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An Aryl Hydrocarbon Receptor Independent Mechanism of JP-8 Jet Fuel Immunotoxicity in Ah-Responsive and Ah-Nonresponsive Mice

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JP-8 jet fuel is handled extensively by personnel in the military and commercial airlines, despite the paucity of information regarding its potential human health effects. JP-8 is a complex mixture primarily consisting of kerosene plus aliphatic and aromatic hydrocarbons. Recent reports indicate that acute JP-8 exposure via inhalation or dermal routes can overtly and persistently impair immune function in mice. Data from preliminary studies in this laboratory assessing the immunotoxicity of JP-8 indicated that oral JP-8 exposure caused an increase in liver weight, a decrease in thymus weight, and a decrease in the PFC response. As these results were similar to classic effects elicited by TCDD, a strong AhR ligand, it was hypothesized that JP-8 may exert immunosuppression via a similar mechanism. To test this hypothesis, an Ah-responsive mouse strain (B6C3F1) and a classically non-responsive mouse strain (DBA/2) bearing a lower affinity AhR were gavaged with JP-8 for 7 days. The results suggest that both mouse strains were equally sensitive to JP-8's toxicity at several endpoints including thymus weight and cellularity, liver weight, and specific IgM antibody responses. Furthermore, JP-8 did not induce CYP1A1 or promote down regulation of the AhR when evaluated by Western blot in either B6C3F1 or DBA/2 mice. *In vitro* studies corroborated these findings as JP-8 did not induce CYP1A1, promote down regulation of the AhR, or activate an XRE-driven reporter gene in murine Hepa-1 cells. These results suggest that JP-8 may exert its toxicity via an AhR-independent mechanism.

Key Words: **JP-8 jet fuel; immunotoxicity; aryl hydrocarbon receptor; CYP1A1/2; DBA/2 and B6C3F1 mice.**

JP-8 jet propulsion fuel is used extensively by the U.S. Air Force, the North Atlantic Treaty Organization (NATO) forces, and commercial airlines. Many industrial and military personnel are exposed to JP-8 on a yearly basis during aircraft refueling and servicing, as well as maintenance, inspection, and cleaning of fuel-storage tanks, yet little is known regarding JP-8's impact on human health (IARC, 1989). Recommended exposure guidelines for JP-8 in the Air Force have been set at 63 ppm (447 mg/m^3 as an 8-h time-weighted average). Similarly, the Navy's occupational and health guidelines are set at 350 mg/m^3 (8-h time-weighted average) to 1800 mg/m^3 for short-term exposures (NAVOSH Standards Update Board, 1992). In spite of these recommendations, fuel handlers, mechanics, and flight line personnel have reported emotional dysfunction, decreased attention spans, fatigue, skin irritation, postural sway imbalances, and adverse effects on sensorimotor speed, liver function, and the respiratory system (Cohen, 1990; Davies, 1964; Dossing *et al.*, 1985; Kinkead *et al.*, 1992; Smith *et al.*, 1997).

Furthermore, recent animal studies indicated that acute or chronic exposure to JP-8 might cause a range of effects including relatively minor toxicological effects in 90-day studies or impaired immune function after a single dermal exposure. For instance, a 90-day inhalation study using male and female rats indicated that JP-8 (0–1000 mg/m^3) had limited toxicity and resulted in no tumor formation (Mattie *et al.*, 1991). In male rats, α_2-microglobulin protein droplet nephropathy was detected after JP-8 exposure; however, this observation appears to be unique in male rats exposed to hydrocarbons and not likely to extrapolate to human toxicity (Mattie *et al.*, 1991). A chronic 90-day, oral exposure to JP-8 (0–3000 mg/kg/day) in rats resulted in no mortality or morbidity with relatively minor or no adverse consequences in liver pathology, serum chemistries, and urological and hematological parameters. Primary effects noted in this chronic study included an increase in serum liver-enzyme levels (alanine aminotransferase and aspartate aminotransferase), perianal irritation, and a dose-dependent decrease in body weight (Mattie *et al.*, 1995). Additional rat studies indicated that JP-8 did not cause fetal malformation after oral JP-8 (0–2000 mg/kg/day) exposure in pregnant rats during gestation days 6–15. Thus, the no-observed-effect level of 500 mg/kg for pregnant dams and 1000 mg/kg for the fetal rat was established (Cooper and Mattie, 1996). Reduced pulmonary function and induced lung pathology has also been reported in rats exposed to JP-8 via inhalation (Hays *et al.*, 1995; Pfaff *et al.*, 1996; Robledo and Witten, 1999). More recently, studies have evaluated immune function in mice after

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administration of JP-8 by way of different exposure routes. Seven-day exposure to aerosolized JP-8 jet fuel ranging from 100-2500 mg/m^3 caused persistent and substantial decreases in spleen and thymus organ weights and cellularity, changes in lymphocytic subpopulations, and compromised T-cell blastogenesis to the T-cell mitogen concanavalin A (Con A; Harris *et al.*, 1997b,c). Similarly, dermal exposure to JP-8 (50 μl/day for 5 days or a 250–300-μl single exposure) suppressed T-cell blastogenesis and impaired delayed and contact hypersensitivity (Ullrich, 1999). Current studies in our laboratory have revealed that 7- or 14-day oral exposures to JP-8 are also immunotoxic (Dudley *et al.*, 2000; unpublished data). Thus, it is apparent from the current literature that profound changes have been noted in pulmonary and immune function by a variety of exposure routes, yet no mechanism has been suggested to account for these effects.

JP-8 is a kerosene-based complex mixture of aliphatic and aromatic hydrocarbons. The composition of JP-8 has been modified from previously used jet propulsion fuels (JP-4 and JP-5), in that it contains additives for static dissipation, anticing, and corrosion inhibition. Chemically, JP-8 is a complex mixture containing approximately 81% alkanes, primarily in the C8–C17 range. The remaining portion includes 10–20% polycyclic aromatic hydrocarbons (PAH) and low levels of benzene, toluene, and xylene (IARC, 1989; reviewed by Zeiger and Smith, 1998).

