IMMUNOTOXICITY OF DERMAL PERMETHRIN AND CIS-UROCANIC ACID: EFFECTS OF CHEMICAL MIXTURES IN ENVIRONMENTAL HEALTH

By

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Abstract

The present study examined adverse effects of sunlight exposure (mimicked by intradermal cis-urocanic acid, cUCA) on local and systemic immune responses, with or without co-exposure to the immunotoxic insecticide permethrin. A single exposure to cUCA caused diminished splenic macrophage phagocytosis that was persistent up to 30 days post-exposure. Five-day exposure to cUCA subtly increased splenocyte proliferation in response to the T cell mitogen Concanavalin A. Four-week exposure to cUCA caused increased splenic lymphocyte cellularity, thymic hypocellularity, and enhanced hydrogen peroxide production by splenic leukocytes. Single exposure to topical permethrin resulted in decreased thymic and splenic weight and cellularity, and inhibited antibody production by splenic B cells. cUCA worsened the negative effect of permethrin on both thymic weight and cellularity, and depressed splenocyte blastogenesis, hydrogen peroxide production, and antibody production. Five-day exposure to either cUCA or permethrin also caused persistent decreased contact hypersensitivity responses, an effect that became more than additive when the chemicals were administered concurrently. Defects in antigen processing and presentation by cutaneous Langerhans cells were evaluated as possible contributing mechanisms to the cutaneous immunosuppression, using mice with deleted genes. Vehicle-exposed IFNγ knockout mice displayed approximately a 22.1% depression in the ear swelling response as compared to control C57BL/6N mice, suggesting that this cytokine may be required for mounting a control-

level hypersensitivity response. Ear swelling in cUCA-exposed IFNγ knockout mice displayed a 21.4% depressed response as compared to cUCA-exposed wild-type C57BL/6N mice, again suggesting that IFNγ is an important cytokine in the contact hypersensitivity (CH) response. TNFαR knockout mice exposed to cUCA displayed 33.9% greater ear swelling than cUCA-exposed wild-type C57BL/6N mice, suggesting that increased TNFα may be involved in inhibited CH by cUCA. TNFαR knockout mice exposed to permethrin displayed 33.9% greater ear swelling than permethrin-exposed C57BL/6N mice, suggesting that increased TNFα may also be involved in inhibited CH by permethrin. C57BL/6N mice exposed to cUCA + permethrin displayed severe reduction of the CH response to 8.7% of the control level. IFNγ knockout mice exposed to permethrin + cUCA showed essentially identical depression of the CH response as IFNγ knockout mice exposed to either permethrin or cUCA alone. These results suggest that IFNγ is required for the greater than additive immunotoxic effect that occurred when these two agents were co-administered. TNFαR knockout mice exposed to cUCA + permethrin displayed 8.7 fold greater ear swelling than similarly exposed C57BL/6N mice, again suggesting that increased TNFα is involved in inhibited CH by both cUCA and permethrin.

DEDICATION

I dedicate this effort to my daughters, Katherine and Christine, my mother Jean, my sister Lisa, and my dear friends and family, for their unwavering support and sense of humor as I completed the last in a long series of educational endeavors. I would like to extend my most sincere appreciation and respect for my major advisor, Dr. Steven Holladay, for his patience and guidance in the successful completion of this project, and to the members of my graduate committee, Drs. Ahmed, Wong, Bender, and Gogal, and external examiner, Dr. Blaylock for their expertise and input throughout my dissertation research.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW 1

A. Chemical Immunotoxicity 1

B. Permethrin 1

C. Urocanic Acid 5

D. Chemical Mixtures 11

E. References 13

CHAPTER 2: SINGLE-DOSE TOPICAL EXPOSURE TO THE PYRETHROID INSECTICIDE, PERMETHRIN IN C57BL/6N MICE: EFFECTS ON THYMUS AND SPLEEN 25

A. Abstract 27

B. Introduction 28

C. Materials and Methods 31

1. Mice 31

2. Permethrin Preparation and Treatment Protocols 31

3. Organ Weights, Cell Preparation, and Cellularity 31

4. Surface Antigen Expression by Flow Cytometry 32

5. Thymocyte Apoptosis and Necrosis: Flow Cytometric And Cytologic Evaluation 33

6. Proliferation of Splenocytes and Thymocytes 33

a. *In-Vitro* Permethrin Exposure 33

b. *In-Vivo* Permethrin Exposure 34

7. Splenic Macrophage Phagocytosis 35

8. Chemiluminescence Response in Splenic Macrophages 36

9. B Lymphocyte Antibody Production: Plaque-Forming Cell Assay 36

10. ^{51}Cr Release Cytotoxicity Assay 37

11. Statistical Analysis 38

D. Results 39

E. Discussion 43
F. Acknowledgement 47
G. References 48

CHAPTER 3: IMMUNOTOXIC EFFECTS OF CIS-UROCANIC ACID EXPOSURE IN C57BL/6N MICE 67
A. Abstract 69
B. Introduction 70
C. Materials and Methods 73
1. Mice 73
2. Cis-Urocanic Acid 73
3. Organ Weights, Cell Preparation, and Cellularity 73
4. Thymocyte Surface Antigen Expression by Flow Cytometry 74
5. Proliferation of Splenocytes and Thymocytes 75
6. Splenic Macrophage Phagocytosis 76
7. Chemiluminescence Response in Splenic Macrophages 76
8. B Lymphocyte Antibody Production: Plaque-Forming Cell Assay 77
9. Cytotoxicity Assay 78
10. Statistical Analysis 79
D. Results 80
E. Discussion 83
F. Acknowledgement 88
G. References 89

CHAPTER 4: CIS-UROCANIC ACID INCREASES BOTH IMMUNOTOXICITY AND LETHALITY OF DERMALLY ADMINISTERED PERMETHRIN IN C57BL/6N MICE 103
A. Abstract 105
B. Introduction 106
C. Materials and Methods 109

1. Mice 109
2. Permethrin and Cis-Urocanic Acid Preparation and Treatment Protocols 109
3. Organ Weights, Cell Preparation, and Cellularity 110
4. Proliferation of Splenocytes and Thymocytes 110
5. Splenic Macrophage Phagocytosis 111
6. Chemiluminescence Response in Splenic Macrophages 112
7. B Lymphocyte Antibody Production: PFC Assay 112
8. Statistical Analysis 113
D. Results 115
E. Discussion 118
F. Acknowledgement 120
G. References 121

CHAPTER 5: MOLECULAR MECHANISMS OF SUNLIGHT AND PERMETHRIN-INDUCED ALTERATIONS IN CUTANEOUS IMMUNITY 134
A. Abstract 136
B. Introduction 138
C. Materials And Methods 141
1. Mice 141
2. Permethrin Treatment Protocol 141
3. Cis-Urocanic Acid 142
4. Contact Hypersensitivity 142
5. Statistical Analysis 143
D. Results 144
E. Discussion 147
F. Acknowledgement 152
G. References 153

CHAPTER 6: CONCLUSIONS 167

APPENDICES 170

I. Splenic Macrophage Phagocytosis Response to Permethrin 170

II. Splenic Chemiluminescence Response to Permethrin 171

III. Splenic Cytotoxic T Lymphocyte Response to Permethrin 172

IV. Thymocyte Proliferative Response to Permethrin 173

V. Thymic Weight Response to Single Exposure to cUCA 174

VI. Thymic Cellularity Response to Single Exposure to cUCA 175

VII. Splenic Weight Response to Single Exposure to cUCA 176

VIII. Splenic Cellularity Response to Single Exposure to cUCA 177

IX. Thymic Weight Response to 5-Day Exposure to cUCA 178

X. Thymic Cellularity to 5-Day Exposure to cUCA 179

XI. Splenic Macrophage Phagocytosis Response to 5-Day cUCA 180

XII. Splenic Antibody Production Response to 5-Day cUCA 181

XIII. Splenic Chemiluminescence Response to 5-Day cUCA 182

XIV. Splenic Cytotoxic T Lymphocyte Response to 5-Day cUCA 183

XV. Splenic Macrophage Phagocytosis to cUCA + Permethrin 184

CURRICULUM VITAE 185

LIST OF TABLES

2.1 Effect of Topical Permethrin on Absolute Thymocyte Subpopulations 58

2.2 Effect of Topical Permethrin on Thymocyte Viability and Apoptosis 59

3.1 Effect of Five-Day Cis-Urocanic Acid on Thymocyte Differentiation 97

LIST OF FIGURES

2.1 Thymic Weight Response to Permethrin 60

2.2 Thymic Cellularity Response to Permethrin 61

2.3 Splenic Weight Response to Permethrin 62

2.4 Splenic Cellularity Response to Permethrin 63

2.5 Antibody-mediated Response to Permethrin 64

2.6 Proliferation Response of Splenocytes to *in-vivo* Permethrin 65

2.7 Proliferation Response of Splenocytes to *in-vitro* Permethrin 66

3.1 Splenocyte and Thymocyte Proliferation Response to cUCA 98

3.2 Splenic Macrophage Phagocytosis Response to cUCA 99

3.3 Thymic Cellularity Response to cUCA 100

3.4 Splenic Cellularity Response to cUCA 101

3.5 Splenic Chemiluminescence Response to cUCA 102

4.1 Effect of cUCA and Permethrin on the Thymic Weight and Cellularity 128

4.2 Effect of cUCA and Permethrin on the Splenic Weight and Cellularity 129

4.3 Effect of cUCA and Permethrin on Splenocyte and Thymocyte Proliferation 130

4.4 Effect of cUCA and Permethrin on Splenic Chemiluminescence 131

4.5 Effect of cUCA and Permethrin on Antibody-Mediated Immunity 132

4.6 Effect of cUCA and Permethrin on Neurotoxic Deaths 133

5.1 CH Response to cUCA and Permethrin in C57BL/6N and SvImJ Mice 163

5.2 CH Response to cUCA and Permethrin in C57BL/6N and TNFαR KO Mice 164

5.3 CH Response to cUCA and Permethrin in C57BL/6N and IFNγ KO Mice 165

5.4 CH Response to cUCA and Permethrin in SvImJ and CD1a KO Mice 166

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1A. CHEMICAL IMMUNOTOXICITY

Toxicological assessment for approval of environmental chemicals and pharmaceutical products typically does not include evaluation of the immune system. The need to incorporate immunotoxicity testing has only been realized in recent years. Risk assessment models for immunotoxicity evaluation have been studied as researchers recognize it is temporally and economically impractical to complete a full evaluation of all arms of the immune system for each compound in question (Luster et al. 1992, 1993). Therefore, a risk assessment model was developed such that individual and pair-wise predictive values were established for the various immunological assays in an attempt to expediently quantitate the possibility of decreased host resistance to disease following chemical challenge using a few selected immune tests. Earlier studies had outlined a tiered approach to full evaluation of the immune system that included immunopathology, cell-mediated and antibody-mediated immune evaluation, nonspecific immunity, and host resistance challenge models (Luster et al. 1988) which supplied the background information necessary for the development of these risk assessment models.

1B. PERMETHRIN

Permethrin (3-phenoxyphenyl methyl (+) cis, trans-3-(2,2-dichloroethenyl)-2,2 dimethylcyclopropane carboxylate) is a synthetic, third generation, type I pyrethroid insecticide that is commonly used in shampoos and topical creams in human and veterinary medicine to eliminate ectoparasites such as fleas, ticks, lice, and mites (Asakawa et al. 1996, Llewellyn et al. 1996, Fuortes 1999). Permethrin is also used in

the prevention of ectoparasite infestation and possible resultant insect-borne disease via direct topical application or permeation of military and hunting clothing (Schreck et al.1986, Schreck and Kline 1989, Sholdt et al. 1989). The degree of topical absorption is species-dependent, and ranged from 2% in human beings and rabbits, 10% in mice, 15% in rhesus monkeys (variation according to isomer and site of application), to as much as 44% in rats. Urinary excretion follows hepatic hydrolysis and either oxidization or glucuronide-conjugation (Miyamoto 1976, Glickman et al.1981, Shah et al. 1981, Taplin and Meinking 1987, Sidon et al. 1988, Snodgrass 1992). The No Observable Effect Level (NOEL) for oral permethrin in rabbits has been reported to be 5 mg/kg/day (Snodgrass 1992). The median lethal oral dose in rats is 4 g/kg, and this level varies slightly according to the age, nutrition, gender, and strain of rat. However, the median lethal oral dose of permethrin in corn oil vehicle is 380 mg/kg. This finding raises concern about the extent of systemic absorption and resulting toxicity with topically-applied insecticides, since most commercially available insecticides are only available as a mixture of active ingredients and inert solvents or vehicles (Metker et al. 1977, McCain et al. 1997).

