Dermal Exposure to Jet Fuel Suppresses Delayed-Type Hypersensitivity: A Critical Role for Aromatic Hydrocarbons

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Dermal exposure to military (JP-8) and/or commercial (Jet-A) jet fuel suppresses cell-mediated immune reactions. Immune regulatory cytokines and biological modifiers, including platelet activating factor (PAF), prostaglandin E2, and interleukin-10, have been implicated in the pathway of events leading to immune suppression. It is estimated that approximately 260 different hydrocarbons are found in jet fuel, and the exact identity of the active immunotoxic agent(s) is unknown. The recent availability of synthetic jet fuel (S-8), which is refined from natural gas, and is devoid of aromatic hydrocarbons, made it feasible to design experiments to address this problem. Here we tested the hypothesis that the aromatic hydrocarbons present in jet fuel are responsible for immune suppression. We report that applying S-8 to the skin of mice does not upregulate the expression of epidermal cyclooxygenase-2 (COX-2) nor does it induce immune suppression. Adding back a cocktail of seven of the most prevalent aromatic hydrocarbons found in jet fuel (benzene, toluene, ethylbenzene, xylene, 1,2,4-trimethylbenzene, cyclohexylbenzene, and dimethylnaphthalene) to S-8 upregulated epidermal COX-2 expression and suppressed a delayed-type hypersensitivity (DTH) reaction. Injecting PAF receptor antagonists, or a selective cyclooxygenase-2 inhibitor into mice treated with S-8 supplemented with the aromatic cocktail, blocked suppression of DTH, similar to data previously reported using JP-8. These findings identify the aromatic hydrocarbons found in jet fuel as the agents responsible for suppressing DTH, in part by the upregulation of COX-2, and the production of immune regulatory factors and cytokines.

Key Words: aromatic hydrocarbons; COX-2; immunotoxicity; jet fuel.

During the past 20 years, the United States Air Force completed a gradual transition to a new jet fuel, known as jet propulsion-8 (JP-8). JP-8 was formulated to be a safer and more efficient fuel. It has a higher flash point, a lower vapor pressure, and a lower freezing point than previously used aviation fuels. The result is a fuel that is less combustible, more resistant to exploding, and one that performs better at high altitudes (Ritchie et al., 2003). JP-8 is closely related to commercial jet fuel (Jet-A). JP-8 is essentially commercial jet fuel supplemented with an anti-icing agent, an anticorrosive agent, and an antistatic agent to meet the military’s performance specifications. Estimates suggest that over 2 million people worldwide are exposed to 60 billion gallons of military and/or commercial jet fuel annually. All branches of the United States military use JP-8, where it is used to fuel jet aircraft, tanks, fighting vehicles, ships, helicopters, portable heating, and air conditioning units. Today, the military uses JP-8 as its universal fuel (Ritchie et al., 2003).

Standard toxicological studies suggested that JP-8 caused minimal adverse effects (Cooper and Mattie, 1996; Kinkead et al., 1992; Mattie et al., 1991, 1995). As the conversion to JP-8 continued, reports of health problems (nausea, respiratory infections, skin irritations) after dermal or respiratory exposure, the main routes of human exposure, prompted a further review of JP-8 toxicity. Harris and colleagues introduced JP-8 to mice and rats via the respiratory tract and reported that immune function was particularly susceptible to the toxic effects of JP-8 (Harris et al., 1997a,b, 2000, 2002). Using a mouse model, we found that dermal exposure to JP-8 suppressed the immune response to a recall antigen (Ramos et al., 2002; Ullrich, 1999). Cell-mediated immune reactions were more sensitive to the immune modulatory effects of jet fuel. Contact hypersensitivity, delayed-type hypersensitivity (DTH), and T-cell proliferation were suppressed by dermal application of jet fuel, whereas antibody formation in vivo was not suppressed (Ullrich, 1999; Ullrich and Lyons, 2000). Both primary and secondary immune reactions were suppressed following the dermal application of jet fuel (Ramos et al., 2002). One of the first steps in jet fuel-induced immune suppression is the binding of platelet activating factor (PAF) to its receptor, which induces prostaglandin E2 production. Blocking PAF receptor binding with a series of selective PAF receptor antagonists, or inhibiting prostaglandin secretion with a selective cyclooxygenase-2 (COX-2) inhibitor, blocked jet fuel-induced suppression of DTH (Ramos et al., 2004). Moreover, PAF activates the transcription of interleukin (IL)-10 (Walterscheid et al., 2002), which is found in the serum of JP-8–treated mice.

