_4 levels in adulthood.

Key Words: **immunological parameters; host resistance; jet propulsion fuel-8; JP-8, thyroid hormones.**

JP-8 jet propulsion fuel (JP-8) is used extensively by the U.S. Air Force and the North Atlantic Treaty Organization (NATO) forces. Between these two organizations, it is estimated that 5 billion gallons of JP-8 are used annually (Henz, 1998). Military and industrial personnel are exposed to JP-8 on a daily basis during aircraft refueling, takeoff, servicing, inspection, and during cleaning of fuel storage tanks (Pleil, 2001). Civilian populations near military or commercial airfields may also have opportunities for exposure due to environmental contamination, as jet fuel from spills and fuel tank leaks may enter nearby soil and water resources (EPA, 2003). Environmental contamination can also occur at fuel test facilities where the fire suppressive properties of various fuel additives are evaluated (EPA, 2003).

Similar to most petroleum distillates, JP-8 is a heterogeneous, kerosene-like mixture. It contains approximately 81% alkanes, primarily in the C8–C17 range. The remaining portion consists of 10–20% polycyclic aromatic hydrocarbons (PAHs) with relatively low levels of benzene, toluene, and xylenes (IARC, 1989; ATSDR, 1998). The composition of JP-8 was modified from previously used jet propulsion fuels (JP-4 and JP-5), in that it contains additives for static dissipation, anticing, and corrosion inhibition.

Initial toxicological studies with JP-8 examined toxicity from 90-day exposures. For instance, F-344 rats and C57BL/6 mice that were exposed to JP-8 (0–1000 mg/m^3) via inhalation did not exhibit tumor formation and displayed minimal treatment-related adverse effects (Mattie *et al*., 1991). Male rats did acquire α_2-microglobulin nephropathy, but this was considered unique to this species and not extrapolatable to humans (Mattie *et al*., 1991). This study was followed by another 90-day experiment examining the effects of oral exposure to neat JP-8 (0–3000 mg/kg/day) in Sprague-Dawley rats (Mattie *et al.*, 1995). Observations included perianal irritation, gastritis, α_2-microglobulin nephropathy, and a dose-dependent decrease in body weight. No mortality or morbidity occurred even after exposure to high levels (3000 mg/kg) of JP-8 for 90 days, and no adverse consequences in liver pathology were noted. However, examination of hematological parameters identified significant increases in peripheral blood neutrophils and corresponding decreases in blood lymphocytes at all treatment levels (750–3000 mg/kg/day of JP-8).

Subsequent investigations evaluated JP-8's effect on immune function and identified more profound and persistent effects. Immunotoxicity and pulmonary pathology were re-

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ported after 7-day exposures to aerosolized JP-8 jet fuel ranging from 100–2500 mg/m^3 (Harris *et al.*, 1997a,b,c, 2000; Hays *et al*., 1995; Pfaff *et al*., 1996; Robledo and Witten, 1999). Immunological deficits in spleen and thymus organ weight and cellularity, lymphocytic subpopulations, and T-cell blastogenesis to the mitogen concanavalin A (Con A) were long lasting (Harris *et al.*, 1997b,c). Dermal exposure to JP-8 (50 μl/day for 5 days or 250–300 μl as a single exposure) also suppressed T-cell blastogenesis while impairing delayed and contact hypersensitivity in mice (Ullrich, 1999). Suppression of T-cell proliferation was noted 3–4 days after a single dermal exposure to JP-8, and this effect persisted for approximately 3 weeks postexposure (Ullrich and Lyons, 2000). Modulation of inflammatory cytokines has been suggested as a potential mechanism of JP-8-induced immunosuppression, as Ullrich (1999) identified elevated interleukin-10 levels in the serum of dermally exposed mice. Moreover, our laboratory has also demonstrated immunotoxicity, including a decrease in thymus weight and cellularity and substantial deficits in humoral immune responses, after oral exposure to JP-8 for 7 days (Dudley *et al*., 2001).

Few studies have evaluated the impact of JP-8 exposure during development. Cooper and Mattie (1996) established a no observed adverse effect level of 1000 mg/kg for a reduction in fetal rat body weight. Dermal exposure of pregant dams to kerosene, a primary component of JP-8, resulted in a live pup birth index of 97% with no significant changes in birth weight, mean number of live pups per litter, and weight gain in pups from treated and control groups (Schreiner *et al*., 1997). Immunological function after gestational exposure to JP-8, however, has not been assessed in any study to date.

To address the impact of JP-8 on the developing immune system, this study examined a battery of immunological endpoints in mice following *in utero* exposure to JP-8. Assessment of immune function was comprehensive and included evaluation of immunological parameters at weaning (age 3 weeks) and at adulthood (age 8 weeks). This study also evaluated thyroid hormone levels and host susceptibility to bacterial or tumor cell challenge at adulthood.

MATERIALS AND METHODS

Chemicals. JP-8 jet fuel (JP-8; lot no. 3509) was provided by Capt. Thomas Miller of AFRL/HEST (Operational Toxicology Branch) at Wright Patterson Air Force Base, OH. The JP-8 contained the following additives: 15 mg/l corrosion inhibitor (DCI-4A), 0.1% v/v icing inhibitor (DI-EGME), and 2 mg/l static inhibitor (Statis 450). It was filtered at 0.45 microns for animal research and stored at room temperature in sealed glass containers in a dry and explosive-proof cabinet.

Mice. Adult, timed-pregnant female C57BL/6N mice mated with C3H/HeJ male mice were purchased from Harlan-Teklab, Madison, WI. Pups generated from this mating were B6C3F1, a common strain used in immunotoxicology testing. The time-pregnant mice were transported via air-conditioned truck on day 3 or 4 of gestation to an AAALAC approved animal facility at the Medical University of South Carolina. Mouse chow (Tek Lab Sterilizable Rodent Diet, formula no. 8656; Harlan Tek Lab, Madison WI) and water were provided *ad libidum*. Pregnant dams were housed 4 per cage and then housed individually 1 day prior to birthing. Bedding, food, and water were changed twice a week, and mice were observed daily.