In mice, it is suggested that the manifestations of halogenated aromatic hydrocarbon (HAH) exposure, (i.e., polychlorinated biphenyls, dibenzo-*p*-dioxins, and dibenzofurans) including thymus involution, suppression in the splenic antibody response to TNP-LPS and SRBC, and liver hypertrophy are mediated through AhR (aryl hydrocarbon receptor) signal transduction (Gonzalez *et al.*, 1998; Kerkvliet *et al.*, 1990; Staples *et al.*, 1999). Furthermore, studies have revealed that some strains of mice (C57BL/6 and B6C3F1) are more sensitive to the effects of aromatic hydrocarbons than other strains (DBA/2; Forkert, 1997; Poland *et al.*, 1974; Vecchi *et al.*, 1983). It is now understood that DBA/2 mice differ at the Ah locus (substitution of a valine for alanine at amino acid 375) resulting in a lower affinity cytosolic receptor (Poland *et al.*, 1994). Since this difference in sensitivity appears to reside solely at the Ah locus, any effects that are mediated by AhR signal transduction should occur at a lower dose in Ah-responsive mice and would only occur in Ah-nonresponsive mice at a higher, saturating dose. Understanding this difference has been advantageous in evaluating the role of the AhR in immunosuppression following exposure to a number of different PAHs (Lubet *et al.*, 1984; Silkworth *et al.*, 1984, 1995; White *et al.*, 1985).

The mechanisms of PAH-induced immunotoxicity are not clear; however, several possibilities have been postulated, which include interaction with the Ah-receptor, membrane perturbation, altered interleukin production, modulation of intracellular Ca^{+2} mobilization, and metabolic activation to reactive metabolites (reviewed by White *et al.*, 1994). Many PAHs are carcinogenic and their ability to cause cancer has been linked to the Ah-receptor (Kerkvliet *et al.*, 1990; Okey *et al.*, 1984a,b; Piskorska-Pliszczynska *et al.*, 1986) and the immunotoxicity of PAHs is thought to be related to their carcinogenic potency (White and Holsapple 1984; White *et al.*, 1985, 1994). Generally, the bay-region diols (diol epoxides) of the parent are the most toxic metabolites and are considered responsible for the carcinogenic effects, and it has been suggested that these metabolites may mediate the observed immunosuppression as shown by the diol epoxides of benzo[a]pyrene (BaP) and 7, 12-dimethylbenz[a]anthracene (DMBA; White *et al.*, 1994). However, metabolic activation of parent PAHs is not always required for immunosuppression. BaP was shown to suppress *in vitro* Ab production directly (White and Holsapple, 1984) and it has been suggested that immunosuppression by chrysene is not related to metabolites (Silkworth *et al.*, 1995). Regardless, current thought centers around the correlation between PAH carcinogenicity and immunotoxicity and the role of the AhR and bioactivated metabolites in PAH-induced immunosuppression.

The immunotoxicity of HAHs like 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD), which are resistant to enzymatic metabolism, is most likely due to effects or products resulting from activation of the Ah gene complex. However, the immunotoxicity of PAHs that bind the AhR is probably due to an increased formation of bioactivated metabolites. Quantitative studies to evaluate protein expression of the AhR and CYP1A1 following *in vivo* and *in vitro* TCDD exposure reveal a rapid reduction in the AhR and increased levels of CYP1A1 in total cell and tissue lysates (Davarinos and Pollenz, 1999; Pollenz, 1996; Pollenz *et al.*, 1998). For example, Sprague-Dawley rats given a single oral dose of TCDD demonstrated a reduction in AhR protein levels in spleen, liver, lung and thymus 8 h after exposure (Pollenz *et al.*, 1998). This is consistent with reduced AhR protein in the palate of developing mice (Abbott *et al.*, 1994) and in reproductive organs from rats treated with TCDD (Roman *et al.*, 1998). It is possible that other HAHs and PAHs that are strong AhR ligands may result in similar AhR reduction. Studies in our laboratory have indicated that oral exposure to JP-8 causes a reduction in thymic mass, an increase in liver mass, and suppression of antibody responses that are considered in HAH exposures to be mediated via AhR signal transduction. Moreover, many PAHs are known to bind to the AhR (Piskorska-Pliszczynska *et al.*, 1986). Currently, there are few reports that evaluate a complex mixture of aliphatic and aromatic hydrocarbons like JP-8 and its role as a possible AhR ligand. To address this issue, experiments were undertaken to evaluate the effects of JP-8 on thymus weight and cellularity, liver weight, specific IgM antibody responses, and the expression of the AhR and CYP1A1 in AhR-responsive and AhR-nonresponsive mice. Additionally, a murine hepatic cell line was used to evaluate the

expression of AhR, CYP1A1, and a TCDD-inducible reporter gene following *in vitro* JP-8 administration.

MATERIALS AND METHODS

Animals. Female B6C3F1 and DBA/2 mice were supplied by Charles River Laboratories (Raleigh, NC). The mice were 7–8 weeks of age and housed 3–4/cage on a 12-hour light/dark cycle at 22°C and 65% relative humidity in an AAALAC accredited facility. Mice were given water and standard rodent laboratory diet (Harland-Teklab, Madison, WI) *ad libitum*.

Treatment. Following a one-week acclimation period after shipment, mice were randomly assigned to control or treatment groups (4–5 mice/group). The control group was administered a vehicle of olive oil while the treatment groups were administered 1000 or 2000 mg/kg/day of JP-8 (kindly provided by Wright-Patterson Air Force Base) prepared in the vehicle. The exposure period was 7 days and the route of exposure was via gavage. Mice used for the positive control were treated with TCDD (Radian International, Austin, TX). TCDD was dissolved in olive oil and mice were gavaged with 15 μg/kg for 3 consecutive days. The IACUC of the Medical University of SC approved all animal procedures.

