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The Humoral Immune Response of Mice Exposed to Simulated Road Paving-Like Asphalt Fumes

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Asphalt is a complex mixture of organic molecules, including polycyclic aromatic hydrocarbons (PAH), which have been reported to cause serious adverse health effects in humans. Workers in manufacturing and construction trades exposed to asphalt are potentially at risk for being exposed to asphalt fumes and PAHs. Epidemiological investigations have collected mounting evidence that chemicals found in asphalt fumes present carcinogenic and possibly immunotoxic hazards. Studies evaluating the immunotoxic effects of asphalt fume are limited due to the large number of variables associated with asphalt fume exposures. This work investigates the immuno-toxic effects of road paving-like asphalt fume by analyzing the *in vivo* IgM response to a T-dependent antigen after exposure to whole, vapor, and particulate phase road paving-like asphalt fumes and asphalt fume condensate. Systemic exposures via intraperitoneal injection of asphalt fume condensate (at 0.625 mg/kg) and the particulate phase (at 5 mg/kg) resulted in significant reductions in the specific spleen IgM response to SRBC. Pharyngeal aspiration of the asphalt fume condensate (at 5 mg/kg) also resulted in significant suppression of the IgM response to SRBC. A significant reduction in the specific spleen IgM activity was observed after inhalation exposure to whole asphalt fumes (35 mg/m^3) and the vapor components (11 mg/m^3). Dermal exposures to the asphalt fume condensate resulted in significant reductions in the total (at 50 mg/kg) and specific (at 250 mg/kg) spleen IgM response to SRBC. These results demonstrate that exposure to road paving-like asphalt fumes is immunosuppressive through systemic, respiratory, and dermal routes of exposure in a murine model and raise concerns regarding the potential for adverse immunological effects.

Keywords Paving asphalt, asphalt fumes, immunotoxicity, PFC assay, SRBC

INTRODUCTION

Asphalt, the product of the nondestructive distillation of crude oil in petroleum refining, is a complex mixture of paraffinic, aromatic hydrocarbons and heterocyclic compounds containing sulfur, nitrogen, and oxygen. These organic molecules vary widely in composition from non-polar, saturated hydrocarbons to highly polar condensed aromatic rings (Levin et al., 1994; Speight, 1999). Asphalt production is dictated by performance specification rather than by a specific chemical composition; therefore, no two asphalts are chemically identical. The exact chemical content of asphalt depends on the chemical complexity of the original crude petroleum. Elemental analysis demonstrates that most asphalt molecules contain (by weight) 79–88% carbon, 7–13% hydrogen, trace–8% sulfur, 2–8% oxygen, and trace–3% nitrogen (Speight, 1999). Asphalt also contains bitumen, which is a high boiling point mineral oil fraction with a complex chemical composition including polycyclic aromatic hydrocarbons (PAH) (Jarvholm, 2006).

Workers in manufacturing and construction trades using asphalt are potentially at risk for exposure to asphalt fumes (Hicks, 1995). Most of the asphalt produced in the United States is used in the roofing (11%) and paving (87%) industries (AI, 1990). Currently, about 11,000 contractors employ nearly 300,000 employees in the United States (NIOSH, 2003). Several million tons of asphalt are used each year by the paving industry. The potential for occupational exposure to asphalt fumes is high, although the specific health effects resulting from exposure still requires investigation. The major route of occupational exposure to asphalt is by inhalation, but its components may also be absorbed through the skin (Wolff et al., 1989). Epidemiological investigations have established mounting evidence that chemicals found in asphalt fumes present carcinogenic and possibly immunotoxic hazards (Campo et al., 2006). Exposure to asphalt fumes has been suggested as a cause of lung cancer, and other studies have reported cancers of the digestive tract, stomach (Maizlish et al., 1988; Hansen, 1989, 1991; Engholm et al., 1991; Partanen and Boofetta, 1994) and the urinary system (Bender et al., 1989; Hansen, 1989) among workers in road paving operations. A high occurrence of respiratory diseases

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such as bronchitis, emphysema and asthma identified in asphalt workers suggests that asphalt fumes contain immunotoxic compounds (Maizlish et al., 1988; Hansen, 1991; Norseth et al., 1991; Gamble et al., 1999).