Exposure and experimental design. Pregnant dams were gavaged on gestation days 6–15 daily with 1000 or 2000 mg/kg JP-8 (100 μl gavage volume) in a carrier of olive oil. Control animals received olive oil only (100 μl gavage). At least ten dams were randomly assigned to each treatment group. Typical litter size ranged from 4 to 7, and pups remained with dams until age 21 days, when they were weaned, separated by gender, and housed 4 per cage. In the litters born, gender distribution was approximately 49% male and 51% female in control and treatment groups. A male and female from each litter of each treatment group were selected for testing for various assays at either 3 or 8 weeks of age. Select immunological, hematological, or thyroid parameters were assessed in B6C3F1 pups either at weaning (age 21 days; 3 weeks old) or at maturity (age 56 days; 8 weeks old). Typically, each experiment involved 4–6 mice per treatment group, depending on the distribution of male and female mice/litter available for the assays. Experiments were repeated at least twice unless otherwise stated.

Body weight. Body weight was measured weekly in pups from birth to 8 weeks of age. Known standard weights were used to verify balance calibration prior to use (DI-800, Denver Instruments, Arvado, CO).

Organ weight and total cellularity. Kidney, liver, thymus, and spleen weights were measured at weaning (3 weeks) or at adulthood (8 weeks). Bone marrow cells were aseptically collected from the medullary cavity of one femur from each mouse. Peritoneal macrophages were obtained via sterile peritoneal lavage. Total cellularity was determined for spleen, thymus, bone marrow, and from peritoneal lavage using a Coulter Model ZM particle counter (Coulter Electronics, Hileah, FL). The Coulter counter was calibrated with cell suspensions that were verified using a hemacytometer.

Flow cytometric evaluation of thymic and splenic lymphocytic subpopulations. Spleen or thymus cells were labeled with fluorescent (phycoerythrin or fluorescein isothiocyanate) mouse IgG_2 monoclonal antibodies specific for murine CD4 or CD8. In addition, splenocytes were separately labeled with rat anti-mouse B220 to semiquantitate the percentage of B-cells in the spleen. Details of this labeling procedure are described previously (Peden-Adams *et al*., 2001). Splenic and thymic CD4/8 lymphocytic populations were assessed at weaning (3 weeks) and at adulthood (8 weeks), while anti-B220 populations in the spleen were determined at adulthood only. Data are represented as absolute number of cells as determined by multiplying the percent gated cells by the total number of nucleated cells obtained by the Coulter Counter.

Hematology. Whole blood in EDTA collection tubes was sent to Antech Diagnostics (Farmington, NY) for complete blood count (CBC) analysis with white blood cell (WBC) differential. CBC analysis included erythrocyte and leukocyte number, hemoglobin, and hematocrit. Hematology was assessed at weaning (3 weeks) and at adulthood (8 weeks).

Stem cell assay. This procedure was based on previous studies assessing granulocyte-macrophage progenitors and erythrocyte progenitors (Bradley *et al*., 1966; Phillips *et al*., 1997). Briefly, bone marrow cells were aseptically washed and collected from the medullary cavity of one femur from each mouse. Cell counts were determined using Coulter counter and 4.5×10^4 total cells were added to Methocult™ medium (GF M3434, Stem Cell Technologies, Inc., Vancouver, BC) for the colony assay. Cultures were performed in duplicate and incubated at 37°C and 5% CO_2 for 10 days. Total numbers of colonies were counted using an inverted microscope. A colony was defined as 50 cells or more. Total colony forming units (CFU) per 10^5 bone marrow cells were reported. This parameter was assessed in F_1 adults only.

Natural killer cell (NK) activity. An *in vitro* cytotoxicity assay using ^{51}Cr-labeled Yac-1 cells (TIB 160, ATCC, Manassas, VA) was used as based on a previous study by Duke *et al.* (1985) and modified slightly as described in detail by Peden-Adams *et al.* (2001). The results were expressed in lytic units per 10^7 splenocytes, using 10% lysis as the reference point (Bryant *et al*., 1992). NK activity was assessed in F_1 adults only.

Nitric oxide production by peritoneal macrophages. Peritoneal macrophages were aseptically isolated by peritoneal lavage and incubated for 24 hours with 10 μg/ml of lipopolysaccharide (LPS) and 500 Units/ml of inter-

feron-gamma (IFN-γ). These methods are described previously (Keil *et al*., 1995). Nitric oxide was indirectly measured by spectrophotometrically quantitating nitrite, a stable endproduct of nitric oxide generation. A standard nitrite curve was established for each experiment, and data were expressed in μM of nitrite production. Control cells contained macrophages only without any stimulant and background was always less than 0.5 μM. This endpoint was assessed at 8 weeks only.

Phagocytosis. Peritoneal macrophages were aseptically isolated by peritoneal lavage. The cell density was determined and adjusted to 2×10^5/well. Macrophages were added to sterile Lab-Tek tissue culture chamber/slides (NalgeNunc International, Naperville, IL.) and incubated for 1 hour in a humidified incubator set at 37°C and 5% CO_2. Cells were washed with prewarmed medium to remove nonadhered leukocytes. Washed *Listeria monocytogenes* (serotype 4 of strain 19303) was added at an approximate ratio of 10 bacterial cells per 1 macrophage. Sterile normal mouse serum (10%) was added to permit opsonization of bacteria. After 4 hours of incubation, the macrophages were aseptically washed 10 times to remove nonphagocytized bacteria. The remaining cells were fixed, stained with Wright's stain, and then a coverslip was applied. The number of bacteria associated per macrophage was determined using a light microscope. To reduce subjectivity in the reading process, at least 2 independent reviewers enumerated bacteria per macrophage. Phagocytosis was assessed at 8 weeks only.