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(Ullrich, 1999). IL-10 plays an essential role in jet fuel-induced immune suppression, as illustrated by the fact that injecting anti-IL-10 into JP-8-treated mice reversed immune suppression (Ullrich and Lyons, 2000).

What remains unclear is the exact identity of the active agent, or class of agents found in jet fuel responsible for activating immune suppression. Because JP-8 is essentially Jet-A to which refineries add an antifreeze (diethylene glycol monomethyl ether), an antistatic reagent (Stadis 450), and an anticorrosive reagent (DCI-4A) (Ritchie et al., 2003), initial studies focused on the role of the additives in the induction of immune suppression. However, because both JP-8 and Jet-A suppressed DTH, with nearly identical dose response curves (Ramos et al., 2002), and similar mechanisms of action (Ramos et al., 2004), it became apparent that the immunosuppressive properties of JP-8 and Jet-A were a function of the base kerosene fuel.

Recently, a synthetic jet fuel (S-8) produced from natural gas using the Fischer–Tropsch reaction was provided to the United States Air Force and it is currently being tested in flight conditions (http://www.af.mil/news/story_print.asp?id=123026906). Because S-8 is devoid of aromatic hydrocarbons, the availability of this fuel allowed us to experimentally examine the relative roles of aromatics and aliphatics in suppressing DTH. We report here that S-8, unlike JP-8, does not upregulate COX-2 expression in the skin and does not induce immune suppression. When, however, a cocktail of the seven most prevalent aromatic hydrocarbons found in JP-8 were added back into S-8, this mixture upregulated COX-2 expression and suppressed DTH. Agents that prevent PAF receptor binding or inhibit COX-2 activity blocked immune suppression. Moreover, applying a neat solution of the aromatic cocktail (not diluted in S-8) to the skin induced COX-2 and suppressed DTH. These findings support the hypothesis that the aromatic hydrocarbons found in jet fuel induce immune suppression.

**MATERIALS AND METHODS**

**Animals.** Specific pathogen-free female C3H/HeNC (MTV−) mice were obtained from the National Cancer Institute Frederick Cancer Research Facility Animal Production Area (Frederick, MD). The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, in accordance with current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and National Institutes of Health. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee. Within each experiment all mice were age matched. The mice were 8–10 weeks old at the start of each experiment.

**Reagents.** The selective PAF receptor antagonist, PCA-4248, was purchased from Biomol Research Labs, Plymouth Meeting, PA. The selective COX-2 inhibitor, NS-398 was purchased from Cayman Chemicals (Ann Arbor, MI). Stock solutions of both PCA-4248 and NS-398 were prepared in 50% phosphate buffered saline (PBS)/dimethyl sulfoxide and then diluted further (approximately 1:5000, vol/vol) in PBS immediately prior to use. The mice received intraperitoneal injections of 0.2 μg of NS-398 or 200 nmol of PCA-4248 1 h prior to jet fuel treatment.

**Application of jet fuels.** JP-8 (lot # 3509) was acquired from the Operational Toxicology Branch, Air Force Research Laboratory, Wright Patterson Air Force Base, Dayton, OH. Synthetic jet fuel (S-8), produced from natural gas using the Fischer–Tropsch reaction was generated by the Syntroleum Corporation (Tulsa, OK) and supplied to us by the Operational Toxicology Branch. The S-8 fuel contains no measurable sulfur and is devoid of aromatic compounds (www.syntroleum.com/tech Specifications.aspx). The fuels were stored and used in a chemical fume hood. Nitrile rubber-based gloves (Touch N Tuff, Fisher Scientific, Co., Pittsburgh, PA) were used in the place of normal latex gloves due to their superior performance in preventing the penetration of the fuels. Approximately, 300 μl of the undiluted fuel was applied (not occluded) to the shaved dorsal skin (≈ 8 cm²) of the animals as described previously (Ramos et al., 2002). A portion of the jet fuel (approximately 100 μl) was applied at one time and rubbed into the skin with the pipette tip; this was repeated two more times to get a final volume of 300 μl. Three hundred microliters of JP-8 contains 240 mg, and 300 μl of S-8 contains 227 mg. The mice were held individually in the hood for 3 h after exposure to prevent cage mates from grooming and ingesting the fuel. Also, the jet fuel was placed high up on the back of each mouse, immediately behind the head to prevent the animals from grooming themselves and ingesting the fuel. After 3 h, all the residual fuel was either absorbed or evaporated and the animals were returned to standard housing in a specific pathogen-free barrier facility.