The high molecular weight hydrocarbons present in asphalt are often PAHs. The 2-3 ring PAH (anthracene, naphthalene, phenanthrene) are considered irritants while 3–6 ring PAH (benzo[b,j,k]fluoranthene, benzo[a]pyrene, chrysene, and heterocyclic compounds) have been found to be mutagenic and possibly carcinogenic causing serious adverse health effects in humans (Knecht and Woitowitz, 1989; Machado et al., 1993, Watts et al., 1998). Animal studies investigating the immunomodulatory effects of asphalt fumes are limited. Exposure to road paving-like asphalt was not found to affect bacterial clearance of *Listeria monocytogenes* from the lungs of rats (Antonini et al., 2003), but asphalt fume related increases in lymphocyte secretion of interferon-γ, interleukin (IL)-6, and IL-10 were observed. Davila et al. (1997), made the general observation that the humoral arm of the immune system is the most sensitive to modulation by PAHs.

The studies presented here were undertaken to evaluate the immunotoxic effects of asphalt fumes (whole, particulate phase, and vapor component) and asphalt fume condensate on the murine humoral immune response using systemic, respiratory and dermal routes of exposure.

MATERIALS AND METHODS

Animals

Female B6C3F1 mice were purchased from Taconic (Hudson, NY) at 7–8 wk of age. Upon arrival, the animals were allowed to acclimate for a minimum of 5 days in an environmentally controlled barrier facility. The animals were housed 5 per cage in ventilated plastic shoe box cages with hardwood chip bedding, fed modified NIH-31 6% irradiated rodent diet (Harlan Teklad - item #7913), and given tap water from water bottles *ad libitum*. A standard light/dark cycle was maintained on 12-hr intervals. The room temperature was maintained between 18–26°C (65–78°F) and the humidity between 20–60%. Cages were cleaned and sanitized weekly. The NIOSH animal research facility is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

General Experimental Design

All animals were randomly assigned to treatment groups and weighed before the start of the study. A preliminary analysis of variance on body weights was performed prior to the start of the study to insure homogeneous distribution of animals across treatment groups (systemic, aspiration, and dermal exposure, n = 5; inhalation exposure, n = 10).

Test Articles

Asphalt Fume Condensate. Asphalt fume condensate (880-mg/ml), graciously provided by Heritage Research Group (Indianapolis, IN), was used for systemic, pulmonary and dermal studies. Asphalt fume condensate was collected at the top of a paving storage tank at Asphalt Materials Inc. (Indianapolis, IN). The paving asphalt, a PG 64-22 used on the I-65 project, was collected at 160°C. Asphalt fume condensate was diluted to different concentrations with 7% dimethyl sulfoxide (DMSO) in phosphate buffered saline (PBS) for the *in vivo* studies. The condensate concentrations tested in these studies were based on toxicity range finding studies and were limited by dilutions of the concentration provided by the manufacturer.

Asphalt Fumes. The asphalt fume generation and inhalation exposure system used in this study has been previously described and characterized (Law et al., 2006). Briefly, for inhalation exposures of whole asphalt fumes, an asphalt fume generation system (Heritage Research Group) was used to provide consistent test atmospheres emulating road asphalt paving conditions. Hot performance grade asphalt (PG 64-22, Asphalt Materials, Inc.) was preheated to 170°C in an oven, transferred to a reservoir, and passed through a heated pipe and onto a heated plate with the temperature maintained at 150°C at the inlet and 120°C at the outlet resulting in a mean temperature of 25.6 ± 0.9°C for the exposures. Humidity and temperature-controlled air was passed over the plate to mix with asphalt vapor. The mixture was then transported through a heated stainless steel pipe into the animal exposure chamber. Several PAHs including naphthalene, fluorene, and pyrene were present at concentrations of 146.3 ± 12.8 $\mu g/m^3$, 8.7 ± 4.4 $\mu g/m^3$ and 3.3 ± 5.9 $\mu g/m^3$, respectively, when a generation temperature of 150°C was used (Law et al., 2006).

Vapor Component. Animals were exposed to the vapor component of road paving-like asphalt fumes by the same process described above except a glass fiber filter was placed at the chamber inlet to allow only the vapor components into the chamber.

Particulate Phase. The particulate phase of road paving-like asphalt fumes trapped by the glass fiber filters were extracted from the filter with 10 ml/filter of dichloromethane (CH_2Cl_2). CH_2Cl_2 was removed by evaporation under a stream of nitrogen at room temperature. The purity was analyzed by NMR spectroscopy to ensure that there was no CH_2Cl_2 left in the solution. The particulate phase was mixed with 7% DMSO solution in PBS for these studies. DMSO is a common carrier used for dosing carcinogens such as PAHs.

