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CAPT RAMOS GERARDO

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Dedication

I would like to dedicate this thesis to my lovely wife, [redacted]y wonderful children [redacted] to my parents [redacted] [redacted] to [redacted] my brothers and sister for believing in me. Thank you to the Air Force of the United States of America for giving this Captain the opportunity to attend graduate school while on active duty. And to God for giving me such a wonderful family.

IMMUNOSUPPRESSIVE EFFECTS OF JETFUEL AND ITS MECHANISM OF ACTION

A

THESIS

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Gerardo Ramos, M.S.
U.T.M.D. Anderson Cancer Center
Houston, Texas

May, 2002

IMMUNOSUPPRESIVE EFFECTS OF JET FUEL AND ITS MECHANISM OF ACTION

Publication No._____

Gerardo Ramos, M.S.

Supervisory Professor: Stephen E. Ullrich, Ph.D.

Applying military jet fuel (JP-8) to the skin of mice activates systemic immune suppression. In all of the previous experiments, JP-8 was applied to immunologically naïve mice. The effect of jet fuels on established immune reactions, such as immunological memory, is unknown. The focus of the experiments presented here was to test the hypothesis that jet fuel exposure (both JP-8 and commercial jet fuel; Jet-A) suppresses established immune reactions. Mice were immunized with the opportunistic fungal pathogen, *Candida albicans* and at different times after immunization (10 to 30 days) various doses of undiluted JP-8 or Jet-A were applied to their skin. Both the elicitation of delayed-type hypersensitivity (DTH), as measured when mice were challenged 10 days after immunization and immunologic memory (mice challenged 30 days after immunization) were significantly suppressed, in a dose-dependent manner. Dermal exposure to JP-8 and/or Jet-A, either multiple small doses (50 µl over 4 days) or a single large dose (≈200-300 µl) suppressed DTH to *C. albicans*. Also, the ability of splenic T-lymphocytes from JP-8 and/or Jet-A treated mice to proliferate to plate-bound anti-CD3 monoclonal antibody was significantly suppressed. The mechanism by which dermal application of JP-8 and Jet-A suppresses immunological memory involves the release of immune biologic response modifiers. Blocking the production of prostaglandin

E_2 (PGE_2) by a selective cyclooxygenase-2 (COX-2) inhibitor (SC 236) significantly reversed jet fuel-induced suppression of immunologic memory. Furthermore, others have shown that platelet-activating factor (PAF) receptor binding induces COX-2 expression in mouse keratinocytes. Because PAF upregulates the production of immunomodulatory compounds, including (PGE_2), we also tested the hypothesis that PAF plays a role in jet fuel induced immune suppression. The Jet A and JP-8 induced suppression of DTH was blocked by injecting the mice with PAF receptor antagonists. Our findings demonstrate that military jet fuel suppresses immunologic memory. Further, they demonstrate for the first time that dermal application of commercial jet fuel (Jet A) induces immune suppression. Finally our data suggests that one of the first steps in the pathway leading to systemic immune suppression is PAF induced PGE_2 production.

"The views expressed in this article are those of the author and do not reflect the official policy of position of the United States Air Force, Department of Defense, or the U.S. Government.'

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Introduction

Beginning in the early 1990's, the United States Air Force and the air forces of the NATO allies began a gradual conversion to a new jet fuel, JP-8. Kerosene-derived commercial and military jet fuels are complex mixtures of hydrocarbons whose exact compositions are not rigorously defined, but rather the product is refined to meet a series of performance specifications (White 1999). JP-8 is refined to have a higher flash point, lower vapor pressure and lower freezing point than JP-4, the fuel it replaced (for review of fuels see Table 1). The net result is a fuel that performs well at high altitudes and is less combustible, thereby providing an added measure of safety for personnel exposed to the fuel during normal and combat situations. In addition, the higher vapor pressure of JP-8 minimizes evaporative losses during handling and storage, a serious logistical advantage. Because of its advantages compared to previous fuels, all branches of the US Military are converting to JP-8 as the primary fuel for helicopters, aircraft, tanks and fighting vehicles.

Although initial toxicological screening indicated minimal effects following JP-8 exposure (Mattie, Alden et al. 1991; Kinkead, Salins et al. 1992; Mattie, Marit et al. 1995), an unexpected consequence of the use of this safer fuel is increased exposure. When JP-8 is spilled on the skin due to being a heavier fuel, instead of quickly evaporating away, as is the case with JP-4, some JP-8 is absorbed into the skin (Riviere, Brooks et al. 1999; McDougal, Pollard et al. 2000). Similarly, inhalation of aerosolized JP-8 is a significant problem (Pfaff, Parton et al. 1995). As the conversion to JP-8 progressed reports of health problems by personnel

Table 1. A. Fuels Comparison Chart (Navy Environmental Health Center, 2001)

NAVY ENVIRONMENTAL HEALTH CENTER

FUELS COMPARISON CHART

	JP-8	Jet A	JP-5	JP-4	Regular Gasoline, Unleaded
USES	*DOD Jet Fuel since 1991, used predominately by the Army and the Air Force to power aircrafts and land vehicles. Also now used by the Navy at land based activities.	Commercial Airline Jet Fuel	Jet Fuel used aboard ships, Navy vehicles and equipment from about 1952.	DOD Jet Fuel from 1951. Was phased out beginning in 1991 and completely in 1996. Used to power Navy aircrafts and Marine Corps land vehicles.	Used in commercial automobiles by the general public.
SPECIFICATION**	MIL-T-83133	ASTM D 1655	MIL-T-5624	MIL-T-5624	ASTM D 4814
Primary Constituents (typically > 98% total volume)- All petroleum products are made from *crude oil*. Crude oil contains primarily hydrocarbon compounds made up of mostly carbon and hydrogen. In hydrocarbon compounds, the carbon atoms link together in chains of different carbon lengths. In a refinery, these chains are separated by heating (distillation). The crude oil is heated and the different compounds separate into groups based on their boiling points and density. The boiling point and density ranges of these groups are also related to the number of linked carbons the individual compounds contain. *Gasoline* is a blend of some of the shorter chain molecules that boil off first in the refining process. The chain lengths range from four to twelve, C4 – C12. *Kerosene* contains more of the middle distillate, or middle boiling point compounds in the C6 to C18 range. Kerosene is followed by *diesel fuel* and heavier fuel oils (like heating oil for houses) that contain longer chain compounds. Gasoline typically contains more benzene and higher amounts of aromatic (benzene containing compounds) than the kerosene based jet fuels. Crude oil composition varies depending on its source.					
	> 98 % Kerosene containing compounds in the C7 through C18 range	> 98% Kerosene containing compounds in the C7 through C18 range	> 98% Specially blended Kerosene containing compounds in the C8 through C17 range	> 98% Mixture of 65% Gasoline and 35% Petroleum Distillates – mixture contains compounds in the C5 through C14 range	> 98% Refined Petroleum Hydrocarbon containing compounds in the C4 through C12 range
Additives (combined typically < 2% total volume) - Additives are used in Jet Fuel to improve its performance under varying conditions. Typical additives to Jet Fuels and Gasoline include antioxidants, metal deactivators, static dissipator, corrosion inhibitors, fuel system icing inhibitors, octane enhancers, ignition controllers, and detergents/dispersants. These additives are used only in specified amounts, as governed by the military (MIL) and or commercial (ASTM) specification. The specification will decide which additives are required and which may be OPTIONAL. Whether an additive is optional or required, if it is added, it must be chosen from one of the chemical listed below. The chemicals listed below for each additive are not all used at once but represent the lists from which to choose.					
ANTIOXIDANT[1]	OPTIONAL **may contain one or more of the following:** • 2,6-di-tert-butyl-4-methylphenol • 2,6-di-tert-butylphenol • 2,4-dimethyl-6-tert-butylphenol • 75% min-2,6-di-tert-butylphenol 25% max tert-butylphenols and tri-tert-butylphenols • 72% min 2,4-dimethy-6-tert-butylphenol 28% max tert-butyl-methylphenols and tert-butyl-dimethylphenols • 55% min 2,4-dimethyl-6-tert-butylphenol 15% min 2,6-di-tert-butyl-4-methylphenol 30% max mixed methyl and dimethyl tert-butylphenols	OPTIONAL **may contain one or more of the following:** • 2,6-di-tert butyl-4-methyl phenol • 2,6-di-tert butyl phenol • 2,4-dimethyl-6-tert-butylphenol • 75% min-2,6-di-tert-butylphenol 25% max mix tert-butylphenols and tri-tert-butylphenols • 72% min 2,4-dimethyl-6-tert-butyl phenol 28% max tert-butyl-methylphenols and tert-butyl-dimethylphenols • 55% min 2,4-dimethyl-6-tert-butylphenol 15% min 2,6-di-tert-butyl-4-methylphenol remainder: monomethyl and dimethyl tert-butylphenols	REQUIRED **contains one or more of the following:** • 2,6-di-tert-butyl-4-methylphenol • 2,6-di-tert-butylphenol • 2,4-dimethyl-6-tert-butylphenol • 75% min-2,6-di-tert-butylphenol 25% max tert-butylphenols and tri-tert-butylphenols • 72% min 2,4-dimethy-6-tert-butylphenol 28% max tert-butyl-methylphenols and tert-butyl-dimethylphenols • 55% min 2,4-dimethyl-6-tert-butylphenol 15% min 2,6-di-tert-butyl-4-methylphenol 30% max mixed methyl and dimethyl tert-butylphenols	OPTIONAL **may contain one or more of the following:** • 2,6-di-tert-butyl-4-methylphenol • 6-tert-butyl-2,4-dimethylphenol • 2,6-di-tert-butylphenol • 75% min-2,6-di-tert-butylphenol 25% max tert-butylphenols and tri-tert-butylphenols • 72% min 6-tert-butyl-2,4-dimethyphenol 28% max tert-butyl-methylphenols and tert-butyl-dimethylphenols • 55% min 2,4-dimethyl-6-tert-butylphenol 15% min 2,6-di-tert-butyl-4-methylphenol 30% max mixed methyl and dimethyl tert-butylphenols	REQUIRED **contains one or more of the following:** • N,N-dialkylphenylenediamines • 2,6-dialkylphenols • 2,4,6-trialkylphenols • butylated methyl phenols • butylated ethyl phenols • butylated dimethyl phenols • triethylene tetramine di(monononylphenolate)

October 2001

Table 1. B. Fuels Comparison Chart (Navy Environmental Health Center, 2001)

FUELS COMPARISON CHART

	JP-8	Jet A	JP-5	JP-4	Regular Gasoline, Unleaded
METAL DEACTIVATOR[2]	OPTIONAL N,N-disalycylidene-1,2-propanediamine	OPTIONAL N,N-disalycylidene-1,2-propanediamine	NOT USED	OPTIONAL N,N-disalycylidene-1,2-propanediamine	**REQUIRED contains one or more of the following:** • N,N-disalicylidene-1,2-ethanediamine • N,N- disalicylidene-propanediamine • N,N- disalicylidene-Cyclohexanediamine • Disalicylidene-N-methyl dipropylene triamine
STATIC DISSIPATOR[3]	REQUIRED Stadis 450 containing: • 50-65% Toluene • <1% Benzene • 5-10% Heavy Aromatic Naptha • <5 % Isopropyl Alcohol • 1-10% Dodecylbenzenesulfonic Acid • 10-20% Trade secret • 1-10% Trade secret	OPTIONAL Stadis 450 containing: • 50-65% Toluene • <1% Benzene • 5-10% Heavy Aromatic Naptha • <5 % Isopropyl Alcohol • 1-10% Dodecylbenzenesulfonic Acid • 10-20% Trade secret • 1-10% Trade secret	NOT USED	REQUIRED Stadis 450 containing: • 50-65% Toluene • <1% Benzene • 5-10% Heavy Aromatic Naptha • <5 % Isopropyl Alcohol • 1-10% Dodecylbenzenesulfonic Acid • 10-20% Trade secret • 1-10% Trade secret	NOT USED
CORROSION INHIBITOR[4]	REQUIRED Organic Acid	NOT USED	REQUIRED Organic Acid	REQUIRED Organic Acid	**REQUIRED contains one or more of the following:** • Organic acids • Phosphoric acids • Sulfonic acids
FUEL SYSTEM ICING INHIBITOR[5]	REQUIRED Diethylene glycol monomethyl ether and 50 to 150 ppm by weight of either • 2,6-ditert-butyl-4-methylphenol • 2,4 dimethyl, 6-tert-butyl-2,4-dimethylphenol • 2,6-di-tert-butylphenol • 75% min-2,6-di-tert-butylphenol 25% max tert-butylphenols and tri-tert-butylphenols	OPTIONAL Diethylene glycol monomethyl ether 0.10 – 0.15%	REQUIRED Diethylene glycol monomethyl ether and 50 to 150 ppm by weight of either • 2,6-ditert-butyl-4-methylphenol • 2,4 dimethyl, 6-tert-butyl-2,4-dimethylphenol • 2,6-di-tert-butylphenol • 75% min-2,6-di-tert-butylphenol 25% max tert-butylphenols and tri-tert-butylphenols	REQUIRED Diethylene glycol monomethyl ether and 50 to 150 ppm by weight of either • 2,6-ditert-butyl-4-methylphenol • 2,4 dimethyl, 6-tert-butyl-2,4-dimethylphenol • 2,6-di-tert-butylphenol • 75% min-2,6-di-tert-butylphenol 25% max tert-butylphenols and tri-tert-butylphenols	REQUIRED Isopropyl alcohol
OCTANE ENHANCER[6]	NOT USED	NOT USED	NOT USED	NOT USED	**REQUIRED contains one or more of the following:** • Methyl t-butyl ether • t-butyl alcohol • ethanol • methanol
IGNITION CONTROLLERS[7]	NOT USED	NOT USED	NOT USED	NOT USED	REQUIRED Tri-o-cresylphosphate

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Table 1. C. Fuels Comparison Chart (Navy Environmental Health Center, 2001)

FUELS COMPARISON CHART

	JP-8	Jet A	JP-5	JP-4	Regular Gasoline, Unleaded
DETERGENTS/ DISPERSANTS[8]	NOT USED	NOT USED	NOT USED	NOT USED	REQUIRED contains one or more of the following: • Alkylamine phosphates • Poly-isobutene amines • Long chain alkyl phenols • Long chain carboxylic acids • Long chain amines

* DOD switched from JP-4 to JP-8 because it is safer to use. JP-8 has a higher flash point and lower vapor pressure, which makes it less likely for an aircraft to explode if damaged in combat. Also, because of its lower volatility, less volatile organic compounds (VOCs) are released into the atmosphere preventing pollution. In 1997, the Defense Energy Support Center (DESC) completed a comprehensive air emission survey of the DoD bulk petroleum storage infrastructure. The purpose of the survey was to quantify the reduction in emissions of VOCs resulting from the conversion from JP-4 to JP-8 jet fuel. The study considered all DoD bulk petroleum storage facilities subject to the pollution prevention goals established by the President pursuant to Executive Order 12856, Federal Compliance with Right-to-Know Laws and Pollution Prevention Requirements. The study identified approximately 210 installations with a total of 1,880 tanks. The study found that from FY 92 to FY 97 annual emissions of VOCs at DoD facilities subject to the requirements of E.O. 12856 decreased from 858,000 lb/yr. to less than 100,000 lb/yr. Although emissions from loading operations were not calculated, we would expect a similar decrease in VOC emissions from these sources also.