**Suppression of the elicitation of DTH by jet fuel exposure.** On day 0, the mice were immunized by the subcutaneous injection of 10⁶ formalin-fixed *Candida albicans* into each flank. Nine days after immunization, the mice were treated with JP-8 or S-8 as described above. Previous studies indicated that applying the jet fuel on days 6–9 postimmunization would suppress the elicitation of DTH (Ramos et al., 2002). One day after jet fuel treatment, the thickness of each hind footpad was measured with an engineer’s micrometer (Mitutoya, Tokyo, Japan) and then challenged by the intradermal injection of 50 μl of *Candida* antigen (Alerchek, Inc., Portland, ME). Eighteen to 24 h after challenge, the thickness of each foot was remeasured, and the mean footpad swelling for each mouse was calculated (left foot + right foot ÷ 2). The background footpad swelling (negative control) was determined in a group of mice that were not immunized but were challenged. The positive control group are mice that are immunized, shaved, and challenged. Specific footpad swelling was calculated by subtracting the background response generated in the negative controls from the mean footpad swelling found in mice that were immunized and challenged. There were five mice per group; the mean footpad thickness for the group ± SE of the mean was calculated. Statistical differences between the control and experimental groups were determined using a one-way ANOVA followed by the Dunnett’s multiple comparison test (GraphPad Prism Software V4, GraphPad, Inc., San Diego, CA). Percentage immune suppression was determined by the following formula: % immune suppression = 1 – [specific footpad swelling of the jet-fuel–treated mice + specific footpad swelling of the positive control] × 100.

**Real-time polymerase chain reaction.** Six hours after the final JP-8 treatment, the dorsal skin of the treated mice was removed and snap frozen in liquid nitrogen. The skin samples were then pulverized with a mortar and pestle. Total RNA was then extracted with Trizol (Invitrogen, Carlsbad, CA). The isolated RNA was then further purified by using the RNeasy RNA cleanup procedure (Qiagen, Valencia, CA). The concentration of the isolated RNA was measured, and 0.5–1.0 μg was converted to complementary DNA (cDNA) using the Retroscript RT kit (Ambion, Austin, TX). cDNA (25–50 ng) was subjected to real-time PCR (Model ABI Prism 7500, Applied Biosystems, Foster City, CA) using TaqMan Universal PCR mix, and primers and fluorescent probes specific for COX-2 (Psg52) (Mm 00478374-m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Taqman Gene Expression Assay reagents, Applied Biosystems). Threshold cycle (Cₜ) values for COX-2 were normalized to GAPDH using the following equation: 10^[ΔΔCₜ (GAPDH – COX-2)] × 10,000, where ΔΔCₜ is the Cₜ of the GAPDH control, COX-2 is the Cₜ of COX-2, and 10,000 is an arbitrary factor to bring all values above one. There were four mice in each treatment group; RNA was isolated from
each individual mouse. The means and the standard deviation for each treatment group were calculated and statistical differences between each experimental group were determined by using a one-way ANOVA followed by the Student–Newman–Keuls Multiple comparison test (GraphPad Prism Software V4).

**Immunohistochemical detection of COX-2.** Nine hours after the last jet fuel treatment, the dorsal skin was removed, and the dorsal region was used. The skin samples were fixed in 10% neutral formaldehyde, and 5-μm tissue sections were cut. The sections were deparaffinized, and the slides were then dehydrated using a series of alcohol solutions (ETOH; 100%, 95%, 85%, 75%). Slides were then placed in a humid chamber, and endogenous peroxidase was blocked with 6% hydrogen peroxide for 30 min. Slides were incubated with protein blocking buffer (Pierce Biotechnology, Rockford, IL) for 30 min at room temperature, and then treated with rabbit antinimous COX-2 polyclonal antibody (1:400 dilution; Abcam, Cambridge, MA). After 20 min at room temperature the slides were washed and incubated for 1 h with donkey antirabbit peroxidase-conjugated antibody (1:800 dilution; Jackson Immuno Research, West Grove, PA). The diaminobenzidine substrate (Invitrogen, Carlsbad, CA) was added and the slides were incubated for 6 min at room temperature. Isotype controls for each sample were stained with rabbit polyclonal IgG (Abcam, Cambridge, MA). Sections were counterstained with hematoxylin and then mounted with Eukitt quick-hardening mounting media (Fluka, Germany). There were three mice per group, slides from each animal were assayed individually. Representative data are presented here.