Organ weights and cellularities. After mice were euthanized by $C0_2$ asphyxiation, thymus, lung and liver were carefully dissected and weighed using a Mettler Toledo balance. The liver and lung were placed immediately on ice and then frozen at −80°C. The thymus was transferred to 3 ml complete media and a single cell suspension was made by grinding the organ between two sterile glass slides. Thymic cell counts were measured using a Coulter Counter (Model ZM) and are reported as total cellularity for each respective organ. Liver and thymus weights were reported as a somatic index (organ weight/body weight × 100).

Jerne plaque-forming assay. Four days prior to euthanization, mice were injected intraperitoneally with 0.1 ml of a 20% sheep red-blood-cell suspension (SRBC, Biowhittaker) in PBS. All sheep red blood cells for the experiments were drawn from a single, donor animal. As described above, mouse spleens were homogenized, cell densities counted, and suspensions diluted to 2×10^6 cells/ml in complete media. The number of plaque-forming cells was determined using the Cunningham modification of the Jerne plaque assay (Cunningham and Szenberg, 1968). Data are reported as plaque-forming cells (PFC)/million cells.

Cell culture. Wt Hepa–1c1c7 cells were added in 12-well plates at a density of 5×10^6 cells/ml in DMEM and allowed to grow to confluence. Plates were placed in a 37° C incubator at 5% CO_2. JP-8 was dissolved in dimethyl sulfoxide (DMSO) (Fisher, Fair Lawn, NJ) and delivered in a volume of 10 μl to achieve a final DMSO concentration of 0.05%. The JP-8 concentrations used were 500, 100, or 10 ppm. 3-methylcholanthrene (3MC, Lot 88H3402, Sigma) served as the positive control and was prepared in DMSO and added at a concentration of 5 μM/well. Cells only and DMSO control wells were included in all experiments.

Preparation of tissue and whole-cell lysates. Tissue and whole cell lysates were prepared as previously described by Pollenz (1996). Briefly, a portion of the frozen lung was thawed, separated, and weighed. Approximately 0.1 g of tissue was homogenized in 1 ml of 1X lysis buffer. Four hundred and fifty μl were removed and a total volume of 500 μl was achieved by adding 10% Nonidet P-40. Samples were sonicated on ice 3 times for 10 s each, and 50 μl was removed for protein determination, using the Bio-Rad Bradford Reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. The remainder was combined with an equal volume of 2X sample buffer, heated at 95°C for 5 min, and stored at −80°C.

For whole cell lysates, cell monolayers were washed once with cold phosphate-buffered saline (PBS) (Mediatech, Cellgro), trypsinized, and centrifuged at 340g for 5 min. After decanting the supernatant, cell pellets were redissolved in 0.5 ml PBS and centrifuged at 1000 × *g* for 2 min. Pellets were then suspended in 0.1 ml of 1X lysis buffer and sonicated for 10 s. A small portion of the homogenate was removed for protein determination and the remainder was brought to equal volume with 2X sample buffer. Samples were heated at 95°C for 5 min and then stored at −80°C.

Microsomal fraction preparation. The livers were weighed and placed in pre-chilled tubes. Homogenization buffer was added at 3 times the tissue weight. Next, samples were completely homogenized, transferred to pre-chilled centrifuge tubes, and spun at 20,000 × *g* (Beckman JA-20) for 10 min (4°C). The supernatant was transferred to another set of pre-chilled centrifuge tubes and spun at 10,000 × *g* for an additional 20 min. This supernatant was again transferred to pre-chilled tubes and spun at 105,000 × *g* (Beckman L5-50 Ultra) for 1 h. Finally, the supernatant (cytosolic fraction) was discarded and the remaining pellet (microsomal fraction) was washed with ice-cold wash buffer and resuspended in 0.5 ml resuspension buffer. The pellet was then homogenized using a drill-driven pestle at low speed. A small amount was removed for protein determination and the remainder was brought to equal volume with 2X sample buffer, heated at 95°C for 5 min, and stored at −80°C until needed.

Buffers and antibodies. Unless otherwise specified, all reagents used in these experiments were purchased from Sigma (St. Louis, MO). The following buffers were used. Gel loading buffer (2X) (125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4mM EDTA, 0.5ml β mercaptoethanol, 0.005% bromophenol blue), TBS (50 mM Tris, 150 mM NaCl, pH 7.6), TTBS (50 mM Tris, 0.2% Tween, 150 mM NaCl, pH 7.5), TTBS+ (50 mM Tris, 0.5% Tween 20, 300 mM NaCl, pH 7.5), Blotto (5% dry milk in TTBS), 2X lysis buffer (50 mM HEPES, 40 mM Na molybdate, 10 mM EGTA, 6 mM $MgCl_2$, 20% Glycerol, pH 7.4), 1X trypsin-EDTA, homogenization buffer (250 mM sucrose, 20 mM Tris, pH 7.4), wash buffer (250 mM sucrose, 80 mM Tris, 25 mM KCl, pH 7.4), and resuspension buffer (250 mM sucrose, 80 mM Tris, 25 mM KCl, 20% glycerol, pH 7.4). Tissue culture media used were complete media (RPMI-1640 with 10% fetal calf serum and 50,000 IU/l pen/50 μg/l strep) and Dulbecco's modification of Eagle's Medium (DMEM) (with 5% fetal calf serum and 50,000 IU/l pen/50 μg/l strep) (Cellgro, Mediatech). The antibodies used for the Western blots were affinity-purified anti-mouse AhR raised against a bacterial expressed portion of the mouse AhR (amino acids 1–416), anti-rat polyclonal CYP1A1/2 or monoclonal CYP1A1 (Xenotech, Kansas City, KS), and anti-β actin. The secondary antibody was goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (GAR/GAM-HRP) (Santa Cruz Biotech).

