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14. ABSTRACT
The initial focus of this work was to test the hypothesis that dermal application of jet fuel induced immune suppression. Using a mouse model of dermal exposure we noted that applying JP-8 to the skin induced immune suppression. Both primary and recall immune reactions were suppressed by applying JP-8. Cytokine production by JP-8-treated keratinocytes particularly prostaglandin E_2 and interleukin-10 drive immune suppression. During the current funding period we made three important discoveries. First, we found that the aromatic compounds within jet fuel drive immune suppression. When synthetic jet fuel (S-8), which is totally devoid of aromatic compounds, was applied to the skin, no immune suppression was noted. Adding back a cocktail of the 7 most prevalent aromatic compounds found JP-8 to S-8, rendered it immune suppressive. Second, we found that JP-8 activated cytokine production in skin cells by activating the production of reactive oxygen species, which in turn activated NF-κβ, which led to cytokine production and immune suppression. Finally we found that applying JP-8 to the skin activated the migration of mast cells from the skin to the lymph nodes. Blocking the migration, by interfering with the signals that regulate mast cell migration, blocked immune suppression.

15. SUBJECT TERMS
JP-8, immune suppression, cytokines, mast cells, oxidative stress, NF-κβ

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1. Technical Summary:

A. Immune modulation following dermal application of jet fuel. Using a mouse model of dermal exposure, we observed that applying JP-8 to the skin induced immune suppression (Ullrich, 1999). Our initial experiments were designed to characterize what types of immune reactions were affected by dermal application of jet fuel. We observed the following:

First, applying jet fuel to the skin preferentially affects cell-mediated immune reactions. We observed that delayed type hypersensitivity (DTH), contact hypersensitivity (CHS), and T cell proliferation, but not antibody production was suppressed by JP-8 treatment (Ullrich, 1999; Ullrich *et al.*, 2000). Both primary and secondary immune reactions are suppressed by applying JP-8 (Ramos *et al.*, 2002). In our early experiments we used one application of a relatively large amount of JP-8 (200 to 240 mg/mouse), however, we also noted that repeated exposure to small doses of jet fuel (40 to 80 mg over a 4 to 5 day period) will also induce immune suppression (Ramos, et al., 2002). Doses of JP-8 that suppressed in mice compared well to dermal exposures doses reported in Air Force personnel. Using a comparison of body surface area, we estimated that applying a single dose of 300 µl to a 20 gram mouse would be roughly equal to applying 100 ml of JP-8 to 6 foot tall human (Ullrich, 1999). Similarly, we noted significant immune suppression when as little as 50 µl of undiluted JP-8 was applied to mice over the course of 4 to 5 days. This would be approximately equal to a human dermal exposure of 16 to 20 ml per day. After a single exposure to JP-8, the immune suppression persists for approximately 3 weeks (Ullrich, et al., 2000).

Second, activation of cytokine production by JP-8-treatment plays an important role in the activation of immune suppressive pathways. Blocking PGE_2 production with a selective cyclooxygenase (COX)-2 inhibitor, or neutralizing IL-10 activity with a monoclonal antibody, blocked JP-8-induced immune suppression (Ullrich, et al., 2000).

Third, in regard to immune suppression, we noted that there was no difference between JP-8 (military jet fuel) and the fuel used by commercial airlines, Jet-A. JP-8 is Jet-A, supplemented with an anti-corrosive agent (DCI-450), and anti-icing agent (Diethylene glycol monomethyl ether), and an anti-static agent (Statis 450). Early on, attention was focused on the additive package as the agent responsible for inducing immune suppression. However, experimental data suggests that this is not the case. First, the dose response curve for immune suppression induced by JP-8 and Jet-A are identical (Ramos, et al., 2002). Second, the mechanisms involved are similar. Both PAF and PGE_2 are involved in the immune suppression induced by JP-8 and Jet-A (Ramos *et al.*, 2004; Ramos, et al., 2002). While these findings indicate that the base kerosene fuel and not the additive package, induces immune suppression, it leaves unanswered what chemical or class of chemicals in jet fuel cause the immunotoxicity.

