Immune Function Determination in Mice Dermally Exposed to Permethrin

by

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Thesis submitted to the Faculty of the Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

IN

VETERINARY MEDICAL SCIENCES

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October 25, 1999

Blacksburg, Virginia

Keywords : Permethrin, Pyrethroids, Insecticides, Immunotoxicity

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(ABSTRACT)

Inhibited immune responses have been observed following occupational, inadvertent, or therapeutic exposure to chemically diverse xenobiotics. In the present studies, preliminary data were generated showing limited but significant systemic immunotoxicity following low-level topical exposure to the pyrethroid insecticide, permethrin (formerly not considered an immunotoxicant). Permethrin was applied to the shaved dorsal interscapular region of C57Bl/6N mice at doses of 0.5, 1.5, or 5.0 μl/day. The highest of these doses was approximately equal to 215 μg/kg/day, which is about seven times the estimated daily human exposure in individuals wearing permethrin treated clothing for insect protection. Mice were thus exposed to permethrin daily for 10 or 30 consecutive days, or every other day for 7 or 14 exposures. Body weight was not affected by the treatment. However thymic weight was decreased and splenic weight increased 2 days after termination of the topical exposure. Histopathology of immune organs showed no significant changes. Splenic macrophages showed significantly depressed chemiluminescent responses up to 10 days following termination of exposure, but macrophage phagocytic activity was not affected. Cell surface markers of thymocytes, splenocytes and bone marrow cells were not affected. Antibody production as shown by plaque forming cell (PFC) assay decreased significantly at 10 days after dosing termination. Taken together, these data indicate that low-level topical permethrin exposure may produce systemic immunotoxicity.

ACKNOWLEDGEMENTS

I would like give my deepest thanks to my advisor, Dr. Steven Holladay, for all his help, guidance, and patience. I would also like to thank the other members of my committee, Dr. Bonnie Smith and Dr. John Robertson, for their advice and encouragement.

Special thanks to all those in the lab who have been a great help to me over the years: Delbert Jones, Kristel Fuhrman, and Lioudmila Sharova.

I also want to thank Joan Kalnitsky who assisted in flow cytometry, and persons at laboratory animal resourses who took care of all experimental mice.

Special thanks to all those who have been my support outside the lab.

This work was supported by grants from the U.S. army.

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BaP	benzo[*a*]pyrene
Ca^{2+}	Calcium
CA	chromosome aberration
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
Con A	concanavalin A
CTL	cytotoxic T lymphocyte
DDT	dichlorodiphenyltrichloroethane
DEET	N,N-diethyl-*m*-toluamide
DMBA	dimethylbenzanthracene
DOD	Department of Defense
DTH	delayed type hypersensitivity
EPA	Environmental Protection Agency
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
γ-GPT	γ-glutamyl transpeptidase
HIV	human immunodeficiency virus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP_3	inositol triphosphate
LPS	lipopolysaccharide
mAbs	monoclonal antibodies
MCA	methylcholanthrene
MHC	major histocompatibility complex
MLR	mixed leukocyte response
NIEHS	National Institute of Environmental Health Sciences

NK	natural killer
NOEL	No-Observable Effect Level
NTP	National Toxicology Program
PAHs	polycyclic aromatic hydrocarbons
PBB	polybrominated biphenyls
PCB	polychlorinated biphenyls
PFC	Plaque forming cell assay
$PLC_{\gamma 1}$	phospholipase $C_{\gamma 1}$
PTK	protein tyrosine kinase
SERCA	sarcoplasmic/endoplasmic reticulum calcium-ATPase
SRBC	sheep red blood cell
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TNF	tumor necrosis factor
USDA	U.S. Department of Agriculture

CHAPTER 1.0 : BACKGROUND AND LITERATURE REVIEW

1.1 INTRODUCTION

Immunotoxicity produced by a variety of chemical exposures has been reported for several years, and the number of recognized immunotoxicants is increasing (Luster and Rosenthal, 1993). The types of effects shown to occur are often chemical-specific as well as species-specific and include immunosuppression, targeting either systemic or local immunity (e.g., lung or skin), hypersensitivity disease, manifested as respiratory tract allergies or contact dermatitis, and in certain instances autoimmunity. Immunotoxicants of concern have included not only environmental pollutants but also certain therapeutics, consumer products, and biologicals (e.g., the therapeutic use of recombinant materials). Besides, interest has also focused on such diverse materials as silicone implants and pollutants common to the indoor environment. The latter include both chemical agents and bioaerosols such as viruses, bacteria, fungi, algae, and protozoa that have the potential to act as either sensitizing agents or mediators of infectious disease.

Permethrin, a synthetic pyrethroid insecticide, has been used extensively in several fields, and may produce immunotoxicity in humans. In agriculture, it is very effective against a variety of crop-eating insects. In human medicine, it has been shown to be effective and safe for treatment of body lice, head lice and scabies (Taplin and Meinking, 1990). In veterinary medicine, many products for treatment of external parasites such as fleas and ticks contain permethrin. Because it was believed to be safe for use on the human body, the U.S. army chose permethrin along with another chemical, N,N-diethyl-*m*-toluamide (DEET), to impregnate into military clothing as an insect repellent in order to protect the soldiers from insect-borne disease. Efficacy tests conducted by the U.S. Department of Agriculture (USDA) and the Department of Defense (DOD) have shown that permethrin and DEET-treated clothing affords nearly 100% protection against bites from most insect vectors (Snodgrass, 1992).

Soldiers exposed to multiple chemicals in the recent war between the United States and Iraq have developed symptoms related to the exposure (i.e., Gulf War Syndrome). In particular, increased incidences of infections disease and hypersensitivity responses (asthma) have been suggested (Proctor et al., 1998; Coker et al., 1999; Das et al., 1999). The toxicity of a variety of chemicals used in the mission was therefore examined. Permethrin, previously considered not immunotoxic, was one of the chemicals that soldiers contacted during the mission in the gulf war, thus this chemical is under investigation for contributing to the syndrome.

A study by Blaylock et al. (1995) using oral exposure to permethrin in a mouse model demonstrated immunotoxicity of permethrin in the form of inhibited T lymphocyte cytotoxic activity. However, the route of exposure was oral rather than the topical exposure experienced by soldiers, thus it was difficult to extrapolate such results from an animal study to a human effect.

The present study was supported by the U.S. army in order to determine if permethrin impregnated in military uniforms has a possibility of causing immunosuppression to the soldiers wearing those clothes. The experiments were designed by imitating the actual topical exposure. The highest exposure level used in the present experiments was about seven times the amount that humans received when wearing treated clothes (Snodgrass, 1992). The latter author determined that human exposure from wearing permethrin-treated clothing was about 34 μg/kg/day. Because soldiers may wear treated clothing intermittently or for several days consecutively, different exposure protocols were designed corresponding to chemical exposures these soldiers might receive.

The parameters using for determination of immune function were selected from a well-accepted immunotoxicity testing battery, which was developed by National Toxicology Program (NTP) (Luster et al., 1988). Each of these tests has different estimated predictive values for immunosuppression, and when combining two or more tests, give higher predictive values (Luster et al., 1992). These tests included thymus/body weight ratio (predictive value = 0.68), spleen/body weight ratio (predictive

value = 0.61), surface marker analysis (predictive value = 0.83), and antibody plaque-forming cell (PFC) assay (predictive value = 0.78). Combinations of two of these tests gave predictive values ranging from 0.73 (thymus/body weight ratio with spleen/body weight ratio) to 0.91 (PFC assay with surface marker analysis). In addition to these tests, histopathologic changes of thymus and spleen were also used for evaluation. Macrophage functions were also included as a measure of non-specific immunity, including phagocytic ability and chemiluminescent activity of macrophages isolated from the spleen.

The results of this study indicated that dermal exposure to low levels of permethrin daily for a period of time is able to cause alterations of systemic immune functions in mice. These alterations included a decrease of thymus weight/body weight ratio, an increase of spleen weight/body weight ratio, a decrease in the macrophage chemiluminescent response, and a decrease in antibody production. These immune changes were present for a short time after permethrin exposure, and then all immune parameters examined returned to control levels.

1.2 CHEMICAL-INDUCED IMMUNOTOXICITY

Immunotoxicity of chemicals has been of interest for many years as a new endpoint for human risk assessment, and a large number of chemicals have been identified to cause immunotoxicity (Luster and Rosenthal, 1993). These chemicals include the following: polyhalogenated aromatic hydrocarbons (i.e., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD], polychlorinated biphenyls [PCB], and polybrominated biphenyls [PBB]); metals (i.e., lead, cadmium, and arsenic); aromatic hydrocarbons (i.e., benzene and toluene); polycyclic aromatic hydrocarbons (i.e., dimethylbenzanthracene [DMBA], benzo[*a*]pyrene [BaP], and methylcholanthrene [MCA]); pesticides (i.e., trimethyl phosphorothioate, carbofuran, and chlordane); organotins (i.e., dibutyltin chloride); aromatic amines (i.e., benzidine, and acetylaminofluorene); oxidant gases (i.e., NO_2, O_3, and SO_2); particulates (i.e., asbestos, silica, and beryllium); mycotoxins (i.e., T-2 toxin and ochratoxin); therapeutic drugs (i.e.,

cyclosporin, methotrexate, and diphenylhydantoin); and abuse drugs (i.e., cocaine, alcohol, and marijuana).

Interaction of xenobiotics with the immune system may result in undesirable effects of three principle types (Luster et al., 1988). The first type is immunosuppression which is the most common one. The second type is autoimmunity or immune response against the host itself. The last one is an allergic reaction or contact hypersensitivity in which there is a response directed against the chemical.

Immunosuppression caused by chemical agents can result in clinical diseases (Luster and Rosenthal, 1993). Such diseases would more likely be manifested as an increase in the frequency or severity of infections and increased incidences of certain cancers such as Kaposi's sarcoma or non-Hodgkin's lymphoma. The association between increased incidence of recurrent infections and cancerous diseases and the chronic low-level use of immunosuppressive agents has been recognized (Ehrke and Mihich, 1985). Such agents presumably act by inducing moderate levels of immunosuppression. An increasing number of reports has described various immune changes in individuals who have been inadvertently or occupationally exposed to chemical agents. These range from unconfirmed reports with putative non-immunosuppressive compounds such as trichloroethylene and methyl isocyanate to more substantiated studies with PCB, asbestos, and silica. In addition, the results from in vitro and in vivo experimental studies have suggested that many environmental chemicals can inhibit the immune system and alter host resistance to infectious agents or tumor cells (Luster and Rosenthal, 1993).

The duration of immunosuppressive states might be transient or long-lasting depending on the severity and site of the specific xenobiotic effect (Tucker III, 1994). However, there are no confirmed reports of long-lasting immunosuppression due to xenobiotics in humans. The immune impairment which results from continued specific drug therapy with immunosuppressive agents or human immunodeficiency virus (HIV) infection are the only examples of long-lasting acquired immunodeficiency in humans. In fact, studies that have reported acquired deficiency of immune function as a result of xenobiotics or radiation have demonstrated the marked capacity for self-restoring activity

of the immune system, so that once the toxic agent has been cleared from the host, the various cellular components return to a normal state (Tucker III, 1994).

A number of environmental chemicals and therapeutic agents produce autoimmune responses, which in some cases lead to autoimmune diseases in experimental animal models and humans (Luster and Rosenthal, 1993; Bigazzi, 1988). Evidence for drug-induced autoimmunity is more compelling than environmentally related autoimmunity, as typified by reports of penicillamine- and procainamide-induced lupus. Autoimmunity from hydrazine occurs in a fairly restricted population (i.e., slow acetylators). This chemical is found in various natural products including tobacco, mushrooms, and alfalfa seeds. There have been other reports of drug-induced autoimmune hemolytic anemia or thrombocytopenia, the most notable being methyldopa. Similar responses have also been reported in patients receiving chlorpropamide, procainamide, carbamazepine, and interferon therapy (Bigazzi, 1988). Evidence of autoimmunity induced by environmental chemicals, especially in humans, is limited. Occupational or inadvertent exposure to vinyl chloride and/or quartz has been linked to a disorder resembling scleroderma. Certain metals, such as mercury, cause immune complex glomerulonephritis in humans, although the extent of mercury-induced autoimmune disease in humans is unknown. It is well established that low levels of mercury administered to susceptible strains of mice and rats result in immune-complex glomerulonephritis and nuclear autoantibodies (Tubbs et al., 1982).

A number of chemical agents has the capacity to produce contact hypersensitivity, a widely recognized environmental and occupational problem (Luster and Rosenthal, 1993). The examples of these chemicals include polycyclic aromatic hydorocarbons, platinum salts, cotton dust, formaldehyde, ethylenediamine, and organophosphate insecticides. The major characteristic that sets allergic responses apart from immune responses involved in host defense is that the reaction is excessive and often leads to tissue damage. Chemical-induced hypersensitivities fall into two categories distinguished not only mechanistically but temporally: delayed-type hypersensitivity, a cell-mediated response that occurs within 24-48 hr after challenge, and immediate hypersensitivity,

which is mediated by immunoglobulin, most commonly IgE, and manifests within minutes after exposure to an allergen. The type of immediate hypersensitivity response elicited (i.e., anaphylactic, cytotoxic, Arthus or immune complex) depends on the interaction of the sensitizing antigen or structurally related compound with antibody. In contrast, delay-typed hypersensitivity responses are characterized by T-lymphocytes bearing antigen-specific receptors, which, on contact with cell-associated antigen, respond by secreting cytokines.

Molecular mechanisms underlying chemical immunotoxicity have been reviewed by Holsapple et al. (1996). For the vast majority of immunotoxic compounds thus far identified, disruption of normal immune function is clearly mediated through direct interaction between the agent, or its metabolite, and immunocompetent cells. Regardless of whether this interaction occurs at the level of the cell membrane or at intracellular sites, basic regulatory processes mediated by second messengers are often altered. These alterations can ultimately result in immunologic dysfunction, which is most often manifested as immunosuppression. This mechanism of action includes effects on signaling systems controlled by protein tyrosine kinase (PTK)-link receptors that are well known regulators of cell growth and differentiation, effects on guanine nucleotide binding protein (G-protein) regulated pathways, and effects on calcium signaling or calcium regulation.