Systemic Exposure to Asphalt Fume

For systemic exposures, intraperitoneal injections (IP) of either asphalt fume condensate (0–5 mg/kg) or the particulate phase (0–40 mg/kg) of asphalt fumes were given every other day in a 0.2 ml volume for 1 wk. Asphalt fume dilutions were made with 7% DMSO in PBS.

Aspiration Exposures to Asphalt Fume Condensate

Mice were exposed to asphalt fume condensate by pharyngeal aspiration as follows: under light isoflurane anesthesia (Abbott

Laboratories, North Chicago, IL), mice were held vertical by their incisor teeth against an angled restraining device (Rao et al., 2003). The tongue was then gently extended with padded forceps to prevent swallowing, and the test solution was pipetted directly into the oropharynx. The tongue was maintained in extension until the solution was aspirated into the lungs. Control groups received 7% DMSO solution in PBS. Mice were dosed every fifth day for 3 weeks (4 doses) with asphalt fume condensate in doses ranging from 0–5 mg/kg.

Inhalation Exposure to Asphalt Fume

Depending on study design, mice were exposed 3.5 hr/d, 5 d/wk, for either 1 (5 days) or 2 wk (10 days) to either asphalt fume or the vapor component of asphalt fumes. Mice were placed one or two per cage in the exposure chamber. Analytical values for the test atmosphere concentrations were obtained daily for each exposure. Teflon filters, having a diameter of 37 mm and a pore size of 0.45 μm, were used for gravimetric analysis of the fume in the exposure chamber. The filters were weighed immediately following the end of each sampling period during animal exposure to fume concentration. Fume concentration in the exposure chamber was determined by averaging the fume weight from each sampling period throughout the exposure period. During exposures, mean whole asphalt fumes and the vapor component of asphalt fumes concentration measured 35 mg/m^3 and 11 mg/m^3, respectively (Law et al., 2006).

Air-exposed mice were placed in the inhalation chamber and provided an air sham exposure according to the same protocol as asphalt fume-exposed mice. The concentrations used for the inhalation experiments were based on the capabilities of the asphalt fume generator, toxicity range finding studies, and previous research (Antonini et al., 2003; Ma et al., 2003). For the exposure periods, mean temperature and humidity in the animal exposure chamber for the inhalation of whole asphalt fumes and the vapor component of asphalt fumes were 25.6 $\pm$ 0.9°C and 37 $\pm$ 3.6%, and 24.7 $\pm$ 0.6°C and 38.7 $\pm$ 4.5%, respectively. Minimal stress to the animals was expected using whole body exposures.

Dermal Exposure to Asphalt Fume Condensate

Mice were exposed topically to 7% DMSO or increasing concentrations (1 mg/kg–500 mg/kg) of asphalt fume condensate on the dorsal surface of each ear (25 μl per ear) for 4 consecutive days. On the 5th day, mice were immunized with sheep red blood cells (SRBC) according to the procedure described below.

IgM PFC Response to a T-dependent Antigen, SRBC

Studies were done to examine the IgM plaque forming cell (PFC) response to intravenous challenge with SRBC after pretreatment with asphalt fumes. The primary IgM response to SRBC was enumerated using a modified hemolytic plaque assay of Jerne and Nordin (1963). Mice were immunized 4 days prior to sacrifice with 7.5×10^7 SRBC by intravenous injection in a 200 μl volume. On the day of sacrifice, mice were euthanized by CO_2 asphyxiation and spleens were removed. Single cell suspensions of the spleen from individual animals were prepared in HBSS by disrupting the spleen between the frosted ends of microscopic slides.

To identify the total number spleen cells, 20 μl of cells were added to 10 ml of isoton buffer (1:500) and 2 drops of Zap-o-globin (Beckman Coulter, Fullerton, CA) were added to lyse red blood cells. Cells were then counted using a Coulter counter (Z2 model, Beckman Coulter, Fullerton, CA). 1:30 and 1:120 dilutions of spleen cells were made. Then, 100 μl of the dilutions were added to a test tube containing a 0.5 ml warm agar/dextran mixture (0.5% Bacto-Agar, DIFCO; and 0.05% DEAE dextran, Sigma), 25 μl of 1:1 ratio of SRBC suspension, and 25 μl of 1:4 dilution (1 ml lyophilized) guinea pig complement (Cedarlane Labs, Hornby, Ontario). Each sample was vortexed, poured into a petri dish, covered with a microscope slide cover slip, and incubated 3 hr at 37°C.