** Specifications define the required results, but do not mandate the method(s) for achieving the results.
"MIL" indicates a Military Specification standard for products used in the military.
"ASTM" (American Society for Testing and Materials) indicates a Commercial technical specification standard.

ADDITIVES
1. Antioxidants prevent the formation of deposits in aircraft engine fuel systems.
2. Metal deactivators suppress fuel oxidation.
3. Static dissipator is used primarily to reduce the hazardous effects of static electricity generated by movement of fuel through high flow-rate fuel transfer system.
4. Corrosion inhibitors protect metals from corrosion in fuel handling systems.
5. Icing inhibitors prevent any water in the fuel tank from freezing at high altitudes.
6. Octane enhancer provides a more complete and thorough burn of fuel mixture.
7. Ignition controller is used as a lubricant in the ignition system.
8. Detergents/dispersants remove and prevent deposits such as carbon in the engine's intake system.

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exposed to JP-8, including nausea, headaches, fatigue, blocked nasal passages, ear infections and skin irritation, prompted additional reviews of the health effects of JP-8. These health hazards are what prompted the study of JP-8. For the same reasons, the commercial airlines were concerned about their flight line employees. Although the studies performed were aimed at delineating the effects of the military grade JP-8, the commercial airline industry quickly came in line with their own concerns.

Military installations around the world employ diverse group of individuals whose jobs are to ensure logistical precision to maintain flight schedules to support many contingencies. The personnel involved in keeping an aircraft in the air and making sure it is ready for its next sortie who are at increased risk of being exposed to jet fuel include fuelers, fuel handlers, jet engine mechanics, fuel tank maintenance personnel, and spill cleanup crews. The ground crew around the aircraft during engine starts are also exposed to raw fuel. During engine starts, especially in cold climates, there is a significant amount of uncombusted fuel that passes through the engines and is aerosolized. The aerosolized jet fuel during cold starts forms a plume that exposes the ground crew standing 200-500 feet away from the aircraft to jet fuel. Ground troops are also at risk to jet fuel exposure because JP-8 is also the fuel source for many stoves and heaters used in the field.

Those at highest risk for dermal exposure are the ones that come in direct contact with the fuel at its charging and discharging destinations. For example, the fuel tank maintenance individuals in some instances have to wear an anti-static cotton suit to prevent any chance of creating a static environment that is conducive to igniting the jet fuel fumes. Although wearing cotton suits provides protection against ignition it does not

provide adequate protection against dermal exposure. Before these individuals climb into the fuel tanks they are emptied, but small puddles of fuel are always left behind. Also as the fuel tank is opened residual fuel is displaced and may come into contact with the fuel tank maintenance personnel, a common problem with commercial airline fuelers. During these tasks fuelers may take many precautions (ie. protective clothing, gloves etc...) but getting jet fuel on their skin is almost inevitable.

It is now clear that JP-8 has toxic effects (Hays, Parliman et al. 1995; Pfaff, Parton et al. 1995; Pfaff, Tollinger et al. 1996; Smith, Bhattacharya et al. 1997), and that immune function is particularly susceptible to JP-8 induced toxicity (Harris, Sakiestewa et al. 1997; Harris, Sakiestewa et al. 2000). In our laboratory, we have been studying the immunotoxic effect of dermal exposure to JP-8. Employing a mouse model, we found that dermal exposure to JP-8 induces immune suppression. Classic delayed type hypersensitivity, contact hypersensitivity and T cell proliferation were all significantly suppressed by JP-8-exposure. Cellular immune reactions appear to be more susceptible to the suppressive effects of JP-8 than humoral immune reactions, as antibody formation *in vivo* was not affected by JP-8-treatment. The mechanism underlying JP-8-induced immunotoxicity involves the secretion of biological response modifiers, presumably by JP-8-treated keratinocytes (Allen, Riviere et al. 2001; Kabbur, Rogers et al. 2001). Blocking the production of prostaglandin E_2 (PGE_2) with a selective cyclooxygenase (COX)-2 inhibitor totally reversed JP-8-induced immune suppression. A COX-2 inhibitor was selected because it acts on the inducible form of cyclooxygenase. It selectively inhibits COX-2 and does not affect the constitutively expressed COX-1. Historically, inhibition of COX-1 has deleterious gastrointestinal effects (Siebert et. al.

1994). Moreover, the immune regulatory cytokine, interleukin (IL)-10, was found in the serum of JP-8-treated mice and injecting neutralizing anti-IL-10 antibodies into JP-8-treated mice blocked JP-8-induced immunotoxicity (Ullrich 1999; Ullrich and Lyons 2000).

Although our previous data support the conclusion that JP-8 is a dermal immunotoxin, important questions remain in regard to its mode of action. In all of our previous experiments the JP-8 was applied to naïve animals prior to immunization (i.e., suppressing the induction of immunity). Of equal concern, however, is the ability of immunotoxin exposure to suppress established immune responses. Perhaps the most important medical advance of the twentieth century was the reduction, and in some cases the complete eradication (i.e., smallpox) of certain microbial infections through the widespread use of childhood vaccinations. If dermal JP-8 exposure can suppress established immune reactions, such as immunological memory, it may suggest that protection against infectious disease could be compromised in exposed individuals.

A second major concern, not addressed in our previous studies, involves the question of why JP-8 is immune suppressive. JP-8 is essentially commercial jet fuel (Jet-A) with three additives (Table 1), an anti-freeze (Diethylene glycol monomethyl ether), an anti-static agent (Stadis 450) and an anti-corrosive reagent (DCI-4A) (Kanikkannan, Jackson et al. 2000). It is not clear if the immunosuppressive properties of JP-8 are a function of the base kerosene fuel or results from one or more of the components the military adds to Jet-A.

This uncertainty is primarily due to the general lack of information regarding the effects of Jet-A on the immune response. To resolve these questions we tested the

hypothesis that applying jet fuels, JP-8 and Jet-A, to the skin of previously immunized mice suppresses established immune reactions, including the elicitation of DTH and immunological memory.

A second major focus of this work is to determine the molecular events that trigger jet-fuel induced immune suppression. From previous work (Ullrich and Lyons 2000) it is apparent that cytokines and biological response modifiers, such as IL-10 and PGE_2 are involved. Some have implicated platelet-activating factor (Walterscheid, Ullrich et al. 2002) in up regulation of COX-2 gene expression and PGE_2 synthesis in keratinocytes (Pei, Barber et al. 1998), which suggests PAF may have a role in jet fuel induced immunosuppression. PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a phospholipid mediator. It has strong biological effects as it is involved in activating platelets and many other cell types, including monocytes, mast cells, and polymorphonuclear leukocytes. It is also involved in chemotactic recruitment of granulocytes to site of inflammation or injury. PAF responsive cells contain a G protein coupled receptor. This receptor recognizes PAF and various PAF-like molecules. There are two pathways to PAF synthesis. The first pathway involves the oxidation of phosphatidylcholine which produces a PAF-like molecule that can bind and activate the PAF receptor. The other pathway (De Novo synthesis) involves the activation of PLA_2 which acts upon the sn-2 side chain of phosphotidylcholine to make an inactive LysoPAF and the PGE_2 precursor arachidonic acid. Arachidonic acid is cleared to PGE_2 through a COX-2 initiated mechanism. LysoPAF goes on to become PAF through the action of an acetyl transferase. It is unclear how the PAF and PAF-like molecules pass themselves through the plasma membrane to access the PAF receptor and initiate the cascade. Like

many biological signaling pathways there is a critical need to achieve a balance between production and degradation of the molecule. One of the regulatory mechanisms is through the actions of PAF-acetyl hydrolases (PAF-AH). An intracellular and an extracellular PAF-AH is in part responsible for maintaining this balance (see Figure 1)

Binding to the PAF receptor leads to downstream effects that include activation of the mitogen activated protein (MAP) kinase pathway, activation of phospholipases, upregulation of certain cytokines and prostaglandins (Ishii and Shimizu 2000). PAF receptor activation plays a role in cellular communication in various organ systems, including the vascular system, the central nervous system, the endocrine system and the gastrointestinal system.

PAF receptor binding and its role in the immune suppression induced by other dermal immuno-toxins has been investigated. Waltersheid et. al. used a DTH mouse model to show that the PAF receptor was involved in UV induced suppression. The immunosuppression is mediated by PAF binding to the PAF receptor on other PAF receptor containing cells. This starts a cytokine cascade which results in immune suppression. COX-2 and IL-10 gene transcription is activated by treating keratinocytes with UV radiation and PAF. Also it was discovered that COX-2 and IL-10 gene transcription was inhibited by treating keratinocytes with PAF receptor antagonists (Waltersheid, J.P. et al, 2002). Injecting mice with a PAF analog suppressed DTH in vivo and, a selective COX-2 inhibitor abrogated the suppression, indicating that PAF receptor activation is upstream of PGE_2 production.

PAF Synthesis

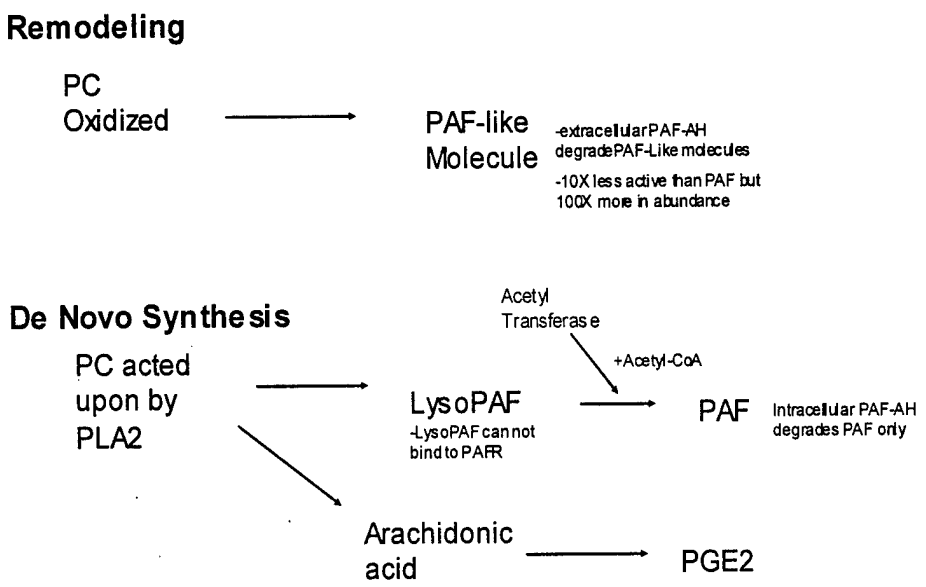

Figure 1. PAF Synthesis Pathways

The mechanism of UV induced suppression seems to be similar to the jet fuel induced suppression. From studies with UV-induced immune suppression it has been shown that an early step in the cascade is the production of PGE_2. This causes downstream effects such as the up regulation IL-4 and IL-10 in the serum (Shreedhar, Giese et al. 1998). The target of these cytokines is the dendritic cell. As the skin is the first line of defense against external contaminants it contains a dendritic cell network that it made up of Langerhans cells that act as antigen presenting cells and present captured antigen to the T cells. UV-irradiation affects the antigen presentation and leads to a suppressed response by the T cells (Ullrich, 1999). The result of the UV irradiation on the function of the dendritic cell is suppressed secretion of IL-12. Antigen presenting cells isolated from lymphoid organs of UV irradiated mice fail to present antigen to TH1 clones (Ullrich 1994) (See Figure 2.)

Because both UV radiation and jet fuel induce PGE_2 (Shreedhar, Giese et al. 1998; Ullrich and Lyons 2000) secretion, the second hypothesis tested here states jet fuel induced immune suppression results from PAF activation and the cascade of events that PAF upregulation activates. The specific aims of this research are

1) Does JP-8 suppress established immune reactions in mice?
2) Does Jet A suppress established immune reactions in mice?
3) What are the mechanisms involved?

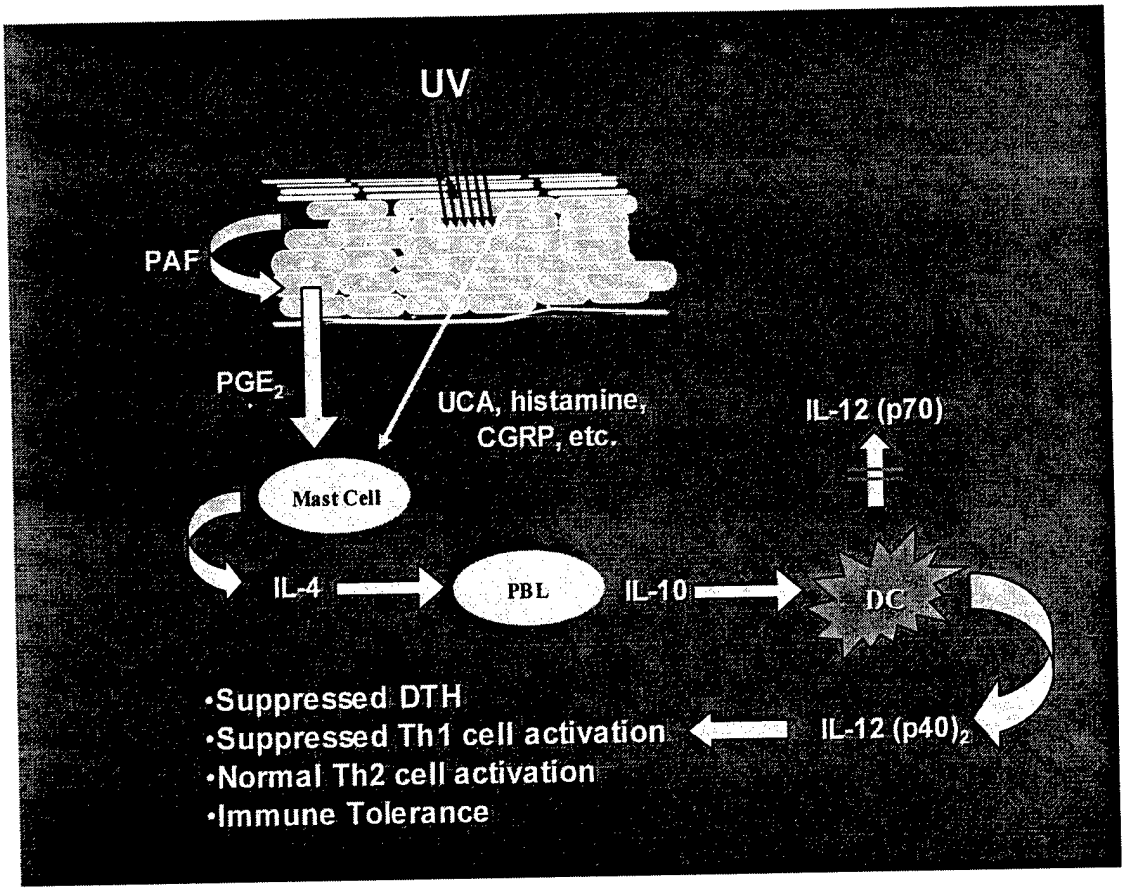

Figure 2. Mechanism of UV Induced Suppression of DTH

Results

Suppression of immunological memory by JP-8 and Jet-A.