**Western analysis to detect COX-2 protein.** Nine hours after the final JP-8 treatment, the dorsal skin of the treated mice was removed and snap frozen in liquid nitrogen. Skin samples from individual mice (N = 3) were pulverized with a mortal and pestle. Protein was isolated from mouse skin by lysis in buffer (50 mM Tris/HCl, 150 mM NaCl, 0.2% sodium dodecyl sulfate, 1% Nonidet, and 1% Triton X-100). Samples were centrifuged (15 min, 12,000 × g) to eliminate debris. Protein concentration was determined using a commercial reagent based on the Bradford dye-binding procedure (Bio-Rad Laboratories, Hercules, CA). Bovine serum albumin was used to generate a standard curve. Equal amounts of cellular protein were loaded onto a 10% polyacrylamide and separated by electrophoresis (100 V for 90 min). Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (25 V for 1 h), and the membrane was subsequently blocked with 5% nonfat dry milk overnight at 4°C. The PVDF membrane was then probed with a rabbit antinimous polyclonal antibody (anti-Cox-2, 1:1000; Cayman Chemicals) or a mouse antinimous monoclonal antibody (anti-Actin, 110,000; Sigma-Aldrich, Co.) for 1 h at room temperature. Peroxidase-labeled secondary antibodies were used for detection. Antibody detection was performed by enhanced chemiluminescence (Perkin–Elmer, Waltham, MA) per manufacturer’s specifications. Specificity of the anti-COX-2 antibody was confirmed by the use of purified ovine COX-2 (Cayman Chemicals). Films and photographs of the western blots were scanned at 300 dpi using a Cannon CanoScan LiDE 500F scanner. Densitometric analysis was performed using ImageJ for the Macintosh (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**RESULTS**

**Failure of Synthetic Jet Fuel to Induce Immune Suppression**

Synthetic jet fuel produced by the Fischer–Tropsch reaction is essentially devoid of aromatic hydrocarbons. This provides an excellent vehicle to test the hypothesis that the aromatic hydrocarbons present in JP-8 suppress DTH. Previously, we demonstrated that applying 240 mg of JP-8 to mice 9 days after immunization suppressed the elicitation of a DTH reaction (Ramos et al., 2002), so we repeated that experiment here and compared the immunosuppressive properties of JP-8 and S-8 (Fig. 1). As before, applying 240 mg of JP-8 to the skin significantly suppressed DTH (p < 0.01 compared with the positive control). On the other hand, applying S-8 to the skin, at all doses tested, did not suppress DTH. This experiment has been repeated in excess of eight times, and in no instance did we find that applying S-8 suppressed DTH.

Jet-fuel-treated keratinocytes secrete prostaglandin E2 (Ramos et al., 2004), and blocking prostaglandin production with a selective COX-2 inhibitor abrogates JP-8–induced immune suppression (Ramos et al., 2002). Next we asked if dermal application of S-8 can upregulate COX-2 expression in the skin. Mice were treated with 300 μl (240 mg) of JP-8 or 300 μl (227 mg) of S-8. RNA was isolated from the skin 6 h after treatment, and the expression of COX-2 messenger RNA (mRNA) was measured by real-time PCR. As can be seen in Fig. 2, JP-8 induces significant COX-2 expression, whereas no upregulation of COX-2 mRNA was observed after treatment with S-8. These data indicate that S-8 does not upregulate mRNA for COX-2 and does not suppress DTH.

**Adding a Cocktail of the Most Prevalent Aromatic Hydrocarbons Found in JP-8 to S-8 Converts S-8 into an Immunosuppressive Fuel**

The findings reported above strongly suggest that the aromatic hydrocarbons in JP-8 contribute to its immune suppressive properties. If true, it is reasonable to expect that adding back aromatic hydrocarbons to S-8 would render it immunosuppressive. We consulted the literature and found that the aromatic hydrocarbons present in JP-8 at the highest concentrations are benzene, toluene, ethylbenzene, xylene, 1,2,4-trimethylbenzene, cyclohexylbenzene, and dimethyl-naphthalene (Yang et al., 2006). We added the aromatic hydrocarbons to S-8 at the concentration normally found in JP-8 (S-8 + 1×), or at two times (S-8 + 2×), five times (S-8 + 5×), or 10 times (S-8 + 10×) the normal concentration. The