The plaques (representing antibody-forming B-lymphocytes) were viewed and quantified after this incubation. Results were expressed as specific activity (IgM PFC per spleen cells). Positive control mice were injected IP with 25 mg/kg of cyclophosphamide (CP) for 4 consecutive days before sacrifice for the dermal, aspiration and intraperitoneal exposure studies. CP is a well-known immunosuppressant and the positive control of choice for the PFC assay (Anderson et al., 2006, 2007).

Toxicity after Exposure to Asphalt Fumes

For all studies, the mice were sacrificed by CO_2 asphyxiation, weighed, and examined for gross pathology at the end of the experiment. The following organs were removed, cleaned of connective tissue and weighed: liver, spleen, kidneys, and thymus.

Statistical Analysis

The design structure of these experiments was a completely randomized design, and the treatment structure utilized a one-way layout with animals randomly assigned to a vehicle control, test article, or positive control group. Comparisons of endpoints between the control group and each treatment level were carried out using a one-way ANOVA with Dunnett's test (Dunnett and Crisafio, 1955). If the assumptions were not able to be met by parametric analysis, the nonparametric Kruskal-Wallis k-sample test (Kruskal and Wallis, 1952) was utilized followed by the Mann–Whitney U-test for pair-wise comparisons with the control. Linear trend analysis was performed to determine if asphalt fume exposure had a dose responsive effect on the IgM PFC response to SRBC. Differences were considered significant if $p < 0.05$ as compared to the vehicle control.

RESULTS

Systemic Exposure

For hazard identification, initial systemic exposures were conducted using intraperitoneal injections. Systemic exposure of asphalt fume condensate resulted in a dose responsive trend ($p < 0.01$) with statistically significant suppression of the specific IgM response to SRBC, ranging between 36% and 57% (Figure 1A). Intraperitoneal injection of the particulate phase resulted in a dose-responsive decrease ($p < 0.01$) of the PFC response after exposure to 5, 10 or 40 mg/kg with 69% suppression observed at the high dose (Figure 1B). No overt toxicity as indicated by changes in body or organ weight was observed for any of the exposure groups (data not shown). The PFC assay positive control (CP) caused significant suppression (94%) of the PFC response.

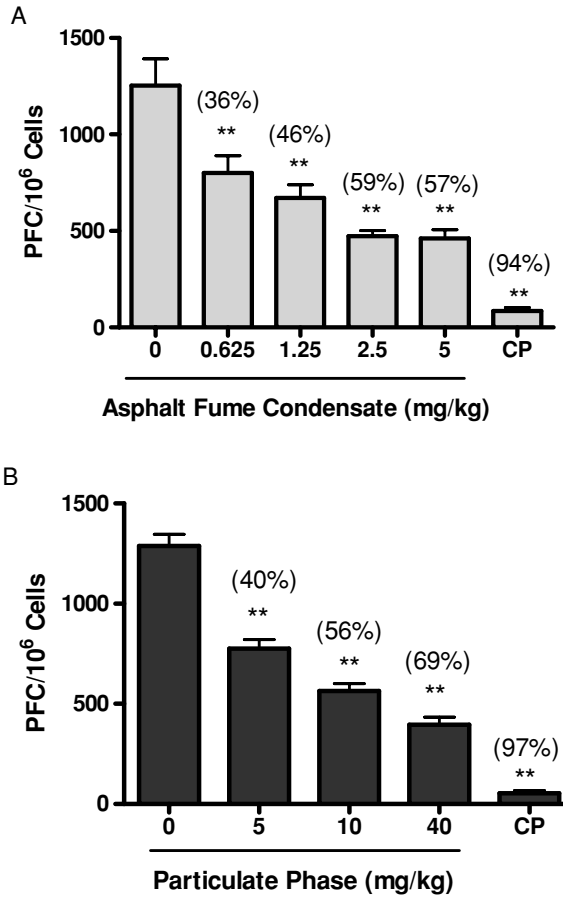

FIG. 1. IgM response to SRBC after systemic exposure of road paving-like asphalt fumes. The spleen IgM response is illustrated after intraperitoneal injection of asphalt fume condensate (A) or the particulate phase of asphalt fumes (B). CP (25 mg/kg) was used as a positive control. Values represent the mean ± SE derived from 5 animals in each group. Numbers above the bars represent percent suppression compared to vehicle control. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle.