The type I pyrethroid insecticides, including permethrin, cause neurotoxic effects that have been well characterized in the literature and include aggression, sparring, tremor, and hyperthermia. These effects are very distinct from the effects of type II pyrethroid insecticides such as cypermethrin and deltamethrin, which cause pawing, burrowing, salivation, hypothermia, and lowered motor activity (McDaniel and Moser 1993). These effects of permethrin are thought to be the result of alterations in synaptic membrane potential and resulting neuroexcitation (Eells et al. 1992). This

neuroexcitation has been demonstrated in laboratory rodents to result from increased sodium influx through voltage-sensitive sodium channels into synaptic terminals (Lees 1998), producing membrane depolarization, excess glutamate uptake, and neurotransmitter (acetylcholine) release (Eells et al. 1992, Vaccari et al. 1998). Permethrin has also been shown to inhibit acetylcholinesterase (AchE) activity in rat brain cortex by 97-98% within 45 minutes of *in-vitro* (5 ppm) exposure or by 50% within 30 minutes following *in-vivo* (250 mg/kg body weight) administration, which would exacerbate permethrin's neurotoxic effects (Bandyopadhyay 1982). The maximum plasma concentrations are achieved at 3.52 hours (Anadon et al. 1991), peak neurotoxic effect of permethrin is at 5 hours post oral administration in rodents, and result in changes in biogenic amines and their metabolites: 3-methoxy-4-hydroxyphenylglycol (MHPG), 5-hydroxyindoleacetic acid (5-HIAA, a serotonin metabolite), and aspartate levels in homogenized brain tissue (Hudson et al. 1986). Permethrin is also thought to diminish energy coupling by inhibiting glutamate and succinate respiration in mitochondria (Gassner et al. 1997). Larger (approximately a quarter of the acute oral LD50 value, 300 mg/kg/day) or more prolonged exposure (5-7 days) to oral permethrin and two type II pyrethroid insecticides, cypermethrin and deltamethrin, results in sparse axonal peripheral nerve demyelination and Wallerian degeneration, which results in abnormal locomotion; the potential mechanism of action includes upregulation of β-glucuronidase and β-galactosidase in laboratory rodents (Rose and Dewar 1983, Cavaliere et al. 1990). Further, there was enhanced neurotoxicity when permethrin was administered concurrently with other pesticides such as DEET (N,N-diethyl-m-toluamide) and pyridostigmine bromide, which is likely attributable to competition for liver and plasma

esterases, leading to decreased insecticide breakdown and increased transport of the parent compound to nervous tissues (Abou-Donia et al. 1996).

Low doses of permethrin cause mild neurological signs and immunomodulation. Higher doses of permethrin result in more severe peripheral and central nervous system clinical signs including hyperactivity, convulsions, paralysis, and even death (Hansen et al.1994). Permethrin and several analogs (cypermethrin, bioallethrin, and deltamethrin) have demonstrated immunomodulatory effects in murine and human models, including diminished natural killer cell cytotoxicity, T cell and antibody-mediated immunomodulation, alterations in class II MHC cells, modified cytokine levels, and variations in thymocyte numbers, distribution and function (Puig et al. 1989, Enan et al. 1996, Santoni et al. 1997, 1998, Diel et al. 1998-1, 2, 1999, Murphy et al.1999, Zhang et al. 1999). These data suggest that this class of insecticides may significantly alter local and systemic immunity.

Limited information is available about precise mechanisms of immunomodulation caused by permethrin. Recent studies have demonstrated that low dose subacute topical exposure (10 days) to permethrin causes diminished antibody-mediated immunity in mice, and oral cypermethrin decreases antibody production in both rats and mice (Desi et al. 1985, Tamang et al. 1988, Punareewattana et al. 2001). A potential mechanism of action has been demonstrated to be inhibited production of cytokines (IFNγ and IL-4) necessary for antibody production (Diel et al. 1998). Further, subacute topical permethrin exposure in mice depressed splenic macrophage hydrogen peroxide production, and the speculated mechanism included permethrin-induced inhibition of mitochondrial complex I in the electron transport chain, which may interfere with

leukocyte respiratory burst development (Gassner et al. 1997, Punareewattana et al. 2001). Other authors have noted that intraperitoneal administration of permethrin or deltamethrin results in a calcium/calmodulin-dependent alteration of the protein kinase-phosphatase cascade, leading to increased apoptosis of thymocytes and resulting thymic atrophy (Rashatwar and Matsumura 1985, Enan et al. 1996). These reports have demonstrated a few potential mechanisms by which permethrin affects the immune system, but clearly more information is needed in order to more fully understand the molecular mechanisms of permethrin's immunotoxicity.

1C. UROCANIC ACID

Urocanic acid (2-propenoic acid, 3-[1H-imidazol-4(5)-yl] was originally found in dog urine more than a century ago (Jaffe 1874) and was later detected in animal sweat and skin (Zenisek and Kral 1953). This led to the proposal that UCA may act as an endogenous sunscreen or photoprotectant against UVB-induced DNA damage. Urocanic acid is found predominantly in the stratum corneum and the liver, and is synthesized via deamination of histidine. Urocanic acid represents about 0.7% of the dry weight of the epidermis, and is present predominantly in the trans isomer in skin protected from sunlight (Tabachnick 1957). The concentration of cUCA in nonirradiated murine skin is 0.2 ug/cm^2 (4% of total UCA in the skin), and rises to 15 μg/cm^2 upon irradiation at 96 mJ/cm^2 or 80 μg/mouse after 42 mJ/cm^2 (Noonan et al. 1988). Only 1 μg is necessary to reduce contact hypersensitivity (CH) responses, and it has been demonstrated in BALB/c mice that only 21-26 minutes of sunlight exposure, regardless of latitude and with normal stratospheric ozone concentrations, results in sufficient conversion of urocanic acid to

result in a 50% drop in skin immune responses (DeFabo et al. 1990). Urocanic acid is produced by epidermal keratinocytes and is stored in the trans isomeric form (tUCA) in keratohyalin granules in the stratum corneum. The poorly soluble tUCA is racemized to the highly water soluble cis isomer when exposed to low-wavelength (280-320 nm) UVB irradiation. Cis-urocanic acid is then absorbed systemically and is thought to be excreted unmetabolized in the urine (Anglin and Batten 1968, Morrison and Deibel 1986).

Ultraviolet-B irradiation causes protein denaturation and DNA damage via induction of cyclobutane pyrimidine formation, strand breaks, and production of adducts which is thought to be an initiating step into the development of some cutaneous neoplasms. Ultraviolet-B irradiation has been implicated as a risk factor for development and progression of several skin cancers, including basal cell carcinoma, malignant melanoma, and squamous cell carcinoma in human beings, and the percentage of UVB-sensitive human beings is estimated to be approximately 40-45% in both the Caucasian and non-Caucasian races (Streilein 1993). Ultraviolet-B irradiation exposure has been known for over 20 years to predispose mice to development of immunogenic cutaneous tumors, and it is thought that direct DNA damage (such as mutations to the p53 gene), in conjunction with immune suppression, leads to the development and progression of immunogenic skin cancers (Kripke 1983, Majewski and Jablonska 1995). Cis-urocanic acid is thought to act as a DNA protectant by absorbing the majority of the UVB rays and undergoing transformation from the trans isomer to the cis isomer (Norval et al. 1995). Therefore, UCA has been proposed to act as a natural cutaneous photoprotecting agent against DNA damage caused by UVB irradiation. Recent reports have examined the intensity of UVB exposure necessary to evoke immune suppression in mice, and have

found that under normal stratospheric ozone concentrations at nearly any latitude, 50% cutaneous depression of contact hypersensitivity immune response in the BALB/c mouse would occur following 21-26 minutes of sunlight exposure. These data suggest that induction of murine immune suppression requires low levels of sunlight that can be experienced almost anywhere in the world (DeFabo et al. 1990).

However, increased epidermal concentrations of UCA increases sensitivity to UVB immune suppression and resulting skin cancer development (DeFabo et al. 1997). Consequently, the immunologic effects of sunlight exposure are elicited in the laboratory by the intracutaneous administration of cUCA. The immunologic defects in animals previously exposed to UVB or cUCA were discovered to be two-fold: deficiency of conventional antigen-presenting cells in the spleen and altered populations of regulatory T cells that selectively impaired anti-tumor immunity. Ultraviolet-B irradiation indirectly, through the actions of cUCA, causes upregulation of suppressor T lymphocytes in spleen and lymph nodes, which prevents immunologic rejection of UV tumors transplanted into syngeneic recipients, and also significantly reduce the latent period of tumor appearance during UV carcinogenesis (Fisher and Kripke 1978, 1982).

The beneficial immunomodulatory effects of cUCA have been applied to grafting, and two reports have suggested that cUCA can prolong the survival of allogeneic MHC disparate tail and corneal grafts in BALB/c mice (Gruner et al. 1992, Guymer and Mandel 1993). These findings suggest future development of cUCA or an analog for therapeutic use in the long-term survival of transplanted organs, or in the prevention of graft-versus host disease. Fetal mice have been shown to contain a low quantity of UCA in the skin that is all in the trans isomeric form. Within one day of birth, the quantity of

UCA increases 20 fold, and a high percentage is converted (11%) to the cis isomer. As previously mentioned, older mice contain only 4% cUCA in UVB-protected skin. It is thought that this high percentage of cUCA in neonatal mice may serve as a protective role to suppress immune responses to epidermal antigens while the animal is regulating immune responses to self-antigens, including epidermal antigens (Norval et al. 1989).

However, circulating cUCA has been shown to also confer harmful local and systemic immunomodulation via diminished T cell-mediated immunity and decreased antigen presentation, which may actually contribute to rather than protect against the clinically relevant end-point of increased risk of infectious or neoplastic disease. It has been well documented that exposure to acute, low-dose UVB that qualitatively and quantitatively resembles typical human sun exposure results in diminished CH and induces antigen-specific tolerance by increasing suppressor T cells and converting immunogenic antigen presenting cells to tolerogenic cells (Streilein et al. 1994, Vink et al. 1996). These effects can also be mimicked by intracutaneous injections of cUCA or TNFα (Streilein et al. 1994), suggesting that there are chemical intermediaries at least partially involved in sunlight-induced immunomodulation.

Immunosuppressive effects of cUCA include inhibition of CH (DeFabo and Noonan 1983), delayed allograft rejection (Gruner et al. 1992), and prevention of graft vs. host disease (Gruner et al. 1992). *In-vivo* UVB radiation or intracutaneous administration of cUCA in human beings causes a defect in cutaneous antigen presentation by abrogating the function of $CD1^+$ Langerhans cells (LC), and inducing $CD1^-$ macrophages that activate suppressor T lymphocytes and diminish contact hypersensitivity (Baadsgaard et al. 1990). Another study substantiated these findings, and

showed that following a single exposure of 4 minimal erythemal dose in human beings, CH was significantly reduced. While numbers of $CD1a^+$ LC were significantly diminished and remaining LC were rendered ineffective functionally, numbers of $CD1a^-$ macrophages were increased in the skin (LeVee et al. 1997). Cis-urocanic acid also induces e-selectin expression from endothelial cells of dermal microvessels, which causes release of constitutive TNFα from mast cell granules. Therefore, DNA damage causes direct mutagenesis to skin cells, and also induces the expression and release of TNFα from many mammalian cells (Kibitel et al. 1998). Tumor necrosis factor-alpha decreases vimentin expression in LC, which results in blunting of LC cytoplasmic processes and decreased ability of the compromised cells to acquire and process antigens to present to T lymphocytes. Tumor necrosis factor-alpha is also chemoattractant to LC, so increased concentrations in the skin prevent migration of LC to local lymph nodes and thus decrease antigen presentation.

UVB is thought to suppress cutaneous and systemic immune responses in an indirect manner, following UVB exposure to the two cutaneous chromophores urocanic acid and DNA. Several mediators have been implicated in this process including prostaglandins, interleukin-10, and TNFα (Norval et al. 1995). Epidermal cell-derived thymocyte activating factor (ETAF, or IL-1) is also depressed following UVB exposure, which depresses the activation of T lymphocytes that are associated with antigen (Norval et al. 1989). Ultraviolet-B irradiation -induced increased release of TNFα contributes to failed CH induction (Alard et al. 2001). Tumor necrosis factor-alpha is also thought to induce production and activation of matrix metalloproteinases that may contribute to tumor progression and metastasis (Han et al. 2001). It is now known that for

development of the full extent of murine contact hypersensitivity, activation of both tumor necrosis factor receptors (type I p55TNFR and type II p75TNFR) is necessary. Mice deficient for p55TNFR had a defect in antigen uptake but showed normal migration into regional lymph nodes, whereas mice dendritic cells lacking in p75TNFR showed diminished migration into regional lymph nodes after antigen uptake, but the antigen uptake itself was not affected (Becke et al. 2001).

Ultraviolet-B irradiation also increases macrophagic release of TGFβ1, which is also involved in the release of TNFα from local mast cells and local mononuclear cells and differentiation/maturation of $CD1^+$ DC. (Majewski and Jablonska 1995). Signaling mediators of the TGFβ superfamily, called SMADS, are normally expressed at high levels in the epidermis, but during skin carcinogenesis, mutations in SMADS resulted in loss of growth inhibition mediated by TGFβ, thus resulting in tumor progression (He et al. 2001).