Mitogen-induced lymphocyte proliferation. Splenic lymphocyte proliferation was measured by the standard ^{3}H-thymidine uptake assay as described in detail by Peden-Adams *et al.* (2001) with specific modifications. The final concentration of concanavalin A was 5 μg/ml, and the cells were incubated for 48 hours before a 12-hour tritiated thymidine pulse. Results are reported as the net cpm (cpm stimulated wells - cpm unstimulated wells).

Plaque forming cell response to T-dependent antigen, sRBC. The plaque-forming cell (PFC) response was assessed in F_1 adult mice only by using the Cunningham modification of the Jerne assay (Cunningham and Szenberg, 1968; Jerne and Nordin, 1963). This method is described in detail by Peden-Adams *et al.* (2001). Results are reported as PFCs/million splenocytes.

B16F10 melanoma tumor challenge model. At 8 weeks of age, mice were injected intravenously with B16F10 melanoma cells (1×10^5 total cells/mouse; ATCC, Manassas, VA) (Holsapple *et al.*, 1988). Fourteen days following tumor challenge, mice were euthanized, and the lungs were removed and placed in labeled vials containing Bouin's fixative. The number of nodules was enumerated using a dissecting microscope by at least two independent readers.

Listeria monocytogenes challenge model. A stock of *Listeria monocytogenes* (serotype 4 of strain 19303) was used to challenge mice. Bacterial colony counts were performed on the liver and spleen of mice 4 days postintravenous challenge with *Listeria*. The liver and spleen are major sites for *Listeria* replication, and bacteria can be detected as soon as 24–72 hours after challenge. In previous studies with mice, an LD_8 was reported at 2000 colony forming units (CFU) (Bleavins *et al*., 1995). Therefore, a target challenge range of 2000 CFU/mouse was determined using the information from previous studies in addition to range-finding studies performed in this laboratory. This challenge level was also consistent with levels of septicemia in range-finding studies 4 days postchallenge. To determine levels of septicemia, liver and spleen were aseptically removed and weighed. Premeasured portions of each organ were diluted in sterile saline, 1:10, $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$. One ml of each dilution was added to prewarmed brain heart infusion agar and poured into a sterile petri dish. These plates were incubated overnight at 37°C and enumerated after 24-hour incubation. F_1 adult mice were challenged with *Listeria* at 8 weeks of age and euthanized 4 days post challenge.

Thyroid hormones. Serum thyroid hormone levels were measured in 8-week-old, adult mice exposed *in utero* to JP-8. Total serum T3 and T4 were measured by means of RIA commercial kits for canine T3 and T4 measurement (Diagnostic Products Incorporated, Los Angeles, CA) and were conducted in accordance with the kit/manufacturer's instructions. These kits are recommended by the manufacturer for murine testing, as sufficient cross reactivity between murine and canine thyroid hormones has been reported. Other published studies have utilized these kits when evaluating murine thyroid hormone levels (Goya *et al*., 1995; Hotz *et al*., 1997; Sagartz *et al*., 1997; Wagle *et al*., 1994).

Statistical analysis. Using JMP 4.0.2 (SAS Inst. Inc., 2000), data were tested for normality (Shapiro-Wilks) and homogeneity (Bartlett's), and if needed, appropriate transformations were made. Because differences between sexes could be expected and could skew mean values, differences were tested for sex by treatment and sex within treatment interaction. If no sex-dependent effect was observed, then male and female data were combined. If significant sex by treatment (sex × treatment) or sex within treatment (sex[treatment]) differences were observed ($p \leq 0.05$), these data were separated by gender and analyzed for treatment effects via ANOVA. Statistical significance was determined using a one-way ANOVA ($p \leq 0.05$). When significant differences were detected by ANOVA, Dunnett's Comparison was used to compare treatment groups and controls.

RESULTS

Body and Organ Weight, Organ Cellularity

There were no signs of overt toxicity in any of the mice as indicated by weight loss or lack of activity. When evaluated at weaning (3 weeks), no significant changes were observed in body, thymus, spleen, or liver weights (Table 1). At adulthood (8 weeks), spleen weight was increased in both male and female F_1 mice following exposure to either treatment level, while thymus weight was decreased in males only at the 1000 mg/kg treatment level (Table 1). Body and liver weights were not altered by JP-8 treatment in adult mice. As might be expected, there were statistical differences in male and female body weight, organ weights, and organ cellularity at adulthood. Therefore, these data could not be statistically combined and were separated by gender. Splenic and thymic total cellularity was not altered by treatment in 3-week-old pups (data not shown). However, in 8-week-old mice thymic total cellularity was significantly decreased in both male and female mice at 2000 mg/kg (Fig. 1), while splenic cellularity was not altered (data not shown).

Peripheral Blood Counts

In both 3- and 8-week-old mice, no significant changes were observed in white or red blood cell numbers, hemoglobin, hematocrit, or WBC differentials following *in utero* exposure to JP-8 at either dose (data not shown).

Thymic and Splenic Lymphocytic Subpopulations

A significant increase was noted in the absolute number of thymic CD4-/CD8+ lymphocytes of 3-week-old male mice exposed *in utero* to 2000 mg/kg JP-8 (Table 2). There were also several differences noted between male and female mice in the thymic CD4-/CD8+ subpopulation at both 3 and 8 weeks of age. No significant alterations in splenic CD4/8 lymphocytic subpopulations were detected at weaning or adulthood in F_1 mice (data not shown). Additionally, the absolute number of splenic B-cells (B220+) was not altered in adult F_1 mice (data not shown).

TABLE 1
Body and Organ Weight in 3- and 8-Week-Old B6C3F1 Mice Exposed *in Utero* (Gestational Days 6–15) to Either the Carrier Control (Olive Oil Only), 1000, or 2000 mg/kg JP-8.