B. Mechanisms underlying jet fuel-induced immunotoxicity. In our initial experiments, we observed systemic immune suppression following dermal jet fuel exposure. That is to say, when we applied the jet fuel to the dorsal skin of a C3H/HeN mouse, and then applied a contact allergen to the unexposed ventral skin, we suppressed the CHS reaction. Similarly, applying JP-8 to the dorsal skin of a mouse suppressed the induction of DTH to microbial antigens, such as *Candida albicans* or *Borrelia burgdorferi* injected into the subcutaneous space at a distant site (Ramos, et al., 2002; Ullrich, 1999). The systemic nature of the immune suppression, coupled with the fact that primarily T cell mediated immune reactions were suppressed (Ullrich, et al., 2000) drew our attention to cytokines and biological response modifiers that suppressed cell-mediated immune reactions. We found that dermal jet fuel exposure activated the production of interleukin (IL)-10 (Ullrich, 1999), a cytokine known to suppress cell-mediated immune reactions (Moore *et al.*, 2001). Injecting JP-8-treated mice with monoclonal anti-IL-10 blocked the induction of immune suppression (Ullrich, et al., 2000). Moreover, injecting JP-8-treated mice with recombinant IL-12, a cytokine that counteracts the activity of IL-10 (Schmitt *et al.*, 2000), blocked the induction of immune suppression (Ullrich, et al., 2000). Treating cultured keratinocytes with JP-8 activated the production of prostaglandin E_2, (Ramos, et al., 2004) and injecting JP-8-treated mice with a selective cyclooxygenase-2 (COX-2) inhibitor (Seibert *et al.*, 1994), blocked JP-8-induced immune suppression (Ullrich, et al., 2000).

C. Cytokines and immune-modulatory factors involved in immune suppression. A critical early step in jet fuel induced immune suppression is the production of PGE_2 by JP-8-treated keratinocytes (Ramos, et al., 2004). A critical step in PGE_2 secretion, and perhaps one of the earliest steps in the cascade of events leading to immune suppression is the secretion of the lipid mediator of inflammation, Platelet activating factor (PAF). As the name implies, PAF activates a wide variety of cells including platelets, monocytes, mast cells and polymorphonuclear leukocytes. In addition to activating platelets it also activates monocytes, mast cells, and polmorphonuclear leukocytes. PAF plays a role in cell communication. Cells that are responsive to PAF express a seven transmembrane spanning G-coupled protein receptor. Binding of PAF to its receptor activates of a variety of intracellular events such, increased calcium flux, activation of mitogen-activated protein kinase pathways, the activation of phospholipase (PLA_2) and the transcriptional activation of a variety of genes, including cyclooxygenase-2 (COX-2) (Ishii *et al.*, 2000), and IL-10 (Walterscheid *et al.*, 2002). PAF is secreted in response to oxidative stress, and is secreted by epidermal cells almost immediately following trauma (Alappatt *et al.*, 2000). Because PAF up-regulates the production of PGE_2 (Pei *et al.*, 1998) we tested the hypothesis that JP-8-induced PAF activates cytokine production and initiates immune suppression. To test this hypothesis we pre-treated mice with a series of PAF receptor antagonists and then applied JP-8. The PAF receptor antagonists totally reversed jet fuel induced immune suppression. We noted reversal of immune suppression regardless of whether JP-8 or Jet-A was used to induce immune suppression. At the cellular level, we observed that treating JP-8 and/or Jet-A-treated keratinocytes with a PAF receptor antagonist blocked keratinocyte-derived PGE_2 secretion. We also found that treating jet fuel exposed mice with anti-oxidants, such as Vitamin C, Vitamin E and beta-hydroxyl toluene (BHT), blocked immune suppression (Ramos, et al., 2004). Because jet fuel treatment induces oxidative stress (Rogers *et al.*, 2001), which has been associated with increased PAF production (Alappatt, et al., 2000), these findings indicate that JP-8-induced oxidative stress induces PAF production, which then initiates a cascade of events, including PGE_2 and IL-10 secretion (Shreedhar *et al.*, 1998), that ultimately suppresses cell mediated immune reactions, such as DTH, CHS and T cell proliferation.

D. The aromatic compounds in jet fuel drive immune suppression. An important issue concerning jet fuel induced immunotoxicity is identifying the chemical, or family of chemicals present in jet fuel that activates immune suppression. Jet fuel is made up of over 260 aliphatic and aromatic different compounds (C-6 to C-17) (Ritchie *et al.*, 2003). Initially, those interested in determining what components in jet fuel induced immune suppression focused their attention on the additive package. As mentioned above, JP-8 is essentially Jet-A plus three additives, an antifreeze (Diethylene glycol monomethyl ether), an antistatic agent (Statis 450) and an anticorrosive regent (DCI-4A). Because the dose response curves for JP-8 and Jet-A induced immune suppression are identical, and the mechanisms of actions are similar (Ramos, et al., 2004; Ramos, et al., 2002), it appears that the immunosuppressive properties of jet fuel are inherent to the base kerosene fuel.