Prolonged exposure to cUCA in rodents results not only in cutaneous immune suppression, but also systemic immunomodulation including thymic atrophy and thymic hypocellularity, upregulation of systemic suppression T cells, and a shift of Th1/Th2 cytokine profiles (El-Ghorr et al. 1997). Several molecular mediators have been implicated. Upregulation of IL-10 following low-dose UVB irradiation is thought to mediate systemic tolerance. It is thought that cUCA also mediates inhibition of NK activation, as a result of uncoupling of NK receptors from phospholipase C-mediated phosphoinositide hydrolysis. Cis-urocanic acid down-regulates induction of cAMP, which may affect the second messenger system of the NK cell (Norval et al. 1995). Additionally, monocytes down-regulate NK cell activity by reducing the expression of

CD16 and CD56 surface antigens on NK cells, suggesting that cUCA acts indirectly on NK cells via monocytes or dendritic cells. Also, monocytes normally produce IL-12 in the development of Th1 responses and in the activation of NK cells. This causes NK cells to synthesize IFNγ. Ultraviolet irradiation promotes Th2 responses and depresses Th1 responses, and the cUCA immunomodulation of NK activity has been proposed to be the result of IL-12 and IFNγ downregulation or UV-induced macrophage production of IL-12p40 homodimer, a natural antagonist of biologically active IL-12, leading to IFNγ suppression (Norval et al. 1995, Reeve et al. 1999, Schmitt and Ullrich 2000).

1D. CHEMICAL MIXTURES

Although the effect of concurrent exposure to permethrin and sunlight is a highly relevant concern in human, especially pediatric medicine, there is currently a paucity of information over how this chemical mixture specifically affects the cutaneous and systemic immunity against the development of infectious or neoplastic disease. The alterations in murine systemic immune competence that result from subacute to chronic administration of cUCA seem to be localized primarily to cell-mediated immunity, and are manifested as thymic atrophy, thymic hypocellularity, and altered CH. Preliminary experiments have demonstrated immune modulation following subacute exposure to topical permethrin in both the cellular and antibody-mediated immune systems. However, the effects of shorter duration exposures are not known. Therefore, the present study proposes that acute topical exposure to permethrin or 5-day intradermal cUCA would also result in systemic modulation of cellular and antibody-mediated immunity. Further, it is an attempt to determine potential molecular mechanisms of this modulation

with the use of combination therapy on genetically altered strains of mice to determine whether cUCA and permethrin have similar or divergent molecular mechanisms. The molecular mechanisms of the cutaneous and systemic immunotoxicity of permethrin and cUCA are currently poorly understood, although reports in the literature suggest local cutaneous alterations in cytokines (TNFα, TGFβ, IL-1, IL-2, IL-10) following chemical exposure. The end-points to determine functional effects of the chemicals included splenic and thymic organ weights and cellularity, splenic phagocyte function, splenic leukocyte chemiluminescence assay, splenic and thymic cell surface antigen evaluation and cytometry, plaque forming cell assay, cytotoxic T lymphocyte assay, *in-vitro* proliferation of thymocytes, quantitation of apoptosis, and CH.

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CHAPTER 2: SINGLE-DOSE TOPICAL EXPOSURE TO THE PYRETHROID INSECTICIDE, PERMETHRIN IN C57BL/6N MICE: EFFECTS ON THYMUS AND SPLEEN

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2A. ABSTRACT

Immunomodulatory effects of single topical exposure to permethrin were evaluated in four-to-five-week-old female C57BL/6N mice. Mice exposed to 5-25 μL permethrin (equivalent to 220-1100 g/kg body weight) on the shaved interscapular space were evaluated 48 hours later for: changes in body weight; splenic and thymic organ weight and cellularity; thymocyte cell surface expression, cellular apoptosis, and necrosis; splenic macrophage phagocytosis and H_2O_2 production by chemiluminescence; splenic B cell antibody production and T cell cytolytic activity; and mitogen-induced proliferation of splenocytes and thymocytes after *in-vivo* or *in-vitro* permethrin exposure. Topical application of permethrin caused significant inhibition of splenic T cell proliferation, but did not appear to affect leukocyte function in the other assays evaluated. A dose-related decrease in thymic cellularity was seen in the permethrin-exposed mice. The $CD4^+CD8^+$ thymocyte subpopulation was most severely diminished, suggesting possible chemical-induced apoptosis as a mechanism leading to thymic atrophy. Apoptosis was significantly increased in $CD4^-8^-$ and $CD4^-8^+$ thymocytes. Cellularity of the spleen was also reduced by permethrin, an effect that may relate to inhibited proliferation or reduced seeding from the hypocellular thymus.

2B. INTRODUCTION

Permethrin (3-phenoxyphenyl methyl (+) cis, trans-3-(2,2-dichloroethenyl)-2,2 dimethylcyclopropane carboxylate) is a synthetic, third generation, type I pyrethroid insecticide that is commonly used in human and veterinary medicine for the prevention and treatment of ectoparasites such as fleas, ticks, lice, and scabies (Schreck et al. 1986, Schreck and Kline 1989, Sholdt et al. 1989, Asakawa et al. 1996, Llewellyn et al. 1996, Fuortes 1999). This insecticide has been considered relatively safe due to its high activity against a variety of insects and low mammalian toxicity (Papadopoulou-Mourkidou 1983). Permethrin is degraded quickly in the liver by hydrolysis and either oxidization or glucuronide-conjugation, and then the metabolites are excreted in the urine (Miyamoto 1976, Glickman et al. 1981, Shah et al. 1981, Taplin and Meinking 1987, Sidon et al. 1988, Snodgrass 1992). However, despite its presumed relative safety as a topical insecticide, recent reports suggest that low levels of permethrin (34 μg/kg/day topically in treated military clothing) may contribute to the persistent local and systemic immunotoxicity referred to as the "Persian Gulf Syndrome" (Snodgrass 1992). Furthermore, commercially available permethrin is typically formulated as an insecticide/solvent mixture, which results in log-fold higher median LD50 (380 mg/kg) versus pure permethrin (4 g/kg in rats), suggesting greater systemic absorption and resulting increased risk of toxicity (Metker et al. 1977, McCain et al. 1997).

Low doses of permethrin cause mild neurological signs; higher doses result in more severe peripheral and central nervous system clinical signs including hyperactivity, convulsions, paralysis, and even death (Hansen et al. 1994).

Mechanisms of such neurotoxicity have been studied for permethrin and include prolonged sodium ion channel opening, which causes increased sodium influx and protracted axonal depolarization. More recent reports suggest that pyrethroids also disrupt mitochondrial energy coupling, which is thought to exacerbate permethrin's neurotoxic effects (Gassner et al. 1997). Beyond neurotoxicity, subacute to chronic oral exposure to permethrin and its analogs cypermethrin, bioallethrin, and deltamethrin have been reported to cause local and systemic immunomodulation in mice. These immune effects include diminished natural killer cell cytotoxicity and variations in thymocyte numbers, distribution and function (Enan et al. 1996, Santoni et al. 1997, 1998). Preliminary observations in humans exposed by the topical route to permethrin, as occurs with insecticide use or treatment for lice or mites, suggest these individuals may also suffer immune modulation. Specifically, T cell and antibody-mediated immunomodulation, alterations in class II MHC cells, and modified cytokine levels, have recently been reported in permethrin-exposed humans (Puig et al. 1989, Murphy et al. 1999, Zhang et al. 1999). Topical absorption of permethrin is rapid in mice compared to other insecticides, as measurable quantities can be detected in the blood within five minutes after topical application (Shah et al. 1981). Rate and extent of topical absorption is also species-dependent and ranges from 2% in human beings and rabbits to 10-25% in mice, 15% in rhesus monkeys, and 44% in rats (Shah et al. 1981, Grissom et al. 1987, Sidon et al. 1988, Franz et al. 1996). These data suggest that absorption of permethrin across the skin could result in systemic immune effects, similar to oral exposure where uptake from the gut is limited (McCain et al. 1997). In this regard, we recently reported immune modulation

in mice treated daily with permethrin for 10 - 30 days on the shaved interscapular skin. These mice displayed reduced size and cellularity of the spleen and thymus, and inhibited antibody production and contact hypersensitivity responses (Punareewattana et al. 2000, 2001). In the present studies, a broader panel of assays was used to more closely examine immune effects in mice receiving single-dose topical permethrin treatment, which may be more comparable to typical human exposure. Specific assays were selected based on Luster et al. (1992, 1993) to evaluate multiple arms of immune system function, and included in spleen and thymus: organ weights and cell counts; cell-surface antigen expression; and lymphocyte proliferation. Functional assays were evaluated in splenic leukocytes and included: macrophage phagocytosis and chemiluminescence; B cell antibody production; and lytic activity of cytotoxic T cells. Similar organ weight effects occurred as were seen previously, including significant thymic involution. Cytometric evaluation further indicated that $CD4^{+}8^{+}$ thymocytes were markedly depleted by permethrin, suggesting the possibility of enhanced apoptosis. Direct chemical effects on thymocytes were observed following *in-vivo* or *in-vitro* treatment with permethrin, including increased apoptotic and necrotic cells, and may relate to thymic involution caused by topical permethrin.

2C. MATERIALS AND METHODS

1. MICE. Five-week-old female C57Bl/6N mice were used in these studies. Mice were purchased from Charles River Laboratories (Portage, MI). Mice were acclimated one week and maintained under controlled conditions of temperature (22±1˚C), humidity (40-60%), and light (12/12-hour light/dark cycle). Food and water were provided ad libitum. Mice in all experiments were humanely treated, in accordance with the guidelines of the Virginia Tech Institutional Animal Care and Use Committee (VT IACUC).

2. PERMETHRIN PREPARATION AND TREATMENT PROTOCOLS. Permethrin was provided by the US Army Center for Health Promotion and Preventive Medicine (Aberdeen Proving Ground, MD) from stock purchased immediately before by the Army from Coulston Industries (Coulston Products, Easton, PA). The permethrin consisted of a 91.6% pure mixture of 57.7% trans and 42.3% cis permethrin, respectively. Mice were exposed to a single dose of 5, 10, 15, or 25 μL permethrin (which exceeds typical human exposure by approximately 10-50x) on the shaved interscapular skin, to mimic the most common route of human exposure at levels that typically did not result in neurotoxicity (Snodgrass 1992). Control groups received identical volumes of vegetable oil (vehicle) in the interscapular space, 48 hours prior to sacrifice. Mice were sacrificed by cervical dislocation 48 hours following dosing.

3. ORGAN WEIGHTS, CELL PREPARATION, AND CELLULARITY. The thymus and spleen from each mouse were collected by dissection and placed,

individually, into pre-weighed 60 x15 mm culture dishes (Fisher Scientific, Norcross, GA). Wet organ weights were immediately obtained using an Ohaus analytical scale (TS 120S, Florham Park, NJ). Eight mL cold incomplete culture medium (RPMI-1640, Fisher) were added to petri dishes with organs, after which thymocytes and splenic cells were gently dissociated into the culture medium using a wire mesh screen (Sigma, St. Louis, MO) and curved forceps. Suspended cells were washed twice in incomplete culture medium, resuspended in 2 mL culture medium, and counted using a Scharfe CASY-1 electronic cell counter (Scharfe System, GmbH, Germany). The accuracy of the cell counter was verified in each experiment by counting 2-3 samples on a hemocytometer using Natt Herrick's vital stain.

4. SURFACE ANTIGEN EXPRESSION BY FLOW CYTOMETRY.

Thymocytes from control and permethrin-exposed mice were suspended in PBS at 5 x 10^6 cells/mL. Expression of CD4 and CD8 surface antigens on thymocytes was determined by incubating 100 µL cell suspension containing 5 x 10^5 cells with 0.5 µg fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8a (clone 536.7) and 0.5 µg phycoerythrin (PE)-conjugated anti-mouse CD4 (clone H129.19) monoclonal antibodies (BD Pharmingen, San Diego, CA) in a 96-well round-bottom tissue culture plate (Corning, NY). Following a 30-minute incubation in the dark at 4°C while mixing on a Gyrotary Shaker Model G2 orbital mixer (New Brunswick Scientific, New Brunswick, NJ), cells were washed twice in PBS and 5,000 events from each sample were analyzed using an Epics XL flow cytometer (Coulter, Hilaeh, FL). Cell viability, cell size and granularity were verified by forward and side-angle light scatter and viability was determined to be greater than 95% in all samples. Dead cells

were excluded from analysis with electronic gates, and data were analyzed using the Immuno-4 software program.

5. THYMOCYTE APOPTOSIS AND NECROSIS: FLOW CYTOMETRIC AND CYTOLOGIC EVALUATION. Thymocytes isolated from mice previously treated with topical permethrin at 4, 8 and 12 hours prior to sacrifice were dissociated according to the procedures outlined above, and then were cultured in 96 well polystyrene plates, 5 x 10^5 cells per well at 37˚C, 5% CO_2. Cells were first stained with the CD4 and CD8 monoclonal antibodies according to the procedure described above. Cells were then incubated with 7-amino-actinomycin D (7-AAD, 1 μg/5 x 10^5 cells, Molecular Probes, Eugene OR) for identification of apoptotic cells, or annexin V (Pharmingen) staining for late apoptosis/necrosis 30 minutes prior to analysis on the flow cytometer. This procedure permitted quantitation of viable, early apoptotic, and late apoptotic/necrotic cells in each thymocyte subpopulation.