Treatment (mg JP/kg)	Age (wk)	Sex	Body weight	Spleen weight	Thymus weight	Liver weight
Control	3	M	11.3 ± 0.9	0.472 ± 0.02	0.558 ± 0.03	5.708 ± 0.10
1000	3	M	10.8 ± 0.5	0.485 ± 0.04	0.608 ± 0.02	5.454 ± 0.23
2000	3	M	11.7 ± 0.4	0.491 ± 0.02	0.560 ± 0.02	5.736 ± 0.13
Control	3	F	9.6 ± 0.7	0.485 ± 0.01	0.622 ± 0.02	5.684 ± 0.16
1000	3	F	10.9 ± 0.2	0.480 ± 0.01	0.628 ± 0.01	5.824 ± 0.13
2000	3	F	10.4 ± 0.1	0.510 ± 0.02	0.632 ± 0.03	5.637 ± 0.16
Control	8	M	24.1 ± 1.4[a]	0.326 ± 0.03[a]	0.204 ± 0.01[a]	5.770 ± 0.16
1000	8	M	25.8 ± 0.9[a]	0.451 ± 0.01[a]*	0.147 ± 0.01[a]*	6.048 ± 0.11
2000	8	M	23.9 ± 0.5[a]	0.381 ± 0.01[a]*	0.222 ± 0.001[a]	5.313 ± 0.10
Control	8	F	20.0 ± 0.4[b]	0.407 ± 0.03[b]	0.399 ± 0.03[b]	5.744 ± 0.18
1000	8	F	21.6 ± 0.6[b]	0.495 ± 0.05[b]*	0.308 ± 0.03[b]	5.644 ± 0.15
2000	8	F	19.4 ± 1.1[b]	0.528 ± 0.03[b]*	0.319 ± 0.03[b]	5.671 ± 0.20

Note. Organ data are normalized by body weight [(organ weight/body weight) × 100]; $n = 4$ for all treatment groups except 8-week male controls, where $n = 5$. Data are reported as mean ± SEM and are representative of 2 trials. A significant difference due to treatment is noted with an asterisk ($p \leq 0.05$). Due to the fact that there were significant differences ($p \leq 0.05$) between 8-week-old male[a] and female[b] mice within treatments (i.e., sex[treatment] interaction) the data are shown separated by gender.

Splenic Lymphocyte Proliferation

There was a significant suppression of B-cell lymphocyte proliferation evident at 3 weeks of age following *in utero* exposure to 2000 mg/kg JP-8 (Fig. 2). Both male and female 3-week-old mice were uniformly affected. There were no significant alterations in T- lymphocyte proliferation at 3 weeks of age (Fig. 2). At 8 weeks of age no significant change was observed in splenic T- or B-cell mitogen-induced proliferation at either treatment level or by gender (data not shown).

Macrophage Cellularity, Nitric Oxide Production, and Phagocytosis

Macrophage parameters were assessed in F_1 mice at 8 weeks of age only. Peritoneal cellularity was not significantly affected by *in utero* exposure to 1000 or 2000 mg/kg JP-8 (data not shown). Although significant alterations in phagocytosis (control and 1000 mg/kg) and macrophage nitric oxide production (control only) were observed in male versus female F_1 mice, these endpoints were not altered by treatment with JP-8 (data not shown).

IgM Antibody Plaque-Forming Cell (PFC) Assay

In F_1 adult mice, decreases of 46% and 81% in the IgM plaque-forming cell response were observed in the 1000 and 2000 mg/kg treatment groups, respectively. Both male and female mice were uniformly affected (Fig. 3). This parameter was not assessed at 3 weeks of age.

Bone Marrow Cellularity and Stem Cell Proliferation

At 8 weeks of age, bone marrow parameters were evaluated in F_1 mice. No significant changes were observed in bone

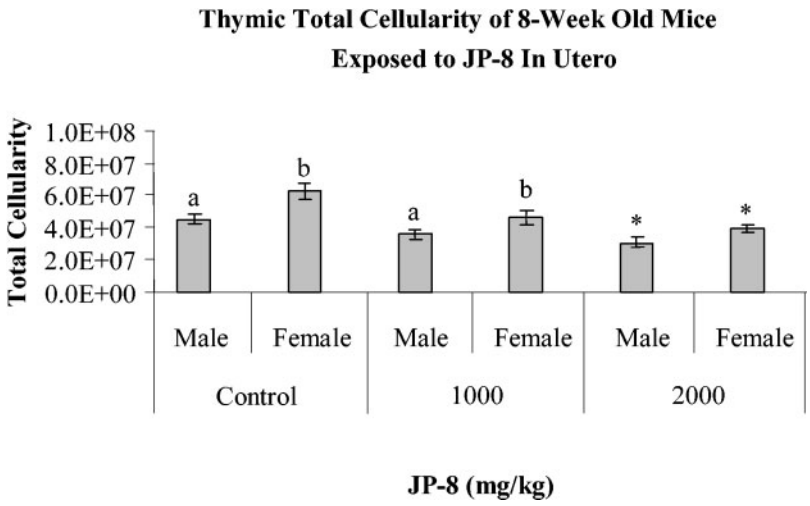

FIG. 1. Total thymic cellularity in 8-week-old B6C3F1 mice exposed *in utero* (gestational days 6–15) to either the carrier control (olive oil only), 1000, or 2000 mg/kg JP-8. Data are representative of 2 trials and are reported as mean ± SEM. Significant differences due to treatment are noted with astericks (*) ($p \leq 0.05$). Due to the fact that there were significant differences (≤ 0.05) between male[a] and female[b] mice within treatments at 8 weeks of age (i.e., sex-[treatment] interaction) the data are shown separated by gender; $n = 4$ for males and females in each treatment or control group.