To experimentally address this question, we took advantage of the fact that the United States Air Force is currently flight-testing a synthetic jet fuel (hereafter called S-8) that can be produced from coal or natural gas using the Fischer-Tropsch reaction. Because S-8 is devoid of aromatic hydrocarbons (www.syntroleum.com/tech_specifications.aspx), testing its immunosuppressive properties provided an excellent opportunity to determine the role of the aromatic and aliphatics components of jet fuel in the induction of immune suppression. The literature suggests that there is a difference in the penetration (McDougal *et al.*, 2000; Riviere *et al.*, 1999) and toxicity (Chou *et al.*, 2003; Rogers *et al.*, 2004) between aromatics and aliphatics, and that certain aromatics are immune suppressive (Abadin *et al.*, 2007). Therefore, we began a series of studies to determine if applying S-8 to the skin of mice induces immune suppression. We found that applying S-8 to the skin, at all doses tested, did not induce immune suppression. Also, unlike JP-8, applying S-8 to the skin did not up-regulate COX-2 expression. To confirm the role of the aromatic compounds in activating immune suppression, we added to S-8 a cocktail of the 7 most common aromatic compounds found in jet fuel (Benzene, Toluene, Ethylbenzene, Xylene, Trimethylbenzene, Cyclohexylbenzene & Dimethylnaphthalene) at the same concentration found in JP-8. When we applied the supplemented S-8 to the skin, we noted up-regulation of COX-expression and suppressed DTH. We also found that if the cocktail of the aromatic compounds was added directly to the skin, (i.e., not diluted in S-8) it induced immune suppression. Finally, when the mice treated with the supplemented S-8, or the aromatic compounds alone, were injected with either a PAF receptor antagonist, or a COX-2 inhibitor, no immune suppression was noted (Ramos *et al.*, 2007).

These experiments indicate that the aromatic compounds in jet fuel induce immune suppression, and they do it using a mechanism identical to that already described for JP-8 and Jet-A.

Our findings add to the observations made by McDougal *et. al.* and Riviere *et. al.* concerning differences in the deposition and toxicity of aliphatic and aromatic hydrocarbons after dermal exposure. Aromatic compounds rapidly penetrate through the skin, whereas aliphatic compounds are absorbed by the skin and have an increased residence time in the epidermis (McDougal, et al., 2000; Riviere, et al., 1999). Also, the aromatics compounds are more potent in inducing keratinocyte cell death. Aromatic cytotoxicity in this context has been reported to be a function of the number of side chains and the number of rings in which the more complex the ringed structure the more its cytotoxic potential (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 pro-inflammatory cytokines by keratinocytes (Muhammad *et al.*, 2005). Our findings indicate a differential effect of aromatic and aliphatic hydrocarbons on the induction of immune suppression.

E. Dermal mast cells transmit the immunosuppressive signal from the skin to the immune system. The skin is the largest organ of the body and its main purpose is to provide barrier function for the preservation of body homeostasis. However, contained within the skin are specialized elements that provide immunological function. Langerhans cells and dermal dendritic cells serve as antigen presenting cells that can initiate and/or regulate immune reactions (Fukunaga *et al.*, 2008; Kaplan *et al.*, 2005; Kissenpfennig *et al.*, 2005). Epidermal keratinocytes secrete a wide variety of immune regulatory cytokines that have diverse effects on immune reactivity (Ullrich, 1995). Dermal mast cells also contribute to the immunological function of the skin. Due to their abundant expression of Fcε receptors and their ability to secrete histamine following IgE binding, mast cells have been traditionally associated with allergic-type immune reactions. However, newer findings indicate that mast cells influence a wide variety of non-allergic immune responses (Bischoff, 2007) and participate in inducing immune regulation and tolerance (Lu *et al.*, 2006). For example, Mast cells, and in particular, mast cell migration, plays a critical role in the immune suppression induced by UV radiation (Byrne *et al.*, 2008; Hart *et al.*, 1998; Ullrich *et al.*, 2007).