Gel electrophoresis and Western blotting. Tissue, cell lysates, or microsomes were electrophoresed on a 4–12% Tris/glycine gel (Novex, San Diego, CA) and transferred to nitrocellulose membranes. Membranes were blocked in 2 changes of BLOTTO for 30 min and stained overnight at 4°C with primary antibodies (anti-mouse AhR (A-1A), anti-rat CYP1A1/2, or anti-β-actin). The next day, membranes were washed in 2 changes of BLOTTO for 15 min and then incubated for 1 h with GAR/GAM-HRP at room temperature. Next, membranes were washed in 2 changes of BLOTTO and 2 changes TTBS+ for 15 min each. Following a final 5-min wash in TBS, membranes were subjected to electrochemiluminesence (ECL) according to the manufacturer's specifications (NEN, Boston, MA).

Eukaryotic transfection and reporter gene assays. Approximately 5×10^6 Hepa-1c1c7 cells were placed into 60-mm culture dishes and incubated at 37°C for 16–24 h. PgudLuc1.1 (200 ng) (Garrison *et al.*, 1996) and 500 ng pSV-β galactisidase (Promega, Madison WI) were than transfected into cells with LipofectAMINE reagent, as detailed by the manufacturer (Gibco, Gaithersburg, MD). PGudLuc1.1 contains a 484-bp fragment from the mouse CYP1A1 promoter that contains 4 XRE sequences upstream of a luciferase reporter gene. Following a 24-h recovery period, cells were incubated in the presence of TCDD (2 nm), DMSO (0.02%), or JP-8 for an additional 8 h. Cells were then scraped from plates in reporter lysis buffer (Promega) and β-Gal luciferase activities were determined as specified by the manufacturer. Normalized luciferase activity is reported as a percentage of control and the SEM for 3 replicates.

Statistics. All experiments were repeated at least twice. The data were evaluated for normality and homogeneity prior to performing a one-way

TABLE 1
Changes in Body Weights (Final – Initial) in B6C3F1 and DBA/2 Mice Exposed to JP-8 Orally for 7 Days

	Control	1000 mg/kg/day	2000 mg/kg/day	TCDD
B6C3F1	1.25 ± 0.21	2.72 ± 0.61*	1.96 ± 0.19*	1.35 ± 0.21
DBA/2	1.1 ± 0.45	1.5 ± 0.58	1.12 ± 1.23	1.3 ± 0.14

Note. Data are reported as mean ± standard error of the mean (SEM). Sample size: 6 for control, 5 for the JP-8 treatment groups, and 2 for the TCDD treatment group.

* Significantly different from control ($p < 0.05$).

analysis of variance followed by Dunnett's statistical test ($p < 0.05$). Statistical analysis was performed using Minitab statistical software (Version 12.1). Statistics were not calculated for mice exposed to TCDD, due to the small sample size ($n = 2$).

RESULTS

Effects of JP-8 on Body and Liver Weight and Thymus Weights and Cellularities in B6C3F1 and DBA/2 Mice

JP-8 jet fuel significantly increased body weight change in the B6C3F1 but not the DBA/2 mice following a 7-day gavage (Table 1). The mean initial body weights for the B6C3F1 mice in the control group were 21.2 ± 0.39 g, 21.3 ± 0.57 g in the 1000 mg/kg JP-8 group, and 22.6 ± 1.11 g in the 2000 mg/kg JP-8 group. The mean initial body weights for the DBA/2 mice in the control group were 20.2 ± 0.34 g, 20.3 ± 0.52 g in the 1000 mg/kg JP-8 group, and 17.9 ± 0.83 g in the 2000 mg/kg JP-8 group.

There was a dose-dependent increase in liver weights in both the B6C3F1 and the DBA/2 strains. B6C3F1 and DBA/2 mice indicated a 30% increase at the 1000 mg/kg JP-8 dose and 46% and 49% increases at the 2000 mg/kg dose, respectively (Fig. 1). TCDD served as a positive control and also increased liver weight.

Both the B6C3F1 and DBA/2 mice exhibited decreases in thymus weight and cellularity (Fig. 2). B6C3F1 mice demonstrated a 40% reduction in cellularity at 2000 mg/kg, while the DBA/2 mice exhibited a 37% reduction in cellularity at 2000 mg/kg JP-8. Both mouse strains exhibited statistically significant decreases in thymus weight after treatment with 2000 mg/kg JP-8. TCDD caused decreases in thymus weight and cellularity in B6C3F1 and DBA/2 mice.

Effects of JP-8 on the Plaque-Forming Cell Response

Both the B6C3F1 and DBA/2 mice exhibited significant decreases in PFC activity (Fig. 3). In B6C3F1 mice, exposure to 1000 and 2000 mg/kg JP-8 resulted in 68% and 90% reductions in the number of PFCs, respectively. Similarly, DBA/2 mice exposed to 1000 and 2000 mg/kg JP-8 demonstrated 50% and 74% reductions in the number of PFCs,