In the first set of experiments we tested the hypothesis that dermal application of jet fuels suppresses immunological memory. Adult female C3H/HeN mice were immunized with *C. albicans*, on day 0, and boosted with antigen on day 7. Thirty days after the initial immunization, the mice were challenged with *Candida* extract. Delayed type hypersensitivity was measured 18 to 24 hours later. Two different protocols were used to determine if jet fuel treatment suppresses immunological memory. In the first, a single dose of jet fuel was applied (day 29). In the second, the mice were treated with jet fuel on four successive days (days 26 through 29). The data from this experiment are found in Figure 3.

In Fig. 3A, the effect of a single application of jet fuel is shown. Mice were treated with 100 to 300 µl of undiluted JP-8, or Jet-A, 29 days post immunization. Significant ($p < 0.02$ versus the positive control) and substantial (50 to 70%) immune suppression was observed when 200 to 300 µl of JP-8 or Jet-A were applied to the skin of immunized mice.

Next we wished to determine if smaller does of JP-8 and/or Jet-A, given repeatedly over a period of time, would suppress immunological memory. Mice were treated with 25 to 100 µl of jet fuel on 4 successive days immediately prior to antigenic challenge. From the data presented in Figure 3B, it is apparent that repeated exposure to small doses of jet fuel will activate immune suppression. In Figure 3A, a single exposure to 100 µl of JP-8 or Jet-A was not immune suppressive. However, when as little as 25 µl was given over 4 successive days, substantial (52% suppression JP-8; 73% suppression Jet-A) and

significant (JP-8 $p = 0.016$; Jet-A $p = 0.0027$, versus the positive control) immune suppression was observed. When smaller amounts of JP-8 or Jet-A were applied (i.e., 10 µl over 4 days), no immune suppression was observed (data not shown). These data indicate that military jet fuel (JP-8) can suppress immunological memory. They also indicate, for the first time, that dermal application of commercial jet fuel (Jet-A) suppresses the immune response.

Does prostaglandin E_2 production contribute to jet fuel induced immune suppression?

Previously, we reported that the induction of biological response modifiers by JP-8, particularly prostaglandin E_2 (PGE_2) play a critical role in jet fuel induced immune suppression. Furthermore, the use of drugs that suppress PGE_2 secretion *in vivo*, such as selective cyclooxygenase-2 inhibitors, block immune suppression in JP-8-treated mice (Ullrich and Lyons 2000). To determine if similar mechanisms are involved in suppressing immunological memory the following experiments were done. Mice were immunized with *C. albicans* as described above and treated with 200 µl of JP-8 on day 29. Two hours prior to the jet fuel treatment the selective cyclooxygenase-2 inhibitor, SC 236 was diluted in phosphate buffered saline and injected into the peritoneal cavity. The effect that the selective cyclooxygenase-2 inhibitor has on JP-8-induced immune suppression is found in Figure 4A. Compared to the positive control, applying JP-8 significantly suppresses the memory response ($p = 0.0001$; 83% immune suppression). Injecting SC 236 blocks immune suppression, in a dose-dependent fashion. At the lowest dose used (0.2 µg) we saw no effect.

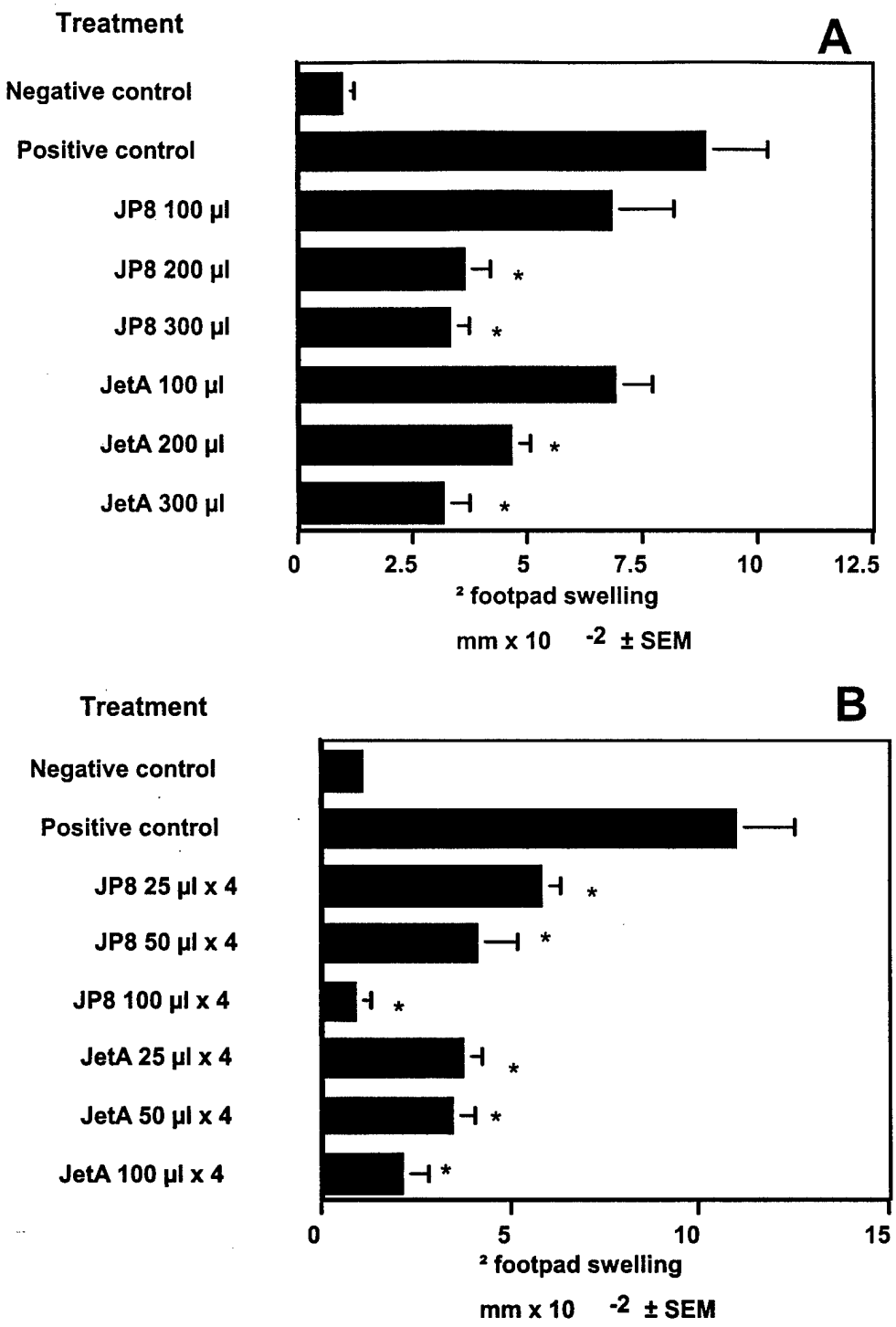

Figure 3. Jet fuel exposure suppresses immunological memory

Figure 3. Jet fuel exposure suppresses immunological memory. Mice were immunized with *C. albicans* on day 0, again on day 7, treated with JP-8 or Jet-A on day 29 (panel A) or on days 26 through 29 (panel B), and challenged with antigen on day 30. * Significant difference ($p < 0.02$; two-tailed Student's t-test, $N = 5$) versus the positive control.

When, however, the JP-8-treated mice were injected with higher doses of SC 236, the amount of immune suppression observed was diminished. When the JP-8-treated mice received 1 μg of SC 236 less immune suppression was noted (44%). Moreover, although the magnitude of the DTH reaction observed in these mice was still significantly different from that observed in the positive control ($p = 0.02$), there was a significant difference between the response found in mice treated with JP-8 and SC 236 and mice treated with JP-8 only ($p = 0.01$). When the mice received 2 μg of SC 236, prior to JP-8 treatment, the magnitude of the DTH reaction observed was indistinguishable from that found in the positive control ($p = 0.136$). These data imply that JP-8-induced PGE_2 production plays a critical role suppressing immunological memory.

Next we wanted to determine whether injecting SC 236 would block Jet-A induced immune suppression. The effect that the selective cyclooxygenase-2 inhibitor has on Jet-A induced immune suppression is found in Figure 4B. In this experiment, the mice were treated with Jet-A once, 29 days post immunization (Jet A 1x) or 4 times on 26 through 29 days post immunization (Jet A 4x). Two hours prior to each Jet-A treatment, the mice received an intraperitoneal injection of SC 236. The magnitude of the DTH reaction observed in mice that received a single injection of SC 236 (0.2, 1.0 or 2.0 μg/mouse) prior to application of 200 μl Jet-A, was not significantly different from the response found in the positive control, indicating a total reversal of immune suppression. A similar situation was found after multiple Jet-A treatments (4x). Although the magnitude of the DTH reaction observed in mice receiving 0.2 and 1 μg of SC 236 was still significantly different from that observed in the positive control ($p = 0.04$), there was a significant difference between the response found in mice simply treated with Jet-A and

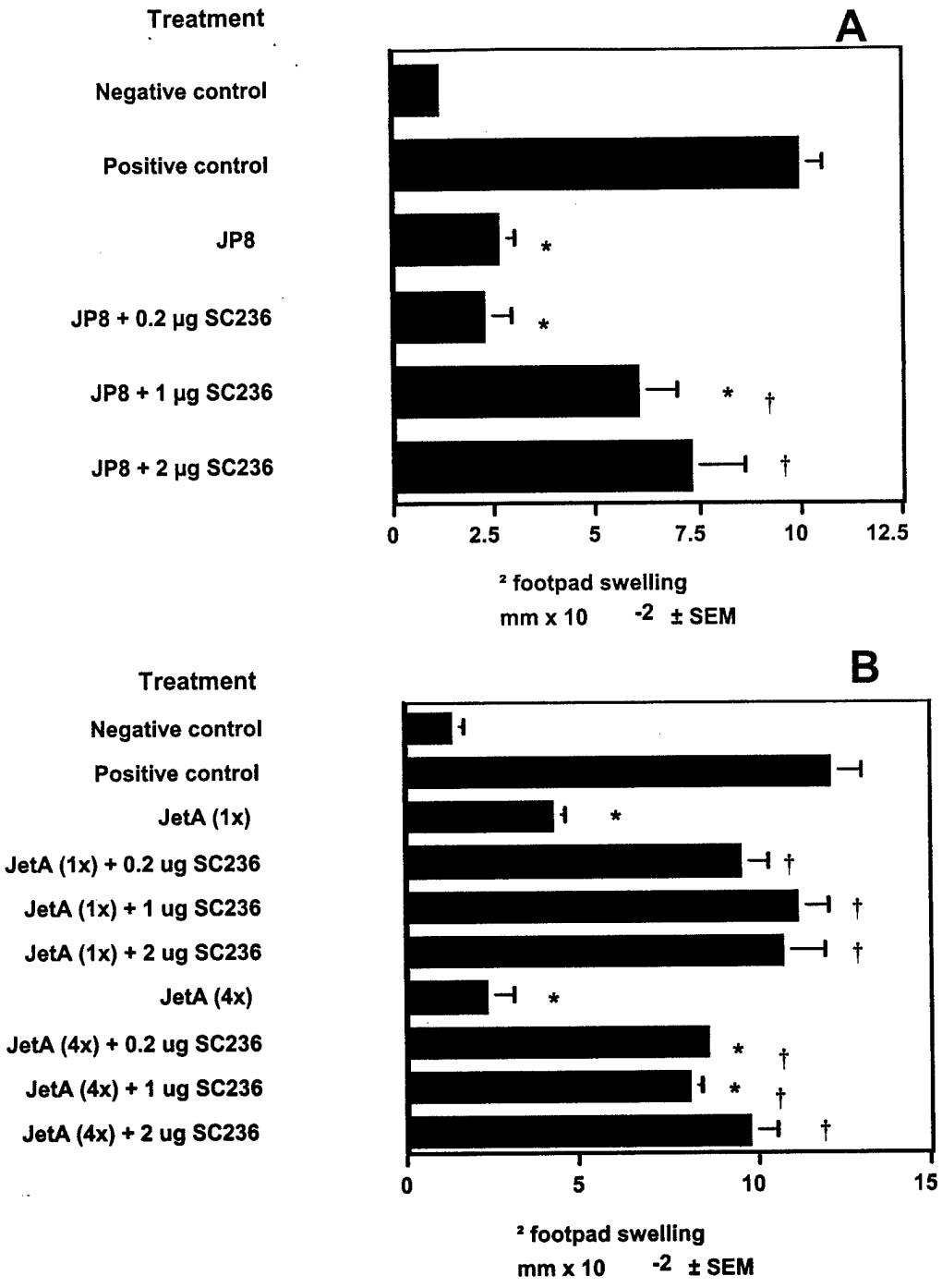

Figure 4. A selective cyclooxygenase-2 inhibitor blocks jet fuel-induced suppression of immunological memory

Figure 4. A selective cyclooxygenase-2 inhibitor blocks jet fuel-induced suppression of immunological memory. Mice were immunized with *C. albicans* on day 0, again on day 7, and treated with 200 µl of JP-8 on day 29. Two hours prior to JP-8 treatment the mice were injected with SC 236 (panel A). In panel B, immunized mice were treated with 200 µl of Jet-A on day 29 (1x) or on days 26 through 29 (4x). Two hours prior to each Jet-A treatment, the mice were injected with SC 236. * Significant difference ($p < 0.02$; two-tailed Student's t-test, $N = 5$) versus the positive control. † Significant difference ($p < 0.004$; two-tailed Student's t-test, $N = 5$) versus the jet fuel only control.

mice treated with Jet-A and injected with SC 236 ($p = 0.0006$). At the highest dose of SC 236 used (2 μg), the magnitude of the DTH reaction generated was not significantly different from that observed in the positive control, demonstrating total reversal of immune suppression. These data imply that Jet-A-induced PGE_2 production plays a critical role suppressing immunological memory.

Jet fuel treatment suppresses the elicitation of DTH to C. albicans.

The focus of our studies is to determine the effect of jet fuel exposure on established immune reactions. Another model system that is often used to measure the effect of immunosuppressive agents on an established immune response is the suppression of the elicitation of DTH (Magee, Kripke et al. 1989; Moyal, Courbière et al. 1997; Damian, Halliday et al. 1998; Nghiem, Kazimi et al. 2001). In these experiments mice are immunized with *C. albicans* on day 0, treated with jet fuel on days 6 through 9, challenged on day 10 and DTH is measured on day 11. Dose response curves for jet fuel-induced suppression of the elicitation of DTH are shown in Figure 5.

The results from these experiments are very similar to the findings presented above. Both JP-8 and Jet-A, applied after immunization, suppress DTH in a dose dependent fashion. Furthermore, as demonstrated previously, repeated exposure to small doses of jet fuel over 4 successive days will activate immune suppression.