6. PROLIFERATION OF SPLENOCYTES AND THYMOCYTES:

a. *IN-VITRO* PERMETHRIN EXPOSURE. Splenocytes and thymocytes from untreated 5-week old female C57BL/6N mice were collected and dissociated using sterile technique. Erythrocytes were removed from splenocyte cell suspensions in ACK lysing solution (0.015 M NH_4Cl, 1.0 mM $NaHCO_3$, 0.1 mM EDTA) for five minutes at room temperature. Splenocytes and thymocytes were then suspended in the presence of either 25 or 100 μM permethrin in DMSO or vegetable oil control. Blastogenesis of the splenocytes and thymocytes was quantified in the presence and absence of the T cell mitogen, concanavalin A (ConA, Sigma) at 0.1 or 1.0 μg/well

using the nonradioactive colorimetric/fluorometric assay (Ahmed et al. 1994 and Gogal et al. 1997). Briefly, 100 µL aliquots of 5 x 10^6 lymphocytes/mL were added to quadruplicate wells containing 100 µL of either medium alone or medium plus ConA. After 24 hours of culture for splenocytes or 48 hours of culture for thymocytes at 37°C and 5% CO_2, 20 µL of Alamar blue dye (Accumed International, Inc., Westlake, OH) was added to each well of the plate and returned to the incubator. Twenty-four hours after the dye was added, the absorbance at 570 nm and 600 nm was measured with a kinetic microplate reader (Molecular Devices, Menlo Park, CA). The dye, when added, is in an oxidized (blue color) form that is reduced (red color) as the cells proliferate. The 570 nm absorbance measures the reduced form and 600 nm measures the oxidized form. Because there is some degree of overlap between the two absorbances, it is necessary to subtract the 600 nm absorbance from the 570 nm absorbance to obtain the specific absorbance, which reflects the specific level of proliferation. The specific absorbance of unstimulated cells (in media alone) was compared to the specific absorbance of cells incubated with the mitogens, and was expressed as percent of control.

b. *IN-VIVO* PERMETHRIN EXPOSURE. Splenocytes and thymocytes from mice topically treated with either 25 µL topical permethrin or vegetable oil were aseptically isolated and dissociated 48 hours after treatment, as described above. Following resuspension in complete medium, 100 µL aliquots (5 x 10^5 cells/100 µl) was added to triplicate wells containing ConA at a concentration of 1.0 µg/well. A ten-fold range-finding dose of ConA was used in the *in-vitro* studies, and the optimal

dose was assessed to be 1.0 μg/well; subsequent *in-vivo* studies used just this concentration for optimal proliferation. Ability to proliferate in the presence of the mitogen was assessed by the colorimetric change of Alamar blue by spectroscopy, as described above. Cells cultured in wells containing complete medium without ConA were classified as unstimulated wells and served to measure spontaneous proliferation.

7. SPLENIC MACROPHAGE PHAGOCYTOSIS. Splenic macrophages were dissociated and suspended as above in culture medium. Erythrocytes were removed from splenocyte cell suspensions in ACK lysing solution, as described above. Cells were washed twice in culture medium, resuspended in 2 mL standard buffer (Hank's balanced salt solution, HBSS, Fisher), and counted electronically as described above. The phagocytic capacity of splenic macrophages was determined by a modification of the method of Dunn and Tylor (1981). Briefly, 10 μL of PBS-washed and disaggregated (Ultrasonic Cell Disrupter, 30 sec at 35%, Misonix, Inc., Farmingdale, NY) Fluoresbrite microspheres (1.16 μm; Polysciences, Inc., Warrington, PA) were added to 5 x 10^5 splenic cells/well to result in approximately 50 microspheres/cell per well. Following coincubation of microspheres and splenic macrophage suspensions at 37˚ C and 5% CO_2 for 18 hours, cells were washed twice to remove non-phagocytosed microspheres and resuspended in PBS prior to flow cytometric analysis. For each sample, 5,000 events were collected and the number of cells ingesting fluorescent particles was expressed as a percentage of 5,000, as previously described (Hart et al. 1997).

8. CHEMILUMINESCENCE RESPONSE IN SPLENIC MACROPHAGES. The production of hydrogen peroxide (H_2O_2) in phorbol-12-myristate 13-acetate (PMA) – stimulated splenic macrophages was determined by the method of Bass et al. (1983). Briefly, splenic cell suspensions that were prepared as above and diluted to 5 x 10^5 cells in complete culture medium were incubated with 5 µL of 5 mM dichlorofluorescein-diacetate (DCF-DA; Molecular Probes, Eugene, OR) for 15 minutes at 37°C, 5% CO_2. Following incubation with DCF-DA, cells were stimulated by the addition of 10 µL of 100 ng/mL PMA (Sigma) in a subsequent 30-minute incubation period at 37°C and 5% CO_2. 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.

9. B LYMPHOCYTE ANTIBODY PRODUCTION: PLAQUE-FORMING CELL ASSAY. The plaque forming cell assay (PFC) was used to quantify the ability of splenic lymphocytes to mount an antibody-mediated immune response to the T-dependent antigen, sheep red blood cells (sRBC) (Roitt and Delves 1992). Four days prior to sacrifice, mice received by intraperitoneal injection 0.5 mL 10% sRBC that had been previously washed twice and resuspended in PBS. Four days following sRBC administration, mouse spleens were isolated and dissociated as described above. After two washings, splenocytes were counted and suspended to a concentration of 2 x 10^7 cells/mL in RPMI. Agar was prepared from 500 mg agar dissolved in 110 mL HBSS, with pH adjusted to 7.3, and 1.6 mL DEAE-dextran

solution obtained from a stock of 150 mg DEAE-dextran dissolved in 5 mL saline. Agar was kept in a 47°C water bath, and 0.8 mL was used per sample, into which the following was added: 30 µL 30% sRBC, 100 µL splenic cell suspension, and 20 µL guinea pig complement. This mixture was mixed and poured into a 35 mm petri dish top, and immediately the lower part of the same dish was placed on top of the agar to ensure even and thin spreading of the agar. Samples were performed in duplicate, and the solidified plates were incubated (37°C, 5% CO_2) for a minimum of 4 hours. Lysed red cells appeared as foci of clearing in the agar. These areas of clearing (plaques) indicated the location of splenocytes that were producing IgM against the foreign red cell antigens. The plaques were enumerated microscopically and expressed as a ratio of number of IgM-producing splenocytes per 1,000 splenocytes as an indicator of the animal's ability to respond to foreign antigen with an antibody-mediated immune response.

10. ^{51}Cr RELEASE CYTOTOXICITY ASSAY. Cytotoxic T lymphocyte activity was measured in splenocytes sensitized in vitro with mitomycin c-treated P815 mastocytoma cells (Brunner et al. 1968). Following ACK lysis of erythrocytes, splenocyte responder cells from experimental mice were suspended at 2 x 10^7 cells/mL. Sensitizing cells were log phase P815 mastocytoma cells (American Type Culture Collection, Manassas, VA) that had been raised in cell culture, washed, and incubated at 37°C for one hour with 100 µCi ^{51}Cr (ICN Pharmaceutical Inc., Irvine, CA) at a concentration of 5 x 10^6 cells/mL in complete medium, and then mixed with responder cells at ratios of 50:1, 25:1, and 12.5:1 to assess the level of splenocyte

cytotoxic T cell activity. Chromium labeled P815 cells were also added to quadruplicate wells containing medium only to determine spontaneous release, and to quadruplicate wells containing 0.1% triton x-100 (RPI, Elk Grove Village, IL) to determine total release. The cultures were centrifuged and then incubated at 37°C and 5% CO_2 for 44 hr, after which 0.2 mL of the supernatant was harvested using a Skatron harvester (Skatron, Sterling, VA). Determination of released label was made using a Cobra gamma counter (Packard Instruments, Chicago, IL). CTL activity was calculated from the formula:

$$\frac{\text{cpm (experimental - cpm (spontaneous release)}}{\text{cpm (total release) - cpm (spontaneous release)}} \times 100$$

11. STATISTICAL ANALYSIS. Data were expressed as arithmetic mean ± SEM. For the thymocyte apoptosis study, a univariate 2-way analysis of variance was performed and for the remaining experiments, a one-way analysis of variance was completed; treatment structure for the thymocyte apoptosis study only was a two by three way factorial design, with a simple, random sampling structure. A randomized complete block design was used for error control, with restricted randomization in the *in-vitro* studies. Dunnett's post-hoc t-test was used to establish significant differences in treatment groups versus controls, and a Bonferroni correction was utilized in the *in-vitro* studies to account for relationships between the thymocyte subgroups. Results described as different in this paper indicate significantly different at $p < 0.05$ (*) or $p<0.01$ (**).

2D. RESULTS

1. ORGAN WEIGHTS AND CELLULARITY. The single application of topical permethrin at 5, 10, 15 or 25 μL (equivalent to approximately 220-1100 mg/kg permethrin) did not alter body weight in any of the aged-matched and weight-matched treatment groups. However, topical permethrin caused a dose-related decrease in thymic organ weight and cellularity 48 hours following exposure. Mean thymic organ weight was numerically reduced at all permethrin concentrations and significantly diminished at the 15 μL and 25 μL doses (p=0.0011 and p=0.0008, respectively; n=10/treatment group, Fig. 2.1). Thymocyte cellularity diminished in a manner parallel to thymic weight, with significance again occurring at 15 and 25 μL permethrin (p=0.0028 and p=0.0023, respectively; n=10/treatment group, Fig. 2.2). Topical exposure to the three higher doses of permethrin (10, 15 or 25 μL) caused a similar dose-related decrease in spleen weight relative to control (p=0.007 at 10 μL, p=0.00004 at 15μL and 0.0001 at 25 μL, n=19/treatment group). The lowest volume of topical permethrin (5 μL) resulted in a numerically lower splenic organ weight that was not statistically significant (p=0.24, n=5; Fig. 2.3). The effect of permethrin on splenic leukocyte counts paralleled that of spleen weight, being dose-related and significant at the higher doses (p=0.0021 at 15 μL and p=0.0001 at 25 μL, n=19/treatment group; Fig. 2.4).

2. SURFACE ANTIGEN EXPRESSION BY FLOW CYTOMETRY. Permethrin caused a significant depletion of thymocytes. Therefore, flow cytometric evaluation of thymocyte subpopulations defined by CD4 and CD8 antigen expression was examined. The percentage of cells in the major $CD4^+CD8^+$ positive (double

positive; DP) thymocyte population was markedly diminished at both 15 and 25 μL permethrin (p=0.0162 for 15 μL and p=0.001 for 25 μL; n=5/treatment group). The percentage of cells in each of the remaining populations defined by these antigens, $CD4^-8^-$ (double negative; DN) $CD4^-8^+$ (CD8 single positive; SP) and $CD4^+8^-$ cells (CD4 SP) increased as permethrin dose increased, possibly the result of the decreased percentages of cells in the major DP population. The absolute numbers of cells in each thymocyte population (calculated as total thymic cellularity X percentage in each phenotype) was therefore calculated. The total number of cells in all phenotypes was reduced by permethrin, with DP cells being most affected (Table 2.1).

3. THYMOCYTE APOPTOSIS AND NECROSIS. The decreased number of thymocytes in phenotypes defined by CD4 and CD8 antigens, and more particularly the decreased DP cells, suggested the possibility that permethrin may directly target thymocytes, increasing apoptotic or necrotic cell death. Therefore, thymocytes from mice exposed to 25 μL topical permethrin were isolated at several time points post-exposure, and evaluated cytometrically for viability, apoptosis, and necrosis. Data were expressed as percentages of viable, apoptotic, and necrotic cells from total thymocytes. The proportion of viable, apoptotic, and necrotic cells from each subpopulation was also considered to determine if sensitivity of thymocyte subpopulations varied across phenotypes in response to permethrin exposure. Total thymocyte numbers did not change over the 8-hour exposure. Permethrin treatment caused a numerical decrease in total viable thymocyte numbers; however, statistical significance was not achieved in any experiment. In contrast, the viability of DN and CD4 and CD8 SP thymocytes was significantly decreased by permethrin (Table

2.2A). Apoptosis of total thymocytes, DN, and CD8 SP was significantly increased, while apoptosis of CD4 SP and DP thymocytes increased numerically but not statistically (Table 2.2B). Similar to 7-AAD, annexin-V staining demonstrated late apoptosis/necrosis that was significant in CD4 and CD8 SP thymocytes, and numerically increased in all thymocyte subpopulations (Table 2.2C).

4. FUNCTIONAL ASSAYS: MACROPHAGE ACTIVITY, B CELL ANTIBODY PRODUCTION, LYMPHOCYTE PROLIFERATION, AND T CELL LYTIC ACTIVITY. The initial experiments demonstrated that permethrin diminished size and cellularity of the spleen and thymus. These results raised questions regarding functionality of leukocytes in the spleen and thymus following permethrin treatment. The ability of splenic macrophages to phagocytose fluorescent microspheres was not significantly altered following permethrin treatment, nor were significant differences seen in hydrogen peroxide production by splenocytes or cytotoxic T cell-mediated lysis assays (Appendices I, II, and II, respectively). A dose-related numerical decrease in the number of antibody-producing splenic B cells from permethrin-treated animals was observed, but was not significant (p=0.117 at the 25 μL dose, n=14/treatment group; Fig. 2.5). Splenocytes from mice previously treated with topical permethrin (25 μL administered to the shaved interscapular space) demonstrated highly significant decreased proliferative response to the T cell mitogen, ConA (p=0.0002, n=8/treatment group; Fig. 2.6). Similarly, splenocytes collected from untreated animals and cultured with permethrin displayed reduced blastogenesis in response to ConA. Proliferation was inhibited significantly at both

25 and 100 μM permethrin, after 72 hours of culture (p=0.013 at 25 μM and p=0.0034 at 100 μM, n=16/treatment group; Fig. 2.7). In repeated experiments, thymocytes isolated from permethrin-treated animals demonstrated non-significant trends toward reduced proliferative ability following 72 hr culture in the presence of ConA (Appendix IV).