**FIG. 1.** Dermal application of synthetic jet fuel (S-8) does not suppress DTH. Mice were immunized with Candida albicans on day 0 and treated with 240 mg of JP-8 or various doses of S-8 on day 9. The mice were challenged with Candida antigen on day 10 and DTH was measured on day 11. *Significant difference (p < 0.01) from the positive control.
composition of the various mixtures used is shown in Table 1. We then applied the different mixtures of the aromatic hydrocarbons diluted in S-8 to the skin of immunized animals (Table 2), and measured the effect this treatment had on DTH (Fig. 3). Compared with the positive control, applying JP-8 caused significant immune suppression \((p < 0.05)\), and as shown above, applying S-8 had no suppressive effect \((p > 0.05)\). Adding the aromatic hydrocarbons to S-8 rendered it immunosuppressive \((p < 0.05)\) vs. the positive control. We saw no clear dose response relationship in this experiment. S-8 supplemented with the level of aromatics normally found in JP-8 appeared to be as immune suppressive as that found when the concentration of aromatic hydrocarbons was 10 times the concentration found in JP-8. This suggests that a threshold level needed to suppress DTH can be achieved with the concentration of aromatic hydrocarbons normally found in JP-8 (18–22%).

In the same experiment we also applied different amounts of the aromatic cocktail mixture (Table 2) directly to the skin (i.e., not diluted in S-8). Somewhat surprising was the observation that as little as 12 \(\mu\)l (10.67 mg) of the cocktail of the aromatic hydrocarbons could by itself, suppress DTH (Fig. 3, \(p < 0.05\) vs. the positive control). These data further support the hypothesis that the aromatic hydrocarbons present in jet fuel activate immune suppression.

Next, we examined the upregulation of COX-2 by the aromatic hydrocarbons found in jet fuel (Fig. 4A). Skin samples were taken from mice 6 h after treatment with JP-8, S-8, S-8 supplemented with the cocktail of aromatic hydrocarbons, or the cocktail of aromatic hydrocarbons by themselves. As demonstrated above, JP-8 treatment caused a significant increase in COX-2 mRNA expression but treating the mice with S-8 did not. We saw an increase in COX-2 expression when the mice were treated with S-8 supplemented with five times the concentration of aromatic hydrocarbons found in jet fuel \((5\times)\). We also saw a dramatic increase in COX-2 expression when mRNA was isolated from the skin of mice treated directly with 12 \(\mu\)l (10.67 mg; \(1\times\)), 24 \(\mu\)l (21.35 mg; \(2\times\)), or 60 \(\mu\)l (53.37 mg; \(5\times\)) of the aromatic hydrocarbons (Fig. 4B). At no time tested did S-8 induce COX-2 expression when the mice were treated with S-8 supplemented with five times the concentration of aromatic hydrocarbons found in jet fuel \((5\times)\). These data further support the hypothesis that the aromatic hydrocarbons present in jet fuel immunosuppressive.

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FIG. 2. Dermal application of synthetic jet fuel (S-8) does not upregulate expression of COX-2. Skin samples were isolated from normal mice (no Tx), mice treated with JP-8, and mice treated with S-8. COX-2 expression was measured by real-time PCR. The data are expressed as arbitrary units relative to the amount of GAPDH mRNA. *Indicates a statistically significant difference \((p < 0.01)\) from the normal control.

### TABLE 1

<table>
<thead>
<tr>
<th>Chemical</th>
<th>% in JP-8 (wt/wt)</th>
<th>S8 + 1(^x) (mg)</th>
<th>S8 + 2(^x) (mg)</th>
<th>S8 + 5(^x) (mg)</th>
<th>S8 + 10(^x) (mg)</th>
<th>1(^x) (mg)</th>
<th>2(^x) (mg)</th>
<th>5(^x) (mg)</th>
<th>10(^x) (mg)</th>
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</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>1.0</td>
<td>2.271</td>
<td>4.542</td>
<td>11.36</td>
<td>22.71</td>
<td>2.271</td>
<td>4.542</td>
<td>11.36</td>
<td>22.71</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.1</td>
<td>0.227</td>
<td>0.454</td>
<td>1.136</td>
<td>2.271</td>
<td>0.227</td>
<td>0.454</td>
<td>1.136</td>
<td>2.271</td>
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<tr>
<td>Ethylbenzene</td>
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<td>0.454</td>
<td>0.908</td>
<td>2.271</td>
<td>4.542</td>
<td>0.454</td>
<td>0.908</td>
<td>2.271</td>
<td>4.542</td>
</tr>
<tr>
<td>Xylene</td>
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<td>1.363</td>
<td>2.725</td>
<td>6.813</td>
<td>13.63</td>
<td>1.363</td>
<td>2.725</td>
<td>6.813</td>
<td>13.63</td>
</tr>
<tr>
<td>Trimethylbenzene</td>
<td>1.0</td>
<td>2.271</td>
<td>4.542</td>
<td>11.36</td>
<td>22.71</td>
<td>2.271</td>
<td>4.542</td>
<td>11.36</td>
<td>22.71</td>
</tr>
<tr>
<td>Cyclohexylbenzene</td>
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<td>2.271</td>
<td>4.542</td>
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<td>2.271</td>
<td>4.542</td>
<td>11.36</td>
<td>22.71</td>
</tr>
<tr>
<td>Dimethylnaphthalene</td>
<td>0.8</td>
<td>1.817</td>
<td>3.634</td>
<td>9.085</td>
<td>18.17</td>
<td>1.817</td>
<td>3.634</td>
<td>9.085</td>
<td>18.17</td>
</tr>
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</table>