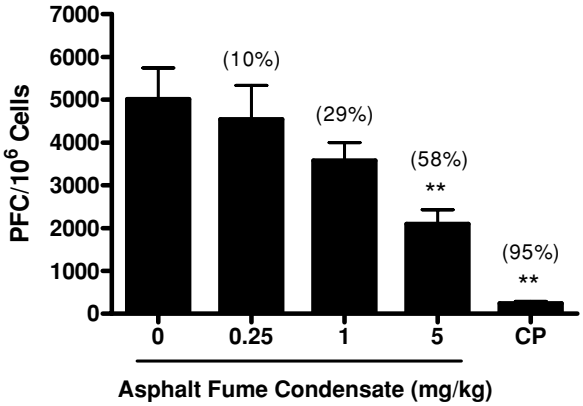

FIG. 2. IgM response to SRBC after pulmonary exposure to road paving-like asphalt fumes. The spleen IgM response is illustrated after pharyngeal aspiration of asphalt fume condensate. CP (25 mg/kg) was used as a positive control. Values represent the mean ± SE derived from 5 animals in each group. Numbers above the bars represent percent suppression compared to vehicle control. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle.

RESPIRATORY EXPOSURE

Pharyngeal Aspiration

Following pharyngeal aspiration of asphalt fume condensate, a statistically significant ($p < 0.01$) dose responsive decrease, ranging between 10% and 58%, in IgM specific PFC/10^6 cells was observed (Figure 2). No overt toxicity as indicated by changes in body or organ weight was observed for any of the exposure groups (data not shown). The PFC assay positive control, CP, resulted in significant suppression (95%) of the PFC response.

Inhalation Exposure

Female B6C3F1 mice were exposed to air, whole asphalt fumes (35 mg/m^3) or the vapor component of asphalt fumes (11 mg/m^3) using the method of whole body inhalation for 5 or 10 days. Exposure to whole asphalt fumes suppressed the ability to mount a normal IgM antibody response to SRBC. Figure 3A demonstrates 53% suppression compared to air control in PFC/10^6 cells after a 1-week exposure and 41% suppression compared to air control in PFC/10^6 cells after a 2-week exposure compared to air control. Although suppression was seen following 2 weeks of exposure to whole asphalt fumes, it was not statistically significant due to the variability in the air-exposed mice. Consistent with whole asphalt fume exposure, a 2-week time course inhalation exposure to the vapor component of asphalt fumes (Figure 3B) resulted in a 30% suppression compared to the air control of the specific IgM response to SRBC following a 1-week exposure and a 23% suppression after a 2-week exposure compared to the air control. No overt toxicity as indicated by changes in body or organ weight was observed for any of the exposure groups (data not shown).

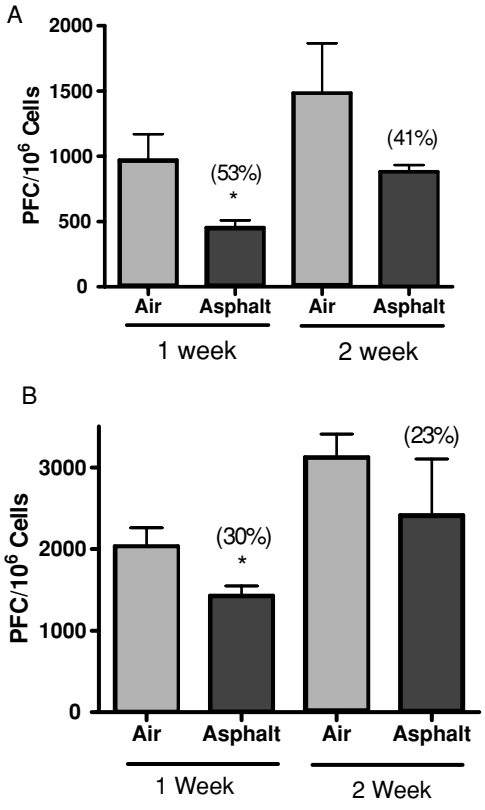

FIG. 3. IgM response to SRBC after inhalation exposure to road paving-like asphalt fumes. The spleen IgM response is illustrated after inhalation with whole asphalt fumes (A) or the vapor component of asphalt fumes (B). Mice were exposed in an inhalation chamber to 35 mg/m^3 of whole asphalt fumes or 11 mg/m^3 of the vapor component as asphalt fumes for a 5 d (1 wk) or 10 d (2 wk) period. Numbers above the bars represent percent suppression compared to vehicle control. Values represent the mean $\pm$ SE derived from 10 animals in each group. $^{*}p < 0.05$ and $^{**}p < .01$ vs. vehicle.