Disruption of PTK signal transduction pathways in lymphocytes can be caused by oxidative stress (Holsapple et al., 1996). Oxidative stress in lymphocytes causes reduced responses to mitogens or antigens and is associated with immune dysregulation. Inhibitory effects of oxidative stress occur at multiple levels because depletion of glutathione has been found to inhibit interleukin-2 (IL-2) production by mitogen-activated T cells and to inhibit their proliferation in S phase. In addition, cells sorted on the basis of glutathione levels show differences in their ability to respond to polyclonal mitogens, demonstrating the close relationship between redox regulation and the potential for cellular growth. The molecular basis for these inhibitory effects is that oxidative stress disrupts PTK-dependent signals in lymphocytes by receptor-independent activation of

target genes (ZAP70 or Syk) to induce downstream responses through activation of phospholipase $C_{\gamma 1}$ ($PLC_{\gamma 1}$). Oxidative stress also causes a reduction in antigen receptor signal transduction pathways and results in both polyclonal activation and progression towards apoptosis.

Cannabinoids suppress the immune system through the inhibition of adenylate cyclase (Holsapple et al., 1996). Cannabinoid receptors, CB1 and CB2, have been identified in a variety of lymphoid cells derived from human and rodent species. Both cannabinoid receptors belong to the G protein-coupled superfamily of receptors and negatively regulate adenylate cyclase. Engagement of cannabinoid receptors in lymphocytes produced a marked inhibition of adenylate cyclase activity, which in turn lead to a decrease in the formation of cAMP-dependent protein kinase (PKA) and the subsequent phosphorylation events normally mediated by this kinase (i.e., CREB proteins) during T-cell activation.

Immunotoxicity produced by polycyclic aromatic hydrocarbons (PAHs) is mediated in part by the alterations in Ca^{2+}-dependent pathways of B- and T-cell activation (Holsapple et al., 1996). PAHs are an important class of environmental pollutants that have been associated with an increased risk for cancer and that have been shown to exert important suppressive effects on the immune system of animals and humans. The immunotoxicity of PAHs and the potential role of altered Ca^{2+} homeostasis has been recently reviewed by Davila et al (1995). PAHs have been shown to increase intracellular levels of Ca^{2+} in various murine and human B- and T-cell lines and cells obtained from murine lymphoid tissues. Lymphoid cell death via apoptosis has been associated with an increase in intracellular Ca^{2+} produced by PAHs. PAHs disrupt Ca^{2+} homeostasis in murine and human lymphocytes by at least two distinct mechanisms. First, it has been demonstrated that 7,12-dimethylbenz(a)anthracene (DMBA) activates PTKs in T cells leading to tyrosine phosphorylation of $PLC_{\gamma 1}$, the production of IP_3, and the release of Ca^{2+} from intracellular stores. The second mechanism by which PAHs appear to alter Ca^{2+} homeostasis in lymphocytes relates to direct or indirect inhibition of Ca^{2+}-ATPase pumps found in the endoplasmic reticulum (ER) membranes. These Ca^{2+}

pumps, known as SERCAs, play an important role in Ca^{2+} reuptake following release triggered by intracellular agents such as IP_3. Krieger et al (1995) recently showed that PAHs specifically inhibit ATP hydrolysis associated with SERCA enzymes obtained from human T cells and other tissues.

Second messengers such as tyrosine phosphorylation, cAMP and calcium, and their associated signaling pathways, are primary targets for a number of diverse classes of chemicals (Holsapple et al., 1996). Because of the central roles that these signal transduction pathways play in the physiology of immunocompetent cells, toxicant-induced changes can have consequences that range from subtle changes in immune function to marked immunosuppression and can even include an aberrant induction of apoptosis.

1.3 RISK ASSESSMENT IN IMMUNOTOXICOLOGY

Risk assessment is a process in which relevant biological, dose-response and exposure data for a particular agent are analyzed in an attempt to establish qualitative and quantitative estimates of adverse outcomes (Scala, 1991). Such data are sometimes used in the development of standards for regulating the manufacture, use and release of chemicals into the environment. For the most part, risk assessment for chemical agents has focused on estimating the incidence of cancer from lifetime exposures to a chemical agent at some unit dose. The use of non-cancer endpoints including disorders of the developmental and reproductive systems, nervous system and immune system have only recently received attention in this area.

The major focus of risk assessment in immmunotoxicology is the detection and evaluation of unwanted effects of chemicals and drugs on the immune system by way of toxicity tests using rodent species.

A tiered testing approach for screening drugs and chemicals for their potential to alter immune function in the mouse was developed and validated by the NTP at the U.S. National Institute of Environmental Health Sciences (NIEHS) (Luster et al., 1988). This effort involved the participation of four separate laboratories. The testing panel is divided

into two tiers. Tier I tests include the following: immunopathology (i.e., hematology-complete blood count and differential: body, spleen, thymus, kidney, and liver weights; splenic cellularity: and spleen, thymus, and lymph node histology); humoral-mediated immunity (HMI) (i.e., IgM antibody plaque-forming cells to T-dependent antigen and LPS mitogen response); cell-mediated immunity (CMI) (i.e., lymphocyte blastogenesis to mitogens (Con A) and mixed leukocyte response (MLR) against allogeneic leukocytes); and nonspecific immunity (i.e., NK cell activity). Tier II tests include the following: immunopathology (i.e., quantitative of splenic B and T cell numbers); humoral-mediated immunity (i.e., enumeration of IgG antibody response to SRBCs); cell-mediated immunity (i.e., cytotoxic T lymphocyte (CTL) cytotoxicity and delayed type hypersensitivity (DTH) response); non-specific immunity (i.e., macrophage function-quantitation of resident peritoneal cells and phagocytic ability); and host resistance challenge models (i.e., syngeneic tumor models and bacterial, viral, and parasitic infectivity models).

Tier I is a limited effort and has high probability of detecting potent immunotoxicants. The likelihood of detecting weaker immunotoxicants in Tier I, such as those that may affect only a specific cell population or subpopulation, is presumably less. However, based on the report of Luster and colleagues (1988), it was found that the compounds that affected an assay in Tier II always demonstrated some effect in Tier I. Therefore, while Tier I provides little information on the specificity of immune disorders or its relevance to the host, it can readily detect an immune change resulting from chemical exposure. Among the assays in Tier I, the PFC and MLR assays are more sensitive than the others.

Tier II represents an in-depth evaluation which includes additional assays for CMI, HMI, and nonspecific immunity, as well as an assessment for host resistance. Tier II testing is normally included only if functional changes are seen in Tier I and at dose levels which are not overtly toxic (i.e., body weight changes). Immune function tests in Tier II provide information on the mechanism of immunotoxicity and help characterize the nature of the effects.

In this tiered approach, animals are usually only evaluated at one time point; thus the possibility for recovery or reversibility of immunologic changes is not evaluated. In conducting the studies, routinely a 14-day exposure period is employed. However, 30- or 90-day exposure periods have been used based on the pharmacokinetic properties of the chemical being tested.

This testing battery has been utilized to examine a variety of compounds by several laboratories. The database generated from these studies, which consists of over 50 selected compounds, has been analyzed to improve testing strategies and provide information to aid in quantitative risk assessment for immunotoxicity. The sensitivity and predictability of tests in the immunotoxicity testing battery outlined above were recently reported by Luster et al. (1992). Analysis of the results indicated that the tests or test combinations from the panel varied in their ability to identify immunotoxic compounds. Furthermore, the analysis revealed a concordance of more than 90% for the prediction of immunotoxic compounds in mice when certain groups of immune tests were performed. The two immune function tests that showed the greatest association with immunotoxicity for the compounds studied were cell surface marker analysis (83%) and the splenic antibody plaque forming cell response (78%). The combination of either of these two tests with almost any other parameter markedly increased the ability to predict immunotoxicity. On the other hand, several other tests were found to be rather poor predictors of immunotoxicity. These included leukocyte counts (43%), lymphoproliferative response to LPS (50%), and splenic cellularity (56%). In conclusion, these results indicated that examination of only two or three immune parameters may be used to successfully predict immunotoxicants in mice.

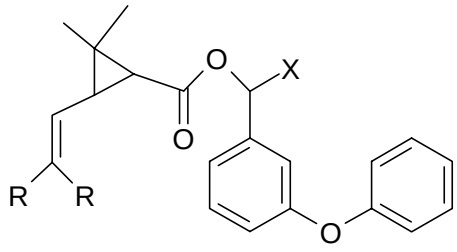
1.4 PERMETHRIN : GENERAL INFORMATION

Permethrin is a broad spectrum insecticide, a chemical used to kill a variety of insects. Permethrin is referred to as a synthetic pyrethroid insecticide because, while manmade, it resembles naturally occurring chemicals with insecticidal properties, called pyrethroids (Figure 1). The first of synthetic pyrethroid insecticides was allethrin, synthesized in

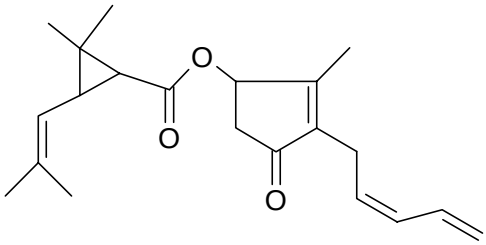
1949 by Schechter and colleagues (Taplin and Meinking, 1990). It had about the same insecticidal activity as the natural pyrethrins, but it was not long before other pyrethroids were developed that had greater insecticidal activity than natural compounds, and less mammalian toxicity. The first of these was bioresmethrin, to be followed by tetramethrin and d-phenothrin (Elliott and Janes, 1978). These early, or first-generation, pyrethroids were unstable to light, thus limiting their use in agriculture and animal husbandry. In 1973, the first photostable pyrethroid, permethrin, was developed by the team at the Rothamsted Experimental Station in the United Kingdom, under the direction of Dr. Michael Elliott (Elliott et al., 1973). This team was able to substitue chlorine for the methyl group in the acid portion of the molecule, and they replaced the previous photolabile site in the alcohol moiety with 3-phenoxybenzyl. This rendered the molecule photostable, and increased the insecticidal activity to around 18 times greater than dichlorodiphenyltrichloroethane (DDT) and almost 4 times as effective as natural pyrethrins against *Anopheles stephensi*. In practical terms, this allowed permethrin to retain activity on plant leaves for 2 weeks or so, even in bright sunlight.

Chemical nomenclature of permethrin is 3-phenoxybenzyl-(1R,S)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (Elliott et al., 1976). It contains four stereoisomers due to the chirality of the cyclopropane ring. The cis:trans isomer ratio is reported to be 2:3 and the optical ratio of 1R:1S is 1:1 (racemic). Thus, permethrin contains the [1R,trans], [1R,cis], [1S,trans], and [1S,cis] isomer in the approximate ratio 3:2:3:2 (WHO, 1990). The [1R,cis] isomer is the most insecticidally active among the isomers, followed by the [1R,trans] isomer. Regarding physical properties, permethrin is an odorless, colorless crystalline solid or a viscous liquid that is white to pale yellow. It is stable to heat, light and air. Permethrin keeps for a year or longer when stored under cool, dry conditions. The technical grade of permethrin is composed of cis/trans isomeric ratio about 40:60, and the purity is not less than 89%.

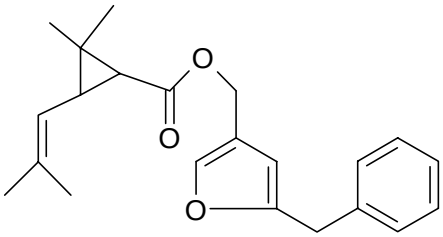
Permethrin has several trade names. For examples, these names include Ambush, BW-21-Z, Ectiban, Eksmin, Exmin, FMC-33297, Indothrin, Kafil, Kestril NRDC 143, Pounce, PP 557, Pramiex, Qamlin and Torpedo. All formulations labeled for agricultural



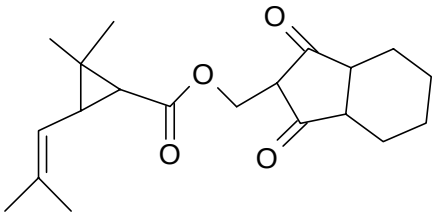
General structure of
a synthetic pyrethroid



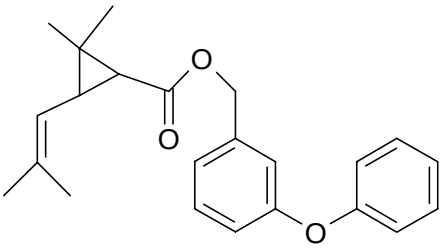
Allethrin



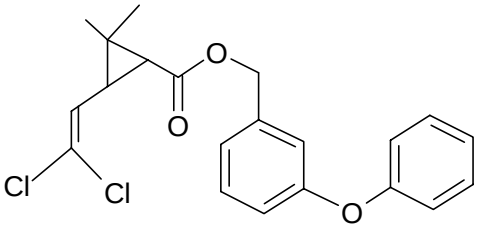
Bioresmethrin



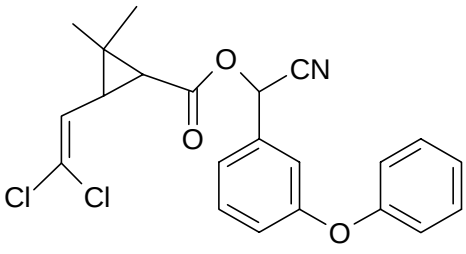
Tetramethrin



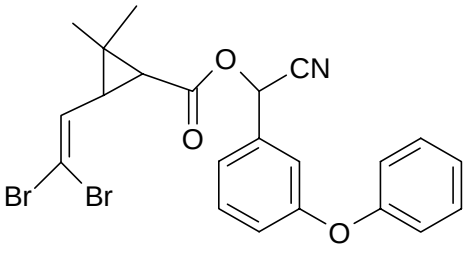
d-Phenothrin



Permethrin



Cypermethrin



Deltamethrin

Figure 1. Chemical structure of synthetic pyrethroids

use, excluding livestock and premises uses, are classified by the U.S. Environmental Protection Agency (EPA) as Restricted Use Pesticides (RUP) because of their possible adverse effects on aquatic organisms (US EPA, 1987).

Permethrin is used against a number of pests, on nut, fruit, vegetable, cotton, ornamental, mushroom, potato and cereal crops. It is used in greenhouses, home gardens and for termite control (Meister, 1992). It is also used for controlling animal ectoparasites, biting flies, and cockroaches. Generally, pyrethroid insecticides work by quickly paralyzing the nervous system of insects, producing a quick knockdown effect on insect pest populations. Permethrin acts as a stomach poison when it is ingested by insects or as a contact poison through direct contact with target pests. It kills adults, eggs, and larvae, and has a slight repellent effect against insects. The insecticidal activity of permethrin lasts up to 12 weeks after application (Hayes, 1982).