\(^x\)The aromatics were added back to S-8 at an amount equal to that found in JP-8 (1\(^x\)); twofold more (2\(^x\)); fivefold more (5\(^x\)); and 10-fold more (10\(^x\)).

\(^b\)The amount needed to make 20 ml of each aromatic mixture (S8 + 1\(^x\), S8 + 2\(^x\), etc.) was calculated and then diluted to final volume with S-8. A separate stock was made with the same concentration of aromatics but not diluted with S-8 (1\(^x\), 2\(^x\), 5\(^x\), and 10\(^x\)) to make an aromatic only solution at a concentration equal to the concentration found in JP-8.
TABLE 2
Amount of Aromatic Hydrocarbons Applied to the Dorsal Skin of Mice

<table>
<thead>
<tr>
<th>Mixture applied</th>
<th>Aromatic/mouse (mg)</th>
<th>S8/mouse (mg)</th>
<th>Total applied/mouse (mg)</th>
<th>% Aromatic per mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>S8 + 1×</td>
<td>10.67</td>
<td>216.4</td>
<td>227.1</td>
<td>4.7</td>
</tr>
<tr>
<td>S8 + 2×</td>
<td>21.35</td>
<td>205.8</td>
<td>227.1</td>
<td>9.4</td>
</tr>
<tr>
<td>S8 + 5×</td>
<td>53.37</td>
<td>173.7</td>
<td>227.1</td>
<td>23.5</td>
</tr>
<tr>
<td>S8 + 10×</td>
<td>106.7</td>
<td>120.4</td>
<td>227.1</td>
<td>46.9</td>
</tr>
<tr>
<td>1×</td>
<td>10.67</td>
<td>0</td>
<td>10.67</td>
<td>100</td>
</tr>
<tr>
<td>2×</td>
<td>21.35</td>
<td>0</td>
<td>21.35</td>
<td>100</td>
</tr>
<tr>
<td>5×</td>
<td>53.37</td>
<td>0</td>
<td>23.35</td>
<td>100</td>
</tr>
<tr>
<td>10×</td>
<td>106.7</td>
<td>0</td>
<td>106.7</td>
<td>100</td>
</tr>
</tbody>
</table>

*The aromatics were added back to S-8 at an equal amount to that found in JP-8 (1×); twofold more (2×); fivefold more (5×); and 10-fold more (10×).

Blocking PAF Receptor Binding and Inhibiting COX-2 Activity Blocks the Immune Suppression Induced by the Aromatic Hydrocarbons

Previously we demonstrated that treating JP-8–treated mice with PAF receptor antagonists (Ramos et al., 2004) and/or a selective COX-2 inhibitor (Ramos et al., 2002) prevented the induction of immune suppression. To determine if similar mechanisms are involved in the immune suppression activated by the aromatic hydrocarbons found in jet fuel, the following experiment was done. JP-8, S-8, S-8 supplemented with the cocktail of aromatic hydrocarbons (S-8 + 5×) or the aromatic hydrocarbons alone (5×) were applied to the skin of mice. Some of the mice received PCA-4248, a PAF receptor antagonist, or NS-398, a selective COX-2 inhibitor prior to jet fuel treatment. The effect that these treatments had on DTH was then measured (Fig. 5). As mentioned above JP-8 treatment, but not S-8 treatment suppressed the elicitation of the DTH reaction (p < 0.05 vs. the positive control). Adding back the aromatic hydrocarbons to the S-8 converted it into an immune suppressive fuel (p < 0.05 vs. the positive control). Similarly, adding the aromatic compounds directly to the skin suppressed the immune response (p < 0.01 vs. the positive control). When, however, PAF receptor binding (PCA-4248), or COX-2 activity (NS-398) was blocked, no suppression of DTH was noted (p > 0.05 vs. the positive control). These findings indicate the mechanism of immune suppression induced by the aromatic hydrocarbons, and that found after applying JP-8 is similar, further supporting the hypothesis that the aromatic hydrocarbons in JP-8 are responsible for suppressing DTH.