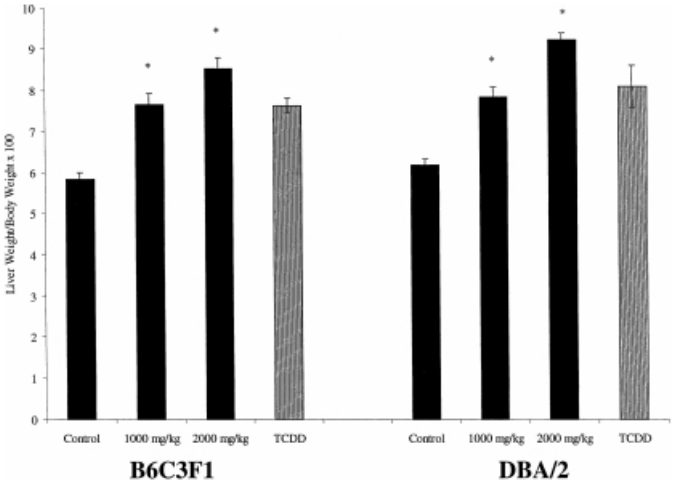

FIG. 1. Liver-to-body weight index in B6C3F1 and DBA/2 mice following a 7-day exposure to JP-8. Mice were treated with olive oil only, 1000 mg/kg/day JP-8 dissolved in olive oil, or 2000 mg/kg/day JP-8 dissolved in olive oil. For the positive control, 2 mice were gavaged for 3 consecutive days with 15 μg/kg TCDD dissolved in olive oil. Error bars represent the standard error of the mean (SEM). Statistically significant results were evaluated by one-way ANOVA and Dunnett's test and are indicated by an asterisk ($n = 6$ for control, $n = 5$ for JP-8-treated mice, and $n = 2$ for TCDD-treated mice; $p < 0.05$).

respectively. As expected, TCDD decreased the plaque-forming cell response in both B6C3F1 and DBA/2 mice.

Western Blots for the Detection of AhR and CYP1A1 in Whole-Tissue Lysates

To more clearly define the role of AhR signal transduction in JP-8's immunotoxicity, Western blots were performed to evaluate the expression of AhR and CYP1A1 protein in whole-tissue lysates prepared from the lungs of JP-8-exposed animals. A previous study has shown that the lung contains the highest

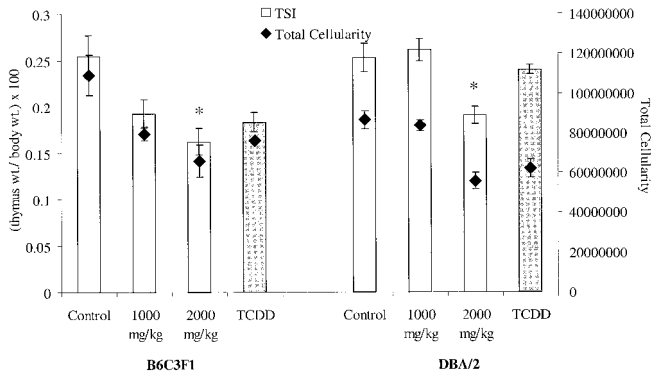

FIG. 2. Thymus weight and cellularity determined in B6C3F1 and DBA/2 mice after exposure to JP-8 for 7 days. Error bars represent the SEM. Significant differences between control and treated animals were determined by one-way ANOVA and Dunnett's test and are indicated by an asterisk ($n = 6$ for control, $n = 6$ for JP-8-treated mice, and $n = 2$ for TCDD-treated mice; $p < 0.05$).

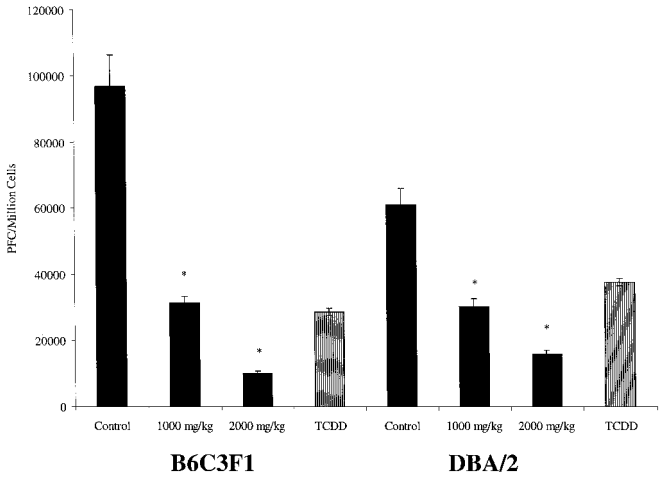

FIG. 3. The plaque-forming cell response as determined by the Jerne PFC method. Results are reported as the number of plaques counted per million cells. Significant differences between the number of plaque-forming cells in control and treated groups were determined by one-way ANOVA and Dunnett's test and are indicated by an asterisk ($n = 6$ for control, $n = 5$ for JP-8-treated mice, and $n = 2$ for TCDD-treated mice; $p < 0.05$).

level of AhR mRNA in the mouse and would therefore be a sensitive target for toxicity (Li *et al.*, 1994). Following a 7-day JP-8 exposure, the AhR was not down-regulated in the lung in either strain of mice and there was no induction of CYP1A1 above control levels (Fig. 4). However, TCDD caused a down-regulation of the AhR and subsequent upregulation of CYP1A1 in both B6C3F1 and DBA/2 mice.

Western Blots for the Detection of CYP1A1 in Liver Microsomal Preparations

Western blots were performed using the liver microsomal fraction from B6C3F1 and DBA/2 mice to determine if JP-8

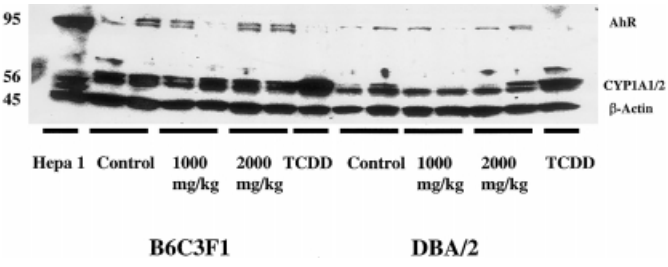

FIG. 4. Western blot of CYP1A1/2 from whole tissue lysates prepared from the lungs of mice exposed to JP-8 *in vivo*. Following tissue preparation and protein assay, 25 μg of protein were resolved on a 4–12% Tris/glycine gel, transferred to nitrocellulose, and incubated overnight with anti-CYP1A1/2 (1:2000), anti-AhR (1 μg/ml) or anti-β actin (1:1000). In the figure, lane 1 is 25 μg of hepa-1 cell lystate, lanes 2–8 correspond to the B6C3F1 mice and are as follows: 2–3, control; 4–5, 1000 mg/kg JP-8; 6–7, 2000 mg/kg JP-8; 8, TCDD. Lanes 9–15 are the DBA/2 mice, as follows: 9–10, control; 11–12, 1000 mg/kg JP-8; 13–14, 2000 mg/kg JP-8; 15, TCDD. Numbers on left are the migration of molecular-weight standards (kDa).