In human medicine, permethrin is used effectively to treat body lice, head lice, and human scabies (Taplin and Meinking, 1990). The first study of permethrin for the treatment of human ectoparasites was conducted in the Fayoum Oasis, Egypt, in 1976. Nassif and Kamel (1977) found permethrin 1% dusting powder to be highly effective against body lice. Two weeks after a single dusting, 99% of the population was louse free. In another study by the same investigators, similar effectiveness was demonstrated for dusting powders containing 0.25% and 0.5% permethrin (Nassif et al., 1980). The authors also reported excellent results against body lice that were no longer sensitive to DDT or lindane, and against *Pulex irritans* and *Xenopsylla cheopsis*, the human and rat fleas. For treatment of head lice, permethrin 1% formulated in a hair conditioning rinse (NIX) applied to the scalp for 10 minutes has proven to be a remarkably safe and effective product, requiring only one treatment for over 95% of subjects (Brandenburg et al., 1986; Taplin and Meinking, 1990). Permethrin 5% cream (Elimite) was approved as a treatment for scabies by the U.S. FDA in September 1989. It was found to be more effective than crotamiton 10% cream (Eurax) (Taplin et al., 1990) and 1% lindane lotion (Schultz et al., 1990).

In addition to direct application to the human skin, several other uses of permethrin that relate to public health and the skin are in use. Bed nets impregnated with permethrin have been used to reduce the incidence of night-biting insects, and thus the transmission of vector-borne disease, particularly malaria. These have been shown to effective in reducing the total number of mosquito bites as well as the number of infective bites (Lindsay et al., 1989). The most impressive reports on the value of impregnated bed nets come from China, where this protection has been offered to over 2.7 million people. In four provinces, in which 22,000 people were issued bed nets treated with permethrin or deltamethrin, the incidence of malaria was reduced 65-92% (Flannigan et al., 1984).

For over 10 years, the US Department of Agriculture, in collaboration with the US Department of Defense, has supported research on the value of permethrin treatment of military uniforms. Similar studies have also been conducted by British military entomologists The results have been impressive. Controlled experiments in the laboratory and with human volunteers in the field show that clothing impregnated or sprayed with permethrin offers considerable protection against a wide range of pestiferous and vector insects. These include mosquitoes (Lillie et al., 1988; Schreck and Kline, 1989), human body lice (Sholdt et al., 1989a), tsetse flies (Sholdt et al., 1989b), and ticks (Schreck et al., 1982), including *Ixodes dammini* the principal vector of Lyme disease and human babesiosis in the United States (Schreck et al., 1986). In one study, in Alaska, permethrin-treated uniforms offered 93% protection in areas where men wearing untreated uniforms received 1188 mosquito bites per hour (Lillie et al., 1988). The greater protection against biting insects is obtained by using permethrin-impregnated uniforms in combination with DEET, an Extended Duration Topical Insect/Arthropod Repellent (EDTIAR), developed by the US Department of Defense and US industry (Schreck et al., 1984). This combination reduced bites from the mosquito *Aedes taeniorhynchus* in Florida from an estimated 2287 bites during 9 hours to only 1.5. The conscientious use of DEET and permethrin-impregnated uniforms appears to offer substantial protection against pestiferous and vector insects and arthropods for troops

stationed in endemic areas, and should greatly reduce the incidence of cutaneous infections, malaria, dengue, and other vector-borne diseases. However, there has been a concern of potential exposure to permethrin as a result of transfer from treated cloth to the skin surface of human. Snodgrass (1992) conducted an experiment in rabbits to quantitate leaching from treated clothing. The studies were performed in which swatches of fabric impregnated with ^{14}C-labeled permethrin were applied to the backs of rabbits for 1 week. At the end of 7-d exposure, about 3.2% of the available permethrin had reached the skin, 2% having been recovered from excreta (absorbed) and 1.2% remaining on the skin surface. The author estimated the exposure dose to humans from wearing permethrin-treated (0.125 mg/cm^2) military clothing to be $6x10^{-4}$ mg/kg/d.

The effects of permethrin on the ecosystem were reassuring. It is rapidly broken down by organic materials and microorganisms in the soil, where it has a half-life of approximately 4 weeks. Permethrin binds very strongly to soil particles and it is nearly insoluble in water, thus it is not expected to leach or to contaminate groundwater. The binding, or adsorption, of permethrin in soil may be limited to organic matter (Wagenet, 1985).

1.5 TOXICOLOGY OF PERMETHRIN

1.5.1 TOXICOKINETICS

The toxicokinetics of permethrin have been studied in the rat by Anadon et al. (1991). A single dose of permethrin was administered by the oral or intravenous route. The kinetics of permethrin after iv administration in rats were best described by a two-compartment open model, with a relatively rapid distribution phase ($t_{1/2\alpha}$ = 0.46 hr) and a more prolonged elimination phase ($t_{1/2\beta}$ = 8.67 hr). A similar kinetic profile was observed in rats orally given permethrin. The apparent volumes of distribution during the elimination phase (V = 0.72 liter) and at steady state (V_{ss} = 0.65 liter) were relatively large. These values, and the high lipid solubility of permethrin, suggest a penetration and distribution of the pyrethroid in body fluids including intracellular water. After a single oral dose, permethrin was both absorbed (T_{max} = 3.52 hr) and eliminated slowly ($t_{1/2\beta}$ =

12.37 hr). The observed low total plasma clearance (CL = 0.058 liter/hr) also explains the slow elimination of permethrin in the rat. In the study by Anadon et al., (1991), the bioavailability of permethrin was relatively low (*F* = 60.69%) following oral administration due to permethrin degradation at the site of absorption and a first pass effect. The (1R,trans)- and (1R,cis)-esters, the active isomers of permethrin, are readily metabolized by ester cleavage, by hydroxylation of the terminal dimethyl group in the acid, or the phenoxy group of the alcohol, and by conjugation of the resulting carboxylic acids and phenols, with *cis*-permethrin being more stable than *trans*-permethrin. The metabolites are quickly excreted and do not persist significantly in tissues (Elliott et al., 1976). In the study by Anadon et al. (1991), the metabolism of permethrin was rapid and both metabolites, *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid, were detected in plasma and tissues. The brain regions also contain the higher areas under the tissue concentration-time curve of the metabolites, mainly *m*-phenoxybenzyl alcohol, indicating a metabolism system may be present; the metabolites could not enter the brain, presumably due to their polarity.

Percutaneous absorption of permethrin has been investigated in rat, rabbit, dog, and man, and the degree of absorption is highly species dependent. When applied in an alcoholic vehicle, 60% is absorbed in the rat, 30% in the rabbit, and 12% in the beagle dog, but less than 2% is absorbed percutaneously in human studies (Taplin and Meinking, 1990). Percutaneous absorption of permethrin was studied in rhesus monkeys and rats by Sidon et al. (1988). ^{14}C radiolabeled *cis*- or *trans*-isomer of permethrin was applied to either the forehead or forearm of rhesus monkeys or to the mid-lumbosacral region of the rat. Urine was collected for 7 or 14 d. The results have shown the following: (a) there were no significant isomeric differences in the skin penetration of permethrin in rats or monkeys; (b) The skin absorption of *cis*- and *trans*-permethrin isomers was greater in the forehead than the forearm of monkeys; (c) Rat skin was more permeable to both permethrin isomers than either the forehead or forearm of monkeys; (d) anotomic site variations as well as interspecies differences are important factors to consider when utilizing animal models to predict percutaneous absorption of permethrin in humans.

Percutaneous absorption of permethrin was compared to lindane in a recent study (Franz et al., 1996). The study was designed to compare the systemic absorption of both agents from their commercial formulations, 5% permethrin cream (Elimite) and 1% lindane lotion (Kwell), which were used for treatment of scabies. In vitro percutaneous absorption of the two scabicides was identical in guinea pig skin, but human skin was 20-fold more permeable to lindane than to permethrin. In vivo guinea pig blood and brain levels of lindane were found to be fourfold greater than permethrin levels. The authors concluded that there were at least three factors favoring permethrin over lindane for the topical treatment of scabies: (1) lower inherent toxicity, (2) lower percutaneous absorption, and (3) lower blood and brain levels.

In a recent study, the percutaneous absorption of permethrin and DEET was investigated when applied simultaneously to the skin as a mixture, the relevant route of exposure in the Persian Gulf (Baynes et al., 1997). Topical application of permethrin-DEET mixture resulted in absorption of DEET (1-20% dose), but no permethrin. Permethrin (1.2-1.7% dose) was detected only when mouse skin was dosed solely with permethrin. This finding suggested that DEET decreased permethrin absorption.

Permethrin is capable of producing a dose-dependent marked enzyme-inducing effect (Anadon et al., 1988). The influence of permethrin on plasma antipyrine kinetics and γ-glutamyl transpeptidase (γ-GPT) activity was studied in rats. Treatment with 190 mg permethrin/kg/day for 3 days decreased antipyrine half-life and the area under the curve, and increased the apparent volume of distribution and the clearance significantly. The γ-GTP activity was significantly increased within 21 days and 14 days after the start of permethrin administration, at doses of 90 and 190 mg permethrin/kg/day respectively.

1.5.2 ACUTE, SUBACUTE AND CHRONIC TOXICITIES

In all species thus far investigated pyrethroids induce toxic signs that are characteristic of a strong excitatory action on the nervous system. Toxic doses of pyrethroids generally cause hypersensitivity to sensory stimuli, and a number of compounds may induce tingling sensations in the skin. In mammals, two distinct toxic

syndromes have been described (Ecobichon, 1991). The T-syndrome is induced by pyrethrins and noncyano pyrethroids, type I pyrethroids, e.g., permethrin, and is characterized by the prominent symptom of whole-body tremors. These compounds initially cause aggressive sparring behavior and increased sensitivity to external stimuli. This is followed by a fine tremor, gradually becoming more severe until the animal finally becomes prostate with coarse whole-body tremor. The CS-syndrome, induced by deltamethrin and most other cyano pyrethroids, type II pyrethroids, is characterized by choreoathetosis (a condition marked by choreic and athetoid movement) and salivation. The CS-syndrome consists of pawing and burrowing, profuse salivation, and coarse tremor progressing to choreoathetosis and clonic seizure. Some pyrethroids produce tremors and salivation, classified as the intermediate TS-syndrome.

Permethrin exhibited extremely low mammalian toxicity. Based on oral LD_{50} values in rats, permethrin is about 3 times less toxic on a milligram per kilogram basis than the organophosphate malathion, 15 times less than the carbamate, carbaryl, and about 40 times less than the organochlorines, lindane or DDT (Taplin and Meinking, 1990).

The amount of permethrin that is lethal to one-half (50%) of experimental animals exposed to it is referred to as the lethal dose fifty, or LD_{50}, of this insecticide. The oral LD_{50} in rats is 430 to 4,000 mg/kg. Aqueous suspensions usually produced the least toxic results, LD_{50} values ranging from 3,000 to >4,000 mg/kg. However, corn oil is the more standard vehicle for pyrethroids and yielded LD_{50} values of about 500 mg/kg for oral administration in rats and mice. The LD_{50} is over 270 mg/kg when injected into the veins. The *cis* isomer has a greater potential for mammalian toxicity than the *trans* isomer, which is more rapidly metabolized and excreted (WHO, 1990).

Following oral administration of permethrin to rats, signs of poisoning became apparent within 2 hr after dosing and persisted for up to 3 days. At lethal levels, these signs included whole body tremors of varying degree from slight to convulsive, which in some cases were accompanied by salivation. Associated signs included hyperactivity and

hyper-excitability to external stimuli, urination and defecation, ataxia and lacrimation (WHO, 1990).

Subacute toxicity of permethrin was studied in mice. The mice were fed permethrin in the diet at levels ranging from 0 to 10,000 mg/kg for 28 days. Mortality, growth, and food utilization were normal for all animals. Animals fed permethrin at 2,000 mg/kg or more showed increased liver weight and liver-to-body weight ratio. Higher weight and organ-to-body weight ratios were also observed in the kidney, heart, and spleen of animals receiving a dose of 10,000 mg/kg. On histopathological examination, regenerating tubules in the renal cortex and hypertrophy of centrilobular hepatocytes with cytoplasmic eosinoplilia, which were not dose related, were observed in all the treated animals (WHO, 1990).

Long-term feeding of pyrethroids resulted in an increase in liver size and excessive formation of bile duct tissue. The 90-day No-Observable Effect Level (NOEL) was 5 mg/kg/day in dogs fed permethrin (Gosselin, 1984). Rats fed 150 mg/kg/day for 6 months, showed a slight increase in liver weights. A chronic-toxicity study of permethrin was conducted in rats and mice by Ishmael and Lithfield (1988). Groups of Alpk:AP (Wistar-derived) rats were fed diets containing 0, 500, 1000 or 2500 ppm permethrin for 2 years and Swiss-derived mice were maintained for their lifetime (80% mortality) on diets containing 0, 250, 1000, or 2500 ppm permethrin. Changes of toxicological significance were confined to the top dose level of 2500 ppm permethrin in both species. Tremors and hypersensitivity to noise were noted in rats at this dose during the first 2 weeks of study but such signs were not seen in mice. Pathological examination of the central and peripheral nervous systems did not reveal abnormalities attributable to permethrin administration. The effect on mice at 2500 ppm permethrin was shown by decreased body weight gain. Liver hypertrophy, associated with increase in liver weight, microsomal enzyme activity, and proliferation of smooth endoplasmic reticulum occurred in the rat with similar but less marked changes in the mouse. This was considered to be an adaptive response of no toxicological significance. No evidence of a carcinogenic effect was seen in the rat study. In the mouse study a slight elevation in benign lung

tumor incidence in males only at 2,500 ppm permethrin was observed but was not considered to represent a carcinogenic effect.

1.5.3: NEUROTOXICITY

Neuroexcitatory symptoms of acute poisoning of vertebrates by pyrethroids are related to the ability of these insecticides to modify electrical activity in various parts of the nervous system (Vijverberg and van den Bercken, 1990). The principal action of pyrethroids in the peripheral nervous system is to induce pronounced repetitive activity. In particular sense organs produce trains of nerve impulses instead of single nerve impulses after exposure to pyrethroids, either *in vitro* or *in vivo*.

Permethrin and other noncyano pyrethroids induce short nerve impulse trains, which contain no more than a few dozen repetitive nerve impulses. On the other hand, the cyano pyrethroids cause long-lasting trains that contain hundreds or even thousands of repetitive nerve impulses. On several occasions, repetitive discharges lasting over 30 s have been recorded. In addition, the duration of nerve impulse trains induced by noncyano as well as cyano pyrethroids increases dramatically as the temperature is lowered. The effect is readily reversed by raising the temperature (Vijverberg and van den Bercken, 1990).

Pyrethroids affect greatly the voltage-dependent conformational changes of sodium channels in excitable membranes. They cause sodium channels to stay open much longer than normal, resulting in a prolongation of the transient sodium current associated with membrane depolarization and a marked, slowly decaying sodium tail current after termination of the depolarization. This effect may account for membrane depolarization, suppression of the amplitude of the nerve impulse, and block of excitation that may occur in various parts of the nervous system (Vijverberg and van den Bercken, 1990).