DISCUSSION

Because of its widespread use, JP-8 represents the single largest potential for chemical exposure for U.S. military personnel (Ritchie et al., 2003). Dermal and respiratory exposure to JP-8, the two most prevalent routes of exposure, suppresses the immune response (Harris et al., 1997a; Ullrich, 1999). Similarly, dermal exposure to commercial jet fuel, Jet-A, which serves as the base kerosene fuel for JP-8, induces immune suppression (Ramos et al., 2002). This latter observation suggests that it is the base kerosene fuel, and not the additive package that renders jet fuel immunosuppressive. Here we tested the hypothesis that the aromatic hydrocarbons in jet fuel are responsible for suppressing DTH. We based this hypothesis on the fact that others have shown that the penetration (McDougal et al., 2000; Riviere et al., 1999) and toxicity (Chou et al., 2003; Rogers et al., 2004) of the aliphatic and aromatic hydrocarbons in jet fuel differ, and that some of the aromatic hydrocarbons found in jet fuel, particularly, benzene, and toluene are immune suppressive (Abadin et al., 2007; Veraldi et al., 2006). These observations, coupled with the availability of synthetic jet fuel, which is devoid of aromatic compounds, made an experimental test of the hypothesis feasible. Here we report that dermal exposure to synthetic jet fuel does not induce the upregulation of COX-2 in the skin and is not immunosuppressive. Adding a cocktail of the most prevalent aromatic hydrocarbons found in JP-8 to S-8 activated COX-2 expression, and rendered the synthetic fuel immunosuppressive. Moreover, when the cocktail of the aromatic hydrocarbons was added directly to the skin (i.e., not diluted in S-8), it also activated COX-2 expression and induced immune suppression. Inhibiting COX-2 function blocked suppression of DTH induced by JP-8, or S-8 supplemented with the aromatic hydrocarbons, and that found

FIG. 3. Adding a cocktail of the most prevalent aromatic hydrocarbons found in JP-8 to S-8 converts it into an immunosuppressive dermal toxin. Mice were immunized with Candida albicans on day 0 and treated with 240 mg of JP-8 or 227 mg of S-8 on day 9. Some mice were treated with S-8 supplemented with the seven most common aromatic hydrocarbons found in JP-8 (S-8 + 1×; S-8 + 2×; S-8 + 5×; S-8 + 10×). Other mice received the cocktail of the aromatic hydrocarbons alone, not mixed in S-8 (1×; 2×; 5×; 10×). The mice were challenged with Candida antigen on day 10 and DTH was measured on day 11. *Indicates a statistically significant difference (p < 0.05) from the positive control.
when the aromatic compounds were applied directly to the skin. These findings support the hypothesis that the aromatic hydrocarbons found in jet fuel, and not the aliphatic hydrocarbons are responsible for suppressing DTH.

These findings extend observations by McDougal et al. and Riviere et al. concerning differences in the deposition and toxicity of aliphatic and aromatic hydrocarbons delivered by dermal exposure. Aromatic compounds rapidly penetrate through the skin, whereas the aliphatic compounds are absorbed by the skin, and their retention times in the skin are longer (McDougal et al., 2000; Riviere et al., 1999). The aromatic compounds are more potent in causing keratinocyte cell death, a function associated with the number and size of the side chains attached to the aromatic ring (Chou et al., 2003; Rogers et al., 2004). The aliphatic hydrocarbons found in jet fuel are responsible for dermal irritation (i.e., erythema, edema, and increased epidermal thickening), and are more potent at inducing the secretion of proinflammatory cytokine by keratinocytes (Muhammad et al., 2005; Yang et al., 2006).

Our findings add to these observations by suggesting that it is the aromatic hydrocarbons in jet fuel that are responsible for activating the release of immune regulatory cytokines and inducing immune suppression.