2E. DISCUSSION

The low neurotoxic risk and the efficacy of permethrin for ectoparasite control have resulted in increased human exposure to this insecticide. A growing database suggests, however, that permethrin and related pyrethroids may target the immune system. The type II pyrethroid insecticide cypermethrin, which differs from permethrin by a single cyano substitution, has been shown to suppress cellular and antibody-mediated immune responses in mice, rats, rabbits, and goats (Desi et al. 1985, Tamang et al. 1988). Recent studies indicate that permethrin also negatively affects the immune system through alterations in cell-mediated, antibody-mediated, and natural killer cell immunity (McCorkle et al. 1980, Blaylock et al. 1995, Santoni et al. 1997, 1998, Diel et al. 1998, 1999, Punareewattana et al. 2001). With limited exceptions, these adverse immune effects have been reported in animals exposed by the inhalational or oral route, rather than by topical exposure, which is the most common route of exposure in humans.

Punareewattana et al. (2000, 2001) recently reported systemic and local immunotoxicity in mice that were exposed by the topical route to permethrin. The systemic immune effects observed by these authors occurred after subacute dosing (10-30 consecutive days) were similar to effects reported in rodents after oral permethrin exposure, and included thymic and splenic atrophy and hypocellularity, and inhibited antibody production and T cell cytolytic activity. The local immune effects persisted to 30 days post-dosing, and were manifested as diminished contact hypersensitivity. It remains unclear if significant immunotoxicity may be caused by single-dose topical exposure to permethrin, which may be more representative of

exposure in humans for control of lice (e.g., shampoo preparations) or mites (e.g., topical creams). Further, little information is currently available regarding mechanisms by which oral or topical permethrin causes systemic immunotoxicity.

Results of the present studies demonstrate that single-dose topical permethrin causes systemic immune changes in mice similar to those seen with longer topical exposures, in the absence of overt toxicity. Decreased thymic weight and thymocyte cellularity in treated mice was particularly noteworthy, with the greatest reduction in thymocyte numbers being observed in the DP thymocyte subpopulation. Apoptotic cell death naturally occurs in such DP thymocytes, as a part of selective processes for recognition of antigen presenting molecules as well as for recognition of self antigens (Cohen et al. 1992, Green and Cotter 1992, Hartley et al. 1993). Together, these observations raised questions regarding the possibility that permethrin may cause thymic hypocellularity, at least in part, by increased apoptosis of thymocytes. In this regard, deltamethrin was reported to cause increased thymocyte apoptosis (Enan et al. 1996).

Thymocytes collected from mice previously exposed to topical permethrin showed limited but significant decreases in viability, consistent with possible increased apoptotic or necrotic cell death. Apoptosis was significantly increased in the DN and SP phenotypes rather than in the DP cells that were most strongly diminished by the topical permethrin treatment. The reason for this observation is unclear, but may relate to relative sensitivity of the different thymocyte phenotypes to chemical-induced apoptosis, as well as to timing of thymocyte collection and apoptosis assay. In this regard, Kamath et al. (1997) recently made a similar

observation in dioxin-treated mice, and hypothesized that the apoptotic DP thymocytes were rapidly cleared by resident macrophages and thus difficult to demonstrate.

Antiproliferative effects may also contribute to thymic or splenic atrophy, thus were also evaluated as mechanisms that may lead to hypocellularity. Splenocytes, but not thymocytes, collected from permethrin-exposed mice displayed reduced proliferative capacity to the mitogen, ConA. These cells were collected 48 hr after permethrin exposure and in the case of thymocytes incubated for 48 hr before proliferation was determined. Thymocytes have been described as metabolically active cells (Holladay et al. 1993), raising questions about the possibility of recovery from an antiproliferative effect of permethrin after dosing and during the incubation. Therefore, proliferation was also examined in thymocytes and splenocytes following in vitro exposure to permethrin, and was significantly diminished in splenocytes but not thymocytes.

In addition to the antiproliferative immunotoxic effects of permethrin, the current study also demonstrated trends towards decreased antibody production following a single dose of permethrin. These data are similar to a previous report, in which longer topical permethrin dosing caused diminished antibody production (Punareewattana et al. 2001). Alterations in antibody-mediated immunity have also been demonstrated with other pesticides such as propanil or pentachlorophenol in human beings (Daniel et al. 2001, McClure et al. 2001), heptachlor in rats (Smialowicz et al. 2001), malathion in fish, mice, rats, and rabbits (Banerjee et al.

1998, Beaman et al. 1999) and the organochlorines DDT and lindane in rats (Koner et al. 1998).

In summary, single dose exposure to topical permethrin caused a variety of immunological changes in mice, with the thymus being more profoundly affected than the spleen. Thymic atrophy and hypocellularity appeared to result, at least in part, from increased thymocyte apoptosis and from antiproliferative chemical effects on thymocytes. Antiproliferative effects in splenic T lymphocytes and reduced cell counts were demonstrated in this organ. In support of this hypothesis, Stelzer and Gordon (1984) previously reported inhibited proliferation of murine T cells exposed in vitro to pyrethroids. The recent report of inhibited cell division in an immortal Chinese hamster lung cell line (V79) following exposure to permethrin (Hadnagy et al. 1999) further supports the observation that this agent may inhibit thymocyte and splenocyte proliferation.

Comparing permethrin doses used to cause thymic and splenic effects in mice to human exposure is difficult, however some estimates can be made. Grissom et al. (1987) calculated that 2.5% of topical permethrin in acetone crossed the skin in mice. Baynes et al. (1997) estimated that 1.7% of permethrin in DMSO crossed mouse skin. Studies using topical Elimite® cream (5% permethrin) for mite control in humans suggest that about 1.25% of the permethrin is absorbed after an 8 - 14 hr treatment (i.e., after treatment according to label) (COT 1994). Based on the estimated 1.25% absorbed rate, Punareewattana et al. (2001) calculated an absorbed permethrin dose in humans treated with Elimite® of about 558 μg/kg. The 5 - 25 μL topical permethrin received by the present mice corresponded to about 220 - 1100 mg/kg topical

permethrin. Assuming the higher 2.5% absorbed rate across mouse skin, this indicates 5.5 - 27.5 mg/kg absorbed permethrin, or about 10 - 50 times estimated human exposure. It is important to note that the absorption rate of permethrin across mouse skin from the present corn oil vehicle is not known, thus the above calculations may not accurately represent systemic permethrin exposure in the present mice.

In conclusion, the present study in mice demonstrates altered immune responses after topical permethrin exposure. The immune responses that were inhibited, including antibody production, have high concordance (predictive values) for immunosuppression in mice (Luster et al. 1992). Beyond immunosuppression, recent data suggest possible increased incidence of allergic disease associated with chronic inhalation exposure to permethrin (Fuortes 1999). This observation may relate to increased histamine release by basophils and decreased IFNγ and IL-4 release by peripheral lymphocytes caused by permethrin. These collective reports verify the need for additional examination of the immunomodulatory effects of permethrin.

2F. ACKNOWLEDGEMENT

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Table 2.1. Effect of Topical Permethrin on Absolute Thymocyte Subpopulations

Permethrin (μL)	Total Thymocytes ($x10^6$)	CD4-CD8- ($x10^6$)	CD4+8- ($x10^6$)	CD4-8+ ($x10^6$)	CD4+CD8+ ($x10^6$)
0	69.86±7.92	7.96±3.19	9.32±1.33	3.85±0.44	48.72±3.94
15	19.94±0.86**	4.70±0.94	3.85±0.37*	1.60±0.18*	9.77±1.66**
25	18.52±0.86**	6.08±0.96	4.14±0.33*	1.54±0.12*	6.73±1.07**

Each measurement is mean ± SEM (n=5 per treatment group). *p<0.05; **p<0.01.

Table 2.2. Effect of Topical Permethrin on Thymocyte Viability and Apoptosis

A. Thymocyte viability

Exposure	Total	$CD4^-CD8^-$	CD4	CD8	$CD4^+CD8^+$
Vehicle	74.9±3.1	66.7±4.1	56.0±3.6	80.9±3.4	80.9±2.7
25 µL perm	72.4±3.1	62.8±3.9	52.0±3.3	74.7±3.3	79.0±2.8

B. Thymocyte apoptosis (7-AAD)

Exposure	Total	$CD4^-CD8^-$	CD4	CD8	$CD4^+CD8^+$
Vehicle	20.4±2.6	22.3±2.9	22.3±1.7	14.0±2.4	12.3±1.6
25 µL perm	22.7±2.7	27.2±2.7*	24.2±1.8	18.2±2.5*	13.7±1.6

C. Thymocyte apoptosis (annexin V)

Exposure	Total	$CD4^-CD8^-$	CD4	CD8	$CD4^+CD8^+$
Vehicle	4.7±0.8	8.9±1.5	21.5±2.5	5.0±1.3	6.6±1.4
25 µL perm	4.9±0.6	9.8±1.4	23.6±2.2	7.1±1.0	7.2±1.4

Numbers are expressed as percentages of viable and apoptotic cells from each thymocyte phenotype. Each measurement is mean ± SEM (n=5 per treatment group). *$p<0.05$.

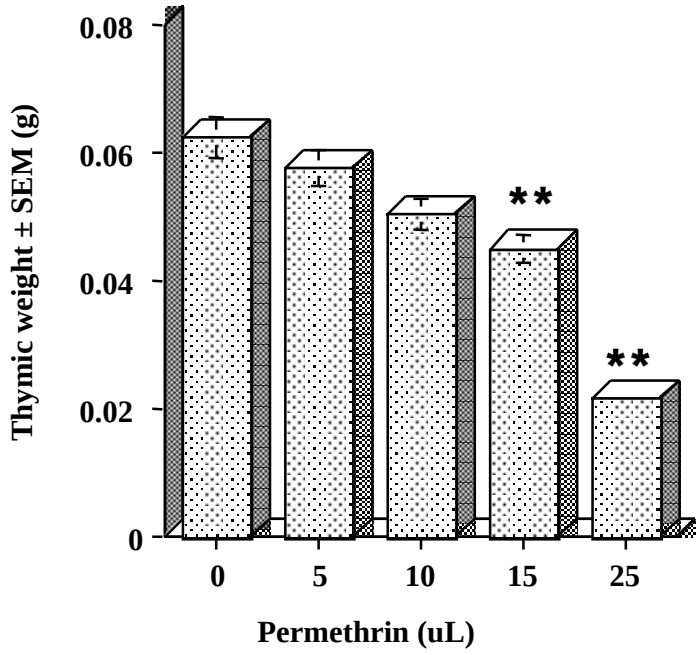

Figure 2.1. Thymic weight response to single exposure to topical permethrin in 5 week-old female C57BL/6N mice. Permethrin was applied to the shaved interscapular space at 0 – 25 µl. Results represent a dose-related decrease in thymic weight at 48 hr following permethrin exposure. Each measurement is the mean ± SEM (n=10 per treatment group); ** $p<0.01$.

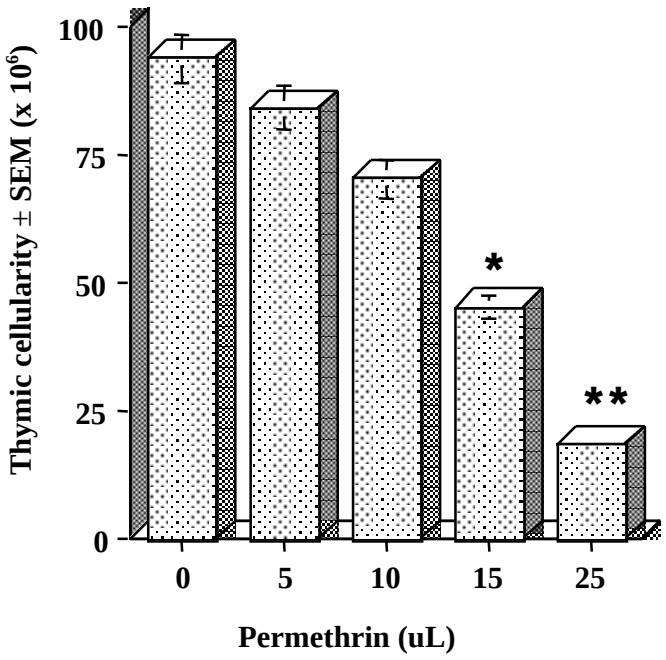

Figure 2.2. Thymic cellularity response to single exposure to topical permethrin in female C57BL/6N mice. Permethrin was applied to the shaved interscapular space at 0 – 25 µL. Results represent a dose-related decrease in thymic cellularity at 48 hr following permethrin exposure. Each measurement is the mean ± SEM (n=10 per treatment group); *p<0.05, ** p<0.01.