Neurobehavioral effects of permethrin were reported by Hudson et al (1986). Permethrin causes a dose-dependent, reversible decrease in the rate of lever pressing during operant behavior at doses well below the LD_{50} without overt toxic signs. The more toxic *cis* isomer of permethrin is more effective in this respect than the *trans*-permethrin.

In the rat noncyano pyrethroids as well as DDT enhance the acoustic startle reflex, but the latency of the startle reflex remains unaffected. These effects are produced in the absence of overt toxic signs. In contrast, cyano pyrethroids produce variable effects on the startle reflex as well as on its latency in addition to a suspected direct effect on muscle. The differences between the behavioral effects on noncyano and cyano pyrethroids might be related to their differential effects on the sensory nervous system (Vijverberg and van den Bercken, 1990).

Neurotoxicity of permethrin co-exposed with pyridostigmine bromide and DEET was investigated in order to study the effects of gulf war combined chemical exposures (Abou-Donia et al., 1996). The study investigated neurotoxicity produced in hens by individual or simultaneous exposure to these agents. The results showed that exposure to single compounds produced minimal toxicity, while combinations of two agents produced greater neurotoxicity than that caused by individual agents. Neurotoxicity was further enhanced following concurrent administration of all three agents. The authors hypothesized that competition for liver and plasma esterases by these compounds leads to their decreased breakdown and increased transport of the parent compound to nervous tissues.

1.5.4: CYTOTOXIC AND CYTOGENOTOXICITIES

Permethrin and other pyrethroids have been investigated for their cytotoxicity and cytogenotoxicity by Hoelleinger et al. (1987). In this study, the effects of pyrethroids on human lymphocytes and L1210 lymphoblastoid mouse cells were examined including cytogenotoxic effects with micronuclei test. Permethrin was found to have a slight cytogenotoxic effect. In another study by Surralles et al. (1995), five pyrethroid insecticides including permethrin were tested for their ability to induce micronuclei in both whole-blood and isolated human lymphocytes cultures, by using the cytokinesis-block method. Permethrin gave mostly negative results, while cyano pyrethroids had a weak effect.

Permethrin did not induce mutation in either bacteria or cultured Chinese hamster V79 cells (Moriya et al., 1983; Pluijmen et al., 1984; Herrera and Laborda, 1988). It did not induce mutations or aneuploidy in *Drosophila melanogaster* (Gupta et al., 1990; Woodruff et al., 1983). It also did not induce primary DNA damage, measured as differential toxicity, in bacteria (Miyamoto, 1976). Permethrin was found to have ability to induce structural chromosome aberrations (CA) in human lymphocyte cultures and Chinese hamster ovary (CHO) cells (Barrueco et al., 1994). It induced chromosome and chromatid-type aberrations. From this study, permethrin was characterized as an S-phase independent clastogenic agent.

Permethrin has been demonstrated to interfere with the mitochondrial respiratory chain (Gassner et al., 1997). It was found that micromolar concentrations of permethrin inhibited glutamate and succinate sustained state 3 respiration in a concentration-dependent manner. This inhibition could in principle be due to an effect of pyrethroids on an essential mitochondrial transport system and/or on a component of the respiratory chain. Complex I was identified as a major site of inhibition by permethrin. Complexes II and IV were not or only marginally affected by the chemical.

The interaction of permethrin, deltamethrin, allethrin, cypermethrin, or DDT with calmodulin and expression of enzyme activities of phosphoesterase was investigated by Rashatwar and Matsumura (1985). Permethrin was found to be the most potent inhibitor affecting both calmodulin and the enzyme. As calmodulin is a universal calcium binding protein, and it is utilized in many Ca^{2+} requiring systems, the inhibition on its activities may affect greatly calcium homeostasis in the cells.

1.5.5: IMMUNOTOXICITY

Permethrin and other pyrethroids have been investigated *in vitro* on the mitogenic responsiveness of murine T lymphocytes to concanavalin A (Con A) and B lymphocytes to lipopolysaccharide (LPS) (Stelzer and Gordon, 1984). Permethrin was shown to inhibit the mitogenic response to Con A over a concentration range of $1x10^{-5}$M to $5x10^{-5}$M. At a concentration of $3x10^{-5}$M, it inhibited the mitogenic response at each of several

concentrations of Con A tested, and did not cause a shift in the lymphocyte mitogenic dose-response to Con A. The mitogenic responsiveness to LPS was found to be inhibited by permethrin at the same concentrations which inhibited the mitogenesis induced by Con A. Results from this study suggest the possibility of immune suppression by permethrin. Another *in vitro* immunotoxicity study of permethrin was conducted recently by Diel et al. (1998). The study showed that permethrin inhibited lymphocyte proliferation induced by phytohaemagglutinine (PHA), and decreased the production of IFN-γ and IL-4 by lymphocytes in a concentration-dependent manner. In a recent *in vivo* study by Blaylock et al. (1995), mice orally exposed to permethrin were shown to have inhibited cellular immune responses. Permethrin caused decreased responses in immune functions requiring specific antigen recognition and/or effector function, mixed lymphocyte response (MLR) to allogenic lymphocytes, cytotoxic T lymphocyte (CTL) and natural killer (NK) cell activities, while non specific mitogen stimulations and body and organ weights were not affected. Other reports on immunotoxicity of pyrethroids have been studied in type II or cyano pyrethroids. Cypermethrin was reported to decrease humoral responses and alter cellular responses in rats and rabbits (Desi et al., 1985). This compound was also reported to suppress both cellular and humoral immune responses in mice and goats (Tamang et al., 1988). Deltamethrin was reported to have immunosuppressive effects on both humoral and cellular immune responses in mice (Lukowicz-Ratajczak and Krechniak, 1992), and to cause thymus atrophy in mice (Enan et al., 1996). In the study by Enan et al (1996), it was found that deltamethrin was able to induce apoptosis in the thymus of treated animals via the alteration of Ca/CaM-dependent protein kinase-phosphatase cascade. Very low levels of permethrin in the diet of chickens (0.1 ppm for 3 to 6 weeks after hatching) have been reported to suppress immune system activity (Hayes and Laws, 1990).

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1.7 SPECIFIC OBJECTIVES

Effect of dermal exposure to permethrin on immune system in mice

The specific goals for determining permethrin effects are to:

1. Determine whether subacute dermal exposure to permethrin affects immune function.
2. Determine the specific time point and duration of immune alterations after exposure to permethrin: 2, 10, or 30 days.
3. Determine the specific duration of exposure to permethrin that would cause immuntoxicity: 10 or 30 consecutive day exposure, every other day for 7 or 14 exposures.
4. Determine the specific immune functions that may be affected by permethrin exposure: organ weight, histopathology, cell-surface antigen expression, macrophage function, or antibody-producing ability.
5. Determine the possibility of immune alterations in humans who expose to permethrin.

CHAPTER 2.0 : EFFECTS OF TOPICAL PERMETHRIN EXPOSURE ON SPLEEN, THYMUS, BONE MARROW : WEIGHTS, CELL COUNTS, HISTOPATHOLOGY, AND CELL SURFACE ANTIGEN EXPRESSION

2.1: ABSTRACT

Permethrin was applied to the shaved dorsal interscapular region of C57Bl/6N mice at doses of 0.5, 1.5, or 5.0 μl/day. The highest of these doses was approximately equal to 215 μg/kg/day, which is about seven times the estimated daily human exposure in individuals wearing permethrin treated clothing for insect protection. Mice were thus exposed to permethrin daily for 10 or 30 consecutive days, or every other day for 7 or 14 exposures. Body weight was not affected by the treatment. However thymic weight was decreased and splenic weight increased 2 days after termination of 10 consecutive days of topical chemical exposure. Histopathology of immune organs showed no significant changes. Cell surface markers did not change in thymocytes, splenocytes and bone marrow cells. Taken together, these data indicate that low-level topical permethrin exposure may produce some systemic immune effects.

2.2: INTRODUCTION

Potential consequences of immunotoxicity in humans may include immunosuppression, hypersensitivity disease, or autoimmune disease (Luster and Rosenthal, 1993). In this regard, immunotoxicity of pesticides has been noted in recent reports (Thomas, 1995; Vial et al., 1996). Continued evaluation of potential immunotoxic effects of these agents is therefore important for public health because of the wide use of pesticides for both domestic and industrial purposes.

Permethrin, a photostable class I pyrethroid insecticide, is very effective against a wide range of insects and has been used in agriculture, animal husbandry, and human medicine (Taplin and Meinking, 1990). Because of increasing popularity for control of head lice and other purposes, a variety of people ranging from children to adults are being exposed to relatively high levels of permethrin (Llewellyn et al., 1996; Asakawa et al., 1996; Fuortes, 1999). Permethrin is also considered as an environmental hazard to the household user (Friedrich et al., 1998). Further, permethrin has been used by the US military to impregnate into clothing along with another chemical, N,N-diethyl-*m*-toluamide (DEET), for highly effective prevention of insect-borne disease (Schreck et al., 1986; Lillie et al., 1988; Schreck and Kline, 1989; Scholdt et al., 1989). Snodgrass (1992) found that wearing such clothing resulted in topical exposure of permethrin at about 34 μg/kg/day.

During the Persian Gulf War, service personnel were concurrently exposed to biological and chemical agents as well as stress. Veterans of this war have reported chronic symptoms including headache, loss of memory, fatigue, muscle and joint pain, ataxia, skin rash, respiratory difficulties, and gastrointestinal disturbances (i.e., Persian Gulf Syndrome) (Murphy et al., 1999). An alteration in immune function has been suspected as a possible contributing cause to some of these illnesses. In support of this possibility, some immune parameters were found to be significantly different between veterans with syndrome and controls (Zhang et al., 1999). These alterations included increased total T cells and major histocompatibility complex (MHC) II-restricted T cells,

decreased natural killer (NK) cells, and increased levels of interleukin-2 (IL-2), IL-10, gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α).

Permethrin has been well studied for its neurotoxicity (Vijverberg and van den Bercken, 1990), but research examining possible immunotoxicity is limited to only a few reports. A study *in vitro* has shown that permethrin inhibited the mitogenic response of murine lymphocytes to Con A and LPS (Stelzer and Gordon, 1984). In another study utilizing *in vivo* exposure (oral gavage), cellular immune responses were suppressed in mice exposed to permethrin, including a decreased mixed lymphocyte response (MLR) to allogenic lymphocytes, and inhibited cytotoxic T lymphocyte (CTL) and NK cell activity (Blaylock et al., 1995). However, the more relevant dermal route of permethrin exposure has not been evaluated for capacity to alter immune responses.

In present study, mice were dermally exposed to permethrin to mimic human exposure. Subacute exposures were used to correspond to the exposure that humans might receive when wearing permethrin-impregnated clothing (Snodgrass, 1992). Immune function was evaluated using tests from immuntoxicity testing battery of National Toxicology Program (NTP) (Luster et al., 1992).

2.3: MATERIALS AND METHODS

2.3.1: Mice

Female C57Bl/6 mice (Harlan Sprague Dawley, Indianapolis, IN) were used in these studies. All mice were quarantined for one week prior to initiation of experiments. Following the quarantine period, mice were numbered by ear-tags, then assigned random numbers and randomized into separate sequentially numbered cages. Mice were anesthetized by brief inhalation exposure to methoxyflurane, and the interscapular area was shaved of hair using electric clippers. Mice were maintained under controlled conditions of temperature (22 ± 1 °C), humidity (40-60%), and lighting (12/12 hr light/dark cycle) and provided with food and water ad libitum throughtout the course of the experiments. The mice were also examined daily for clinical change.

2.3.2: Permethrin preparation and treatment protocols

Permethrin (91.6%) was provided by the U.S. Army Center for Health Promotion and Preventive Medicine (CHPPM) from stock purchased immediately before by the Army from Coulston Industries (Easton, PA). Mice were treated with permethrin using three dose solutions defined as low-dose (0.5 ml permethrin in 4.5 ml corn oil), middle-dose (1.5 ml permethrin in 3.5 ml corn oil), and high-dose (5.0 ml permethrin). Corn oil was used as the control solution. All dosing solutions were stored in the dark at room temperature. Mice were dosed by interscapular topical exposure with 5 µl of respective dosing solutions using an Eppendorf micropipettor. Dosing was daily for 10 or 30 consecutive days, or every other day for 7 or 14 exposures. After dosing termination, mice were sacrificed and tested for effects of the chemical exposure at days 2, 10, or 30.

2.3.3: Thymus/body weight and spleen/body weight ratio

The thymus and spleen were removed by dissection and cleaned of excess adipose and other connective tissues. Organs were weighed individually on an Ohaus TS-120 balance (Fisher Scientific, Pittsburgh, PA), and percent of total body weight calculated.

2.3.4: Cell preparation and cellularity

Thymuses, spleens, and both femurs and tibias from each mouse were collected and placed (separately) in 2 ml of culture medium (RPMI 1640; Mediatech, Cellgro, Herndon, VA) in a 60x15 mm culture dish (Fisher Scientific, Norcross, GA). Thymocytes and splenic cells were gently dissociated in the culture medium using a metallic sieve screen (Sigma) and curved forceps. For bone marrow studies, femurs and tibias were stripped of associated muscles, and the proximal and distal ends of each bone removed. Marrow hematopoietic cells were collected and pooled by gently flushing the marrow cavity of each of the four bones collected per mouse with 2 ml culture medium through a 25-gauge needle. Erythrocytes were removed from thymic, splenic, and bone marrow samples by suspending cells in lysing solution (0.015 M NH_4Cl, 1.0 mM $NaHCO_3$, 0.1 mM EDTA) for 5 min at room temperature. Cells were then washed twice in culture medium, resuspended in 2 ml standard buffer (Hank's balanced salt solution), and counted using a CASY-1 electronic cell counter (Coulter Electronics, Hialeah, FL.).