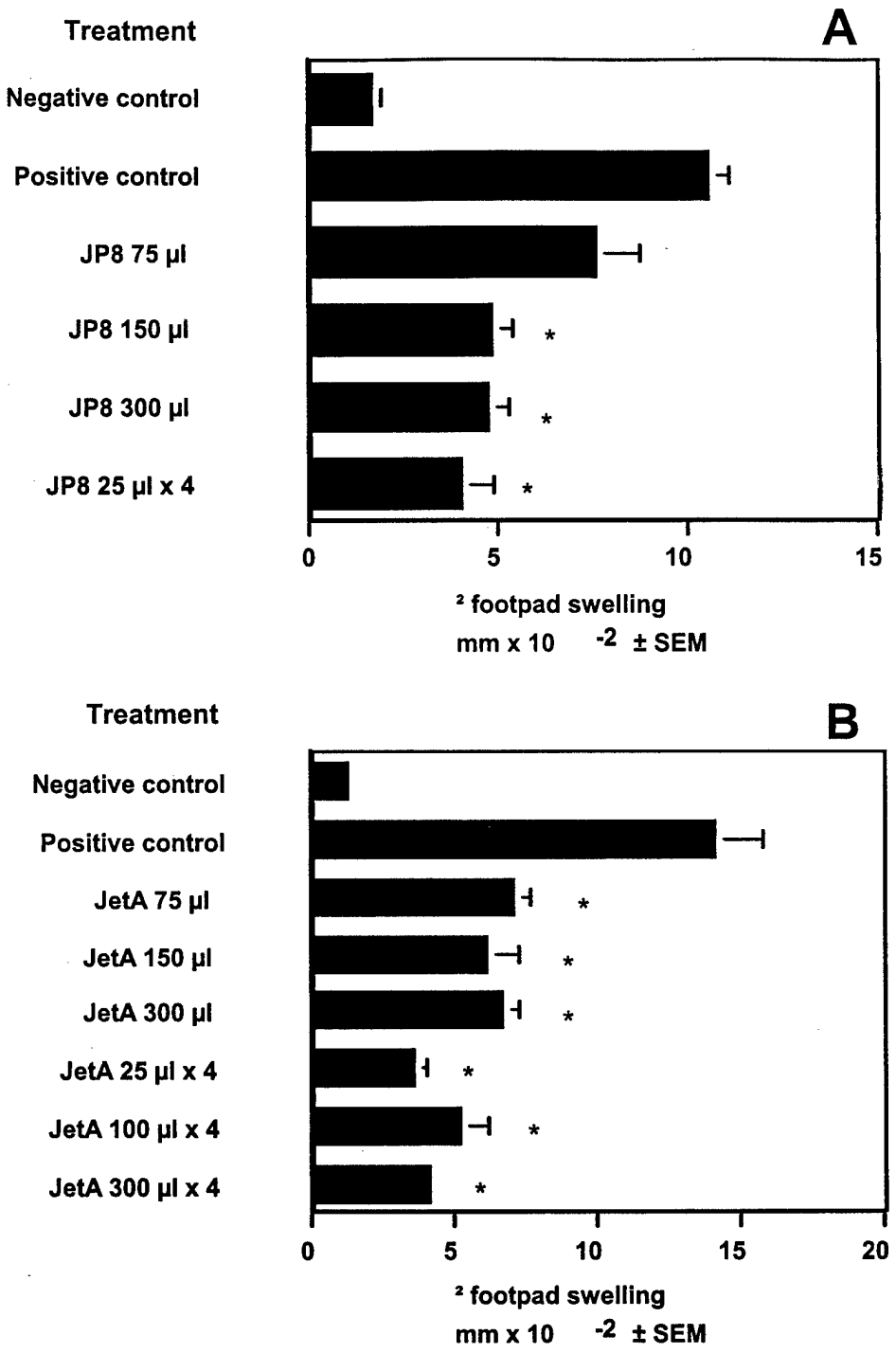

Figure 5. Jet fuel exposure suppresses the elicitation of DTH to C. albicans

Figure 5. Jet fuel exposure suppresses the elicitation of DTH to C. albicans. Mice were immunized with antigen on day 0, treated with JP-8 (panel A) or Jet-A (panel B) on day 9 (single exposure) or days 6 through 9 (multiple exposures). The mice were challenged with *Candida* antigen on day 10 and DTH was measured on Day 11. * Significant difference ($p < 0.008$; two-tailed Student's t-test, $N = 5$) versus the positive control.

The mechanism for suppressing the elicitation of DTH by jet fuel also appears to be similar to that described above (Figure 6). We find that administration of SC 236 blocks immune suppression. For example, we observed a significant difference between the magnitude of the DTH reaction found in JP-8 treated mice († $p < 0.006$) versus that found in JP-8-treated mice receiving 2 μg of SC 236. This effect was noted regardless of whether a single dose of JP-8 was applied (JP-8 1x), or the JP-8 was given over 4 successive days (JP-8 4x; Figure 6A).

Injecting the selective cyclooxygenase-2 inhibitor reversed Jet-A-induced immune suppression. In this case also, there was a significant difference between the DTH reactions found in mice exposed to a single Jet-A treatment versus the reaction observed in mice treated with Jet-A and injected with 1 or 2 μg of SC 236 († $p < 0.05$; Figure 6B). Similarly, when SC 236 was given prior to each Jet A treatment (Jet-A 4x) there was a significant difference between the response found in Jet-A treated mice and mice treated with Jet-A and injected with SC 236 († $p < 0.05$; Figure 6B). These data indicate that similar mechanisms (i.e., PGE_2 production) are involved in jet fuel-induced suppression of the elicitation of DTH and the suppression of immunological memory.

One of the reasons we were interested in determining whether repeated exposure to jet fuels induces immune suppression is because this dosing schedule mimics what is found in the field. During the normal course of their duties, engine mechanics, fuel handlers and fuel tank maintenance personnel are repeatedly exposed to jet fuel (Pleil, Smith et al. 2000). One strategy that we propose for reducing risk due to jet fuel exposure, beside simple avoidance, is the use of cyclooxygenase inhibitors. In all of our previous experiments we gave SC 236 immediately prior to each jet fuel treatment.

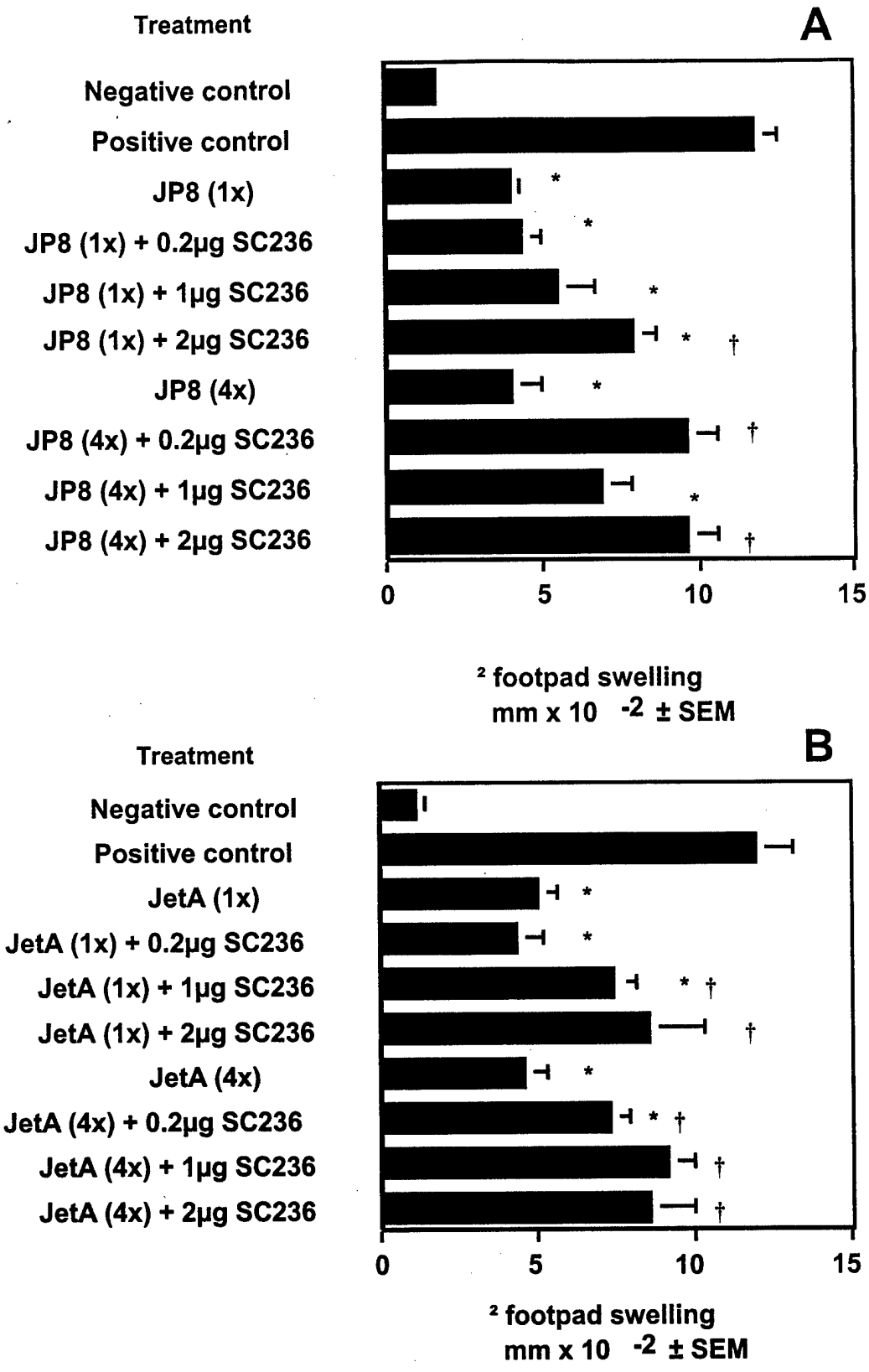

Figure 6. Blocking PGE_2 synthesis *in vivo* blocks the suppression of the elicitation of DTH by jet fuel

Figure 6. Blocking PGE_2 synthesis *in vivo* blocks the suppression of the elicitation of DTH by jet fuel. Mice were immunized with *C. albicans* on day 0, and treated with 200 µl of JP-8 on day 6 (1x) or days 6 through 9 (4x). Two hours prior to each JP-8 treatment the mice were injected with SC 236 (panel A). In panel B, immunized mice were treated with 200 µl of Jet-A on day 6 (1x) or on days 6 through 9 (4x). Two hours prior to each Jet-A treatment the mice were injected with SC 236. * Significant difference ($p < 0.02$; two-tailed Student's t-test, N = 5) versus the positive control. † Significant difference ($p < 0.05$; two-tailed Student's t-test, N = 5) versus the jet fuel only control.

We wanted to determine whether taking the cyclooxygenase-2 inhibitor once, prior to a short course of jet fuel exposure, or once at the end of a short course of jet fuel exposure, would be sufficient to reverse the immunosuppressive effect. To answer these questions 4 groups of mice were exposed to JP-8 (200 μl/treatment; 6 through 9 days post immunization). The first group received no further treatment. The second group of mice was injected with 0.8 μg of SC 236 on day 6 two hours before the first jet fuel exposure. The third group of mice received 0.2 μg of SC 236 on days 6, 7, 8, and 9, similar to what was done above. The fourth group of mice received 0.8 μg of SC 236 2 hours after the last jet fuel exposure. Data from such an experiment are found in Table 2.

As shown previously, repeated treatment with jet fuel, 6 to 9 days post immunization suppresses DTH. Also as indicated above, administering 0.2 μg of SC 236 immediately before each jet fuel treatment reversed immune suppression. The DTH response found in mice treated with jet fuel and given SC 236 before each treatment was significantly different from the response found in the jet fuel-only-treated mice ($p = 0.0006$). When an equivalent amount of SC 236 (0.8 μg) was injected once into the mice on day 6, prior to the start of multiple jet fuel treatments, the degree of immune suppression was reduced (67 vs. 49%) slightly. However, the DTH reaction found in mice given SC 236 only once prior to a short course of jet fuel treatment mice was still significantly different from the positive control ($p = 0.0006$) and not significantly different from the response found in the jet fuel-treated animals ($p = 0.163$). Similarly, injecting 0.8 μg of SC 236 on day 9, after a short course of jet fuel-treatment, reversed the suppressive effect to a degree (56 vs. 67%). Here also, however, the response found in mice given one dose of SC 236 after jet fuel-treatment was still significantly different ($p = 0.0018$) from the

Table 2. Optimal timing for administration of SC 236 to reverse immune suppression after repeated exposure to jet fuel.

Treatment [a]	SC 236 [b]	Δ footpad [c] Swelling	Specific [d] Swelling	% [e] suppression	*p* vs. PC [f]	*p* vs. JP-8 [g]
Negative control	-	0.7 ± .37	-	-	.0001	.0001
Positive control	-	12.5 ± .63	11.8	-	-	.0001
JP-8	-	4.6 ± .79	3.9	67	.0001	-
JP-8	before	6.7± .86	6	49	.0006	.163
JP-8	during	10.1 ± .77	9.4	20	.042	.0006
JP-8	after	5.9 ± 1.3	5.2	56	.0018	.625

Table 2. Optimal timing for administration of SC 236 to reverse immune suppression after repeated exposure to jet fuel.

a. Mice were immunized with *C. albicans* on Day 0, treated with JP-8 on days 6 through 9, and challenged with antigen on day 10. DTH was measured 18 to 24 hours post challenge. Negative control refers to mice that were not immunized but were challenged; positive control refers to mice that were immunized and challenged.

b. Mice received one dose (0.8 μg) of SC 236 on day 6 (before), 0.2 μg of SC 236 on days 6 through 9 (during), or one dose (0.8 μg) of SC 236 on day 9 (after).

c. mm x 10^{-2} ± SEM; N = 5.

d. Change in footpad swelling of the positive control or experimental groups minus the background swelling found in the negative control.

e. % immune suppression = 1-(specific footpad swelling of the experimental groups ÷ specific footpad swelling of the positive control) x 100

f. *p* values determined by two-tailed Student's t-test vs. the positive control

g. *p* values determined by two-tailed Student's t-test vs. the JP-8-treated control

positive control and not significantly different ($p = 0.625$) from the response found in the jet fuel-treated mice. These data indicate that taking a single prophylactic dose of SC 236, before anticipated jet fuel exposure, or taking the cyclooxygenase-2 inhibitor after a short course of jet fuel exposure does not restore the DTH reaction in JP-8-treated mice. Total reversal was only observed when the SC 236 was given before each jet fuel treatment. In a separate experiment, we used a higher dose of SC 236 (2.0 μg/mouse) and obtained identical results (data not shown).

PAF suppresses Delayed-type Hypersensitivity in vivo

Next we wanted to test the hypothesis that PAF is involved in the jet fuel induced suppression. We have shown that by injecting a selective COX-2 inhibitor the suppression can be completely abrogated. Since others have shown that PAF induces COX-2 and PGE_2 and we have shown that these are involved in immune suppression we tested the hypothesis that PAF activates immune suppression. Since PAF is not very stable we used carbomyl-PAF (cPAF). cPAF is metabolically stable and will not degrade as quickly . We injected cPAF intraperitoneal using the identical DTH mouse model as above, but instead of painting mice with jet fuel they were injected with the PAF analog. As can be seen in Fig. 7 cPAF induced significant immune suppression in a dose-dependent manner. As you can see the immune suppression observed when the mice were injected with 10 pmol and 100 pmol was almost identical to the suppression seen with 300 ul of the jet fuel. This data indicates that PAF induces immune suppression and that it is similar to the immune suppression induced by the jet fuels.