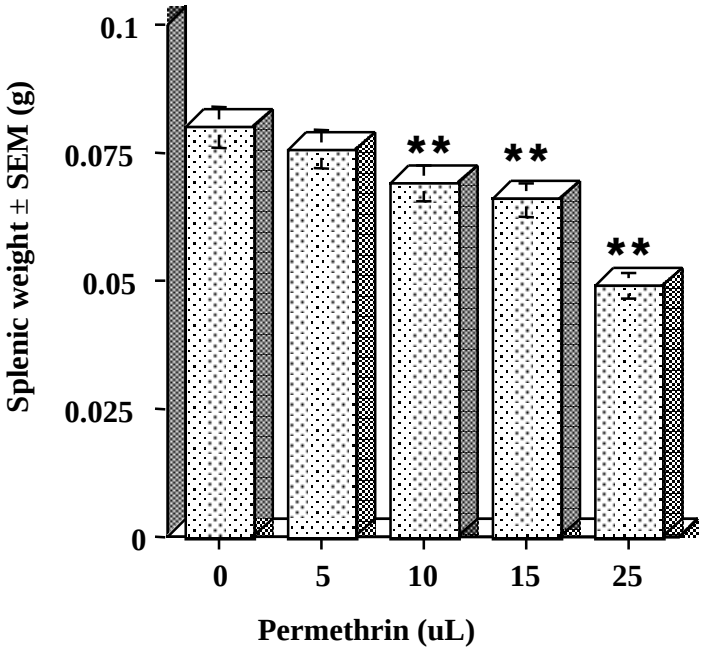

Figure 2.3. Splenic weight response to single exposure to topical permethrin in female C57BL/6N mice. Permethrin was applied to the shaved interscapular space at 0 – 25 μL. Results represent a dose-related decrease in splenic weight at 48 hr following permethrin exposure. Each measurement is the mean ± SEM (n=19 per treatment group); $^{**}p<0.01$.

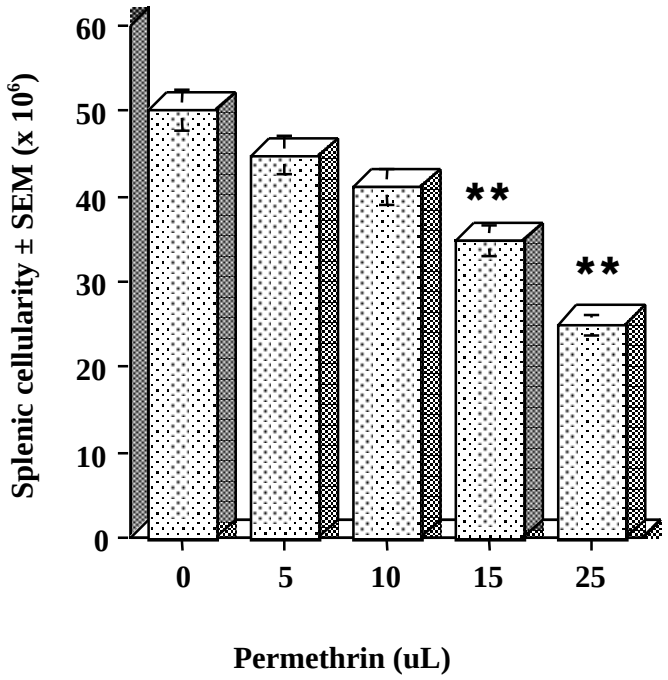

Figure 2.4. Splenic cellularity response to single exposure to topical permethrin in female C57BL/6N mice. Permethrin was applied to the shaved interscapular space at 0 – 25 μL. Results represent a dose-related decrease in splenic cellularity at 48 hr following permethrin exposure. Each measurement is the mean ± SEM (n=19 per treatment group); ** $p<0.01$.

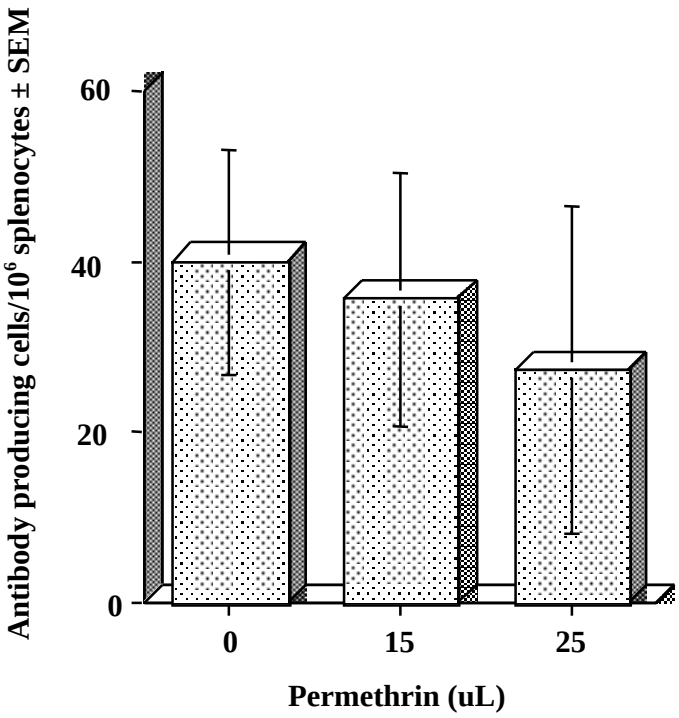

Figure 2.5. Antibody-mediated immune response to single exposure to topical permethrin in female C57BL/6N mice. Permethrin was applied to the shaved interscapular space at 0, 15, and 25 µL. Results represent a dose-related trend towards decreased ability of splenocytes to produce antibodies against allogeneic sheep red cell antigens. Each measurement is the mean ± SEM (n=8 per treatment group).

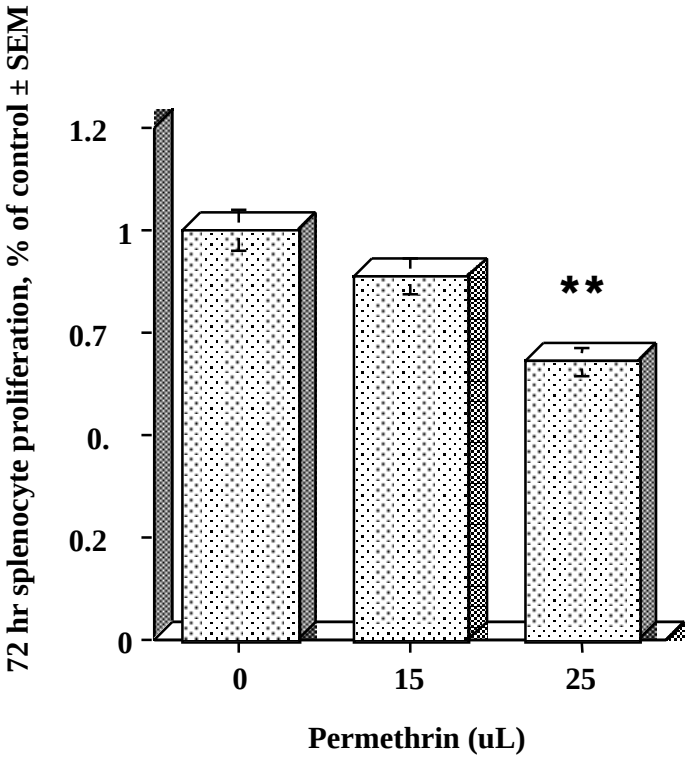

Figure 2.6. Proliferation response of splenocytes from female C57BL/6N mice previously exposed to *in-vivo* 0 or 25 µL topical permethrin in the interscapular space. 72 hr proliferation was measured by colorimetric change in response to a T-cell mitogen, Concanavalin A, and is represented as a percent of control. Each measurement is the mean ± SEM (n=14 per treatment group); ***p*<0.01.

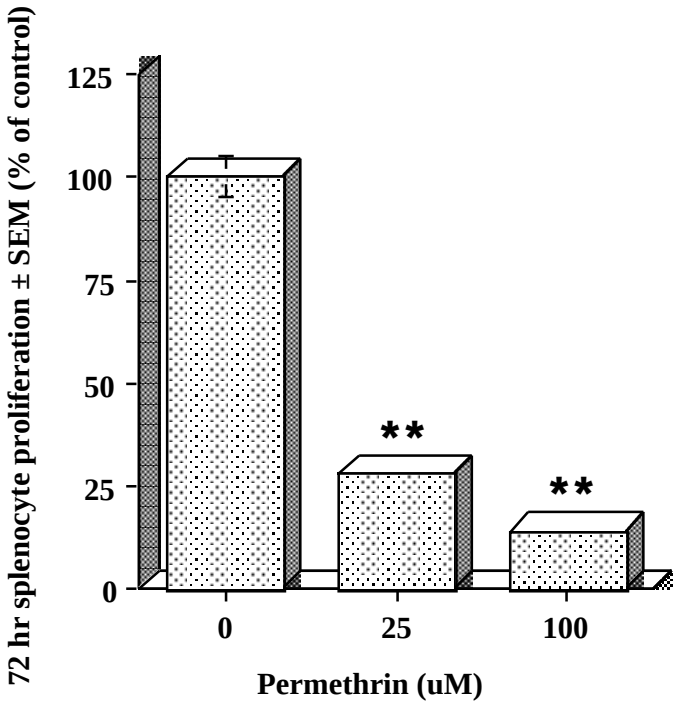

Figure 2.7. Proliferation response of splenocytes from female C57BL/6N mice exposed *in-vitro* to 0, 25, or 100 μM permethrin in DMSO. 72 hr proliferation was measured by colorimetric change in response to ConA, and is represented as a percent of control. Each measurement is the mean ± SEM (n=16 per treatment group); ** $p<0.01$.

CHAPTER 3: IMMUNOTOXIC EFFECTS OF CIS-UROCANIC ACID EXPOSURE IN C57BL/6N MICE

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3A. ABSTRACT

Exposure to ultraviolet radiation results in increased levels of intradermal cis-urocanic acid (cUCA), which in turn causes inhibition of cutaneous immunity by interfering with antigen processing and presentation by Langerhans cells (LC). Reports of systemic immunotoxicity with subchronic cUCA exposure in laboratory rodents have included thymic atrophy, thymic hypocellularity, and decreased T-cell mediated immunity; however, immune effects of short-term cUCA administration, which may better mimic human exposures, are poorly defined. The present study evaluated immune effects of one- and five-day cUCA exposure in C57BL/6N mice. A single administration of intradermal cUCA resulted in decreased splenocyte phagocytosis that persisted 30 days post-cUCA exposure. Five-day consecutive exposure to cUCA caused decreased numbers of $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ phenotypically mature thymocytes and increased $CD4^{+}CD8^{+}$ immature thymocytes, as well as increased splenocyte proliferation in the presence of the T-cell mitogen concanavalin A. Prolonged cUCA exposure (three doses weekly for four weeks) was also examined, and resulted in profound thymic hypocellularity, splenic hypercellularity, and increased splenic macrophage chemiluminescence. Because of the apparent sensitivity of C57BL/6N mice to cUCA, thymic hypocellularity was compared in C57BL/6N and C3H-HeN mice dosed with cUCA, and was more pronounced in the C57BL/6N strain. The latter results may suggest divergent genetic susceptibility in these mouse strains to immunomodulatory effects of sunlight exposure.

3B. INTRODUCTION

Recent literature has demonstrated localized and systemic immunotoxicity in laboratory rodents exposed to ultraviolet (UV) irradiation (Baadsgaard et al. 1990, Gilmour et al. 1993, Streilein et al. 1994, Jeevan et al. 1995, Bacci et al. 1996, El-Ghorr and Norval 1997, Hart et al. 1997, Shreedhar et al. 1998, Kasahara et al. 2001). These effects are thought to be mediated by the cis isomer of the chromophore urocanic acid, which is constitutively produced and stored by epidermal keratinocytes in the inactive trans form, and converted to the cis form upon exposure to UV light. Cis-urocanic acid (cUCA) affects cutaneous, i.e., local, as well as systemic immune system targets with prolonged sunlight exposure, sometimes in a persistent manner (El-Ghorr and Norval 1997). Specific molecular mechanisms of the immunologic sequelae that occur after urocanic acid isomerization are incompletely characterized. A better understanding of such mechanisms is necessary to develop intervention strategies aimed at minimizing deleterious effects on the immune system of excessive UV light exposure.

Urocanic acid [3-(1 *H*-imidazol-4-yl)-propenoic acid] is produced by epidermal keratinocytes as a deaminated product of histidine and is stored in the trans isomeric form (tUCA) in keratohyalin granules in the stratum corneum. The poorly soluble tUCA is converted to the highly water soluble cis isomer when exposed to low-wavelength (280-320 nm) UVB irradiation. Cis-urocanic acid is then distributed systemically and is thought to be excreted unmetabolized in the urine (Anglin and Batten 1968, Morrison and Deibel 1986). Urocanic acid has been proposed to act as a natural cutaneous photoprotecting agent against DNA damage caused by UVB irradiation. However, circulating cUCA has been shown to confer both local and systemic immunomodulation

via diminished T cell-mediated immunity and decreased antigen presentation. These in turn may lead to the clinically relevant end points of increased risk of infectious or neoplastic disease.