2.3.5: Immune cell-surface marker analysis assays

Thymocytes, splenocytes, and bone marrow hematopoietic cell suspensions from each control and permethrin-exposed mouse were adjusted to $5x10^6$ cells/ml in standard buffer. Expression of CD4 and CD8 on thymocytes was determined by incubating these cells in the dark at 4°C for 30 min with 1.0 μg fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 (clone 53-6.7) and 0.2 μg phycoerythrin (PE)-conjugated anti-mouse CD4 (clone H129.19) monoclonal antibodies (Boehringer Mannheim, Indianapolis, IN). Splenocytes were incubated in the dark at 4°C for 30 min with either 1.0 μg PE-conjugated anti-mouse Thy 1.2 (clone 30-H12: Boehringer Mannheim), 1.0 μg PE-conjugated anti-mouse CD45R (Ly-5/B220; clone RA3-6B2; Pharmingen, Sandiago, CA), or 1.0 μg FITC-conjugated anti-mouse Mac1 (clone M1/70: Boehringer Mannheim). Marrow hematopoietic cells were incubated in the dark at 4°C for 30 min with either 1.0 μg FITC-conjugated anti-mouse CD45 (clone IM7; Pharmingen), or 1.0 μg PE-conjugated anti-mouse CD45R. Following incubations cells were washed twice, resuspended in 0.5 ml standard buffer, and immediately analyzed using an Epics XL flow cytometer. Cell viability was verified by forward angle light scatter and ethidium bromide exclusion and was greater than 95% in all samples. The limited number of dead cells present were excluded from analysis with electronic gates. For each sample 10,000 events were collected and analyzed.

2.3.6: Histopathology of spleen and thymus

The spleen and thymus were removed from experimental mice immediately after sacrifice and placed into 10% neutral buffered formalin. After 24 hr of fixation at 4°C, tissues were processed by using routine histological techniques. Sections (6 μm) were made and stained with trichrome. All tissue samples were evaluated using light microscopy.

2.3.7: Statistical analysis

Data were expressed as arithmetical mean ± SEM. Analysis of variance (ANOVA) was used with Dunnett's t test to establish significant differences among

groups. Results described as different in this paper indicate significantly different at $p < 0.05$.

2.4: RESULTS

2.4.1: Mouse body and organ weights

The body weight of experimental mice was not affected by topical permethrin treatment (Table 2.1). Thymic/body weight ratio was decreased by exposure 1 at 2 and 10-days post-exposure (Table 2.2). Thymic cellularity also decreased in exposure 1 mice 2 days after treatment (Table 2.3). Spleen/body weight ratio increased in mice exposed to the middle and high dose levels of permethrin for 10 consecutive days, 2 days after termination of treatment (Table 2.4). Cellularity of the spleen and bone marrow were not affected by permethrin treatment.

2.4.2: Histopathology

In blinded studies, no morphologic effects of the topical permethrin exposure were noted at the light-microscope level. The ratio of the cortex to medulla in the thymus of experimental animals was similar in all groups, as was degree of lymphocyte degeneration (apoptosis) (figure 2.1, figure 2.2). In the spleens, no difference in the ratio of red pulp to white pulp, or in cellular degeneration, was observed (figure 2.3, figure 2.4).

2.4.3: Cell-surface antigen expression

Permethrin did not alter expression of thymocyte CD4, CD8, or CD45R markers. The expression of each CD4, CD8 subpopulation ($CD4^+8^-$, $CD4^+8^+$, $CD4^-8^-$, and $CD4^-8^+$) was not different among control and treated groups in all exposures. Representative data from these experiments are shown in table 2.5. In the spleen, CD45R (identifies B lymphocytes), Thy 1.2 (identifies T lymphocytes), and Mac-1 (identifies macrophages) marker expression was not altered by exposure to permethrin (representative data are shown in table 2.6). In the bone marrow, CD45R (identifies B lineage cells) and CD45 (identifies white blood cells) marker expression was also not altered by permethrin exposure (representative data are shown in table 2.7). Numbers (% positive cells) from

these studies varied somewhat from experiment to experiment based on setting of the cytometer, and data were compared within experiments.

2.5: DISCUSSION

The literature contains extensive information about the neurotoxicity of permethrin, as might be predicted for a pyrethroid compound. Limited reports have examined potential immunotoxicity in animals or humans exposed to this insecticide, however, all of these investigations have demonstrated immune targeting by permethrin. To date, no investigations have studied potential immunotoxicity resulting from dermal exposure to permethrin. Such may be important, given that topical exposure to permethrin is not uncommon in humans and that permethrin has been demonstrated to cross human skin.

Lukowicz-Ratajczak and Krechniak (1992) recently reported that deltamethrin, a cyano-pyrethroid, affected both cellular and humoral immune responses in mice. Enan et al. (1996) further observed thymic atrophy in mice exposed to deltamethrin via a single intraperitoneal treatment. These authers demonstrated that deltamethrin was able to induce apoptosis in the thymus of treated animals via alteration of the Ca/CaM-dependent protein kinase-phosphatase cascade. Permethrin was found to be a potent inhibitor of calmodulin, a universal calcium binding protein, which regulates the Ca^{2+} pump in the plasma membrane and involves in many Ca^{2+} requiring systems (Rashatwar and Matsumura, 1985). It was also found to inhibit mitochondrial complex I, which could interfere with the electron transport chain and process of ATP production (Gassner et al., 1997). These actions of permethrin may lead to a disturbance of Ca^{2+} homeostasis inside the cells, particularly increased intracellular Ca^{2+} concentration, as the Ca^{2+} pump needs both ATP and calmodulin to function properly for pumping Ca^{2+} out of the cells. These results and thymic atrophy found in present study suggest the possibility of increased thymocyte apoptosis caused by permethrin.

The possibility that increased thymocyte apoptosis in the present permethrin-treated mice may have contributed to the observed thymic atrophy was not directly

investigated. However, flow-cytometric evaluation of CD4 and CD8 surface markers did not demonstrate a relative decrease in any specific thymocyte subpopulation defined by these antigens, suggesting selective apoptosis of a responsive phenotype (e.g., $CD4^+8^+$) may not have been involved. Histologic evaluations of the thymus were also conducted, and did not detect increased numbers of apoptotic or necrotic cells.

The possibility that permethrin may have suppressed thymocyte proliferation contributing to thymic atrophy should be considered. As permethrin was found to suppress lymphocyte proliferation stimulated by mitogen (Stelzer and Gordon, 1984), decrease the production of cytokines (IFN_γ and IL-4) (Diel et al., 1998), and suppress cellular mediated immune response (Blaylock et al., 1995), thymic atrophy could also be the result of suppression of cell proliferation. The effect of permethrin on the mitochondrial compartment and calmodulin may play an important role not only in thymocyte apoptosis but also in interference of thymocyte proliferation. Ca^{2+} is an important second messenger involved in many activities inside the cells, thus improper signal resulting from disturbance of Ca^{2+} homeostasis could undoubtly lead to abnormal cell growth.

With different protocols of exposure, thymic atrophy caused by permethrin occurred only after 10 consecutive day exposure, but not after every other day exposure or 30 consecutive day exposure. However, non-significant trends toward reduced thymic weight and cellularity were often seen in the other exposure groups. This phenomenon may be explained by the toxicokinetics of permethrin in animals. Permethrin was metabolized rapidly by ester hydrolysis and oxidation (Casida et al., 1983) resulting in short half-life (12.37 hr after po administration in rat) in rodents (Anadon et al., 1991). Every other day application of permethrin may be less likely to produce any effects. Permethrin was also found to have a hepatic enzyme-inducing ability (Anadon et al., 1988), which could be one of the reasons that 30 day exposure caused less effect.

In this study, permethrin-induced splenomegaly occurred after 10 consecutive days of exposure. The data on cellularity, splenic cell surface markers (CD45R, Thy 1.2, Mac-1) or histopathology did not show significant changes, and did not provide

information suggesting a mechanism of splenomegaly. However, it may be reasoned from these data that splenic weight increased as a result of splenic congestion, but the mechanism is still unknown.

In summary, data in the present study suggest limited but significant systemic alterations may occur in mice following topical exposure to permethrin. It is important to note that these changes were observed at doses not too far above present human exposure. The high dose used in this study was about 7 times human exposure, and the 1.5 μl dose (which also produced significant effects) was only about 2 times human exposure. We did not examine certain aspects of immune function, which may be important including altered skin immune function.

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Table 2.1 Body weight in C57Bl/6 mice topically exposed to permethrin

Mouse body weight (grams, means ± sem)			
Permethrin exposure	**Days after dosing termination**		
	2 day	**10 day**	**30 day**
EXPOSURE 1:			
Control	20.81 ± 0.65	23.20 ± 0.60	22.40 ± 0.30
0.5 µl/day	21.70 ± 0.45	23.20 ± 0.20	23.29 ± 0.47
1.5 µl /day	20.61 ± 0.48	21.10 ± 0.40*	22.29 ± 0.48
5.0 µl /day	20.34 ± 0.43	22.90 ± 0.40	22.70 ± 0.35
EXPOSURE 2:			
Control	19.26 ± 0.43	21.59 ± 0.57	22.40 ± 0.67
0.5 µl /day	19.02 ± 0.56	21.60 ± 0.42	22.60 ± 0.50
1.5 µl /day	19.23 ± 0.64	21.15 ± 0.54	22.80 ± 0.40
5.0 µl /day	20.79 ± 0.60	21.33 ± 0.57	21.90 ± 0.78
EXPOSURE 3:			
Control	20.98 ± 0.60	21.46 ± 0.38	23.14 ± 0.34
0.5 µl /day	21.09 ± 0.22	20.67 ± 0.57	23.31 ± 0.15
1.5 µl /day	21.13 ± 0.52	21.96 ± 0.59	21.86 ± 0.60
5.0 µl /day	22.36 ± 0.50	21.94 ± 0.52	21.48 ± 0.31
EXPOSURE 4:			
Control	20.78 ± 0.51	21.58 ± 0.38	21.07 ± 0.44
0.5 µl /day	20.84 ± 0.49	20.60 ± 0.28	21.14 ± 0.81
1.5 µl /day	21.23 ± 0.51	21.27 ± 0.21	22.10 ± 0.45
5.0 µl /day	20.95 ± 0.59	21.09 ± 0.55	21.79 ± 0.52

Exposure 1 = every day for 10 consecutive days
Exposure 2 = every other day for 7 exposures
Exposure 3 = every other day for 14 exposures
Exposure 4 = every day for 30 consecutive days

* statistically significant ($P < .05$)

Table 2.2 Thymus/body weight ratio in C57Bl/6 mice topically exposed to permethrin

Thymus/body weight ratio (%, means ± sem)			
Permethrin exposure	**Days after dosing termination**		
	2 day	**10 day**	**30 day**
EXPOSURE 1:			
Control	0.266 ± 0.041	0.261 ± 0.017	0.239 ± 0.012
0.5 µl/day	0.253 ± 0.026	0.263 ± 0.018	0.298 ± 0.056
1.5 µl/day	0.142 ± 0.019*	0.217 ± 0.028*	0.250 ± 0.008
5.0 µl/day	0.151 ± 0.012*	0.249 ± 0.015	0.304 ± 0.021
EXPOSURE 2:			
Control	0.306 ± 0.020	0.371 ± 0.023	0.261 ± 0.016
0.5 µl/day	0.353 ± 0.036	0.318 ± 0.018	0.272 ± 0.021
1.5 µl/day	0.242 ± 0.038	0.333 ± 0.019	0.290 ± 0.009
5.0 µl/day	0.256 ± 0.020	0.361 ± 0.016	0.238 ± 0.021
EXPOSURE 3:			
Control	0.282 ± 0.023	0.223 ± 0.012	0.156 ± 0.010
0.5 µl/day	0.301 ± 0.016	0.245 ± 0.016	0.153 ± 0.011
1.5 µl/day	0.281 ± 0.025	0.249 ± 0.015	0.152 ± 0.014
5.0 µl/day	0.252 ± 0.016	0.243 ± 0.014	0.165 ± 0.010
EXPOSURE 4:			
Control	0.243 ± 0.005	0.185 ± 0.015	0.145 ± 0.010
0.5 µl/day	0.209 ± 0.014	0.215 ± 0.020	0.155 ± 0.011
1.5 µl/day	0.211 ± 0.013	0.217 ± 0.019	0.179 ± 0.016
5.0 µl/day	0.225 ± 0.028	0.221 ± 0.007	0.146 ± 0.012

Exposure 1 = every day for 10 consecutive days
Exposure 2 = every other day for 7 exposures
Exposure 3 = every other day for 14 exposures
Exposure 4 = every day for 30 consecutive days

* statistically significant ($P < .05$)

Table 2.3 Thymic cellularity in C57Bl/6 mice topically exposed to permethrin

Thymic cellularity (x 10^7, means ± sem)			
Permethrin exposure	**Days after dosing termination**		
	2 day	**10 day**	**30 day**
EXPOSURE 1:			
Control	5.13 ± 0.78	17.4 ± 1.97	6.87 ± 1.54
0.5 µl/day	3.78 ± 0.58	14.0 ± 1.53	7.28 ± 0.59
1.5 µl/day	1.94 ± 0.34*	14.6 ± 1.69	6.63 ± 0.66
5.0 µl/day	2.49 ± 0.32*	18.1 ± 1.94	5.50 ± 1.09
EXPOSURE 2:			
Control	7.06 ± 1.22	26.04 ± 10.13	
0.5 µl/day	6.87 ± 2.08	13.02 ± 3.06	not
1.5 µl/day	7.22 ± 1.47	13.58 ± 2.04	recorded
5.0 µl/day	8.26 ± 2.24	16.24 ± 2.33	
EXPOSURE 3:			
Control	11.08 ± 1.09	11.33 ± 1.54	14.90 ± 1.96
0.5 µl/day	11.12 ± 0.99	11.09 ± 1.53	9.84 ± 1.07
1.5 µl/day	9.20 ± 2.34	9.06 ± 1.54	15.02 ± 2.14
5.0 µl/day	7.31 ± 1.56	6.85 ± 0.52	11.27 ± 0.79
EXPOSURE 4:			
Control	13.11 ± 1.47	12.08 ± 1.54	10.27 ± 1.93
0.5 µl/day	11.26 ± 0.80	12.21 ± 0.71	7.05 ± 0.85
1.5 µl/day	10.86 ± 1.04	10.42 ± 0.80	7.02 ± 0.83
5.0 µl/day	10.84 ± 1.59	7.72 ± 1.02	6.35 ± 0.69

Exposure 1 = every day for 10 consecutive days
Exposure 2 = every other day for 7 exposures
Exposure 3 = every other day for 14 exposures
Exposure 4 = every day for 30 consecutive days

* statistically significant ($P < .05$)

Table 2.4 Spleen/body weight ratio in C57Bl/6 mice topically exposed to permethrin