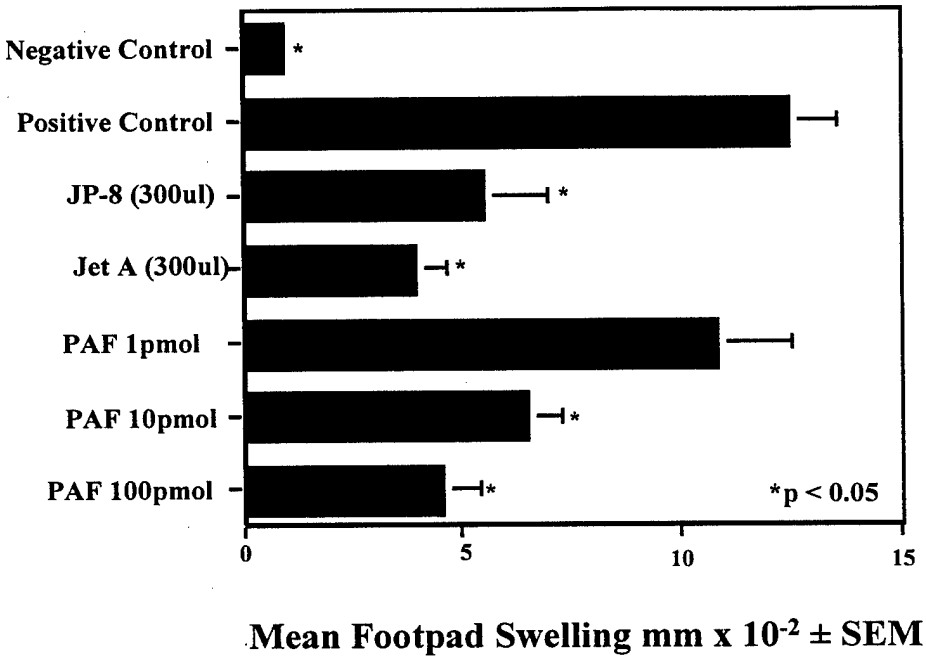

Figure 7. PAF suppresses the elicitation of DTH. Mice were painted with JP-8, Jet A or were injected with cPAF (1-100 pmol) on day 9 after immunization with C. *albicans*. On day 10 the mice were challenged with *Candida* antigen. DTH was measured 24 h after challenge. The background response (negative control) was measured in mice that were not immunized but were challenged. The positive control was measured in mice that were immunized and challenged. Results are expressed as means ± SEM. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the positive control (two-tailed Student's t test, $n = 5$). A representative experiment is shown; this experiment was repeated three times with similar results.

Jet Fuel Induced Immune Suppression is Blocked by a PAF Receptor Antagonist PCA-4248

The previous data indicates that PAF can mimic jet fuel and suppress DTH. To further evaluate the involvement of PAF in the jet fuel induced suppression we used a PAF receptor antagonist to block the PAF signaling. PCA-4248 was chosen because it is reported to be a specific PAF receptor antagonist and was commercially available. Mice were first injected with various doses of PCA-4248 in PBS. The mice were then painted with jet fuel as described above. As seen in Fig. 8, as the concentration of the drug increased so did the abrogation of the jet fuel induced suppression. When JP-8 treated mice were injected with greater than 50 nmol, the DTH response observed was indistinguishable from the positive control (Panel A, $P<0.05$). Similarily PCA-4248 treatment (1000 nmol) totally abrogated Jet A induced immune suppression (Panel B, $P < 0.05$). These data indicated that blocking the PAF receptor signal blocks jet fuel induced immune suppression.

Effects of the PAF Receptor Antagonist on DTH

It was important to determine if the PAF receptor antagonists by themselves had immune regulatory effects. Therefore, we acquired three other structurally diverse PAF receptor antagonists and tested their immunologic properties. As seen in Figure 9, there was no statistical difference between the responses of PCA-4248, CV-3988 and Oct Br when compared to the positive control ($P < 0.05$). Although there is a statistical difference between the response of dioxolane and the positive control there were no statistical difference when the antagonist were compared amongst each other ($P=0.1919$,

ANOVA) These data indicate that the structurally diverse PAF receptor antagonist do not inherently suppress DTH.

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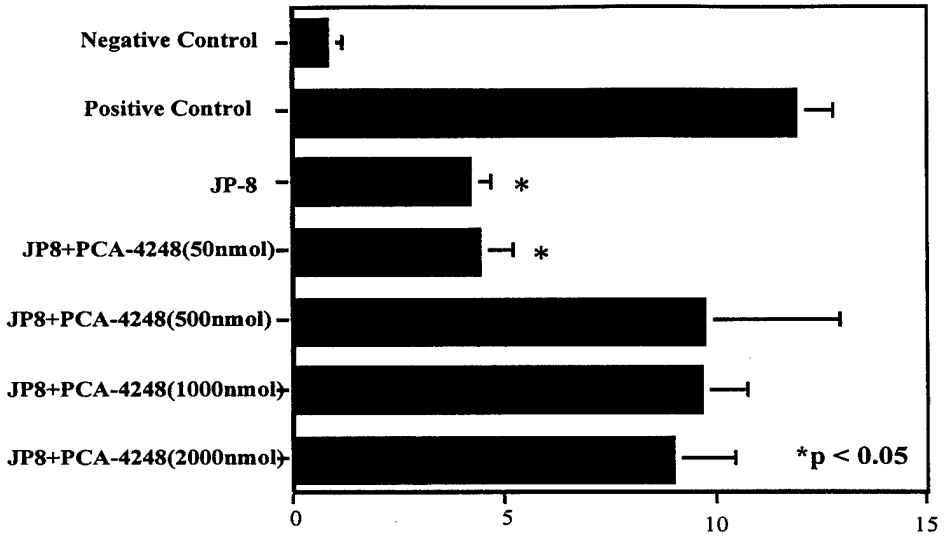
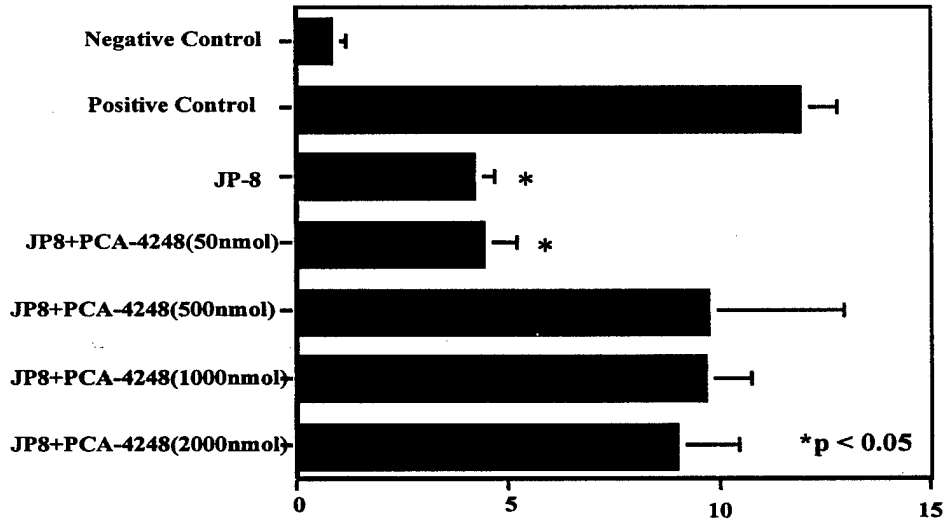


Mean Footpad Swelling mm x 10^{-2} ± SEM

B



Mean Footpad Swelling mm x 10^{-2} ± SEM

Figure 8. Reversal of Jet fuel Induced Immune Suppression by PAF Receptor

Antagonist PCA-4248.

Figure 8. Reversal of Jet fuel Induced Immune Suppression by PAF Receptor Antagonist PCA-4248. Mice were immunized with C. *albicans* on day zero. On day 9 the mice were painted with JP-8 (A) or Jet A (B) in the presence or absence of PCA-4248 (50-2000 nmol). On day 10 the mice were challenged with *Candida* antigen. DTH was measured 24 h after challenge. The background response (negative control) was measured in mice that were not immunized but were challenged. The positive control was measured in mice that were immunized and challenged. Results are expressed as means ± SEM. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the positive control (two-tailed Student's t test, $n = 5$). A representative experiment is shown; this experiment was repeated three times with similar results.

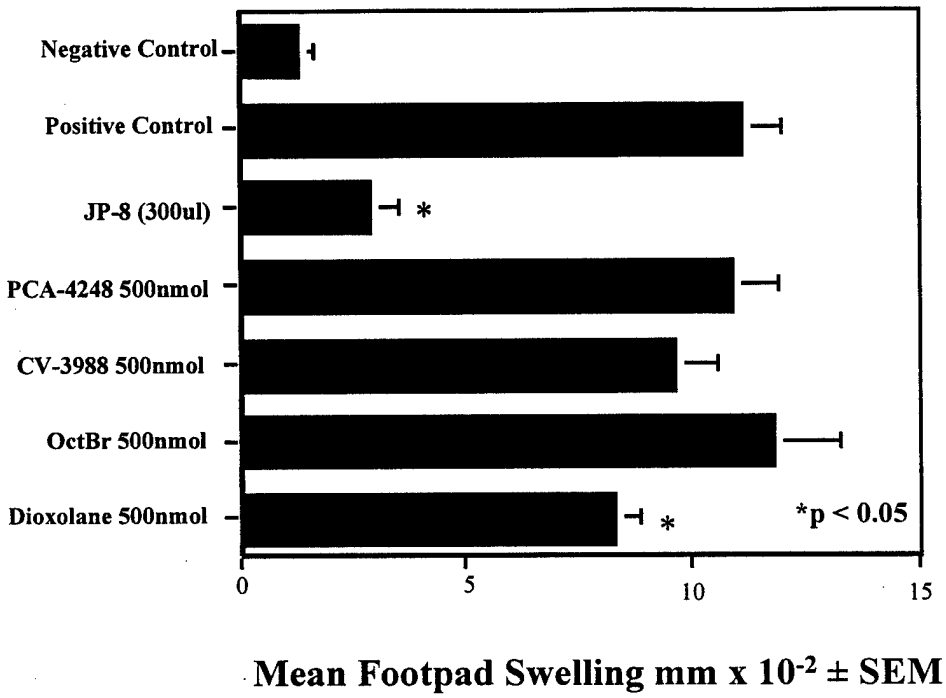


Figure 9. Effects of PAF Receptor Antagonist on DTH

Mice were immunized with C. *albicans* on day zero. On day 9 one group of mice was painted with JP-8 without any antagonist and the other groups were injected with PCA-4248, CV-3988, Octylonium Bromide and Dioxolane at 500 nmol and not treated with JP-8. On day 10 the mice were challenged with *Candida* antigen. DTH was measured 24 h after challenge. The background response (negative control) was measured in mice that were not immunized but were challenged. The positive control was measured in mice that were immunized and challenged. Results are expressed as means ± SEM. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the positive

control (two-tailed Student's t test, n = 5). A representative experiment is shown; this experiment was repeated three times with similar results.

Other PAF Receptor Antagonists Block the Jet Fuel Induced Suppression

Our data indicates that jet fuel immune suppression is mediated by PAF due to the ability of the antagonist PCA-4248 to inhibit the suppression. To ensure that inhibition of the suppression is not unique to PCA-4248 and to confirm the receptor's role in the induction of suppression, we used the other three structurally diverse known PAF receptor antagonist. As introduced before, they are CV-3988, Octylonium bromide, and dioxolane. Groups of mice were injected intra-peritoneal with 500 nmol of CV-3988, Octylonium bromide, and dioxolane and then painted with one dose of JP-8 or Jet A (300 ul) 2 hours after the drug administration. All the PAF receptor antagonists blocked the jet fuel induced suppression, JP-8 Fig. (10A) and Jet A Fig (10B). In fact, the DTH response observed when the mice were treated with jet fuel and treated with PAF receptor antagonist was indistinguishable from the positive control ($P < 0.05$). These results suggest the reversal of jet fuel induced suppression is not unique to PCA-4248 and can be accomplished by other structurally diverse receptor antagonists. These data supports the hypothesis that PAF receptor signaling is required for jet fuel induced suppression.

PAF, JP-8 and Jet A Induced Suppression reversed by PAF Receptor Antagonist PCA 4248

We have observed that a PAF analog suppresses DTH in vivo and we have also seen that the response suppressed by the JP-8 and Jet A jet fuel can be reversed by PAF receptor antagonists. To further evaluate the hypothesis that the PAF and jet fuel

suppression was through a similar mechanism we treated groups of mice with PCA-4248 with subsequent PAF and jet fuel treatments accordingly. The PAF induced suppression was also reversed with treatment with PCA-4248 (Fig. 11). In the groups treated with the antagonist the suppression again was totally abrogated and was indistinguishable from the positive control in the PAF and the jet fuel groups ($P < 0.05$). This data indicates that the mechanism of immune suppression induced by either the PAF analog and the jet fuel is similar. It also further supports the involvement of the PAF receptor in the jet fuel induced suppression.

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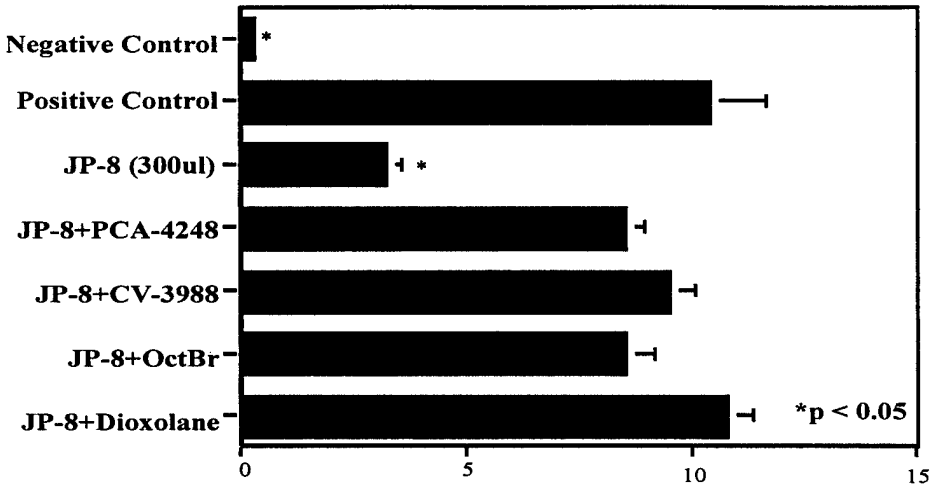
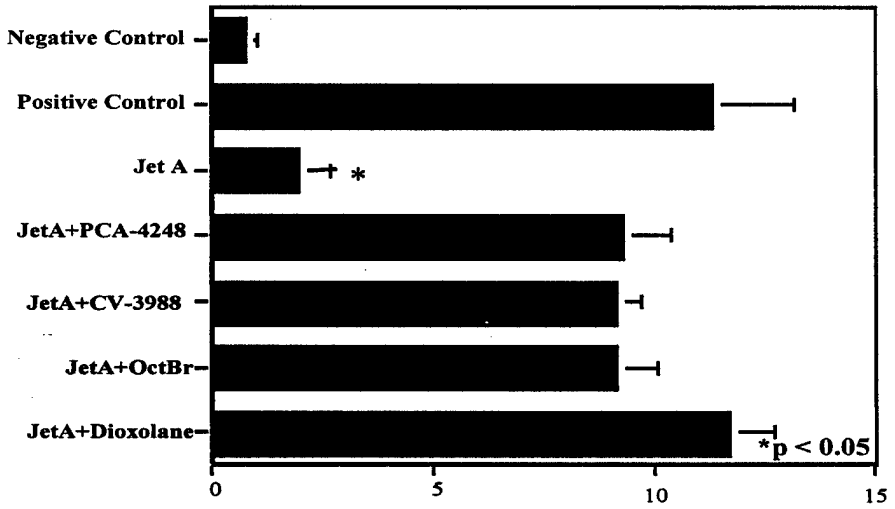


Mean Footpad Swelling mm x 10^{-2} ± SEM

All PAF Receptor Antagonists at 500nmol; All JP-8 treatments at 300ul

B



Mean Footpad Swelling mm x 10^{-2} ± SEM

All PAF Receptor Antagonists at 500nmol; All Jet A treatments at 300ul

Figure 10. Jet Fuel Induced Suppression reversed by other PAF receptor

Antagonists

Figure 10. Jet Fuel Induced Suppression reversed by other PAF receptor Antagonists

Mice were immunized with C. *albicans* on day zero. On day 9 the mice were painted with JP-8 (A) or Jet A (B) and were injected with PCA-4248, CV-3988, Octylonium Bromide, or Dioxolane (500 nmol) two hours before. On day 10 the mice were challenged with *Candida* antigen. DTH was measured 24 h after challenge. The background response (negative control) was measured in mice that were not immunized but were challenged. The positive control was measured in mice that were immunized and challenged. Results are expressed as means ± SEM. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the positive control (two-tailed Student's t test, $n = 5$). A representative experiment is shown; this experiment was repeated three times with similar results.