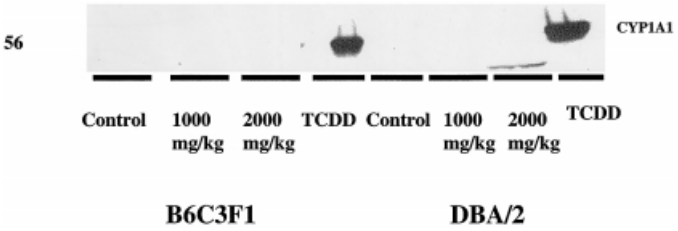

FIG. 5. Western blot of CYP1A1 in liver microsomal fraction in B6C3F1 and DBA/2 mice. Following microsome preparation, 5 μg of protein/track were electrophoresed and transferred to a nitrocellulose membrane. Membranes were incubated with anti-mouse monoclonal CYP1A1 (1:500) overnight. Lanes 1–7 are the B6C3F1 mice, as follows: 1–2 control; 3–4, 1000 mg/kg JP-8; 5–6 2000 mg/kg JP-8; 7, TCDD. Lanes 8–14 are the DBA/2 mice: 8–9, control; 10–11; 1000 mg/kg JP-8; 12–13; 2000 mg/kg JP-8; 14, TCDD.

induced the protein expression of CYP1A1 in liver. The results suggest that JP-8 did not upregulate the levels of CYP1A1 protein in B6C3F1 or DBA/2 mice. However, TCDD caused a robust upregulation of CYP1A1 protein in both strains (Fig. 5). These results corroborate Western blots performed with whole tissue lysates from kidney in B6C3F1 and DBA/2 mice treated with JP-8 (data not shown).

Western Blots for the Detection of the AhR and CYP1A1 Expression in Wt-Hepa 1 Cell Lysates

A 6-hour incubation with 10, 100, or 500 ppm JP-8 did not down-regulate the AhR or induce CYP1A1 (Fig. 6). However, 5 μM 3MC caused both a down-regulation of the AhR and induction of CYP1A1. Even though there was no apparent dose-response relationship for JP-8 administration *in vitro*, time-course experiments were performed to assure that changes were not occurring at very short or long intervals. As a result, JP-8 did not down-regulate the AhR or upregulate CYP1A1 at any of the time points (6, 12, 18, 24, or 48 h) at an exposure of 100 ppm (data not shown). The Hepa-1 studies demonstrate that JP-8 has limited action on AhR-mediated signal transduction at 10, 100, and 500 ppm. A 500-ppm *in vitro* concentration of JP-8 was considered a reasonably high JP-8 concentration for the dose-response and time-course experiments, for 2 reasons. *In vitro* range-finding studies to

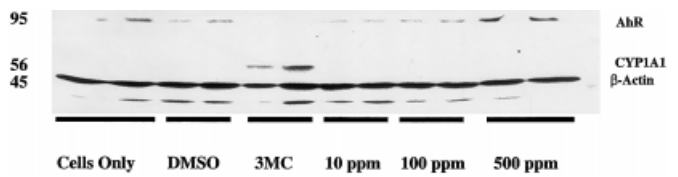

FIG. 6. Western blot of CYP1A1 and the AhR from whole-cell lysates prepared from the Hepa-1 cell line. Approximately 12.5 μg of protein were loaded per track. The figure depicts the dose-response assessment for JP-8 *in vitro* after a 6-h incubation. Lanes are as follows: 1–2, cells only; 3–4, DMSO; 5–6, 3MC; 7–8, 10 ppm JP-8; 9–10, 100 ppm JP-8; 11–12, 500 ppm JP-8. Numbers on left are migration of molecular weight standards (kDa).

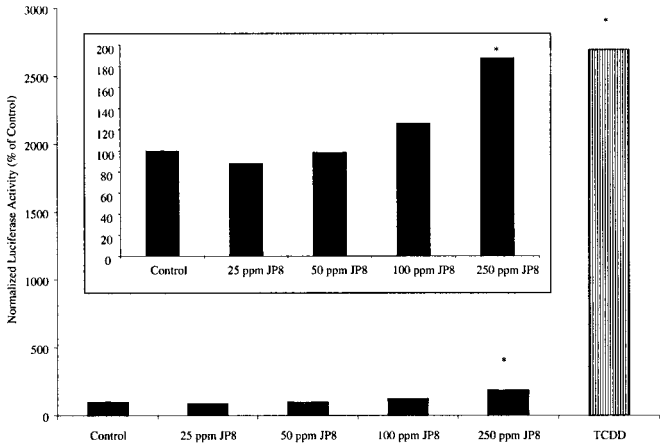

FIG. 7. and inset. TCDD and JP-8-inducible luciferase activity in Hepa-1 cells. Triplicate plates of H1L1.1c2 cells were treated with TCDD (2 nM) or JP-8 at different concentrations for 6 h. Control cells were treated with 0.05% DMSO. After the 8-h incubation, cells were scraped from the plates and lysates were evaluated for protein concentration and luciferase activity. The difference in protein concentration among each treatment group was no greater than 0.01%. The inset depicts the dose-response assessment in the absence of TCDD to correct for distortion. Statistically significant results were evaluated by one-way ANOVA and are indicated by an asterisk ($p < 0.05$). The SEMs were less than 5% of the individual means and are difficult to observe on the graph. This experiment was repeated 3 times.