Spleen/body weight ratio (%, means ± sem)			
Permethrin exposure	**Days after dosing termination**		
	2 day	**10 day**	**30 day**
EXPOSURE 1:			
Control	0.226 ± 0.025	0.317 ± 0.051	0.242 ± 0.035
0.5 µl/day	0.268 ± 0.011	0.320 ± 0.070	0.284 ± 0.051
1.5 µl/day	0.468 ± 0.067*	0.328 ± 0.053	0.253 ± 0.026
5.0 µl/day	0.392 ± 0.050*	0.330 ± 0.065	0.252 ± 0.020
EXPOSURE 2:			
Control	0.208 ± 0.044	0.235 ± 0.020	0.272 ± 0.014
0.5 µl/day	0.244 ± 0.018	0.240 ± 0.029	0.263 ± 0.024
1.5 µl/day	0.316 ± 0.036*	0.224 ± 0.035	0.275 ± 0.054
5.0 µl/day	0.278 ± 0.024	0.200 ± 0.019	0.448 ± 0.112
EXPOSURE 3:			
Control	0.192 ± 0.047	0.212 ± 0.046	0.207 ± 0.025
0.5 µl/day	0.223 ± 0.031	0.227 ± 0.040	0.266 ± 0.073
1.5 µl/day	0.178 ± 0.020	0.188 ± 0.031	0.242 ± 0.040
5.0 µl/day	0.222 ± 0.013	0.249 ± 0.040	0.242 ± 0.031
EXPOSURE 4:			
Control	0.153 ± 0.012	0.176 ± 0.016	0.257 ± 0.044
0.5 µl/day	0.228 ± 0.024	0.159 ± 0.012	0.183 ± 0.024
1.5 µl/day	0.199 ± 0.029	0.218 ± 0.044	0.196 ± 0.017
5.0 µl/day	0.165 ± 0.026	0.192 ± 0.037	0.230 ± 0.032

Exposure 1 = every day for 10 consecutive days
Exposure 2 = every other day for 7 exposures
Exposure 3 = every other day for 14 exposures
Exposure 4 = every day for 30 consecutive days

* statistically significant ($P < .05$)

Table 2.5 CD4 and CD8 expression in of thymocytes in C57Bl/6 mice topically exposed to permethrin

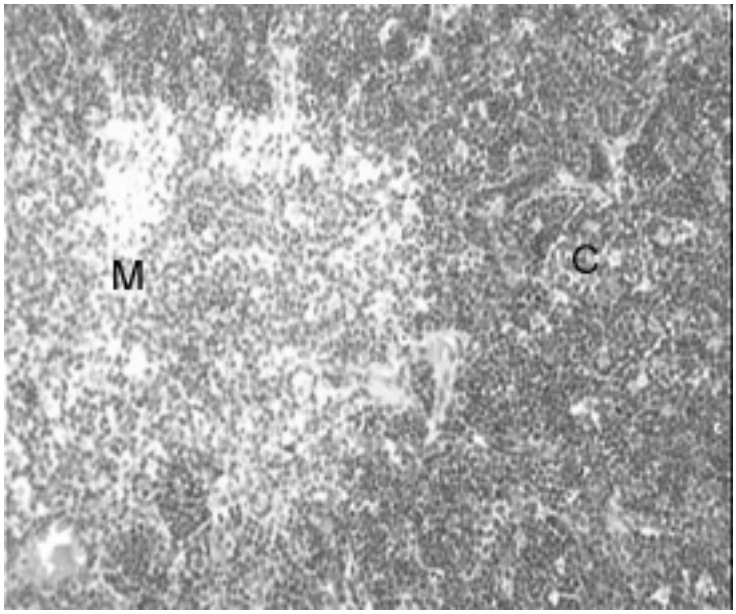
CD4 and CD8 expression (% positive cells, means ± sem)			
Thymocyte markers	**Days after dosing termination**		
	2 day	**10 day**	**30 day**
$CD4^+8^-$			
Control	5.28 ± 0.62	6.98 ± 0.87	9.82 ± 1.14
0.5 µl/day	5.27 ± 0.50	6.90 ± 0.38	10.23 ± 0.65
1.5 µl/day	5.67 ± 0.37	7.46 ± 0.30	8.80 ± 0.90
5.0 µl/day	6.50 ± 0.56	7.15 ± 0.24	11.58 ± 0.64
$CD4^+8^+$			
Control	84.52 ± 0.88	85.68 ± 1.52	80.72 ± 2.43
0.5 µl/day	85.07 ± 0.39	84.25 ± 1.09	82.98 ± 1.18
1.5 µl/day	86.03 ± 0.74	82.08 ± 1.41	81.07 ± 2.28
5.0 µl/day	84.47 ± 0.66	85.32 ± 1.13	80.55 ± 1.18
$CD4^-8^-$			
Control	8.75 ± 0.87	4.63 ± 0.64	7.97 ± 1.79
0.5 µl/day	8.42 ± 0.75	5.40 ± 0.58	5.18 ± 0.61
1.5 µl/day	6.83 ± 0.81	6.70 ± 0.84	8.97 ± 2.02
5.0 µl/day	7.43 ± 0.77	5.35 ± 0.92	5.70 ± 0.55
$CD4^-8^+$			
Control	1.45 ± 0.15	2.78 ± 0.51	1.48 ± 0.31
0.5 µl/day	1.25 ± 0.11	3.42 ± 0.78	1.63 ± 0.24
1.5 µl/day	1.45 ± 0.19	3.78 ± 1.22	1.20 ± 0.18
5.0 µl/day	1.62 ± 0.09	2.15 ± 0.39	2.17 ± 0.38

Table 2.6 CD45R, Thy 1.2 and Mac-1 expression of splenocytes in C57Bl/6 mice topically exposed to permethrin

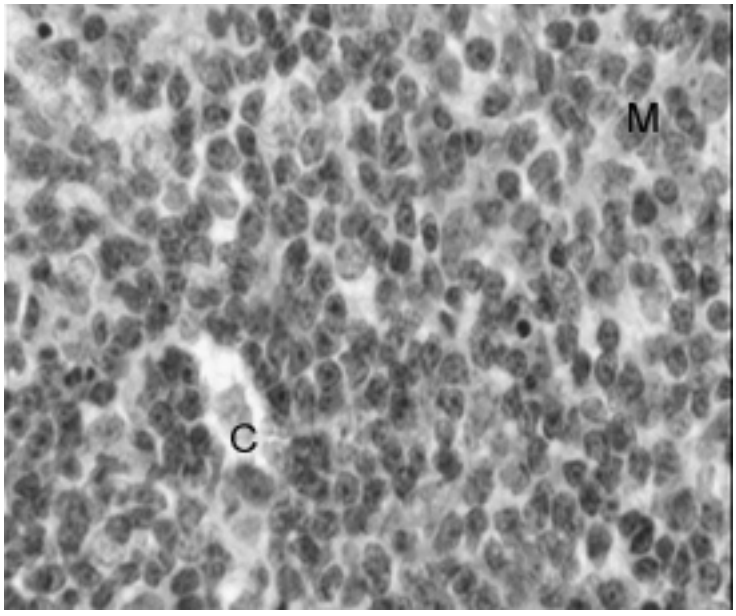
CD45R, Thy 1.2 and Mac-1 expression in spleen (% positive cells, means ± sem)			
Splenic cell markers	**Days after dosing termination**		
	2 day	**10 day**	**30 day**
CD45R			
Control	27.43 ± 3.68	20.47 ± 0.76	24.4 ± 2.45
0.5 µl/day	32.20 ± 2.87	21.72 ± 1.93	29.3 ± 1.30
1.5 µl/day	24.84 ± 6.38	17.80 ± 1.31	27.0 ± 0.69
5.0 µl/day	29.52 ± 2.19	16.53 ± 0.96	24.0 ± 1.78
Thy 1.2			
Control	49.85 ± 1.81	38.35 ± 4.16	34.4 ± 1.53
0.5 µl/day	45.17 ± 1.35	38.38 ± 4.22	33.6 ± 1.17
1.5 µl/day	41.80 ± 8.52	40.53 ± 2.66	39.0 ± 1.38
5.0 µl/day	50.92 ± 1.68	40.20 ± 4.11	38.9 ± 2.59
Mac-1			
Control	5.55 ± 0.29	10.6 ± 0.58	15.77 ± 1.22
0.5 µl/day	7.67 ± 0.68	7.83 ± 0.51	18.15 ± 1.30
1.5 µl/day	8.77 ± 0.52	9.02 ± 0.88	13.92 ± 0.96
5.0 µl/day	6.37 ± 0.36	10.6 ± 0.66	14.57 ± 1.07

Table 2.7 CD45 and CD45R expression of bone marrow cells in C57Bl/6 mice topically exposed to permethrin

CD45 and CD45R expression (% positive cells, means ± sem)			
Bone marrow cell markers	**Days after dosing termination**		
	2 day	**10 day**	**30 day**
CD45			
Control	29.43 ± 2.30	25.07 ± 1.86	40.70 ± 6.64
0.5 µl/day	23.72 ± 2.68	19.60 ± 2.84	59.08 ± 5.19
1.5 µl/day	25.42 ± 2.10	23.96 ± 2.70	54.62 ± 5.22
5.0 µl/day	22.88 ± 2.42	21.70 ± 1.93	43.45 ± 3.75
CD45R			
Control	8.00 ± 0.81	10.87 ± 0.98	9.73 ± 1.08
0.5 µl/day	8.22 ± 0.79	10.63 ± 1.35	9.52 ± 1.20
1.5 µl/day	7.05 ± 0.71	9.28 ± 1.19	8.22 ± 0.83
5.0 µl/day	6.05 ± 0.36	9.82 ± 1.04	8.72 ± 0.89

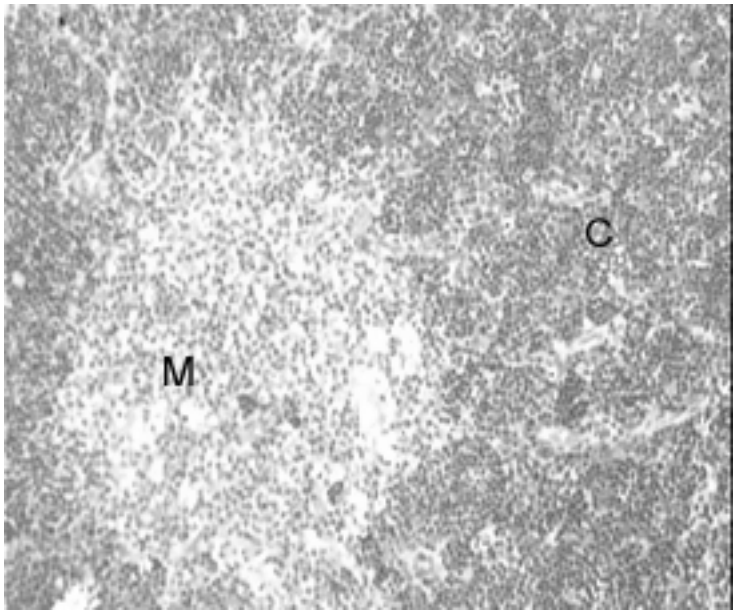


A



B

Figure 2.1 Histology of thymus from control mice. In the sections, cortex (C) and medulla (M) are indicated. (A-original magnification x 25; B-original magnification x 100)



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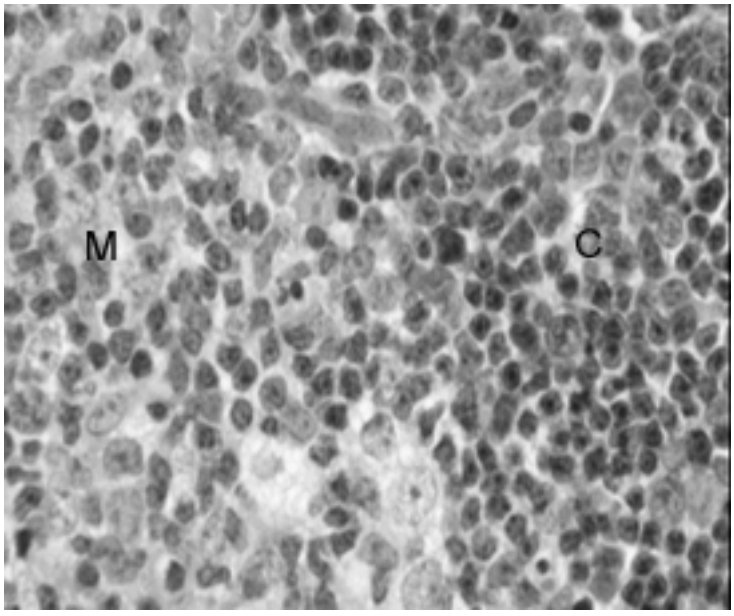
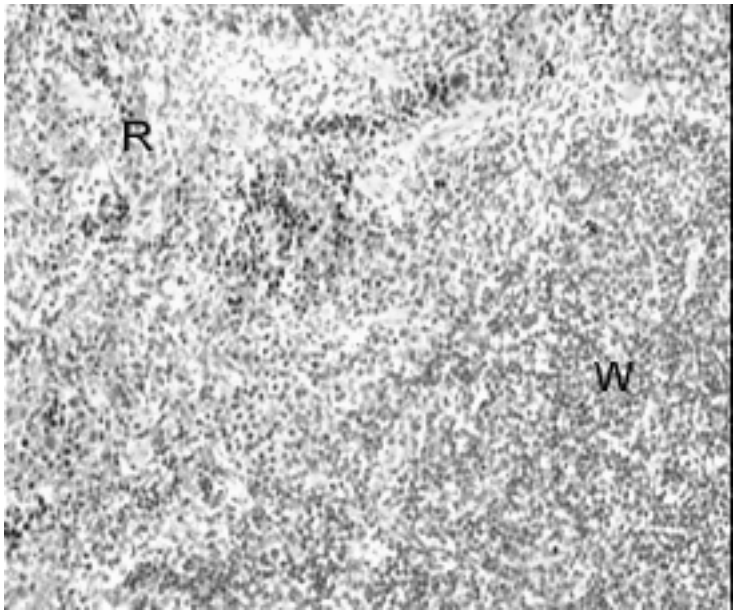
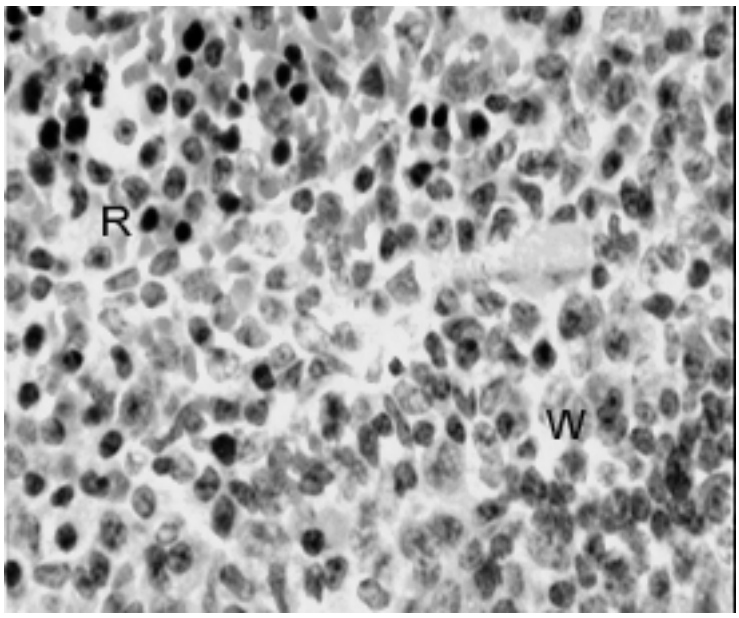