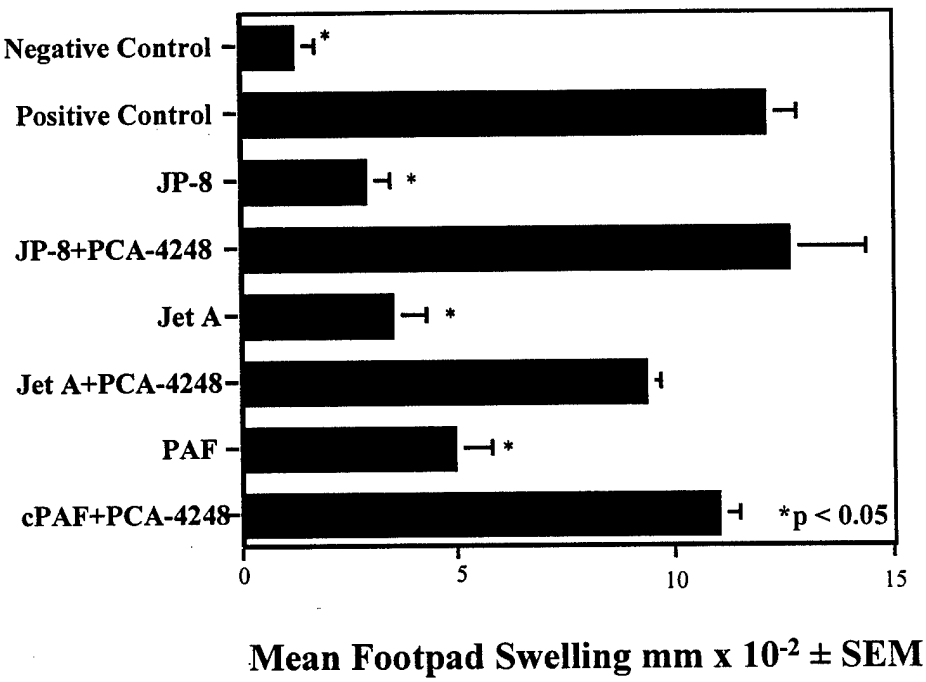


Figure 11. JP-8, Jet A and PAF Induced Suppression Reversed by PCA-4248
Mice were immunized with C. *albicans* on day zero. On day 9 the mice were painted with JP-8, Jet A or injected with cPAF and were injected with PCA-4248 at 500 nmol two hours before. On day 10 the mice were challenged with *Candida* antigen. DTH was measured 24 h after challenge. The background response (negative control) was measured in mice that were not immunized but were challenged. The positive control was measured in mice that were immunized and challenged. Results are expressed as means ± SEM. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the positive control (two-tailed Student's t test, $n = 5$). A representative experiment is shown; this experiment was repeated three times with similar results.

Role of Ant-Oxidants in the Jet Fuel Induced Suppression

The literature indicates that oxidative stress converts phosphatidylcholine into PAF-like molecules. Waltersheid et. al. exposed egg yolk phosphatidylcholine to UV irradiation and when this solution was injected into naive mice DTH was suppressed (Walterscheid, Ullrich et al. 2002). This suggested that UV activated an oxidative stress episode which converted phosphatidylcholine into a PAF like molecule. In the same experiment injecting the un-irradiated phosphatidylcholine did not result in suppression of DTH. To test the hypothesis that an oxidative stress episode was also involved in the jet fuel induced suppression we treated mice with the jet fuel and injected into the peritoneal cavity Vitamin C two hours before the jet fuel treatment. As is found in Figure 12 the mice that were injected with the Vitamin C two hours before the JP-8 treatment had a reversal of the suppression at the 500 nmol and the 5000 nmol concentrations ($P < 0.05$). In fact there was no statistical difference between the groups of mice injected with the two higher doses and the positive control ($P < 0.05$). These data indicates that oxidative stress plays a role in the jet fuel induced suppression and that anti-oxidant treatment blocked the suppression.

Role of Anti-Oxidants in Reversing JP-8 Induced Suppression

To further illustrate the involvement of oxidative stress in the suppression of DTH we tested the hypothesis that other relevant anti-oxidants would also reverse the suppression and that the reversal was not unique to Vitamin C. Rogers et. al. demonstrated the presence of oxidative species in rat skin exposed to JP-8 (Rogers, Gunasekar et al. 2001). In the next two experiments we used two additional anti-oxidants which included

Vitamin E and Beta-Hydroxy Toluene (BHT). Both abrogated the jet fuel suppression of DTH. As can be seen in Figure 13 panel A, the JP-8 treated groups injected with Vitamin C and Vitamin E response was not statistically different from the positive control ($P < 0.05$). We did notice a difference between the group of mice injected with BHT when compared to the positive control ($P < 0.05$). On the other hand panel B shows the DTH response is indistinguishable from the positive control with groups of mice treated with Jet A and injected with the Vitamin C, Vitamin E and BHT ($P < 0.05$). These findings indicate that jet fuel induced suppression is through a ROS activated pathway and that pre-treatment with several anti-oxidants abrogated the suppression.

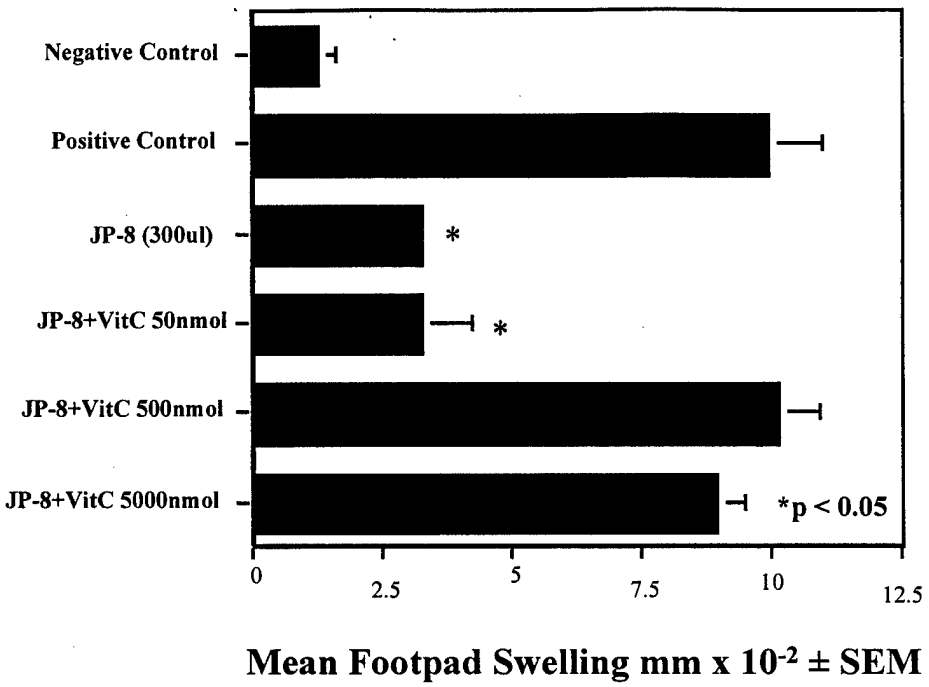


Figure 12. Vitamin C Dose Response Reversing JP-8 Induced Suppression.
Mice were immunized with C. *albicans* on day zero. On day 9 all the groups of mice were painted with 300 ul JP-8 plus or minus Vitamin C (50 – 5000 nmol). On day 10 the mice were challenged with *Candida* antigen. DTH was measured 24 h after challenge. The background response (negative control) was measured in mice that were not immunized but were challenged. The positive control was measured in mice that were immunized and challenged. Results are expressed as means ± SEM. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the positive control (two-tailed Student's t test, $n = 5$). A representative experiment is shown; this experiment was repeated three times with similar results.

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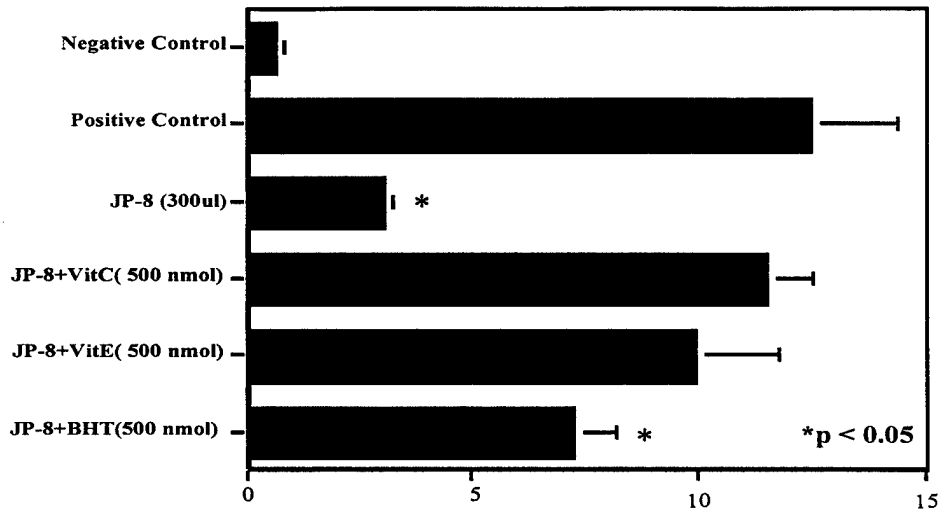
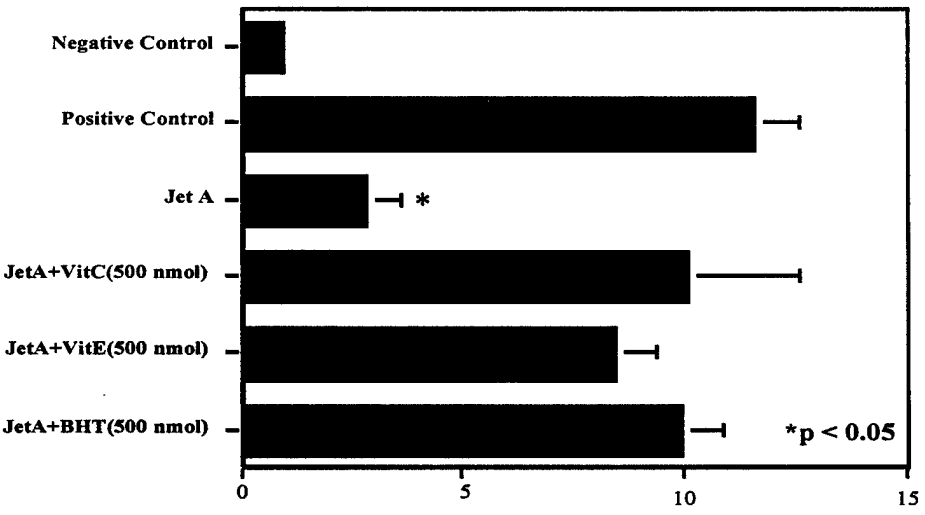


Mean Footpad Swelling mm x 10^{-2} ± SEM

-JP-8+VitC, JP-8+VitE, and JP-8+BHT statistically different from JP-8 group P<0.0124

-JP-8+VitC, JP-8+VitE, and JP-8+BHT not statistically different from each other P=0.1336

B



Mean Footpad Swelling mm x 10^{-2} ± SEM

Figure 13. Reversal of Jet Fuel Induced Suppression by Anti-Oxidant Treatment

Figure 13. Reversal of Jet Fuel Induced Suppression by Anti-Oxidant Treatment
Mice were immunized with C. *albicans* on day zero. On day 9 all the groups of mice were painted with 300 ul of JP-8 (A) or Jet A (B) and a dose of 500 nmol of Vitamin C, Vitamin E or BHT was injected via intra-peritoneal two hours before the jet fuel treatment. On day 10 the mice were challenged with *Candida* antigen. DTH was measured 24 h after challenge. The background response (negative control) was measured in mice that were not immunized but were challenged. The positive control was measured in mice that were immunized and challenged. Results are expressed as means ± SEM. An asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the positive control (two-tailed Student's t test, $n = 5$). A representative experiment is shown; this experiment was repeated three times with similar results.

JP-8 induced suppression of T-Cell Proliferation

As mentioned above Ullrich et. al. showed that jet fuel induced suppression did not affect antibody production (Ullrich and Lyons 2000). To test the hypothesis that jet fuel treatments in fact affected cellular immunity we purified T-cells from spleens of JP-8 treated mice and activated with plate-bound anti CD-3 and measured [^{3}H] thymidine incorporation. The response for jet fuel treated group was significantly suppressed when compared to the anti-CD3 control response ($P < 0.05$) (Figure 14). Also observed is the reversal of the suppression by treatment with the selective COX-2 inhibitor, although there is a significant difference between the jet fuel group with the COX-2 inhibitor and the anti-CD3 control indicating that the suppression was not totally abrogated ($P < 0.05$). These data indicates that another measure of cell mediated immunity is affected by jet fuel treatment and that it can partially be reversed by the treatment with COX-2 inhibitor.