Because of their location in the dermis, and in view of the emerging appreciation that mast cells regulate immune function, we tested the hypothesis that mast cells are involved in the immune suppression activated by dermal chemical exposure. No immune suppression was observed in jet fuel-treated, mast cell deficient mice, and immune suppression was restored by reconstituting the mast cell deficient with wild-type bone marrow derived mast cells. We also noted increased numbers of mast cells in the draining lymph nodes of mice treated with jet fuel. We noted a JP-8-induced increase in mast cell CXCR4 expression, confirming data reported by others generated by microarray analysis (McDougal *et al.*, 2007). Moreover, we found that treating the mice with an anti-leukemia drug, AMD3100, which is a CXCR4 antagonist and has been shown previously to block mast cell migration to skin draining lymph nodes (Byrne, et al., 2008), abrogated the accumulation of mast cells in the lymph nodes and prevented the induction of immune suppression. We conclude that jet fuel-induced immune suppression is dependent on mast cell function, and suggest that mast cells migrate to draining lymph nodes thereby transmitting the immunosuppressive signal from the skin to the immune system *(Limon-Flores, et al., Mast Cells mediate the Immune suppression Induced by Dermal Exposure to JP-8 jet fuel. Submitted for publication, Toxicological Sciences).*

F. The molecular signals underlying JP-8 cytokine induction and immune suppression. Although it is clear that cytokine production is critical for JP-8-induced immune suppression, the molecular events that drive keratinocyte production of prostaglandin E_2 are not entirely clear. Reactive oxygen species (ROS) are known to induce NF-κβ activation, a ROS sensitive transcription factor with the ability to induce COX-2 expression (Hadjigogos, 2003). Moreover, Espinoza *et al* reported prolonged NF-κβ activation in jet fuel treated rat alveolar epithelial cells results in the up-regulation of pro-inflammatory cytokines (Espinoza *et al.*, 2006). In view of these observations, we tested hypothesis that treating keratinocytes with jet fuel induces ROS, which turns on NF-κβ, which activates COX-2 expression, and drives immune suppression.

Treating keratinocytes with JP-8 does activate ROS production. Further, when we treated keratinocytes that over-expressed the ROS scavengers, catalase or superoxide dismutase, ROS induction was significantly

depressed. Similarly, when keratinocytes that over-expressed the ROS scavengers were treated with JP-8, COX-2 expression was substantially depressed. We also demonstrated that antioxidant treatment blocked JP-8-induced COX-2 transcription, and mRNA expression *in vivo*, and blocked JP-8-induced immune suppression

Because ROS activates NF-κβ, and in light of the importance of this transcriptional activator in COX-2 activation, we also measured the role of NF-κβ in JP-8-induced COX-2 production and immune suppression. Treating mouse skin with JP-8 activated NF-κβ, and prior treatment with parthenolide; an NF-κβ inhibitor blocked its activation. Moreover, *in vivo* treatment with parthenolide blocked JP-8-induced COX-2 expression and JP-8 induced immune suppression. Parthenolide treatment also blocked the transcription of the COX-2 gene. In addition, we confirmed the role of NF-κβ in activating COX-2 by showing suppression of JP-8-induced COX-2 induction in keratinocytes treated with Rel A (p65 component of NF-κβ)-specific siRNA. These data indicate that ROS generation and NF-κβ activation play an important role in activating COX-2 expression and immune suppression following dermal JP-8-treatment.

We saw no effect of parthenolide, at the doses and conditions used in our experiments, on ROS activation. This indicates the following scenario: jet fuel interacts with keratinocytes in the skin and activates ROS production. ROS then activates NF-κβ. NF-κβ then induces COX-2 mRNA expression and transcription. This leads to the production and secretion of prostaglandin E_2, which is an essential step in immune suppression. When we use free radical scavengers, such as Vitamin C and N-acetylcysteine, we are blocking an early step in the pathway, ROS production. When we use parthenolide or p65 siRNA to block NF-κβ activation, we are interfering with a later step in the pathway leading to COX-2 expression. Although we primarily focused on COX-2 expression in these experiments, it is known that other cytokines and immunomodulatory factors, such as PAF are involved in JP-8-induced immune suppression (Ramos, et al., 2004). Others have shown the ROS induces PAF (Alappatt, et al., 2000) and PAF induces NF-κβ (Ko *et al.*, 2002), so the pathways we identified here are probably involved in PAF production following JP-8 treatment. *(Ramos, et al.,* JP-8-induces immune suppression via a reactive oxygen species NF-κβ-dependent mechanism. *Submitted for publication, Toxicological Sciences).*

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Ramos, G and Ullrich, S.E. Dermal application of jet fuel suppresses Immunological Memory, 2th International Conference on the Environmental Health and Safety of jet fuel, August 7-10, 2001.