Baynes et al. previously demonstrated that the composition of a complex chemical mixture applied to the skin can alter the dermal disposition of jet fuel components. In their studies, they measured the absorption profiles and deposition of two marker compounds, radiolabeled naphthalene and dodecane, into the skin.
skin when diluted in Jet-A, or JP-8. Significant differences in the absorption and deposition of the marker compounds were observed depending on the composition of the mixture used as the “vehicle” (Jet-A or JP-8) to deliver the marker compounds to the skin (Baynes et al., 2001). We were not surprised that adding the aromatic hydrocarbons to the S-8 induced immune suppression, because the S-8 serves as an excellent “vehicle” for these studies. However, we were somewhat surprised by the observation that applying the cocktail of the seven most prevalent aromatic hydrocarbons found in jet fuel directly to the skin resulted in COX-2 upregulation and suppressed DTH, not because of the inability of aromatic hydrocarbons to suppress the immune response. Benzene and benzene derivatives, toluene and the breakdown products of naphthalene have been shown to be immune suppressive (Abadin et al., 2007; Kawabata and White, 1990; Veraldi et al., 2006). Based on Baynes’ work, we were concerned that the penetration of the mixture of hydrocarbons would not be sufficient to induce immune suppression. This was obviously not the case, applying as little as 10.67 mg (12 μl) of the aromatic cocktail upregulated COX-2 expression and suppressed DTH. Contrary to our results, Peden-Adams et al. (2001) demonstrated that oral feeding of JP-8 does not suppress DTH. Although it is not clear why our results differ, we suggest that the different routes of administration, or the use of a vehicle, which has been shown by others to attenuate JP-8 toxicity (Choua et al., 2003), may be involved.

McDougal et al. performed flux experiments with static diffusion cells to determine the ability of JP-8 and its component hydrocarbons to induce systemic toxicity. They concluded that penetration of JP-8 and its components was insufficient to cause systemic toxicity. For example, they estimated that even holding one’s hands in JP-8 for 8 h would not be sufficient to induce systemic toxicity because the low amount of fuel that can be absorbed through the skin (McDougal et al., 2000). These findings appear at first glance to be inconsistent with the results presented here, especially our observation that applying as little as 12 μl of the cocktail of aromatic hydrocarbons induces immune suppression. We propose that keratinocytes in the skin act as sensors for damage and then amplify the immunotoxic signal by upregulating COX-2, and secreting PAF and prostaglandin E2. These biological modifiers then communicate the suppressive signal to the immune system. We suggest that the effect of JP-8 on systemic immunotoxicity is an indirect effect, one that can be initiated by relatively small amounts of jet fuel. This hypothesis is supported by the data presented here (Fig. 3), and by previous findings showing that we could generate immune suppression after treating mice with repeated small doses of JP-8 (Ramos et al., 2002). Of course, the penetration of chemicals through mouse and human skin is not equal so these types of comparisons must be made with care. On the other hand, studies of fuel-tank maintenance workers, who wear respirators and receive dermal exposure through fuel-saturated cotton overalls, document elevated urinary levels of 2-naphthol, a breakdown product of naphthalene (Chao et al., 2006), raising the concern that systemic immune toxicity with aromatic hydrocarbons may be achieved after occupational dermal jet fuel exposure.

In summary, our data implicate the aromatic hydrocarbons found in JP-8 and Jet-A as the active agents responsible for suppressing DTH, a prototypical cell-mediated immune reaction. These data suggest that processes to generate or refine fuel that removes the aromatic hydrocarbons may be advantageous from the point of view of preventing immune suppression. Currently, the United States Air Force is flight-testing S-8 produced by the Fischer–Tropsch reaction. Synthetic fuel can be made from a variety of carbon feedstocks such as natural gas and coal. The advantage of using the process to produce liquid fuels for petroleum-poor countries is obvious. Similarly, the low sulfur content of fuel produced by the Fischer–Tropsch reaction makes it an environmentally more desirable fuel. Our data suggest that it is also a safer fuel to use, in that our studies demonstrate no immune suppression when this fuel is applied to the skin. Even though S-8 does not suppress DTH, proper chemical handling is still required. S-8 is not a benign chemical. It is made up of a complex mixture of aliphatic compounds that can lead to irritation and induction of other nonimmunological downstream effects. Whether other aromatic free fuels made from this procedure, such as diesel fuel, are safer from an immunologic point of view, remains to be seen.

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REFERENCES