Jet fuel induced suppression of MTT

To evaluate the effects that jet fuel has on cell viability we used an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay with murine keratinocytes. The MTT is cleaved by active mitochondria and forms a dark blue formazon product which can be solubilized in acidic isopropanol and absorbance read at 570 nm. MTT cleavage is directly proportional to cell viability. It provided a simple way of providing a dose response at which jet fuel treated cells are no longer viable. As you can see in Figure 15 there is a significant difference in the mid and highest dose of JP-8 (20 ug/ml) when compared to the cells that were not treated with jet fuel ($P < 0.05$), and not when compared to the lowest dose. Additionally, there is not a significant difference between the low (0.2 ug/ml) and mid dose of Jet A (2.0 ug/ml) when compared to the cells not

treated with jet fuel ($P < 0.05$). However the difference was significant between the higher does Jet A (20 ug/ml) when compared to the cells not treated with jet fuel. In fact, for the highest doses of JP-8 and Jet A there is a decrease in cell viability of 50% when compared to the cells with no jet fuel ($P < 0.05$). These data indicates that at the highest dose (20 ug/ml) the cells are significantly less viable. It suggests that if using this cell line of keratinocytes to perform jet fuel studies, a dose of 20 ug/ml will diminish cells function which could result in limitation of the assay.

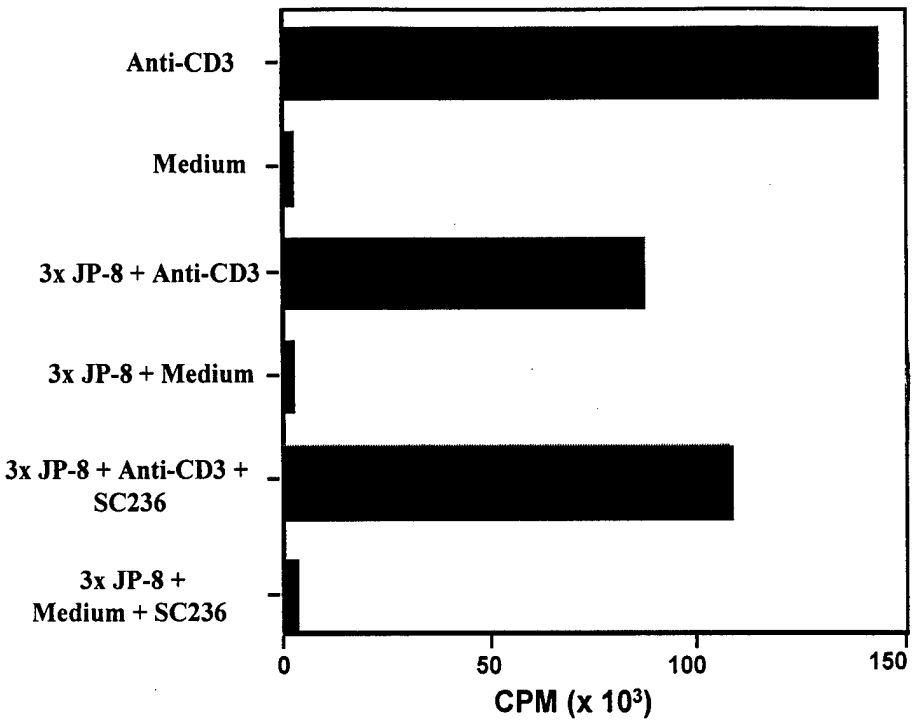


Figure 14. JP-8 Induced Suppression of T-cell Proliferation

Four days after multiple dermal applications of JP-8 (3 consecutive days 200 ul), spleens were removed, and T-cells were isolated by nylon wool filtration. 2 X 10^5 cells were stimulated *in vitro* with plate-bound anti-CD-3 antibody (10 ug/ml) for five days. Background responses were measured in wells containing cells with no antibody (medium). The positive control was T cells isolated from mice not treated with JP-8. T-cell proliferation was determined by incorporation of 3H-Thymidine.

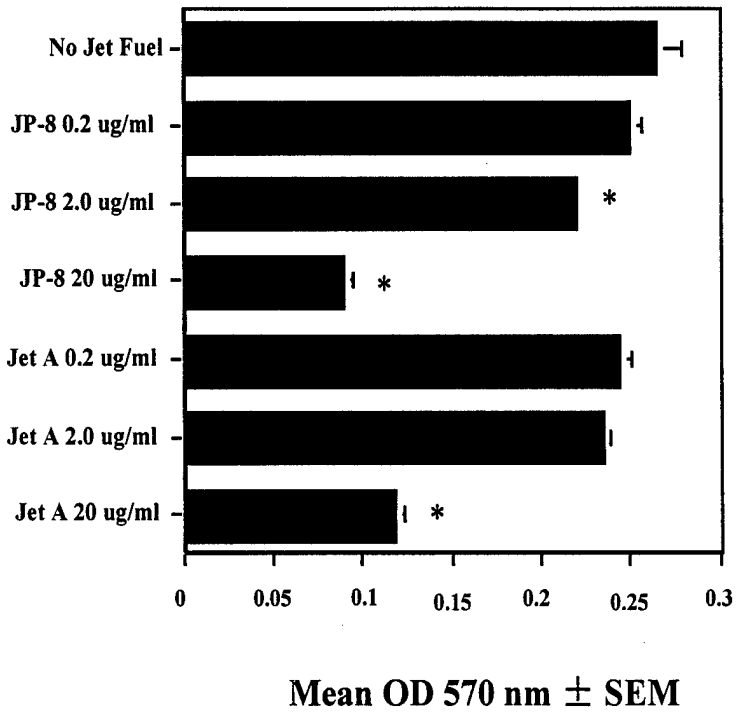


Figure 15. Effects of Jet Fuel on Cell Viability

Murine keratinocytes were plated $5x10^4$ cells/ml in triplicate on 24 well plates. Cells were treated with jet fuel on day 1 and harvested on day 2 with MTT solution. The precipitate was read at 570 nm in acidic isopropanol and a blank in acidic isopropanol was also measured at 570. For the control, cells were plated as described above but not treated with jet fuel. The data is expressed as mean absorbance ± SEM of three separate determinations. An * represents a statistically significant difference ($P < 0.05$) from the control (two-tailed Student's *t* test, $n = 3$)

JP-8 and Jet A DTH Suppression Dose response

Accumulation of DTH suppression data throughout the experiments permitted us to extrapolate a percentage suppression dose response curve for the DTH response. As seen in Figure 16 the JP-8 and Jet A percentage suppression curves are very similar. An observed threshold was noted at approximately the higher doses, after an 800 ul total dose of JP-8 and a 900 ul total dose of Jet A. These data indicates that the immune suppression between the two fuels in inherent in the basic kerosene components of the fuel and not dependent on the components added to make the military grade JP-8.

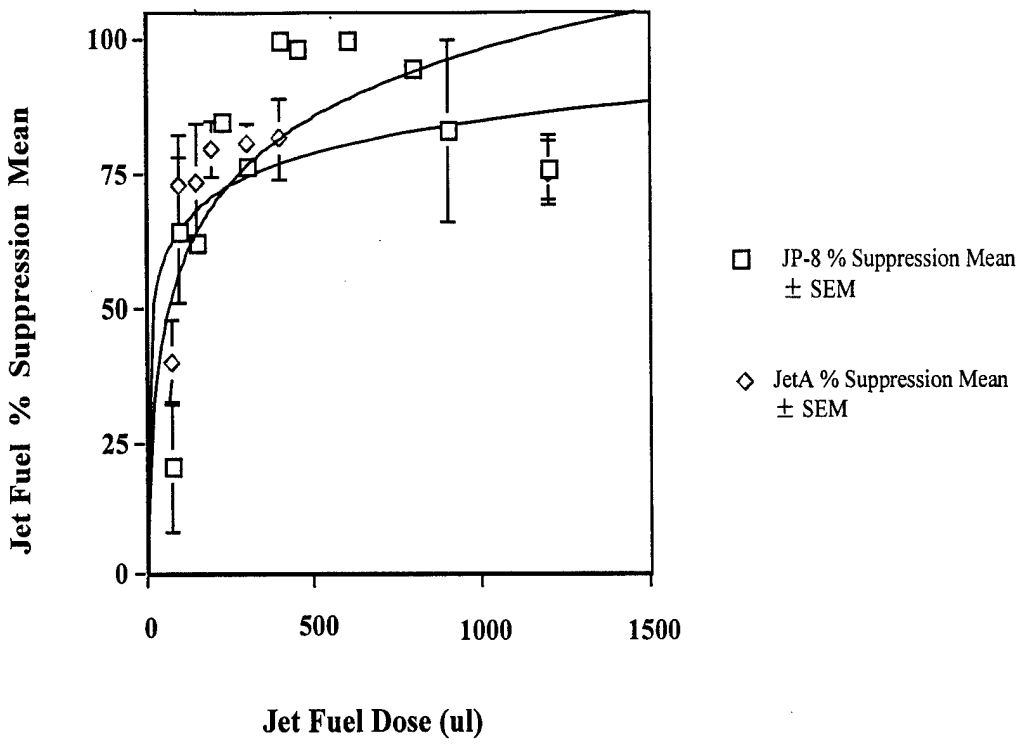


Figure 16. JP-8 and Jet A DTH Suppression Dose Response

Mice were immunized with C. *albicans* on day zero. On day 6 - 9 all the groups of mice were painted with single dose or multiple appropriate dose (50 - 1200 ul total dose) of JP-8 or Jet A. On day 10 the mice were challenged with *Candida* antigen. Data shown as mean percentage suppression ± SEM. Percentage immune suppression = 1-(specific footpad swelling of the experimental groups ÷ specific footpad swelling of the positive control) x 100.

Discussion

The data presented in this thesis expands on our initial observations that dermal exposure to JP-8 induces systemic immune suppression (Ullrich 1999; Ullrich and Lyons 2000), and introduces some novel findings. First, we demonstrate that applying JP-8 to the skin of mice will suppress established immune reactions, such as the elicitation of DTH and immunological memory. Whether jet fuel exposure suppresses ongoing immune reactions is an important question that deserves serious consideration. Perhaps the most successful public health campaign of the twentieth century was the use of wide scale vaccination to prevent morbidity and mortality due to infectious disease. If jet fuel exposure only suppresses the induction of immunity and has no effect on memory, it would suggest that fuel exposure would have little effect on the immune protection afforded by prior vaccination. The data presented here demonstrate that JP-8 exposure suppresses immunological memory and implies that it may decrease vaccine efficacy. They raise the concern that JP-8 exposure may weaken resistance to infectious agents. This observation may be particularly relevant in situations such as combat, where stress and fatigue, coupled with jet fuel-induced suppression may contribute to a weakened immune response.

The second novel observation presented here concerns the immunotoxicity of commercial jet fuel. To the best of our knowledge, this is the first report that dermal exposure to Jet-A induces immune suppression. This is an important finding for a number of reasons. First, it suggests that commercial airline fuel handlers, engine mechanics, fuel tank maintenance mechanics and flight line personnel should take every precaution to minimize dermal contact with Jet-A. Second, as mentioned above, military

JP-8 is essentially Jet-A plus three additives. Our finding that Jet-A by itself is immune suppressive, and the similar dose-response curves for Jet-A and JP-8-induced immune suppression, imply that the ability of JP-8 to induce immune suppression is not a function of the additive package, but is inherent in the base kerosene fuel, the so called straight run middle distillates of crude oil (Broddle, Dennis et al. 1996). Whether other straight run middle distillates induce immune suppression remains to be seen, but the possibility appears to be likely. Third, the other major route of JP-8 exposure is the respiratory tract. Studies by Harris and colleagues have clearly shown that pulmonary exposure to JP-8 is immune suppressive (Harris, Sakiestewa et al. 1997;). Our data suggest that pulmonary exposure to Jet-A should have the same consequence on immune function as pulmonary JP-8 exposure. Although this prediction needs to be confirmed in the future, the data presented here suggest that human dermal or pulmonary exposure to JP-8 and Jet-A, should be minimized.

Of course minimizing jet fuel exposure may not be easy. This is why understanding the mechanism underlying immune suppression is critical. In our previous studies we found that the activation of immune suppression by JP-8 was associated with the production of immune regulatory biological response modifiers (Ullrich and Lyons 2000). Although limited in scope, the experiments performed in this study shed some light on the mechanism underlying jet fuel-induced immune suppression. Suppressing PGE_2 production *in vivo*, with a selective cyclooxygenase inhibitor blocks the suppression of immunological memory and the suppression of the elicitation of DTH. This occurs regardless of whether Jet-A or JP-8 is applied to the skin of immunized mice. This suggests that similar mechanism(s) (i.e., cytokine production *in vivo*) are involved. In

addition, the dose-response curves for SC 236-induced reversal of Jet-A and JP-8-induced suppression of immunologic memory and/or suppression of the elicitation of DTH, are very similar. This also suggests that similar mechanisms are involved and reinforces the idea that the suppressive properties of military jet fuel are inherent in the base kerosene fuel. Also, we find that the dose-response curves for jet fuel (JP-8 and Jet-A) induced suppression of the elicitation of DTH, and jet fuel induced suppression of immunological memory are similar. This indicates that the shorter 11-day DTH assay can serve as a surrogate for the longer 31-day memory experiment. This is particularly attractive because there are future plans to use this assay as the read out for a series of in-depth experiments to determine the mechanisms by which dermal jet fuel exposure suppresses established immune reactions.

Another important aspect of the work presented here is the ability to block jet fuel-induced suppression of immunological memory *in vivo*. We maintain that minimizing human jet fuel exposure will be difficult, if not impossible. Be that as it may, the next best thing is to reverse the immunosuppressive effect. Our data indicates that jet fuel induced immune suppression is a prostaglandin dependent event. Blocking prostaglandin synthesis *in vivo* can be accomplished with cyclooxygenase inhibitors. Our findings suggest that administration of the selective cyclooxygenase inhibitor each time a dermal jet fuel exposure occurs, provides the best method to overcome immune suppression. Although not tested here, we suggest that aspirin will do the same. The real advantage of the selective cyclooxygenase-2 inhibitors is that their side effects are limited and they provide a better alternative for restoring immune function in aspirin-sensitive personnel.

Of course one of the critical questions that remains unanswered is whether jet fuel is a human immunotoxin. The proliferation of T cells to recall antigens *in vitro*, or measuring the DTH reaction *in vivo* after challenge with recall antigens are two methods that can be used to measure immune status in humans. For example the multitest kit, which is commonly used to measure DTH *in vivo* (Moyal 1998), measures the immune response to a variety of antigens initially given during childhood immunization, including tetanus and diptheria toxoids, *Streptococcus*, *Tuberculin*, *Candida*, *Trichophyton* and *Proteus* antigens. This removes the need of immunizing human volunteers to antigens not normally encountered, which is an absolute requirement if one wants to measure the effect of jet fuel on the induction of immunity. Immunizing human volunteers with new antigens after jet fuel exposure raises serious ethical concerns, concerns that may preclude approval by institutional review boards. Based on the data reported here, we suggest that testing immunological memory to recall antigens *in vivo* may be the best way to determine if Jet-A and/or JP-8 is a human immunotoxin.

The data reported here expands our previous findings by demonstrating that JP-8 exposure suppresses the elicitation of DTH and immunological memory. Moreover, we report for the first time, that dermal exposure to commercial jet fuel, Jet-A, suppresses the immune response. The mechanisms underlying Jet-A and JP-8 induced immune suppression appear to be identical. Our data indicate that JP-8 and Jet-A are immunotoxic and suggest that exposure to jet fuels should be minimized. However, in the likely event that jet fuel comes into contact with the skin, our findings demonstrate that drugs that block PGE_2 production *in vivo*, such as cyclooxygenase inhibitors, should be considered. Also, the data reported here suggest that measuring the effect of jet fuel

exposure on the response to recall antigens *in vivo*, may provide one method to determine whether jet fuels are human immunotoxins.