establish a dose response indicated that JP-8 concentrations exceeding 500 ppm were cytotoxic, resulting in morphological changes in cellular architecture and a reduction in total cellular protein in Hepa 1 cells. Furthermore, *in vivo* range-finding studies in our laboratory established that 500 mg/kg of JP-8 orally was the lowest-observable-effect level for immunotoxicity as determined with the PFC assay (unpublished data). Therefore, if PFC responses are mediated via AhR signal transduction at an *in vivo* exposure of 500 mg/kg, it might be expected that an *in vitro* exposure of 500 ppm of JP-8 would cause a down-regulation of the AhR and a corresponding increase in CYP1A1. As this was not observed, our results obtained from Hepa 1 cells exposed to JP-8 *in vitro* were consistent with the JP-8 effects *in vivo*.

Eukaryotic Transfection and Reporter-Gene Assay

Western blots confirmed that JP-8 did not induce CYP1A1 protein expression, nor did it down-regulate the AhR *in vivo or in vitro*. However, it was unknown if JP-8 would induce CYP1A1 activity without appreciable increases in protein expression. Thus, a cell line transfected with the pGudLuc plasmid was utilized to measure increases in CYP1A1 activity through AhR signal transduction pathways. It was determined that while increasing concentrations of JP-8 did result in small increases in CYP1A1 activity, this activity was minimal compared to the levels induced by TCDD. A statistically significant increase (approximately 2-fold) in luciferase activity was observed following treatment with 250 ppm JP-8, while treatment with TCDD resulted in an approximate 25-fold increase in reporter-gene activity compared to control (Fig. 7 and Inset).

DISCUSSION

While recent reports indicate that military and occupational exposure to JP-8 can result in pulmonary, neurologic, and hepatic damage (Mattie *et al.*, 1995; Pfaff *et al.*, 1996; Robledo and Witten, 1999; Smith *et al.*, 1997), the immune system seems to be the more sensitive indicator of JP-8's toxicity (Harris *et al.*, 1997a,b; Ullrich, 1999). The importance of investigating potential mechanisms of JP-8 immunotoxicity is based on 2 observations: (1) the toxicological profile consisting of thymic atrophy, decreased PFC responses, and increased liver weight following exposure to PAHs and TCDD (De Jong *et al.*, 1999; White *et al.*, 1994) is similar to reported effects of JP-8 in this and other studies (Harris *et al.*, 1997a,b); and (2) the PAH content of JP-8 is estimated to be 10–20% of the mixture (IARC, 1989). Thus, it was hypothesized that JP-8 immunotoxicity may be AhR-mediated and this could be delineated by assessing the protein expression pattern of the AhR and CYP1A1 and the differential responses of mice that are either Ah-responsive or nonresponsive.

The use of both responsive and nonresponsive mouse strains and cell lines deficient in the ARNT protein or the AhR have been useful in evaluating the role of the AhR in PAH toxicity. Since nonresponsive strains (DBA/2, AKR, SJL) differ in both the quantity and affinity binding of the cytosolic AhR, comparing the reaction of these strains to a responsive strain following PAH exposure may elucidate the role of the AhR in the toxic response. In particular, two descriptive endpoints have proven reliable and consistent: onset of thymus involution and hepatomegaly (Fernandez-Salguero *et al.*, 1995; Kerkvliet *et al*, 1990; Staples *et al.*, 1999). DBA/2 (nonresponsive) mice typically require 10 times the amount of TCDD to produce an equivalent degree of thymus atrophy or hepatomegaly (Kerkvliet *et al.*, 1990; Poland and Knunston, 1982; Silkworth and Vecchi, 1985; White *et al.*, 1985), and the AhR knockout mouse is resistant to hepatomegaly and liver hypertrophy caused by TCDD (Fernandez-Salguero *et al.*, 1996). Therefore, an agent that operates through AhR-signal transduction pathways typically produces a response in B6C3F1 mice while having no effect in DBA/2 mice. The results of the present study suggest that both B6C3F1 and DBA/2 mice were equally sensitive when comparing either decreases in thymus weight and cellularity or increases in liver weight following JP-8 exposure. Based on these observations, JP-8 may not mediate its toxicological effects via an AhR mechanism.

However, TCDD caused an equivalent increase in liver weight in both B6C3F1 and DBA/2 mice. This is a limitation in the study, because the level of TCDD used (15 μg/kg repeated exposure for 3 days) was high. These results are consistent with a previous report demonstrating that a high dose of TCDD (20 μg/kg) resulted in an increase in liver

weight in both B6C3F1 and DBA/2 mice (Kerkvliet *et al.*, 1990) indicating that this level of exposure is above the dose-response curve. It is expected that the 10-fold difference in sensitivity to TCDD between B6C3F1 and DBA/2 mice would only be apparent at lower doses of TCDD. Nevertheless, TCDD did serve as a positive control in other aspects in that it caused a significant decrease in thymus weight and cellularity in B6C3F1 mice but not in DBA/2 mice, and down-regulated AhR while increasing CYP1A1 protein levels.