Ullrich, S.E. Dermal application of jet fuel (JP-8 & Jet-A) induces immune suppression through a platelet-activating factor dependent mechanism. Air Force Office of Scientific Research Jet fuel Toxicology Workshop, University of Arizona, Tucson, AZ. May 14-16, 2003.

Ullrich, S.E. Mechanisms involved in jet fuel-induced immune suppression. Air Force Office of Scientific Research Jet fuel Toxicology Workshop, University of Arizona, Tucson, AZ. October 13-15, 2004.

Ullrich, S.E. Mechanisms involved in jet fuel-induced immune suppression. Air Force Office of Scientific Research Jet fuel Toxicology Workshop, University of Arizona, Tucson, AZ. November 30 to December 2, 2005.

Ramos, G, and Ullrich, S.E. Immune suppression by dermal exposure to JP-8 and S-8 jet fuels. Air Force Office of Scientific Research Jet fuel Toxicology Workshop, University of Arizona, Tucson, AZ., January 17-19, 2007.

3. Follow-On Uses

None

4. Accomplishments and Successes

- Demonstrated that applying JP-8 to the skin induces immune suppression.
- Documented that cell mediated immune reactions are more sensitive to the immunosuppressive effects of jet fuel.
- Demonstrated a role for JP-8-induced keratinocyte-derived immune suppressive factors and cytokines (platelet activating factor, interleukin-10 and prostaglandin E_2) in activating immune suppression.
- Provided data indicating that both primary and secondary immune reactions are suppressed by jet fuel application. This finding implies that immune protection by prior vaccination may be suppressed by jet fuel exposure.
- Observed that applying Jet-A to the skin also induced immune suppression.
- Demonstrated that the mechanisms underlying Jet-A-induced immune suppression are identical to those described for JP-8.
- Provided evidence documenting that the aromatic compounds found in JP-8 are the agents responsible for activating immune suppression.
- Demonstrated that dermal mast cells are essential for inducing immune suppression and mast cells migrating from the skin to the lymph nodes carry the immune suppressive signal from the skin to the immune system.
- Provided evidence supporting a role for ROS and NF-κβ as molecular triggers for JP-8-induced immune suppression.
- Because avoiding exposure to JP-8 is impractical, a better understanding of the mechanism(s) underlying immune suppression allows for the rationale design of therapies to overcome immune suppression. My research has indicated that anti-oxidants (Vitamin E and C), selective COX-2 inhibitors (Celebrex), platelet activating factor receptor antagonists, NF-κβ inhibitors (Parthenolide) and agents that block IL-10 activity (monoclonal anti-IL-10, or IL-12), all block JP-8-induced immune suppression.

5. Professional Personnel Supported

Names	Professional Category	Full time/part time
Stephen E. Ullrich	Faculty and Principal Investigator	Full time

Heather Lyons	Research Assistant 1	Full time
Nasser Kazimi	Senior Research Assistant	Full time
Jeffrey P. Walterscheid	Graduate Student	Full time
Dat X. Nghiem	Graduate Student	Full time
Gerardo Ramos	Graduate Student	Full time
Alberto Y. Limon-Flores	Post-doctoral fellow	Full time

6. Honors and Awards Received

N/A

7. Professional Activities

Stephen E. Ullrich

Society Memberships:

American Association for the Advancement of Science
American Association of Immunologists
American Society for Photobiology
Council Member: 2003-2006
President-Elect: 2005-2006
President: 2006-2007
Co-Chair of 2008 Scientific Meeting
Society for Investigative Dermatology

Invited to give talks, and chair scientific sessions at both national and international meetings.
Served on a variety of National Institutes of Health Study Sections
Member, Special Emphasis Review Panel of the Interagency Coordinating Committee on the Validation of Alternative Toxicological Methods, NIEHS, 1997 & 2008.

Jeffrey P. Walterscheid

Society Memberships:

American Society for Photobiology
Made Presentations at professional society meetings

Dat X. Nghiem

Society Memberships:

American Society for Photobiology
Made Presentations at professional society meetings

Gerardo Ramos

Society Memberships:

Society of Toxicology
Made Presentations at Professional Society Meetings

Alberto Y. Limon-Flores

Society Memberships:

American Association of Immunologists
Made Presentations at Professional Society Meetings