In-vivo UVB radiation or intracutaneous administration of cUCA in human beings causes a defect in cutaneous antigen presentation by abrogating the function of $CD1^+$ Langerhans cells (LC), and inducing $CD1^-$ macrophages that activate suppressor T lymphocytes and diminish contact hypersensitivity (Baadsgaard et al. 1990). Cis-urocanic acid also induces e-selectin expression from endothelial cells of dermal microvessels, which causes release of constitutive tumor necrosis factor-alpha (TNFα) from mast cell granules and keratinocytes (Kock et al. 1990). Tumor necrosis factor-alpha decreases vimentin expression in LC, which results in blunting of LC cytoplasmic processes and decreased ability of the compromised cells to acquire and process antigens to present to T lymphocytes. Tumor necrosis factor-alpha is also a chemoattractant to LC, so increased concentrations in the skin prevent migration of LC to local lymph nodes and thus decrease antigen presentation. Ultraviolet-B irradiation also increases macrophagic release of transforming growth factor-β1 (TGFβ1), which leads to release of TNFα from local mast cells and local mononuclear cells (Majewski and Jablonska 1995). Prolonged (four-week) exposure to UVB or cUCA results in thymic atrophy and thymic hypocellularity in laboratory rodents, as well as systemic suppression of T cell-mediated immunity (El-Ghorr and Norval 1997) and suppression of natural killer cell activity in human beings (Gilmour et al. 1993). Many of the immunologic effects of sunlight exposure are elicited in laboratory rodents by the intracutaneous administration of cUCA.

The present study evaluated systemic immune modulation in mice following acute or subacute intradermal cUCA administration. Limited information is available regarding effects in thymus or spleen from short-duration cUCA treatment (e.g., single-dose exposure; daily exposure for less than one week), thus these were endpoints of focus. Two immunologic assays with high individual and pairwise predictive values for systemic immunosuppression, antibody production and cytotoxic T lymphocyte activity (Luster et al. 1992, 1993) were used to examine immune function after cUCA dosing. Antigen presenting cells including macrophages have been identified as sensitive targets of cUCA, thus phagocytic and chemiluminescent activities of splenic macrophages were also examined. Thymic cellularity was not reduced with short-term cUCA exposure in C57BL/6N mice as had been reported in C3H-HeN mice after four weeks cUCA exposure (El-Ghorr and Norval 1997). This endpoint was therefore compared in C57BL/6N mice (immunologically sensitive) and C3H-HeN mice (the historic mouse for UV light studies) after four weeks cUCA exposure, and found to be more dramatically affected in the C57BL/6N mice. These results suggest possible underlying genetic differences in C3H-HeN and C57BL/6N mice to UV light, which may be useful for subsequent mechanistic studies of UV light sensitivity.

3C. MATERIALS AND METHODS

1. MICE. Five-week-old female C57Bl/6N and C3H-HeN mice were used in these studies. Mice were purchased from Charles River Laboratories (Portage, MI). Mice were acclimated for one week and maintained under controlled conditions of temperature (22±1˚C), humidity (40-60%), and light (12/12-hour light/dark cycle). Food and water were provided ad libitum. Mice in all experiments were humanely treated, in accordance with the guidelines of the Virginia Tech Institutional Animal Care and Use Committee.

2. CIS-UROCANIC ACID. Cis-urocanic acid was synthesized and purified as previously described (Takahashi and Tezuka 1997), and then evaluated for purity by mass spectroscopy prior to use. Administration of cUCA began as a single suberythremic intradermal dose of 0, 0.5, 5, 50, 100, or 250 μg in PBS (equivalent to approximately 0, 0.033, 0.33, 3.3, 6.6, or 16.5 mg/kg, respectively) in mice arbitrarily assigned to treatment or control groups. With the exception of altered macrophage phagocytosis, single dose cUCA did not produce detectable immune effects. Longer exposure durations were next evaluated, up to and including subacute intradermal dosing (five consecutive daily doses or three times weekly for four consecutive weeks). All assays were repeated in a minimum of three separate experiments unless otherwise stated.

3. ORGAN WEIGHTS, CELL PREPARATION, AND CELLULARITY. The thymus and spleen from each mouse were collected by dissection and placed, individually, into pre-weighed 60 x15 mm culture dishes (Fisher Scientific, Norcross, GA). Wet organ weights were immediately obtained using an Ohaus analytical scale (TS

120S, Florham Park, NJ). Eight mL cold incomplete culture medium (RPMI-1640, Fisher) were added to petri dishes with organs, after which thymocytes and splenocytes were gently dissociated into the culture medium using a wire mesh screen (Sigma, St. Louis, MO) and curved forceps. Suspended cells were washed twice in incomplete culture medium, resuspended in 2 mL culture medium, and counted using a Scharfe CASY-1 electronic cell counter (Scharfe System, GmbH, Germany). The accuracy of the cell counter was verified in each experiment by counting 2-3 samples at random on a hemocytometer using Natt Herrick's vital stain.

4. THYMOCYTE SURFACE ANTIGEN EXPRESSION BY FLOW CYTOMETRY. Thymocytes from control and cUCA-exposed mice were suspended in PBS at 5 x 10^6 cells/mL. Expression of CD4 and CD8 surface antigens on thymocytes was determined by incubating 100 µL cell suspensions containing 5 x 10^5 cells with 0.5 µg fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8a (clone 536.7) and 0.5 µg phycoerythrin (PE)-conjugated anti-mouse CD4 (clone H129.19) monoclonal antibodies (BD Pharmingen, San Diego, CA) in a 96-well round-bottom tissue culture plate (Corning, Corning, NY). Following a 30-minute incubation in the dark at 4°C while mixing on a Gyrotary Shaker Model G2 orbital mixer (New Brunswick Scientific, New Brunswick, NJ), cells were washed twice in PBS and 5,000 events from each sample were analyzed using an Epics XL flow cytometer (Beckman-Coulter, Miami, FL). Cell viability, cell size and granularity were verified by forward and side-angle light scatter and viability was determined to be greater than 95% in all samples. Dead cells

were excluded from analysis with electronic gates, and data were analyzed using the Immuno-4 software program (Beckman-Coulter, Miami, FL).

5. PROLIFERATION OF SPLENOCYTES AND THYMOCYTES. Splenocytes and thymocytes from mice previously treated with 0, 50, or 100 μg intradermal cUCA were aseptically isolated 48 hours after treatment, as described above. Following resuspension in complete medium, 100 μL aliquots (5×10^5 cells/100 μL) were added to triplicate wells containing the T-cell mitogen, concanavalin A (ConA, Sigma). The results of a ten-fold range-finding dose study of ConA determined the optimal dose to be 1.0 μg/well; subsequent studies used just this concentration. Blastogenesis of splenocytes and thymocytes was quantified in the presence and absence of ConA using the nonradioactive colorimetric/fluorometric assay originally described by Ahmed et al. (1994) and Gogal et al. (1997). After 24 hours of culture for splenocytes or 48 hours of culture for thymocytes at 37°C and 5% CO_2, 20 μL of Alamar blue dye (Accumed International, Inc., Westlake, OH) were added to each well of the plate, after which plates were returned to the incubator. After 72 hours of culture, the absorbance at 570 nm and 600 nm was measured with a kinetic microplate reader (Molecular Devices, Menlo Park, CA). The dye, when added, is in an oxidized (blue color) form that is reduced (red color) as the cells proliferate. The 570 nm absorbance measures the reduced form and 600 nm measures the oxidized form. Because there is some degree of overlap between the two absorbances, it is necessary to subtract the 600 nm absorbance from the 570 nm absorbance to obtain the specific absorbance, which reflects the specific level of proliferation. The specific absorbance of unstimulated cells (in media alone) was

compared to the specific absorbance of cells incubated with the mitogens, and was expressed as percent of PBS-treated controls.

6. SPLENIC MACROPHAGE PHAGOCYTOSIS. Splenic macrophages were dissociated and suspended as above in culture medium. Erythrocytes were removed by suspending splenocytes in ACK lysing solution (0.015 M NH_4Cl, 1.0 mM $NaHCO_3$, 0.1 mM EDTA) for 5 minutes at room temperature. Cells were then washed twice in culture medium, resuspended in 2 mL standard buffer (Hank's balanced salt solution, HBSS, Fisher), and counted electronically as described above. The phagocytic capacity of splenic macrophages was determined by a modification of the method of Dunn and Tylor (1981). Briefly, 10 µL of PBS-washed and disaggregated (Ultrasonic Cell Disrupter, 30 sec at 35%, Misonix, Inc., Farmingdale, NY) Fluoresbrite® microspheres (1.16 µm; Polysciences, Inc., Warrington, PA) were added to 5 x 10^5 splenic cells/well to result in approximately 50 microspheres/cell per well. Following coincubation of microspheres and splenic macrophage suspensions at 37˚ C and 5% CO_2 for 18 hours, cells were washed twice to remove non-phagocytosed microspheres and resuspended in PBS prior to flow cytometric analysis. For each sample, 5,000 events were collected and the number of cells ingesting fluorescent particles was expressed as a percentage of the 5,000 events as previously described by Hart et al. (1997).

7. CHEMILUMINESCENCE RESPONSE IN SPLENIC MACROPHAGES. The production of H_2O_2 in phorbol-12-myristate 13-acetate (PMA) – stimulated splenic macrophages was determined by the method of Bass et al. (1983). Briefly, splenic cell

suspensions that were prepared as above and diluted to 5 x 10^5 cells in complete culture medium were incubated with 5 µL of 5 mM dichlorofluorescein-diacetate (DCF-DA; Molecular Probes, Eugene, OR) for 15 minutes at 37°C, 5% CO_2. Following incubation with DCF-DA, cells were stimulated by the addition of 10 µL of 100 ng/mL PMA (Sigma) in a subsequent 30-minute incubation period at 37°C and 5% CO_2. 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.

8. B LYMPHOCYTE ANTIBODY PRODUCTION: PLAQUE-FORMING CELL ASSAY. The plaque forming cell assay (PFC) was used to quantify the ability of splenic lymphocytes to mount an antibody-mediated immune response to the T-cell dependent antigen, sheep red blood cells (sRBC) (Roitt and Delves 1992). Four days prior to sacrifice, mice received an intraperitoneal injection of 0.5 mL 10% sRBC that had been previously washed twice and resuspended in PBS. Four days following sRBC administration, mouse spleens were isolated and dissociated as described above. After two washings, splenocytes were counted and suspended to a concentration of 2 x 10^7 cells/mL in incomplete culture medium (RPMI). Agar was prepared from 500 mg agar dissolved in 110 mL HBSS, with pH adjusted to 7.3, and 1.6 mL DEAE-dextran solution obtained from a stock of 150 mg DEAE-dextran dissolved in 5 mL saline. Agar was kept in a 47°C water bath, and 0.8 mL was used per sample, into which the following was added: 30 µL 30% sRBC, 100 µL splenic cell suspension, and 20 µL guinea pig

complement (Sigma). This mixture was vortexed and poured into a 35 mm petri dish top, and immediately the lower part of the same dish was placed on top of the agar to ensure even and thin spreading of the agar as they solidified. Samples were assayed in duplicate plates, and were incubated (37°C, 5% CO_2) for a minimum of 4 hours. Lysed red cells appeared as clear foci in the agar. These areas of clearing (plaques) indicated the location of splenocytes that were producing IgM against the foreign red cell antigens. The plaques were enumerated microscopically and expressed as a ratio of number of IgM-producing splenocytes per 1,000 splenocytes as an indicator of the animal's ability to respond to foreign antigen with an antibody-mediated immune response.

9. CYTOTOXICITY ASSAY. Cytotoxic T lymphocyte activity was measured in splenocytes sensitized *in-vitro* with mitomycin c-treated P815 mastocytoma cells (American Type Culture Collection, Manassas, VA, Brunner et al. 1968). Following ACK lysis of erythrocytes, splenocyte responder cells from experimental mice were suspended at 2 x 10^7 cells/mL. Sensitizing cells were log phase P815 mastocytoma cells that had been raised in cell culture, washed, and incubated at 37°C for one hour with 100 μCi ^{51}Cr (ICN Pharmaceutical Inc., Irvine, CA) at a concentration of 5 x 10^6 cells/mL in complete medium, and then mixed with responder cells at ratios of 50:1, 25:1, and 12.5:1 to assess the level of splenocyte cytotoxic T cell activity. Chromium labeled P815 cells were also added to quadruplicate wells containing medium only to determine spontaneous release, and to quadruplicate wells containing 0.1% triton x-100 (RPI, Elk Grove Village, IL) to determine total release. The cultures were centrifuged and then incubated at 37°C and 5% CO_2 for 44 hr, after which 0.2 mL of the supernatant was

harvested using a Skatron harvester (Skatron, Sterling, VA). Determination of released label was made using a Cobra gamma counter (Packard Instruments, Chicago, IL). CTL activity was calculated from the formula:

$$\frac{\text{cpm (experimental) - cpm (spontaneous release)}}{\text{cpm (total release) - cpm (spontaneous release)}} \times 100$$

10. STATISTICAL ANALYSIS. Data were expressed as arithmetical mean ± SEM. A one-way analysis of variance was completed, and randomized complete block design was used for error control, with restricted randomization in the *in-vitro* studies. Dunnett's post-hoc t-test was used to establish significant differences in treatment groups versus controls. Results described as different in this paper indicate significantly different at $p<0.05$ (*) or $p<0.01$ (**).

3D. RESULTS

1. THYMIC AND SPLENIC CELLULARITY. No differences in thymic or splenic organ weights or cellularity were observed after a single dose of 0, 5, 50, 100, or 250 μg cUCA at 2 days post-exposure in age-matched and weight-matched C57BL/6N mice (Appendices V, VI, VII, and VIII, respectively). When intradermal cUCA was administered at 0, 50, or 100 μg for five days concurrently to mimic more prolonged human sunlight exposure, a non-significant trend only towards increased splenic cellularity was observed. The 250 μg dose was discontinued for the five-day treatment because this exposure level (16.5 mg/kg) was felt to not be physiologically relevant to prolonged human sunlight exposure, and because solubilizing cUCA in PBS at this level was difficult. There was no change in thymic weight or cellularity with five-day cUCA exposure (Appendices IX and X).