TABLE 2
Flow Cytometric Evaluation of Absolute CD4/CD8 Lymphocytic Subpopulations in the Thymus of 3- and 8-Week-Old B6C3F1 Mice

Treatment (mg JP-8/kg)	Age (wk)	Sex	CD8+ (cells × 10^6)	DP (cells × 10^6)	DN (cells × 10^6)	CD4+ (cells × 10^6)
Control	3	M	1.32 ± 0.21	34.82 ± 2.75	1.17 ± 0.17	4.42 ± 0.44
1000	3	M	0.82 ± 0.17[a]*	38.52 ± 2.15	1.09 ± 0.17	4.11 ± 0.54
2000	3	M	1.58 ± 0.63*	35.45 ± 4.06	1.34 ± 0.29	4.30 ± 0.3
Control	3	F	1.23 ± 0.39	31.67 ± 3.61	1.11 ± 0.23	3.75 ± 0.68
1000	3	F	1.96 ± 0.56[b]	37.29 ± 3.09	1.41 ± 0.27	4.67 ± 0.29
2000	3	F	0.91 ± 0.06	30.0 ± 0.23	0.88 ± 0.06	3.88 ± 0.2
Control	8	M	0.46 ± 0.02[a]	7.52 ± 0.89	0.45 ± 0.06	0.97 ± 0.1
1000	8	M	0.47 ± 0.08[a]	8.39 ± 1.4	0.44 ± 0.11	0.95 ± 0.17
2000	8	M	0.50 ± 0.05	8.14 ± 0.94	0.48 ± 0.07	1.12 ± 0.1
Control	8	F	0.36 ± 0.07[b]	3.82 ± 0.87	0.41 ± 0.02	0.64 ± 0.11
1000	8	F	0.41 ± 0.06[b]	5.2 ± 0.76	0.34 ± 0.03	0.72 ± 0.09
2000	8	F	0.52 ± 0.04	7.3 ± 0.58	0.54 ± 0.11	0.98 ± 0.08

Note. B6C3F1 mice (3- and 8-weeks old) were exposed *in utero* (gestational days 6–15) to either the carrier control (olive oil only), 1000**,** or 2000 mg/kg JP-8. Data from weaned mice are representative of 1 trial, and data from adult mice are representative of 2 trials. Absolute values are reported as (mean ± SEM) × 10^6; $n = 8$ for all treatment groups (4 per sex). Due to the fact that there were significant differences ($p \leq 0.05$) between male[a] and female[b] mice within treatments (i.e., sex[treatment] interaction) the data are shown separated by gender. A significant difference due to treatment is noted with an asterisk ($p \leq 0.05$). DP = double positive, CD4+/CD8+; DN = double negative, CD4-/CD8-.

marrow cellularity or proliferative function (total colony forming units) following *in utero* exposure to JP-8 at either treatment level or by gender (data not shown).

Splenic Natural Killer (NK) Cell Function

No significant change was observed in splenic natural killer cell activity in either male or female F_1 adult mice following exposure to 1000 or 2000 mg/kg JP-8 *in utero* (data not shown).

Listeria Monocytogenes Challenge

At 8 weeks of age, F_1 mice were challenged with *Listeria monocytogenes*. No significant change in susceptibility to this infection was observed in mice exposed to 1000 or 2000 mg/kg

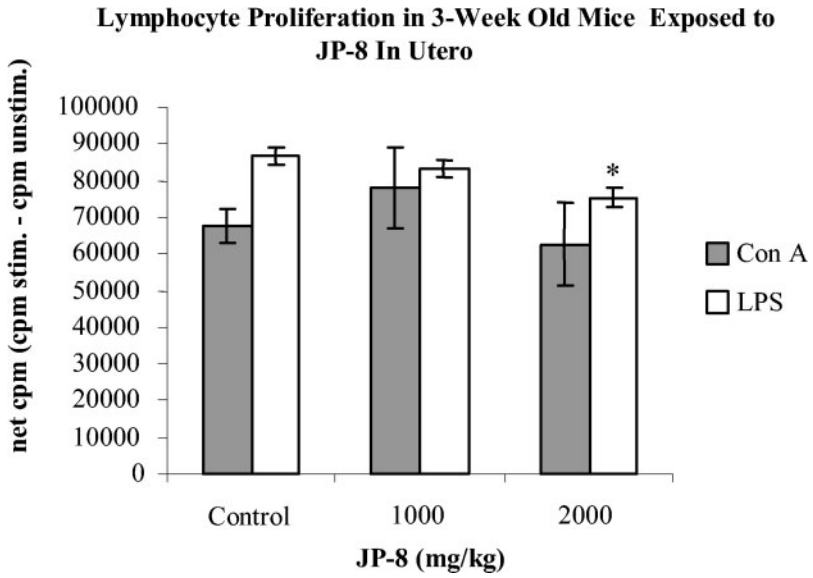

FIG. 2. Lymphocyte proliferation in 3-week-old B6C3F1 mice exposed *in utero* (gestational days 6–15) to either the carrier control (olive oil only), 1000, or 2000 mg/kg JP-8. Concanavalin A (Con A, 5 μg/ml) was used as the T-cell mitogen, and lipopolysaccharide (LPS, 5 μg/ml) was used as the B-cell mitogen. Sample size is 8 for treatment groups and control (4 male and 4 female). A significant (*) difference was observed at 2000 mg/kg JP-8 in B-cell (LPS-induced) lymphocyte proliferation as compared to controls but not between male and female mice within or by treatment ($p \leq 0.05$). Because there was no difference between the response of male and female offspring in this endpoint (as evidenced by sex × treatment and sex[treatment] statistical analysis) these data were not separated by gender. Data are reported as mean ± SEM. A representative graph from of one of 2 trials is shown.