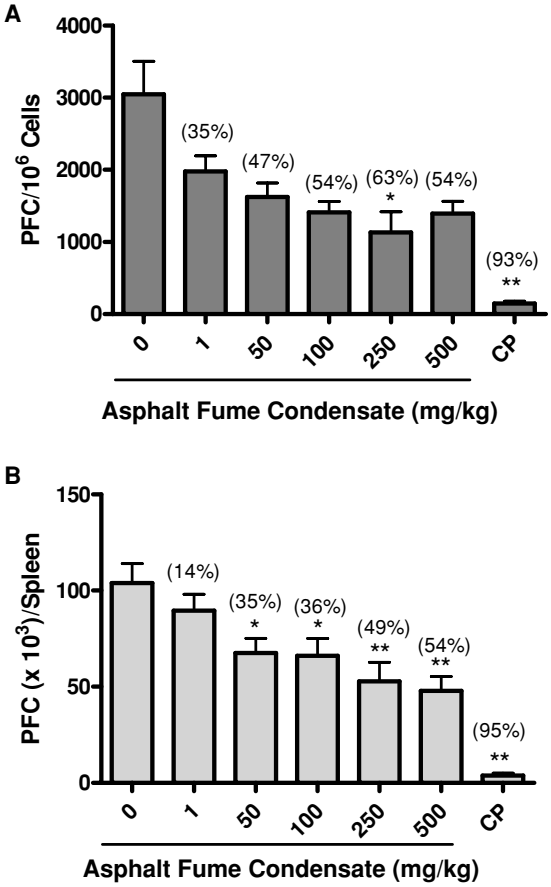

FIG. 4. IgM response to SRBC after dermal exposure to road paving-like asphalt fumes. The specific (A) and total (B) spleen IgM response is illustrated after dermal exposure to asphalt fume condensate. CP (25 mg/kg) was used as a positive control. Values represent the mean $\pm$ SE derived from 5 animals in each group. Numbers above the bars represent percent suppression compared to vehicle control. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle.

Dermal Exposure

Mice were exposed dermally to asphalt fume condensate over a 4-day period. A dose-responsive significant decrease in PFC/10^6 cells (63% suppression) was observed after exposure to 250 mg/kg asphalt fume condensate (Figure 4A). Significant suppression in the total IgM activity was also observed after exposure to asphalt fume condensate concentrations of 50 mg/kg and higher (Figure 4B). The PFC assay positive control, CP, resulted in significant suppression (95%) of the total IgM response and (93%) specific IgM response. No overt toxicity as indicated by changes in body or organ weight was observed for any of the exposure groups (data not shown).

DISCUSSION

Asphalt fumes are known toxicants and the Environmental Protection Agency (EPA) states that asphalt processing and asphalt roofing manufacturing facilities are major sources of hazardous air pollutants such as formaldehyde, hexane, phenol, polycyclic organic matter, and toluene (EPA, 2003). Exposure to these air toxicants may cause cancer, central nervous system problems, liver damage, respiratory problems and skin irritation (AI, 1990).

The majority of research investigating the health effects caused by asphalt fume exposure have focused attention on the PAHs present in the asphalt fumes (Ma et al., 2002, 2003; Antonini et al., 2003). Analysis of PAH content is frequently used to determine levels of occupational asphalt fume exposure and hydroxylated PAH metabolites identified in the urine of asphalt workers have been used as a biomarker of exposure (Buratti et al., 2007). Concentrations of asphalt fumes used in these inhalation studies ranged from 11 to 35 mg/m^3 with PAHs content ranging from 3.3 to 146 μg/m^3.