B

Figure 2.2 Histology of thymus from mice exposed to high dose of permethrin. In the sections, cortex (C) and medulla (M) are indicated. (A-original magnification x 25; B-original magnification x 100)

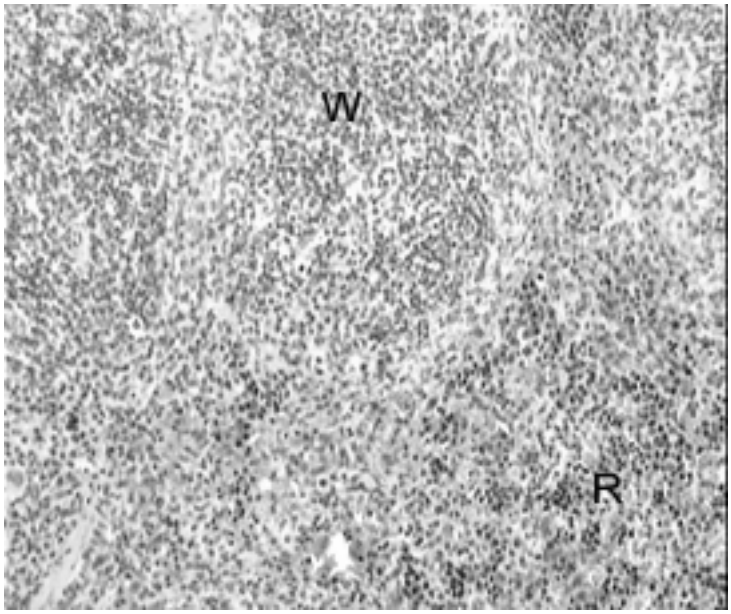


A

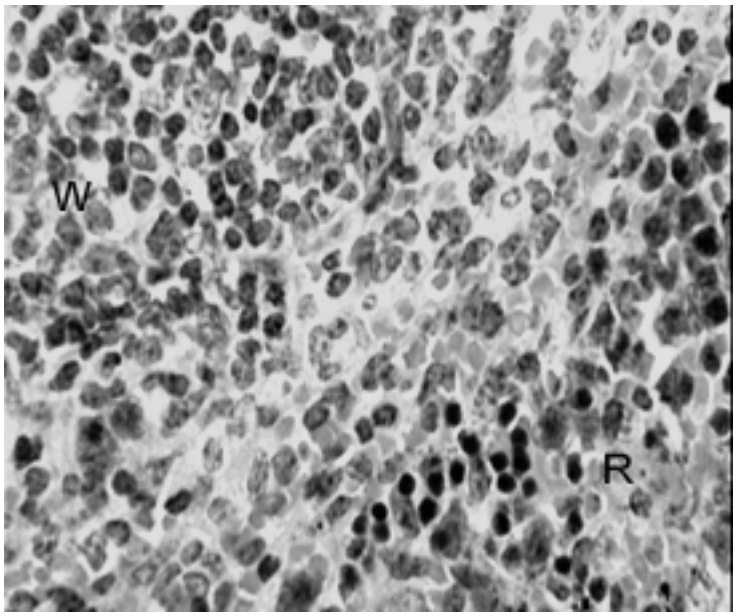


B

Figure 2.3 Histology of spleen from control mice. . In the sections, white pulp (W) and red pulp (R) are indicated. (A-original magnification x 25; B-original magnification x 100)



A



B

Figure 2.4 Histology of spleen from mice exposed to high dose of permethrin. In the sections, white pulp (W) and red pulp (R) are indicated. (A-original magnification x 25; B-original magnification x 100)

CHAPTER 3.0: EFFECTS OF TOPICAL PERMETHRIN EXPOSURE ON MACROPHAGE FUNCTION AND ANTIBODY PRODUCTION

3.1: ABSTRACT

Permethrin was applied to the shaved dorsal interscapular region of C57Bl/6N mice at doses of 0.5, 1.5, or 5.0 μl/day. The highest of these doses was approximately equal to 215 μg/kg/day, which is about seven times the estimated daily human exposure in individuals wearing permethrin treated clothing for insect protection. Mice were thus exposed to permethrin daily for 10 or 30 consecutive days, or every other day for 7 or 14 exposures. The splenic macrophage chemiluminescent response was affected by the treatment at 2 days after exposure to permethrin, but phagocytic ability was not affected. Antibody production as shown by plaque forming cell (PFC) assay decreased significantly after 10 consecutive day exposure to permethrin. Taken together, these data indicate that low-level topical permethrin exposure may produce systemic immune effects.

3.2: INTRODUCTION

Since the end of the Persian Gulf War, numerous studies and reports have described illness of veterans from this war (i.e., Persian Gulf Syndrome) (Doucet, 1994; Unwin et al., 1999; Klaustermeyer et al., 1998; Proctor et al., 1998). The symptoms included headache, loss of memory, fatigue, muscle and joint pain, ataxia, skin rash, respiratory difficulties, and gastrointestinal disturbances (Murphy et al., 1999). It was suggested that this syndrome may result from multiple assaults upon the body's immune system (Doucet, 1994). In support of this possibility, some immune parameters were found to be significantly different between veterans with syndrome and controls (Zhang et al., 1999). These alterations included increased total T cells and major histocompatibility complex (MHC) II-restricted T cells, decreased natural killer (NK) cells, and increased levels of interleukin-2 (IL-2), IL-10, gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α).

Permethrin, a photostable class I pyrethroid insecticide, has been used by the US military to impregnate into clothing along with another chemical, N,N-diethyl-*m*-toluamide (DEET), for highly effective prevention of insect-borne disease (Schreck et al., 1986; Lillie et al., 1988; Schreck and Kline, 1989; Scholdt et al., 1989). These permethrin-impregnated clothing were also used during the Gulf War, and calculated to result in topical exposure of permethrin at about 34 μg/kg/day (Snodgrass, 1992).

Pyrethroid insecticides have been reported to cause immunotoxicity in animal studies. Cypermethrin was reported to decrease humoral responses and alter cellular immune responses in rats and rabbits (Desi et al., 1985). This compound was also reported to suppress both cellular and humoral immune responses in mice and goats (Tamang et al., 1988). Deltamethrin was reported to have immunosuppressive effects on both humoral and cellular immune responses in mice (Lukowicz-Ratajczak and Krechniak, 1992), and to cause thymus atrophy in mice (Enan et al., 1996). In the study by Enan et al. (1996), it was found that deltamethrin was able to induce apoptosis in the thymus of treated animals via the alteration of Ca/CaM-dependent protein kinase-phosphatase cascade.

Permethrin has been well studied for its neurotoxicity (Vijverberg and van den Bercken, 1990), but research examining possible immunotoxicity is limited to only a few reports. Studies *in vitro* has shown that permethrin inhibited the mitogenic response of murine lymphocytes to Concanavalin A (Con A), lipopolysaccharide (LPS) (Stelzer and Gordon, 1984), and phytohaemagglutinine (PHA), and decreased the production of IFN-γ and IL-4 (Diel et al., 1998). In another study utilizing *in vivo* exposure (oral gavage), cellular immune responses were suppressed in mice exposed to permethrin, including a decreased mixed lymphocyte response (MLR) to allogenic lymphocytes, and inhibited cytotoxic T lymphocyte (CTL) and NK cell activity (Blaylock et al., 1995). The more relevant dermal route of permethrin exposure was not evaluated by these authors for capacity to alter immune responses.

In a previous investigation, we reported decreased thymic and increased splenic weight in mice dermally exposed to permethrin (unpublished data). The present study extends these results to include inhibited humoral immune response (antibody production) and suppressed splenic macrophage chemicluminescence (H_2O_2 production) in mice following subacute topical exposure to permethrin.

3.3: MATERIALS AND METHODS

3.3.1: Mice

Female C57Bl/6 mice (Harlan Sprague Dawley, Indianapolis, IN) were used in these studies. All mice were quarantined for one week prior to initiation of experiments. Following the quarantine period, mice were numbered by ear-tags, then assigned random numbers and randomized into separate sequentially numbered cages. Mice were anesthetized by brief inhalation exposure to methoxyflurane, and the interscapular area was shaved of hair using electric clippers. Mice were maintained under controlled conditions of temperature (22 ± 1 °C), humidity (40-60%), and lighting (12/12 hr light/dark cycle) and provided with food and water ad libitum throughtout the course of the experiments. The mice were also examined daily for clinical change.

3.3.2: Permethrin preparation and treatment protocols

Permethrin (91.6%) was provided by the U.S. Army Center for Health Promotion and Preventive Medicine (CHPPM) from stock purchased immediately before by the Army from Coulston Industries (Easton, PA). Mice were treated with permethrin using three dose solutions defined as low-dose (0.5 ml permethrin in 4.5 ml corn oil), middle-dose (1.5 ml permethrin in 3.5 ml corn oil), and high-dose (5.0 ml permethrin). Corn oil was used as the control solution. All dosing solutions were stored in the dark at room temperature. Mice were dosed by interscapular topical exposure with 5 µl of respective dosing solutions using an Eppendorf micropipettor. Dosing was daily for 10 or 30 consecutive days, or every other day for 7 or 14 exposures. After dosing termination, mice were sacrificed and tested for effects of the chemical exposure at days 2, 10, or 30.

3.3.3: Cell preparation

Spleens from each mouse were collected and placed (separately) in 2 ml of culture medium (RPMI 1640; Mediatech, Cellgro, Herndon, VA) in a 60x15 mm culture dish (Fisher Scientific, Norcross, GA). Splenic cells were gently dissociated in the culture medium using a metallic sieve screen (Sigma) and curved forceps. Erythrocytes were removed from splenic samples by suspending cells in lysing solution (0.015 M NH_4Cl, 1.0 mM $NaHCO_3$, 0.1 mM EDTA) for 5 min at room temperature. Cells were then washed twice in culture medium, resuspended in 2 ml standard buffer (Hank's balanced salt solution), and counted using a CASY-1 electronic cell counter (Coulter Electronics, Hialeah, FL.).

3.3.4: Chemiluminescence assay

The production of H_2O_2 in PMA (phorbol-12-myristate 13-acetate)-stimulated splenic macrophages was determined by the method of Bass et al. (1983). Briftly, Splenic cell suspensions ($0.5x10^6$ cells) were incubated with 5 µl dichlorofluorescein-diacetate (DCF-DA; Molecular Probes, Eugene, OR; 5 mM) for 15 min at room temperature. Following incubation with DCF-DA, cells were stimulated by the addition of 10 µl PMA (Sigma, St.Louis, MO; 100 ng/ml) in a subsequent 30 min incubation period. Cells were then placed on ice to stop the reaction and immediately analyzed by flow cytometry.

Background fluorescence, determined using unstained cells from each treatment group, was subtracted from respective populations incubated with the fluorescent probe.

3.3.5: Phagocytosis of fluorescent microspheres

The phagocytic capacity of splenic macrophages was determined by a modification of the method of Dunn and Tylor (1981). Briftly, cells were aliquoted as above in polystyrene round-bottom tubes at $0.5x10^6$ cells in 0.1 ml standard buffer. Fluoresbrite microspheres (1.16 μm; Polysciences, Inc., Warrington, PA) were washed twice in standard buffer, and then sonicated on ice (Ultrasonic Cell Disrupter, Misonix, Inc., Farmingdale, NY) for 30 sec at 35% to disrupt aggregated microspheres. A 10 μl volume of microspheres was added to tubes to give an initial ratio of 50 beads/cell. Fresh culture media then was added to each tube to give a final volume of 2.0 ml/tube. This latter step prevents the development of acidic media conditions resulting from metabolic activity of the cells during incubation. Cells then were incubated overnight (18 hr) at 37°C and 5% CO_2. Following incubation, cells were washed twice with standard buffer to remove non-phagocytized microspheres, after which cells were resuspended in 0.5 ml standard buffer and immediately analyzed by flow cytometry. Before analysis the cytometer was standardized for fluorescence using a sample of cells without beads. Thus, the peak on the fluorescence histogram resulting from cells containing single microspheres could be identified. For each sample 10,000 events were collected and the number of cells ingesting fluorescent particles was expressed as a percentage of 10,000.

3.3.6: Plaque forming cells (PFC) assay

Ability to produce specific antibody was determined by quantitating the plaque-forming cell (PFC) response to the T-dependent antigen, sheep red blood cells (SRBC). Mice were immunized by intraperitoneal injection of $2x10^8$ sheep red blood cells (SRBC) and IgM PFCs were enumerated in spleen cells 4 days later. Splenic cell supensions ($20x10^6$ cells/ml) were prepared in culture medium. Cells of each sample (50 μl, 10^6 cells) were then mixed with 20 μl guinea pig complement (Sigma), 30 μl of 30% SRBC, and 0.4 ml of warmed agar (47°C) in a 35x10 mm culture dish (Fisher Scientific, Norcross, GA), then incubated in 37°C and 5% CO_2 incubator for 4 hr. After incubation,

the number of PFC was counted under a light microscope. The values were expressed as the number of PFC per 10^6 cells.

3.3.7: Statistical analysis

Data were expressed as arithmetical mean ± SEM. Analysis of variance (ANOVA) was used with Dunnett's t test to establish significant differences among groups. Results described as different in this paper indicate significantly different at $p < 0.05$.

3.4: RESULTS

3.4.1: Chemiluminescent response

A dose-response inhibition of H_2O_2 production by splenic macrophages was present in all exposure groups at 2 days after termination of treatment (Table 3.1). A significant decrease in H_2O_2 production continued to 10 days after termination of treatment in Exposures 1 and 4; a trend toward a decrease was present in Exposure 3. Using this procedure, values can be compared within but not between experiments.

3.4.2: Phagocytosis response

Phagocytic ability of macrophages tended to display rather high variability by this assay. A significant difference occurred 2 days after dosing ended, in the low dose group of Exposure 4 (Table 3.2). Values can again be compared within but not between experiments. Given the lack of a dose-response trend in this experiment, and lack of an effect in all other day 2 data, it is doubtful this one effect is biologically meaningful.

3.4.3: PFC response

Permethrin produced a non-significant trend toward decreased plaque number in Exposure 1 mice at 2 days after dosing termination (Table 3.3). This trend became significant in this exposure group at 10 days post-dosing. Values can again be compared within but not between experiments. Of possible importance, Exposure 1 produced the most significant alterations in other immune parameters measured, including decreased thymic weight and increased splenic weight (unpublished data from a previous study). No other exposure regimen significantly altered antibody production.