Another reliable biomarker of AhR activation is CYP1A1 expression. Induction of CYP1A1 is regulated primarily by the AhR, although there are reports that phenobarbital (Sadar *et al*., 1996) and some insecticides (Delescluse *et al.*, 1998) are able to induce CYP1A1 without binding to the AhR. In the present study, the results from the *in vivo* and *in vitro* Western blots identifying the expression pattern of CYP1A1 after JP-8 exposures were not comparable with 3MC or TCDD (known AhR ligands). This would suggest that JP-8 does not exert its toxicity via an AhR-mediated mechanism. However, it could not be precluded that JP-8 may bind minimally to the AhR with enough affinity to cause a small increase in CYP1A1 activity without remarkable increases in CYP1A1 protein expression. Therefore, in the present study, a luciferase reporter gene assay was utilized using Hepa-1 cells integrated with an XRE-driven reporter vector to directly measure CYP1A1 activity via AhR-ligand binding. It was demonstrated that JP-8 (250 ppm) did induce small, yet significant increases in reporter gene activity above control but this activity was well below that induced by TCDD. This may suggest that JP-8 is a weak AhR ligand that binds with enough affinity to cause small increases in CYP1A1 activity without appreciable increases in protein expression. However, without directly assessing the binding affinity of JP-8, this is speculation, because the increase in activity could be due to undefined non-AhR-mediated pathways.

Down-regulation of the AhR has been demonstrated *in vivo* and in different cell culture systems *in vitro* following exposure to TCDD (Pollenz *et al.*, 1998). Additionally, a recent report has shown that both B6C3F1 and DBA/2 mice demonstrated reductions in AhR protein and upregulation of CYP1A1 following exposure to 3MC (Forkert, 1997). Thus, it was hypothesized that if JP-8 were immunotoxic through AhR signal-transduction pathways, a down-regulation of the AhR would be expected in JP-8-exposed animals and in murine Hepa-1 cells. Despite the fact that JP-8 was able to increase luciferase reporter-gene activity, the present study indicates that JP-8 did not down-regulate the AhR at a wide range of exposures. *In vitro* and *in vivo* exposures overlap, in that the highest *in vitro* exposure of 500 ppm may be considered similar to the 500 mg/kg *in vivo* exposure that has been established as the lowest observable immunological effect level in range-finding studies performed in our laboratory. For example, an exposure of 500 mg/kg for 14 days suppressed the PFC response in ranges of 47 to 72% of control (unpublished data). It is unclear if the mechanisms of TCDD and PAH immunotoxicity are related specifically to down-regulation of the AhR, as the low affinity AhR ligand 2,7-dichlorodibenzo-*p*-dioxin produces similar effects to TCDD (Holsapple *et al.*, 1986a,b).

The plaque-forming cell response is a sensitive indicator of immunological disruption and has been widely used to demonstrate immunosuppression following exposure to PAHs and HAHs. Exposure to TCDD has been shown to inhibit IgM secretion through AhR pathways *in vitro* (Sulentic *et al.*, 1998) and via non-AhR-mediated pathways (Davis and Safe, 1991). It has also been demonstrated that from a battery of compounds, only those which were ligands for the AhR caused a decrease in the humoral immune response to sheep red blood cells in B6C3F1 mice (Silkworth *et al.*, 1984). However, AhR-independent pathways have also been described for suppression in the PFC response (Davis and Safe, 1991; Kerkvliet *et al.*, 1990; Lubet *et al.*, 1984). To our knowledge, this is the first study that describes decreases in the PFC response in B6C3F1 and DBA/2 mice following exposure to JP-8, and this further supports observations in other studies that JP-8 is immunotoxic (Harris *et al.*, 1997a,b,c; Ullrich, 1999).

Jet fuel, like most petroleum distillates, is a heterogeneous mixture of kerosene, benzene, toluene, xylenes, and other aliphatics and PAHs. Some of JP-8's components have been previously studied in single-exposure studies. However, identifying the mechanism of toxicity caused by a mixture such as JP-8 is considerably more challenging. Kerosene and benzene are substantial components of JP-8 and are both known to cause hepatic, hematological, and immune toxicity. Specifically, benzene is known to lead to progressive bone marrow degeneration, leukopenia, and disruptions in the drug metabolic system (Abraham, 1996). Similarly, rats exposed to kerosene demonstrated increases in liver weights, decreases in the relative weights of the spleen and thymus, and decreased activity of enzymes (benzo[a]pyrene hydroxylase) involved in the metabolism of environmental chemicals including PAHs (Rao *et al.*, 1984; Upreti *et al*., 1989). Thus, it is important to consider that kerosene and/or benzene may be important contributors to JP-8's induced immunotoxicity, not only by a potential direct effect, but perhaps by an indirect effect via an alteration in metabolic enzymes leading to the formation of more reactive and immunotoxic metabolites.

The recent, large-scale conversion of jet-fuel use from JP-4 to JP-8 in the military environment has increased the incidence of reported human health effects. Thus, our understanding of the molecular mechanisms leading to respiratory or immune dysfunction caused by JP-8 will facilitate how human exposure limits should be assessed. For example, recent work by Witzmann *et al.* (1999) has utilized electrophoretic techniques to examine the expression of cytosolic proteins and their responses to JP-8 jet-fuel exposure. In these studies, it was shown that inhalation of jet-fuel vapors caused increases of GST in liver and kidney homogenates (Witzmann *et al.*, 2000). More studies are needed to determine the metabolic enzymatic profile of this complex mixture of JP-8. This approach would

be more feasible in evaluating health risks of JP-8 as compared to the tedious job of isolating individual components of JP-8 that may or may not cause toxicity.

In conclusion, our most compelling evidence to indicate a non AhR-mediated mechanism of JP-8 immunotoxicity are the results from the Western blots *in vitro* and *in vivo*, demonstrating neither induction of CYP1A1 protein or down-regulation of the AhR. There were minimal increases in XRE-driven luciferase activity following JP-8 exposure of Hepa-1 cells. In further support of these findings, B6C3F1 and DBA/2 mice exposed to moderate to high levels of JP-8 displayed increases in liver weight, suppression of the PFC response, and reduced thymus weight and cellularity. Thus, the immunotoxic effects were similar between the Ah-responsive and -nonresponsive mouse strains, indicating that the AhR is not likely to have played a direct role in JP-8 immunotoxicity following a 7-day oral exposure.

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