Low levels of total PAHs present in asphalt fumes have been detected through environmental and personal sampling during road paving operations. In refinery asphalt processing units, respirable PAH levels have been found to range from non-detectable to 14 mg/m^3, and during paving operation PAH range from < 0.1 mg/m^3 to 2.7 mg/m^3(AI, 1991). Analysis of personal air and dermal samples among asphalt workers found inhalation

(mean 0.3 $\mu g/m^3$) and dermal (5.7 ng/cm^2) exposures to pyrene that were significantly higher than non-asphalt paving workers (McClean et al., 2004). Watts and colleagues (1998) measured the mean concentration of airborne particles (2.5 μm) at three different paving sites in the United States and observed an exposure range of 111–389 $\mu g/m^3$ benzo[α]pyrene.

Most of the PAHs identified in the road paving-like asphalt fumes used in the studies presented here were composed of naphthalene derivatives, not the more carcinogenic four to six ring compounds. Analysis demonstrated naphthalene (2 rings) to be the most abundant PAH in the vapor fraction with additional 2–3 ring PAHs, also being observed. The particulate phase was found to contain 2–4 ring PAHs but 5–6 ring PAHs were not detected (Law et al., 2006). Asphalt application temperatures have been shown to vary from 135–163°C for paving asphalt. Increased temperature has been shown to increase the intensity of PAH emission (Law et al., 2006) potentially influencing the severity of immunosuppression.

These studies were conducted for hazard identification related to immune status following exposure to road paving-like asphalt fumes. The PFC assay was used to evaluate immunotoxicity as this assay has been identified as one of the most sensitive indicators of immune suppression following chemical exposure (Luster et al., 1988). The PFC assay evaluates the complex immune response mediated by the combined actions of a number of cell types, including antigen presenting cells, T-lymphocytes (required for the production and release of lymphokines and cell-to-cell contact) and B-lymphocytes. Chemically induced changes in any of these cell types can result in a decrease in PFC activity (Anderson et al., 2006).

PAHs are known to be immunotoxic and have been shown to exert major toxic effects, including development of cancers in various tissues, cardiovascular diseases, and immunosuppression (Burchiel and Luster, 2001). The carcinogenic and mutagenic effects of PAHs are due to the reactive metabolites produced by the cytochrome P450-dependant monoxygenases system suggesting metabolism is necessary for immunosuppression to occur (Davila et al., 1997; Gao et al., 2005). PAHs have also been shown to be self-regulating and capable of inducing their own metabolism (Grove et al., 2000). This may be an explanation for the results of the time course study presented in Figure 3.

Suppression of the IgM response to SRBC was greater for the 1-wk exposure period compared to the 2-week exposure period for both the whole asphalt fume and the vapor component. Potent immunosuppressive PAHs such as benzo(a)pyrene (BP) and dimethylbenz(a)anthracene (DMBA) have been found to inhibit murine T- and B-lymphocyte proliferation and to alter T-lymphocyte-related cytokine production and B-lymphocyte-mediated antibody production (White and Holsapple, 1984; Blanton et al., 1986; Carlson et al., 2004; Gao et al., 2005). Antigen presentation has also been shown to be affected after exposure to PAHs (Tewari et al., 1979; Blanton et al., 1986; Myers et al., 1987; Woods et al., 2000). The studies referenced above combined with the results of the studies reported here indicate that any of these cell types may be affected by the road paving-like asphalt fumes used for these experiments.

Previous work has documented that asphalt fume exposure does not affect clearance of *L. monocytogenes* from the lungs (Antonini et al., 2003). These studies demonstrated no adverse effect on the innate immune system as evidenced by change in the number of polymorphonuclear neutrophils (PMN) or alveolar macrophage or their function following asphalt fume exposure. However, these investigations demonstrated an elevation in lymphocyte secretion of interferon-γ. Interferon-γ stimulates the differentiation of T4-lymphocytes into T_h1 cells and inhibits the proliferation of T_H2 cells (Oriss et al., 1997) which can lead to a suppression of the T_H2 arm of the immune system. The innate immune response is characterized by the activation of natural killer (NK) cells that have previously been shown to down regulate the antibody response (Abruzzo et al., 1986; Robles and Pollack, 1986). These results, in combination with the results presented in this manuscript, suggest that asphalt fume exposure has a profound effect on the humoral arm of the immune system.

In summary, results from *in vivo* models demonstrate that at concentrations relevant to human exposure, road paving-like asphalt fume exposure is immunosuppressive in a murine model. Suppression was observed following three different routes of exposure: systemic, and the occupationally relevant exposure routes, respiratory and dermal. The high numbers of occupational exposures to asphalt and these findings in animal models further support the potential for adverse human health effects due to asphalt exposure.

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