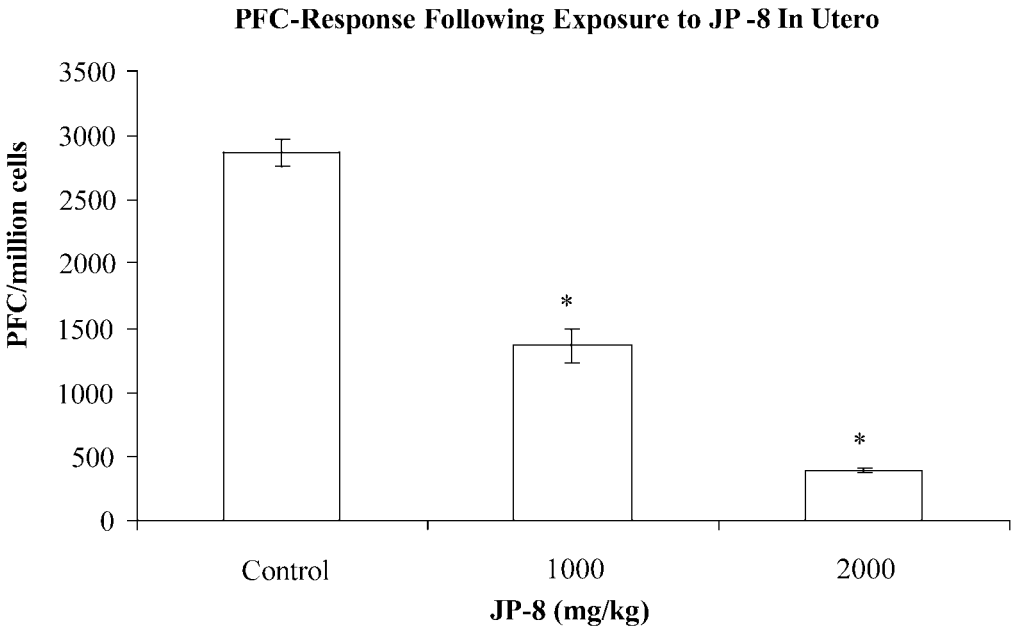

FIG. 3. Sheep red blood cell specific IgM plaque forming cell (PFC) response in adult (age 8 weeks) B6C3F1 mice exposed *in utero* (gestational days 6–15) to either the carrier control (olive oil only), 1000, or 2000 mg/kg JP-8. A representative graph from one of 3 trials is shown. Data are reported as mean ± SEM. Sample size is 8 for treatment groups and control (4 male and 4 female). A significant (*) difference was observed at 1000 and 2000 mg/kg JP-8 as compared to controls but not between male and female mice within or by treatment ($p \leq 0.05$). Because there was no difference between the response of male and female offspring in this endpoint (as evidenced by sex × treatment and sex-[treatment] statistical analysis) these data were not separated by gender.

JP-8 *in utero* as determined by bacterial counts in the spleen and liver on day 4 of infection (Fig. 4). However, male mice had significantly lower splenic infection levels than females.

B16F10 Tumor Challenge

Susceptibility to B16F10 tumor challenge during adulthood was increased. This was indicated by the fact that the number of tumors per lung was increased in F_1 mice exposed to 1000 or 2000 mg/kg JP-8 *in utero* (Fig. 5). Additionally, there was no difference in this response related to the gender of the offspring.

Thyroid Hormones

A significant decrease of 38% in serum thyroxin (T4) levels was observed in the 2000 mg/kg treatment group at adulthood; however, no significant change was observed in serum T3 levels (Fig. 6). Additionally, there was no difference in this response related to the gender of the offspring.

DISCUSSION

The immune system is not typically considered a target when evaluating developmental effects of chemicals. As no validated models to assess developmental immunotoxicity have been established to date (Holsapple *et al.*, 2003), the decision was made to utilize an exposure period encompassing gestation days (GD) 6–15 as adapted from general developmental toxicology models. It was expected that this period would be concurrent with key developmental stages in the ontogeny of the murine immune system (Dietert *et al.*, 2000;

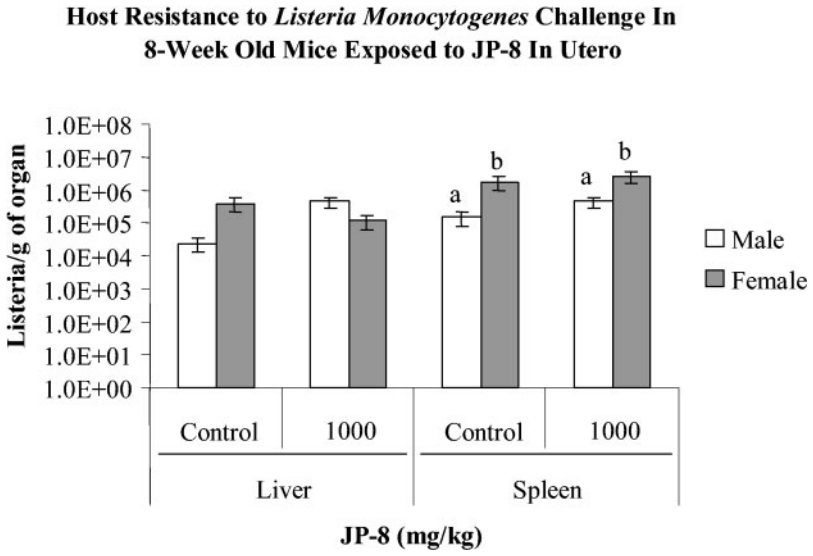

FIG. 4. Challenge with *Listeria monocytogenes* in adult (age 8 weeks) B6C3F1 mice exposed *in utero* (gestational days 6–15) to either the carrier control (olive oil only), 1000, or 2000 mg/kg JP-8. Listeria per gram of liver and listeria per gram of spleen is represented in the graph. A graph from one of three trials is presented and data are reported as mean ± SEM. A significant difference was observed between male[a] and female[b] infection levels in the spleen, but not between treatment and respective control ($p \leq 0.05$). Due to the fact that there were significant differences ($p \leq 0.05$) between male[a] and female[b] mice within treatments (i.e., sex-[treatment] interaction) the data are shown separated by gender.