However, the most important findings expand on the mechanism. Because previous findings indicated that PGE_2 production was an early step in the cascade that leads to the immune suppression (Shreedhar, Giese et al. 1998) we tested that hypothesis with the jet fuel induced suppression. Because the jet fuel induced suppression is similar to the UV induced suppression we suggest the suppression is through a similar mechanism. It has been observed that UV treated keratinocytes secrete PAF (Calignano, Cirino et al. 1988; Sheng and Birkle 1995; Barber, Spandau et al. 1998). Although this has not been measured in jet fuel treated keratinocytes we have blocked the jet fuel induced suppression with a PAF receptor antagonist, suggesting PAF is involved in jet fuel induced suppression. Further, injecting PAF into mice mimics the jet fuel induced suppression and injecting the PAF receptor antagonist abrogates the PAF-induced suppression. These findings suggests that PAF and PAF-like molecules are potent inflammation mediators that activate immune suppression.

As described above the synthesis of PAF is through an enzymatic cleavage of the sn-2 side chain by PLA_2 and then an acetylation of the free hydroxyl. Also fatty acid side chains of phosphatidylcholine can be oxidized leaving a shortened acyl side chain at the sn-2 position (McIntyre, Zimmerman et al. 1999). This oxidized phospholipid by-product are the PAF-like molecules that can also bind and signal through the PAF receptor. This signal is amplified by inducing the production of PAF leading to production of arachidonic acid with subsequent synthesis of PGE_2 through a COX-2 enzymatic driven mechanism. Blocking the jet fuel induced suppression with PAF

receptor antagonist and a selective COX-2 inhibitor indicates the involvement of PAF production in jet fuel induced suppression.

It has been shown that jet fuel skin treatments induce the production of reactive oxygen species (Rogers, Gunasekar et al. 2001). An important pathway of PAF-like molecule production is through the degradation of phosphatidylcholine by reactive oxygen species. The data suggest that jet fuel induces free radical formation that targets cell membrane degradation which initiates the immune suppressive event. Gabrielli et. al. showed that after UV radiation genomic stress the cell must enter a G2/M cell cycle checkpoint which determines if the cell lives repairing its DNA or dies (Gabrielli, Clark et al. 1997). It has also been shown that MAPK activation is crucial in the initiation of the G2/M checkpoint after UV radiation (Bulavin, Higashimoto et al. 2001). MAPK activation is also involved in activation of PLA_2, which is the first step in PAF production (Lin, Wartmann et al. 1993). This suggest that after genomic stress a MAPK dependent mechanism may be involved in PAF synthesis. Although these studies have not been performed with jet fuel, the involvement of the PAF receptor has been elucidated by the studies described above. It has also been observed that a MAPK, p38, is upregulated in response to activated PLA_2 suggesting the involvement of a feedback amplification mechanism. Jet fuel is a lipophilic fuel agent that damages DNA (Grant, Jackman et al. 2001). We suggest that a side-effect of the oxidative stress caused by jet fuel exposure is immune suppression. The degradation of the phosphatidylcholine to a PAF-like molecule initiates a cascade in which this molecules bind to the PAF receptor which results in upregulation of PGE_2 which drives immune suppression. Also reactive oxygen species are well known mediators of DNA damage (Pourzand and Tyrrell 1999). We have shown

the involvement of reactive oxygen species in jet fuel induced suppression by reversing the suppression with anti-oxidant treatment. It has also been observed that jet fuel treated cells undergo genomic stress leading to DNA damage (Grant, Jackman et al. 2001; Rogers, Gunasekar et al. 2001). With this in mind we propose that jet fuel induced genomic stress can activate the MAPK system which is involved in checking the integrity of the cell and the activation of PLA_2. The activation of PLA_2 drives the first step in PAF synthesis, which like the PAF-like molecule bind to the PAF receptor resulting in upregulation of PGE_2 and IL-10 which drives the suppression of DTH (see Figure 17).

In summary, exposure to jet fuel affects the memory response to C. *albicans* antigen, which as described above may have implications for vaccine efficacy. The jet fuel treatments did not affect antibody production but did decrease the response of T-cells to plate-bound anti-CD3. The jet fuel treatments used in these experiments are occupationally relevant. The 300 ul dose on a 20 gm mouse is equal to about 100 ml exposure for a 175 lb individual. These results suggests the jet fuel exposure affects cell-mediated immunity, specifically antigen presentation, by a PGE_2 driven mechanism that affects the dendritic cell's ability to drive a TH1 response. We suggest that jet fuel treated keratinocytes induces PAF which binds to the PAF receptors on adjacent cells and upregulates a cytokine cascasde. This cytokine cascasde ultimately results in systemic immune suppression.

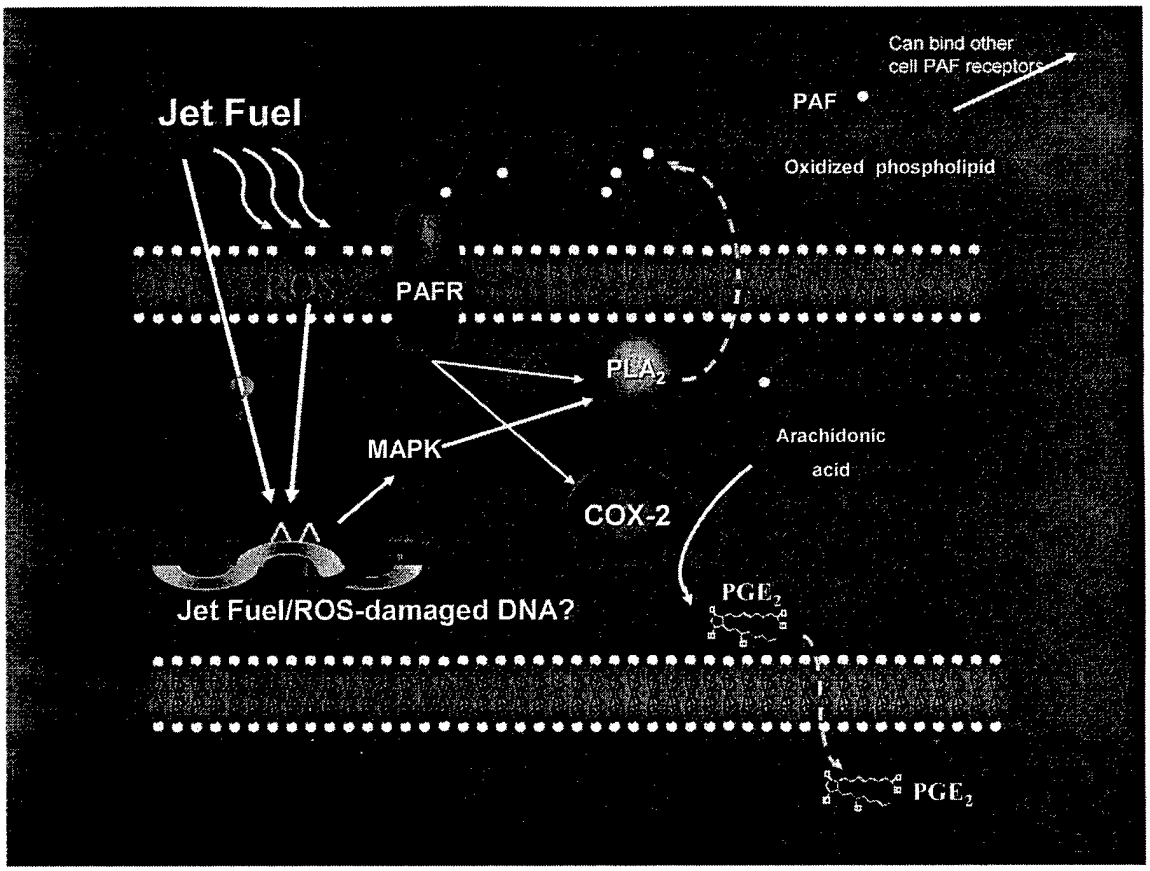


Figure 17. Proposed Pathways for Jet Fuel Induced Immune Suppression

Materials and Methods

Reagents and Cell Lines: The metabolically stable analogue of PAF, carbomyl-PAF (cPAF), and the PAF receptor antagonist PCA-4248 (Fernandez-Gallardo, Ortega et al. 1990), CV-3988 (Terashita, Imura et al. 1985), (±) trans-2,5-bis(3,4,5-trimethoxyphenyl)-1,3-dioxolane (hereafter referred as dioxolane (Corey, Chen et al. 1988), and Octylonium Bromide, were purchased from Biomol. Dr. Peter Isakson (G.D. Searl & Co., St. Louis, MO) provided SC-236 the selective COX-2 inhibitor. Stock solutions of cPAF, PCA-4248, CV-3988, Dioxolane, Octylonium Bromide, and SC-236 were prepared at 5mM concentrations by dissolving each in a 50% DMSO/PBS buffer and diluted further in PBS before injection into mice. A spontaneously transformed mouse keratinocyte cell line JB-6 was obtained from ATCC, American Type Culture Collection , Manassas, VA.

Mice: Specific pathogen-free female C3H/HeNCr (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 United States 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.

Application of jet fuels: Military jet fuel, JP-8 (lot # 3509) or commercial jet fuel, Jet-A, were supplied to us by the Operational Toxicology Branch, Air Force Research Laboratory, Wright Patterson Air Force Base, Dayton, OH. The fuel was stored and used in a chemical fume hood. Nitrile rubber based gloves (Touch N Tuff, Fisher Scientific Co.) were used in the place of normal latex gloves due to their superior performance in preventing the penetration of the fuels. The standard protective clothing worn in the animal facility, Nytex coveralls, surgical bonnets and masks also afforded protection for the investigators against accidental dermal exposure to jet fuel. Different amounts of the undiluted fuel (10 to 300 μl) were applied directly to the dorsal skin of the animals. The mice were held individually in the hood for three hours 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. In every experiment a control group treated with acetone was handled in the identical manner. After three hours 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 immunological memory and established immune responses by jet fuel: Female C3H/HeN mice were immunized by subcutaneous injection of 10^7 formalin-fixed *Candida albicans* into each flank (Moodycliffe, Nghiem et al. 2000). Seven days later the mice were boosted with an identical dose of *C. albicans* into each flank. Thirty days later the mice were shaved and exposed to JP-8 or Jet-A as described above. The next day each hind footpad was measured with an engineer's micrometer (Mitutoyo, Tokyo,

Japan) and then challenged by intra-footpad injection of 50 µl of *Candida* antigen (Alerchek Inc., Portland, ME). Eighteen to 24 hours later the thickness of each foot was re-measured and the mean footpad thickness for each mouse was calculated (left foot + right foot ÷ 2). Generally, there were 5 mice per group, the mean footpad thickness for the group ± the standard error of the mean was calculated. The background footpad swelling (negative control) was determined in a group of mice that were not immunized but were challenged. The specific footpad swelling response was calculated by subtracting the background response observed in the negative controls from the mean footpad swelling found in mice that were immunized and challenged. Each experiment was repeated at least three times. Statistical differences between the controls and experimental groups were determined by use of the two-tailed Student's t-test, with a probability of less than 0.05 considered significant (Prism Statistical Software, 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} x 100.

To determine if jet fuel exposure suppresses the elicitation of DTH, mice were immunized by the subcutaneous injection of 10^7 formalin-fixed *C. albicans*, as described above. Nine days later the immunized mice were shaved and JP-8 or Jet-A was applied to their dorsal skin. The next day each hind footpad was measured and challenged by intra-footpad injection of 50 µl of *Candida* antigen. Footpad swelling was read 18 to 24 hours later.

T-Cell Proliferation to plate-bound anti-CD-3: At various times after jet fuel treatment, the mice were killed, their spleens removed, and single cell suspensions prepared. Contaminating erythrocytes were lysed with ammonium chloride (0.83% in 0.01 M Tris-HCl, Ph 7.2): and the cells were washed and resuspended in complete RPMI-1640 supplemented wit 5% bovine calf serum (Hyclone, Logan, UT). A T-cell enriched population was prepared by passing the spleen cells over a nylon wool column (julius et al., 1973). The cells were then resuspended in medium supplemented with 10% bovine calf serum, 5 X 10^{-5} M 2-mercaptoethanol, 100 units/ml streptomycin, 2 mM L-glutamine, 10% sodium pyruvate, 10mM HEPES buffer, 1 X vitamins and 1 X non-essential amino acids (GIBCO-BRL, Grand Island, NY). The cells (1 X 10^6/well) were then cultured at 37 degrees C in an atmosphere of 5% CO_2, 95% air for 4 days in 96-well tissue culture plated coated with monoclonal anti-CD3 (30ul of a 10ug/ml solution overnight prior to culture; PharMingen, San Diego, CA). During the last 18 hours of culture, 1uCi of tritiated thymidine (ICN Radiochemicals, Irvine, CA) was added to each well. The cells were harvested onto glass-fiber filters (tomtec Harvester, Orange, ,CT) and the incorporation of the radioisotope into newly synthesized DNA was determined by liquid scintillation counting (1205 Betaplate, LKB Wallac, Gaithersburg, MD). Background responses were determined by culturing the cells in wells devoid of anti-CD3. Cells from each different group were cultured in triplicate. The means and standard deviations of the triplicates were calculated, and the statistical differences between controls (acetone-treated) and experimental (jet fuel treated) groups were determined by use of a 2-tailed Student's t-test, with a probability of less than 0.05 considered significant. Each experiment was repeated at least 3 times.

MTT Assay: Murine keratinocytes were plated $5x10^4$ cells/ml in triplicate on 24 well plates. Cells were treated with jet fuel on day 1 and harvested on day 2 with MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma Chemical Co., St. Louis, MO). The precipitate was resuspended in acidic isopropanol and was read at 570 nm (Pharmacia UltroSpec III, LKB Biochrom, England) with acidic isopropanol and a blank with acidic isopropanol was measured at 570. The control was cells plated but not treated with jet fuel. The data is expressed as mean absorbance ± SEM of three separate determinations. An * represents a statistically significant difference ($P < 0.05$) from the control (two-tailed Student's *t* test, $n = 3$)

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Vita

Gerardo Ramos was born in Kokomo, Indiana on [redacted]e Son of [redacted] Although born in Indiana during one of his parents' migrant working season he spent most of his school years in Brownsville, TX. After completing his work at Gladys Porter High School, Brownsville, TX in 1988, he joined the Army. After his Army tour he joined the Air Force Reserves and used the G.I. Bill to pursue a college education and graduated with a Bachelor of Science from University of Texas Health Science Center at San Antonio with a major in Clinical Laboratory Science in 1994. For the next year he worked in a regional hospital in south Texas as a Clinical Laboratory Scientist, while still in the Air Force Reserves. Immediately after joined the Air Force as an officer commissioned as a Biomedical Laboratory Officer in the Biomedical Sciences Corp. While on active duty, in August of 2000 he entered the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences on an Air Force competitive scholarship program from the Air Force Institute of Technology which allows Air Force officers to obtain graduate education that meets the needs of the Air Force.

Address:

1011 Flor del Valle

Brownsville, TX 78521