3.5: DISCUSSION

A limited but growing database in the literature suggests certain pyrethroid compounds may produce immunotoxicity. Brieftly, cypermethrin (a type II or cyano-pyrethroid) was found to inhibit humoral and cellular immune responses in rats, rabbits (Desi et al., 1985), mice and goats (Tamang et al., 1988). Deltamethrin produced thymic atrophy and suppressed humoral and cellular responses in mice (Lukowicz-Ratajczak and Krechniak, 1992; Queiroz, 1993; Madsen et al., 1996). Enan et al. (1996) further reported that deltamethrin induced apoptosis in the thymus of treated animals by altering activity of the Ca/CaM-dependent protein kinase-phosphatase cascade. Permethrin inhibited the mitogenic response of T lymphocytes to Con A and B lymphocytes to LPS (Stelzer and Gordon, 1984). Diel et al. (1998) recently demonstrated a similar inhibition of T lymphoproliferation induced by the mitogen PHA, as well as dose-dependent decreases in IFN_{γ} and IL-4 in mice exposed to permethrin. Blaylock et al. (1995) reported decreased mixed lymphocyte responses (MLR), and inhibited cytotoxic T lymphocyte (CTL) and natural killer (NK) cell activity in mice treated by oral gavage with permethrin.

Collectively, the above reports demonstrate immune effects in experimental animals treated with pyrethroid insecticides. However, none of the above reports examined the possibility that topical pyrethroid (i.e., the typical route of human exposure) may alter function of the immune system. Percutaneous absorption of varying levels of permethrin has been demonstrated in rhesus monkey (Sidon et al., 1988), rat, rabbit, dog, and human (Taplin and Meinking, 1990). Further Snodgrass (1992) estimated that humans wearing permethrin-treated clothing received a topical exposure of about 34 μg/kg/day. These reports raise questions about immune effects resulting from absorbed permethrin in humans wearing treated clothing (i.e., military uniforms; hunting clothing) or following use of permethrin-based shampoos or creams for body lice or scabies. Regarding the latter, a 1% permethrin formulation (NIX®) is widely used in children for treatment of head lice (Brandenburg et al., 1986; Taplin and Meinking, 1990), while a 5%

cream (Elimite®) has been found more effective for treating scabies than 10% crotamiton (Eurax®) (Taplin et al., 1990) or 1% lindane lotion (Schultz et al., 1990).

Results of the present study indicate that topical permethrin may alter both humoral mediated immunity (antibody production) and non-specific immune function (ability of splenic macrophages to produce reactive oxygen species) in mice. Specific mechanisms by which these effects may occur were not investigated in the present experiments. However, the ability of permethrin to inhibit production of cytokines (e.g., IFN_γ and IL-4) essential for antibody production (Diel et al., 1998) and to inhibit B lymphocyte proliferation (Stelzer and Gordon, 1984) may be related to an effect on antibody production.

An effect of permethrin on the ability of macrophages to produce H_2O_2 was clearly evident in this study. The inhibition effect was dose-dependent and persisted up to 10 days after dosing termination. This effect was the only immune effect observed in all topical chemical exposure groups, in this or a previous study, suggesting that the macrophage respiratory burst (i.e., chemiluminescent response) may represent a highly sensitive immune target of permethrin exposure. Reasons for this again remain unclear, however, Gassner et al. (1997) have demonstrated that permethrin inhibits mitochondrial complex I, an effect which may interfere with the electron transport chain, and thus the respiratory metabolic burst of phagocytic cells.

In conclusion, results of the present study support the possibility that topical permethrin exposure may cause systemic immune effects. The effect of this compound on antibody production may be particularly noteworthy, in that this endpoint is one of the strongest predictors of clinically significant immunosuppression (individual predictive value 0.78) (Luster et al., 1992).

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Table 3.1 Chemiluminescent response in C57Bl/6 mice topically exposed to permethrin

Chemiluminescent Response (% positive cells, means ± sem)			
Permethrin exposure	**Days after dosing termination**		
	2 day	**10 day**	**30 day**
EXPOSURE 1:			
Control	33.40 ± 3.00	45.60 ± 4.00	9.20 ± 1.00
0.5 µl/day	25.90 ± 1.70	32.00 ± 1.50	7.90 ± 0.90
1.5 µl/day	22.50 ± 1.40*	27.50 ± 2.10*	9.90 ± 3.50
5.0 µl/day	16.50 ± 1.20*	20.40 ± 1.20*	9.60 ± 1.90
EXPOSURE 2:			
Control	25.60 ± 2.40	19.60 ± 2.60	21.70 ± 1.80
0.5 µl/day	23.80 ± 2.70	23.60 ± 3.30	23.20 ± 1.60
1.5 µl/day	21.40 ± 2.00*	28.00 ± 4.10	24.00 ± 1.60
5.0 µl/day	13.80 ± 1.40*	20.90 ± 4.30	33.60 ± 4.80
EXPOSURE 3:			
Control	30.18 ± 1.26	20.48 ± 0.96	18.07 ± 1.33
0.5 µl/day	23.93 ± 2.32	17.77 ± 2.65	17.87 ± 1.67
1.5 µl/day	23.13 ± 2.48	13.80 ± 2.07	14.65 ± 1.31
5.0 µl/day	20.15 ± 2.08*	14.62 ± 1.67	14.73 ± 0.63
EXPOSURE 4:			
Control	23.37 ± 0.87	29.27 ± 2.63	29.96 ± 2.93
0.5 µl/day	21.92 ± 1.71	21.17 ± 0.59*	22.00 ± 0.98
1.5 µl/day	18.60 ± 1.91*	24.07 ± 1.91	26.00 ± 4.20
5.0 µl/day	17.10 ± 0.96*	23.02 ± 0.89*	21.98 ± 4.33

Exposure 1 = every day for 10 consecutive days
Exposure 2 = every other day for 7 exposures
Exposure 3 = every other day for 14 exposures
Exposure 4 = every day for 30 consecutive days

* statistically significant ($P < .05$)

Table 3.2 Macrophage phagocytosis in C57Bl/6 mice topically exposed to permethrin

Macrophage Phagocytic Activity (% positive cells, means ± sem)			
Permethrin exposure	**Days after dosing termination**		
	2 day	**10 day**	**30 day**
EXPOSURE 1:			
Control	5.92 ± 0.59	16.40 ± 1.80	20.70 ± 3.00
0.5 µl/day	5.57 ± 0.35	18.70 ± 4.20	25.00 ± 3.20
1.5 µl/day	6.69 ± 0.78	16.10 ± 1.90	25.40 ± 2.20
5.0 µl/day	6.31 ± 0.31	20.60 ± 3.30	31.50 ± 5.90
EXPOSURE 2:			
Control	9.10 ± 0.80	3.50 ± 0.50	1.65 ± 0.329
0.5 µl/day	7.50 ± 0.50	3.60 ± 0.90	2.58 ± 0.814
1.5 µl/day	7.80 ± 1.00	3.00 ± 0.40	5.32 ± 1.455*
5.0 µl/day	7.30 ± 0.80	6.30 ± 1.30*	3.63 ± 1.102
EXPOSURE 3:			
Control	3.00 ± 1.580	8.95 ± 6.285	1.52 ± 0.347
0.5 µl/day	1.33 ± 0.220	4.10 ± 1.160	1.20 ± 0.302
1.5 µl/day	1.02 ± 0.110	2.72 ± 0.697	1.25 ± 0.180
5.0 µl/day	0.90 ± 0.060	8.48 ± 3.793	1.23 ± 0.147
EXPOSURE 4:			
Control	0.917 ± 0.147	0.25 ± 0.034	0.36 ± 0.051
0.5 µl/day	0.567 ± 0.056*	0.25 ± 0.043	0.68 ± 0.095
1.5 µl/day	0.933 ± 0.033	0.20 ± 0.026	0.60 ± 0.000
5.0 µl/day	0.650 ± 0.099	0.18 ± 0.040	0.58 ± 0.092

Exposure 1 = every day for 10 consecutive days
Exposure 2 = every other day for 7 exposures
Exposure 3 = every other day for 14 exposures
Exposure 4 = every day for 30 consecutive days

* statistically significant ($P < .05$)

Table 3.3 Plaque forming cell (PFC) assay in C57Bl/6 mice topically exposed to permethrin

PFC Response (plaque numbers/10^6 cells, means ± sem)			
Permethrin exposure	**Days after dosing termination**		
	2 day	**10 day**	**30 day**
EXPOSURE 1:			
Control	60.9 ± 9.67	19.5 ± 3.76	10.8 ± 4.03
0.5 µl/day	67.3 ± 10.43	12.4 ± 4.28	8.9 ± 1.39
1.5 µl/day	47.3 ± 8.33	5.0 ± 1.08*	14.3 ± 2.73
5.0 µl/day	47.5 ± 6.41	4.3 ± 1.24*	12.2 ± 3.50
EXPOSURE 2:			
Control	7.5 ± 2.33	35.6 ± 3.41	32.0 ± 6.14
0.5 µl/day	3.3 ± 0.51	20.1 ± 3.63	22.3 ± 6.53
1.5 µl/day	4.6 ± 1.27	23.2 ± 3.23	31.6 ± 6.70
5.0 µl/day	5.0 ± 1.58	35.2 ± 17.05	32.6 ± 8.74
EXPOSURE 3:			
Control	8.8 ± 1.65	14.3 ± 5.17	12.4 ± 4.42
0.5 µl/day	14.9 ± 6.12	16.8 ± 7.81	11.8 ± 2.09
1.5 µl/day	6.7 ± 1.42	16.7 ± 8.49	6.5 ± 1.22
5.0 µl/day	14.3 ± 7.00	8.8 ± 1.49	13.1 ± 2.86
EXPOSURE 4:			
Control	16.1 ± 5.38	36.5 ± 7.84	44.2 ± 31.27
0.5 µl/day	11.9 ± 2.70	20.6 ± 4.82	12.8 ± 3.85
1.5 µl/day	5.3 ± 0.60	26.3 ± 6.63	9.6 ± 2.33
5.0 µl/day	14.7 ± 5.49	35.8 ± 3.91	7.1 ± 0.76

Exposure 1 = every day for 10 consecutive days
Exposure 2 = every other day for 7 exposures
Exposure 3 = every other day for 14 exposures
Exposure 4 = every day for 30 consecutive days

* statistically significant ($P < .05$)

CHAPTER 4.0: CONCLUSION AND SIGNIFICANCE OF THE CURRENT STUDY

A large number of chemicals have been reported to produce immunotoxicity in humans and animals. The consequences could be immunosuppression, allergic disease, or autoimmune disease. Permethrin, a pyrethroid insecticide, has been used in agriculture, human medicine, and veterinary medicine, thus a variety of people ranging from children to adults have been exposed to this chemical. It was also used by the U.S. Army to impregnate into military uniforms in order to protect soldiers from insect bites. Since Persian Gulf Syndrome occurred, much research has been performed to find the cause of this syndrome. Permethrin, which is usually considered to be a neurotoxic chemical, has been reported to have immunotoxic potential *in vitro* and in animal studies. Therefore, permethrin was evaluated in present study for its potential of producing immunotoxicity by dermal exposure.

This study was designed to evaluate immunotoxicity of permethrin in mice. The experiment design mimicked the actual human exposure. Mice were exposed to permethrin by the topical route daily for 10 or 30 consecutive days, or every other day for 7 or 14 exposures. The highest dosage used was approximately 7 times human exposure. After dosing termination, mice were examined at 2, 10, and 30 days using selected tests from the immunotoxicity testing battery approved by the National Toxicology Program. These tests included thymus/body weight ratio, spleen/body weight ratio, histopathology of thymus and spleen, cell surface marker analysis of thymocytes, splenocytes, and bone marrow cells, plaque forming cell (PFC) assay, macrophage chemiluminescent assay, and macrophage phagocytosis.

In this study, permethrin caused decreased thymus/body weight ratio, increased spleen/body weight ratio, decreased thymic cellularity, decreased macrophage chemiluminescent response, and decreased antibody production. The non-affected immune tests were cell-surface antigen expression, splenic cellularity, macrophage phagocytic ability, and histopathology of thymus and spleen. Most of the effects occurred following the 10-consecutive day exposure, except altered chemiluminescent response

that occurred in all exposures. The effects occurred mostly 2 days after dosing termination.

Permethrin may cause thymic atrophy by increasing thymocyte apoptosis or suppressing thymocyte proliferation. The possibility of permethrin-induced thymocyte apoptosis was supported by the evidence that permethrin was a potent inhibitor of calmodulin, a universal calcium binding protein, and may cause alteration in Ca^{2+} homeostasis. This change in intracellular Ca^{2+} may lead to the process of thymocyte apoptosis. The possibility of permethrin-suppressed thymocyte proliferation was supported by the evidence that permethrin suppressed lymphocyte proliferation stimulated by mitogen, and decreased the production of cytokines. The inhibition effect of permethrin on mitochondrial and calmodulin activities could also contribute to the suppressed-cell growth.

The mechanism of permethrin that caused splenomegaly was unknown. However, the increased splenic weight may come from splenic congestion.

The specific mechanism, which permethrin inhibited antibody production was not investigated in this study. However, this effect may relate to the ability of permethrin to inhibit production of cytokines (IFN_γ and IL-4) essential for antibody production, and inhibit B lymphocyte proliferation.

The inhibition effect on the ability of macrophages to produce H_2O_2 was dose-dependent and persisted up to 10 days after dosing termination. This effect was found in all chemical exposures, suggesting that the macrophage respiratory burst may represent a highly sensitive immune target of permethrin exposure. Permethrin has been shown to interrupt mitochondrial activity and this effect may relate to the inhibited respiratory metabolic burst of macrophages.

The present study has shown that permethrin, considered a safe chemical for using on human body, was able to alter immune functions in a murine study. This was also the first evidence showing immune effects of permethrin by topical exposure. Even though the effects were transient and on only some immune function tests, it offered significant information to the public.

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ABSTRACTS PUBLISHED

Punareewattana, K., Holladay, S.D., Smith, B.J., and Blaylock, B.L. Topical permethrin exposure alters skin immune responses and produces systemic immune effects. The Toxicologist (38th Annual Meeting) March 1999 48:1-S 180.

MANUSCRIPTS (to be submitted)

Punareewattana, K., Holladay, S.D., and Smith, B.J. Effects of topical permethrin exposure on spleen, thymus, and bone marrow: weights, cell counts, cell-surface antigen expression, and histopathology.

Punareewattana, K., Holladay, S.D., and Smith, B.J. Effects of topical permethrin exposure on macrophage function and antibody production.