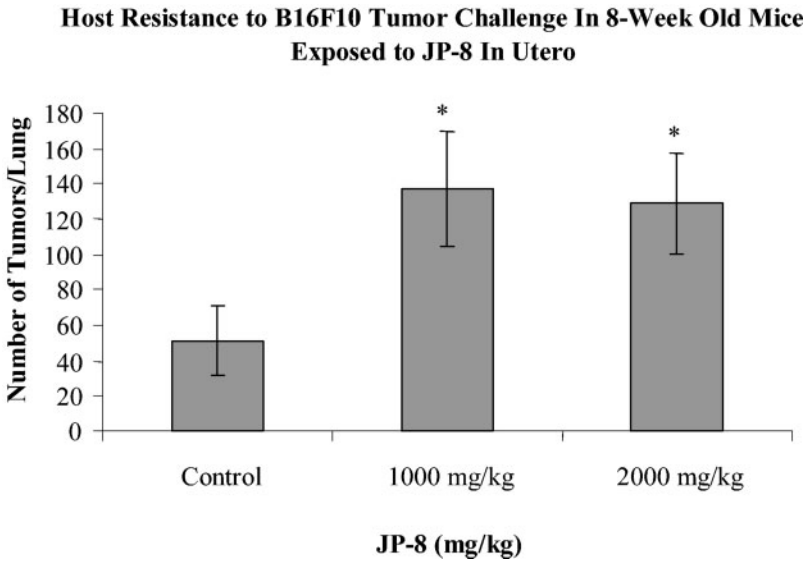

FIG. 5. Challenge with B16F10 tumor cells in adult (age 8 weeks) B6C3F1 mice exposed *in utero* (gestational days 6–15) to either the carrier control (olive oil only), 1000, or 2000 mg/kg JP-8. Number of tumor nodules counted on each lung is represented in the graph. Data are representative of 3 trials and are reported as mean ± SEM. Sample size for all treatment groups is 8 (4 per sex). A significant difference (*) was observed at 1000 and 2000 mg/kg JP-8 as compared to control, but not between male and female mice within or by treatment ($p \leq 0.05$). Therefore, because there was no difference between the response of male and female offspring in this endpoint (as evidenced by sex × treatment and sex[treatment] statistical analysis) these data were not separated by gender.

Holladay, 1999; Holladay and Smialowicz, 2000; Kameyama, 1991; Luebke *et al*., 1986). Gestation days (GD) 7–9 mark the initiation of hematopoiesis, followed by migration of stem cells and expansion of progenitor cells during GD 9–16 (Dietert *et al.*, 2000). GD 13 until birth is the period of colonization and establishment of the bone marrow and thymus (reviewed by Landreth, 2002). Utilizing the exposure schedule of GD 6–15, this study establishes that this developmental period is vulnerable to exposure as confirmed by immunological alterations in the adult offspring.

JP-8 exposure levels and administration route were determined using data from human exposure guidelines (NAVOSH Standards Update Board, 1992), published studies in rodents (Cooper and Mattie, 1996; Mattie *et al*., 1991, 1995), and range-finding experiments performed in this laboratory. Although oral administration is not the most relevant route of exposure in humans, it alone offers delivery of JP-8 in its entirety without prior fractionation of components (reviewed in National Research Council, 2003a). This stems from the fact that JP-8 is a complex mixture of components having extreme variability in chemical and physical properties (e.g., it contains both light and heavy fractions differing in volatility), rendering it difficult to qualitatively and quantitatively assess exposure and dose via inhalation and dermal routes. For example, direct skin application of JP-8 generally results in greater exposure to the higher-molecular-weight fraction of JP-8, while low-molecular-weight, volatile fractions evaporate preferentially. When generating JP-8 atmospheres for inhalation studies, the process used can enrich specific components of JP-8 (reviewed in National Research Council, 2003a). Although much effort has been applied to model inhalation and dermal penetration, technical limitations in measurement technology for these routes make the accurate and comprehensive assessment of actual exposure difficult. As this was the first project to assess the role of JP-8 during immunological development, oral exposure was utilized to consider JP-8 in its entirety.

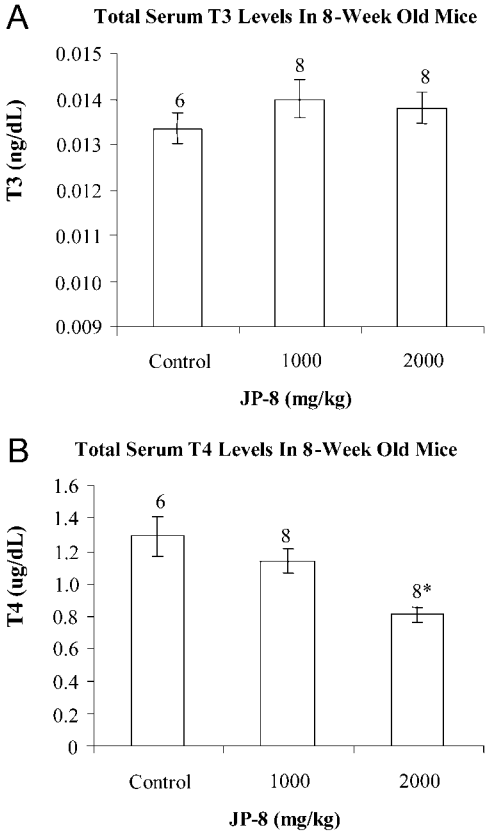

FIG. 6. Total serum T3 (A) and T4 (B) levels in adult (age 8 weeks) B6C3F1 mice exposed *in utero* (gestational days 6–15) to either the carrier control (olive oil only), 1000, or 2000 mg/kg JP-8. The control group consists of 1 female and 5 males, while the 1000 and 2000 mg/kg treatment groups consist of 4 females and 4 males. Data are representative of 1 trial and reported as mean ± SEM. Total sample size for all treatment groups is noted above bar in the graph and a significant difference due to treatment is noted with an asterisk ($p < 0.05$). Due to the fact that there was no difference between the response of male and female offspring in this endpoint (as evidenced by sex × treatment and sex[treatment] statistical analysis) these data were not separated by gender.

Of the immunological effects noted following oral exposure in the pregnant dams, B-cell proliferative responses were transiently suppressed in the developing offspring and fully reestablished by 8 weeks of age. In contrast, previous studies with mature adult mice indicated that JP-8 did not alter B-cell proliferative responses after oral administration of either 500, 1000, or 2000 mg/kg JP-8 for 7 or 14 days (Peden-Adams *et al*., 2001; Scharstein *et al*., 2002). Although not a permanent effect, this does suggest that B-cells may be more susceptible to the effects of JP-8 during *in utero* development.

The converse can be described for T-cells. Proliferative responses with T-cells were unaffected after *in utero* exposure to JP-8, yet there are several reports indicating that T-lymphocytic proliferative responses were suppressed in adult mice after dermal or inhalation exposure to JP-8 (Harris *et al*., 1997a,b; Ullrich, 1999; Ullrich and Lyons, 2000), and this suppression was long-term, as recovery was not evident until 28 days postexposure (Harris *et al.*, 1997c). In contrast to these publications, we have previously reported that oral exposure to JP-8 does not affect T-proliferative responses (Peden-Adams *et al*., 2001; Scharstein *et al*., 2002). Therefore, it might be more reasonable to expect that alterations in this function appear to be contingent on the route of exposure to JP-8.

The PFC response is an immunological parameter that evaluates the effective interaction of antigen-presenting cells, T-cells, and antibody-producing B-cells to mount a primary IgM response. It is considered highly predictive of immunotoxicity, thereby suggesting increased susceptibility to disease (Luster *et al*., 1992). We have previously reported that primary immunological responses to sRBCs were dose-responsively suppressed when adult mice were exposed to JP-8 (Dudley *et al.*, 2001; Peden-Adams *et al*., 2001), but this alteration was temporary, because function was fully restored by 7 days postexposure (Scharstein *et al*., 2002). However, the fact that there was a significant deficit in humoral responses in F_1 mice at 8 weeks is disturbing, demonstrating that this alteration is persistent and the developing immune system appears to be at greater risk from JP-8 exposure.

Despite the substantial decrease in humoral immunity, no increase in susceptibility to *Listeria monocytogenes* was observed after *in utero* exposure to JP-8. This is not surprising, as humoral immunity does not play a large role during the early stages of this infection. During this early window of active infection, immunological defenses depend largely on neutrophils, macrophages, NK cells, and T-cells (Czuprynski *et al.*, 1994; reviewed by Edelson and Unanue, 2000). Furthermore, T-cell, NK, and macrophage cell function were not impaired in the F_1 mice and could effectively contribute to immunological protection. Considering the substantial deficits in humoral immunity caused by JP-8, future studies might assess additional host challenge models that depend largely on humoral immunity for protection, such as the group B Streptococcus bacterial challenge model (Barnes *et al*., 1992).

Immunological resistance to tumors was impaired, and this effect was evident in adult animals. The tumor cell line utilized in this study is syngeneic to one of the parent strains (C57BL/6), and clearance requires the function of CD4(+) T-cells, CD8(+) T-cells, and natural killer (NK) cells (reviewed by Wu and Fleischmann, 2001). This deficit may not be unique to the *in utero* exposure, bacause there are preliminary observations suggesting that B16F10 melanoma tumor growth was less regulated in adult mice exposed to JP-8 via inhalation (National Research Council, 2003b). However, the fact that tumor susceptibility was significantly enhanced when the F_1 offspring were fully mature is striking, indicating long-lasting vulnerability of the developing immune system to the effects of JP-8. Also noteworthy is that, while susceptibility to B16F10 melanoma cells was significantly impaired, NK function exhibited normal levels of cytolytic activity. This study also demonstrates an important role for the incorporation of host challenge models in developmental immunotoxicology evaluations, as resistance to melanoma tumor challenge was impaired with no warning by any of the other immunological endpoints evaluated in this study.

In addition to the immunotoxicological assessment, a cursory evaluation of thyroid function was performed. Not only is proper thyroid function necessary for healthy development, several reports indicate that thyroid hormones can modulate immunological function (Blalock *et al*., 1984; Coutelier *et al*., 1990; Kanazawa *et al*., 1992; Marazuela *et al*., 1995; Montecino-Rodriguez *et al*., 1997). JP-8 exposure during fetal development affected thyroid function, leading to deficits of circulating T_4 levels in serum from adult progeny. While such a deficiency in T_4 may suggest a hypothyroid state, examination of thyroid stimulating hormone (TSH) and thyroid histopathology would be of value in corroborating dysfunction. Within the scope of this study, it is unknown if the alteration in T_4 is linked to any of the changes observed in immunological function, although this would appear to be a worthwhile line of inquiry.

The potential for exposure to JP-8 during pregnancy appears to be substantial, because nearly 200,000 women are on active duty in the Armed Forces (Institute of Medicine, 1995). Of the women on active duty, approximately 94% are of childbearing age (Institute of Medicine, 1995). Potential exposure also extends to the aviation industry, as commercial jet fuel is essentially JP-8 devoid of certain additives that impart properties of military significance. Aside from the fact that women are an ever-increasing population in the military and aviation industry, many civilians reside near environmentally contaminated sites where jet fuel has been reportedly spilled or leaked from supply lines or storage tanks. A list of national priority sites provided by the U.S. Environmental Protection Agency (http://www.epa.gov/superfund/sites/index.htm) describe thousands of gallons of jet fuel contaminating soil and ground water, associated with U.S. Air Force bases bordering residential areas. Thus, it was appropriate to investigate the possible implications of JP-8 exposure during developmental periods in offspring. As this is the first report to demonstrate that the developing immune system is vulnerable to the effects of JP-8,

it is difficult to extrapolate the results of this study to those that might occur in humans exposed *in utero* via the dermal or inhalation routes.

Despite the fact that several immunological and hematological parameters assessed in this study were unaffected, *in utero* exposure to JP-8 caused long-lasting and possibly permanent deficits in humoral immunity, T_4 levels, and tumor resistance. While much work remains to be done, these results suggest that the developing immune system is sensitive to the effects of JP-8, resulting in effects persisting into adulthood.

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