2. THYMIC SURFACE ANTIGEN EXPRESSION BY FLOW CYTOMETRY. Neither one nor five day cUCA exposure caused a decrease in absolute thymocyte numbers as had been reported with subchronic cUCA exposure (El-Ghorr and Norval, 1997). However, there were significant differences in thymocyte flow cytometric surface antigen expression following the five-day cUCA administration, suggesting different sensitivities within thymocyte subtypes ($CD4^-CD8^-$: DN; $CD4^+CD8^-$: CD4 SP; $CD4^-CD8^+$: CD8 SP; and $CD4^+CD8^+$: DP). Absolute numbers of cells in each phenotype were calculated as total thymic cellularity x percentage in the phenotype, and are shown in Table 3.1. The number of CD4 and CD8 SP thymocytes diminished 2.2 and 2.4-fold, respectively. Double negative cells were not significantly altered with a numeric

decrease of less than two-fold, and the number of DP cells significantly increased in the 100 μg cUCA group ($p<0.01$ for SP and DP at 50 and 100 μg cUCA; n=5 per treatment group; Table 3.1).

3. PROLIFERATION OF SPLENOCYTES AND THYMOCYTES. Female C57BL/6N mice that were acutely exposed to intradermal cUCA regardless of dose showed no changes in splenic or thymic lymphocyte proliferation in response to the T-cell mitogen ConA. However the five consecutive day exposure to cUCA resulted in significantly enhanced splenocyte 72 hour proliferation in the presence of ConA at 50 and 100 μg/day cUCA doses; thymocyte proliferation was not affected in any treatment group (n=5 per treatment group, $p<0.05$ for splenocyte proliferation; Fig. 3.1).

4. FUNCTIONAL ASSAYS. Ten days following single intradermal exposure of C57BL/6N mice to cUCA, there was a significant and dose-related drop in splenic macrophage phagocytosis at the 50, 100, and 250 μg doses (n=5 per treatment group, * $p<0.05$, ** $p<0.01$; Fig. 3.2). This effect persisted up to 30 days post exposure, suggesting a long-term diminishment of macrophage function following a single cUCA exposure. Five-day exposure to intradermal cUCA caused no alterations in splenic macrophage phagocytosis (Appendix XI). There were no significant differences between controls and treatment groups in other functional assays including antibody production, splenic H_2O_2 production (chemiluminescence assay), or cytotoxicity assays with five consecutive day dosage regimes (Appendices XII, XIII, and XIV, respectively).

5. THYMIC AND SPLENIC WEIGHT AND CELLULARITY IN C57BL/6N MICE AS COMPARED TO C3H-HEN MICE FOLLOWING SUBCHRONIC cUCA EXPOSURE. The literature indicated significant thymic hypocellularity in C3H-HeN mice dosed with 100 μg cUCA three times per week for four weeks (El-Ghorr et al. 1997). Thymic effects with shorter dosing duration, similar to the present study, or in other mouse strains were not noted. The present C57BL/6N mice did not show thymic involution even when high doses of cUCA were administered as a single dose or daily for five consecutive days. Since previous studies demonstrated thymic involution following subchronic exposure to UVB or cUCA (El-Ghorr et al. 1997), both C57BL/6N and C3H-HeN strains of mice were evaluated in parallel after dosing with cUCA three times per week for four weeks to determine strain differences in immunologic response to subchronic cUCA administration. This exposure to cUCA caused a dramatic decrease in thymic cellularity in both mouse strains. The C57BL/6N strain appeared to be more sensitive to cUCA, showing significant effects at both 50 and 100 μg doses (n=5 per treatment group, Fig. 3.3). In C57BL/6N mice, 50 μg cUCA resulted in thymic cellularity that was 43.7% of control, and 100 μg cUCA resulted in a drop in thymic cellularity to 40.9% of control. In C3H-HeN mice, 50 μg cUCA dose resulted in a less dramatic drop in thymic cellularity to 65.9% of control, whereas the 100 μg cUCA dose resulted in thymic cellularity drop to 57.4% of control. In addition to thymic involution with four-week cUCA exposure, a previously unreported and dose-related and significant increase in splenic cellularity and splenic leukocyte chemiluminescence was present in both mouse strains (n=5 per treatment group; Fig. 3.4 and 3.5).

3E. DISCUSSION

Unlike the localized trans isomer, cUCA is absorbed through the epidermis, circulates systemically and reaches a maximum concentration in the murine serum approximately 25 hr following a single exposure of UVB (6 μg cUCA/mL serum) or 5 hr following a second exposure (30 μg cUCA/mL serum) (Moodycliffe et al. 1993). Such levels of circulating cUCA are thought to act indirectly rather than directly to produce numerous effects on the systemic immune system via cAMP-induced increased release of soluble chemical mediators from cutaneous and immune cells; *in-vitro* studies have shown that cUCA does not directly affect the mononuclear cells of the spleen, suggesting that there are intermediary chemical mediators (such as prostanoids, histamine, etc.) mediating cUCA's systemic immunosuppressive effects (Hart et al. 1997, Bouscarel et al. 1998). These systemic immune effects include increased apoptosis of thymocytes, decreased macrophage phagocytosis, thymic atrophy and hypocellularity, and diminished allograft rejection in corneal and small bowel transplants (Gieseler et al. 1994, El-Ghorr et al. 1997, Filipec et al. 1998).

Thymic atrophy following prolonged exposure to UVB or cUCA is accompanied by an increase in lymph node weight, and may in part be related to diminished capacity for migratory activity and subsequent accumulation of lymphocytes and dendritic cells in lymph nodes (El-Ghorr et al. 1997). An additional postulated mechanism of thymic atrophy and hypocellularity is that UVB irradiation or cUCA upregulates apoptosis via stimulation of the caspase 8, 3, and 1 cascade by increasing expression of CD95 antigen (part of TNFα receptor family) (Takahashi et al. 1999). Apoptotic cell death naturally occurs in $CD4^{+}8^{+}$ DP thymocytes, as a part of selective processes for recognition of

antigen presenting molecules as well as for recognition of self-antigens (Cohen 1992, Green and Cotter 1992, Hartley et al. 1993). Thymocyte numbers in the present C57Bl/6N mice exposed to 100 μg cUCA increased 20% in the $CD4^+8^+$ population; decreased 2.2 and 2.4-fold in the $CD4^+8^-$ and $CD4^-8^+$ populations, respectively; and decreased in the $CD4^-8^-$ nearly 2-fold. It is not known if or to what extent these decreases may have been related to increased apoptotic cell death, or other mechanisms that may have been operating to cause hypocellularity. However, these data suggest that the $CD4^+8^+$ DP cells were more resistant to targeting by cUCA than were the less mature DN cells or the most mature SP cells.

UVB or cUCA may also affect the systemic immune system by suppression of macrophage phagocytosis via diminished IFNγ and increased IL-10 production and release, as was seen in mice exposed 8-9 days prior to a single UVB exposure equivalent to approximately three times the minimal erythemic dose or two topical doses of 200 μg cUCA at 4 hour intervals (Reeve et al. 1999, Dalton et al. 2000). In related experiments, single or multiple low doses of UV radiation decreased clearance of bacteria from murine lymphoid organs, an effect attributed to impaired ability of macrophages to phagocytose bacteria and to produce reactive oxygen species in their intracellular killing (Jeevan et al. 1995). Splenic macrophages isolated from mice in the present experiments showed transient enhanced chemiluminescent responses and highly persistent (to 30 days post-exposure) diminished phagocytosis following exposure to intradermal cUCA. These data support previous studies that suggest macrophage activity may be altered by UV light exposure.

Mechanisms underlying an effect of UVB on macrophage function may be multifactorial, and may involve cUCA-mediated increased gene expression and release of TNFα from mast cells, mononuclear cells, and epidermal keratinocytes, increased release of IL-10 from activated T lymphocytes, and increased release of prostaglandin E_2 (PGE_2) from keratinocytes and monocytes. The above are accompanied by subsequent increased interleukin 4 and 10 (IL-4 and IL-10) release, and decreased interferon-gamma (IFNγ) and IL-12 release (Goettsch et al. 1994, Beissert et al. 1995, Hart et al. 1997, Holan et al. 1998, Shreedhar et al. 1998). These effects inhibit antigen processing and presentation by LC (i.e., monocytic cells in the skin) apparently by decreasing expression of vimentin, the intermediate filament making up the LC dendritic processes (Bacci et al. 1996). More recently, UVB irradiation was reported to alter production of IL-12, IL-1α, and reactive oxygen species in macrophages, resulting in suppression of phagocytosis, without affecting cytokine production or action of neutrophils (Kasahara et al. 2001).

The present observation of increased splenic cellularity in two different strains of mice support El-Ghorr's report of increased secondary lymphoid organ weight (lymph node and numerical increase in splenic weight) following similar chronic cUCA exposure (El-Ghorr et al. 1997). *In-vitro* exposure to cUCA was reported to diminish splenocyte proliferative response to allogeneic cells in a mixed lymphocyte cell assay (Holan et al. 1998, Krulova et al. 1999), an effect that may be mediated by increased production of IL-10 by activated $CD4^+$ T lymphocytes (Holan et al. 1998). In contrast, a limited but significantly increased lymphoproliferative response to the mitogen ConA was observed in the present mice after *in-vivo* cUCA exposure, which was also seen following a one-week exposure to suberythemal doses of narrow band UVB irradiation in rats (Goettsch

et al. 1994). This effect is thought to be mediated by a UVB-induced release of TNFα and IL-1 from irradiated keratinocytes, which have been shown to enhance mitogen-induced proliferation of splenic T cells (Roberts and Carveth 1988). Previous reports have suggested there to be little to no effect of sunlight exposure on circulating immunoglobulins or antibody production, even following four weeks of UVB radiation exposure, and have indicated that the effects of UVB are primarily targeted to cellular immunity rather than antibody-mediated immunity (Hersey et al. 1983, Livden et al. 1987). The present negative results in the antibody PFC assay support these reports.

And finally, there have been suggestions in the literature that there may be divergent susceptibilities to the effects of cUCA immunosuppression in various mouse strains. Norval et al. demonstrated that in order to diminish CH in most strains of laboratory mice, up to 200 μg epicutaneously administered cUCA was required. However, in the C3H-HeN strain, as little as 1 μg cUCA per mouse results in dramatic depression of CH, suggesting that C3H-HeN is an immunosensitive strain to the effects of cUCA (Norval et al. 1989). El-Ghorr supported this idea by demonstrating dramatic thymic atrophy following a four-week cUCA exposure in this strain. The present results verify that the C3H-HeN strain demonstrates T cell-mediated immunosuppression following cUCA exposure, and suggest that the C57/BL/6N strain may be even more sensitive to cUCA's immunomodulatory effects. Ultraviolet-B irradiation immunosensitivity is thought to be a dominantly inherited trait, since F1 crosses of immunosensitive and immunoresistant strains yield immunosensitive progeny (Streilein 1993). The immunogenetic basis of UVB immunosensitivity seen in C3H-HeN and C57BL/6N strains is within the locus that encodes responsiveness to bacterial

lipopolysaccharide. Mice with one or more of this LPS^n allele respond to LPS by massive upregulation of TNFα by macrophages, which is thought to be a contributing factor to the poor CH response following UVB exposure (Streilein 1993). However, a mutation at this locus results in an immunoresistant strain, as is seen in C3H-HeJ mice. However, even mice with the LPS^n allele may be UVB-resistant if they possess the D end of H-2 (murine major histocompatibility complex), which suggests that there is another locus that is involved in conferring UVB-resistance. Finer mapping of the H-2 chromosome reveals that only mice homozygous for $TNF\alpha^d$ are UVB-resistant, whereas all other combinations of TNFα alleles are UVB-sensitive (Streilein 1993). In support of the hypothesis that TNFα is integrally involved in mediation of CH suppression, nanogram intradermal injections of TNFα are non-inflammatory, yet alter epidermal LC cytoskeleton and reduce their mobility (Streilein 1993). Further characterization of the molecular mechanisms will be necessary to better define cUCA's effects on the cutaneous and systemic immune system.

In summary, with the exception of a decrease in splenic macrophage phagocytosis, a single exposure to intradermal cUCA did not produce detectable changes in the selected immune parameters or immune function assays. Thymic atrophy could not be produced after single dose cUCA or after daily dosing for 5 consecutive days, but was dramatic after four weeks dosing. This effect would not appear to be corticosteroid (stress) -mediated, in that the most sensitive $CD4^+8^+$ thymocytes were least affected by cUCA. Thymic atrophy was also more severe in C57Bl/6N mice than in C3H-HeN mice, suggesting underlying differences in sensitivity of these mouse strains to UVB irradiation. Finally, mice dosed daily for five days with cUCA did not show altered T

cell cytolytic ability or depressed antibody production, suggesting these mice are likely to respond at a control level to immunologic challenge.

3F. ACKNOWLEDGMENTS. Supported by NIH RO1 ES09642-02

3G. REFERENCES

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Table 3.1. Effect of 5-day cUCA on Thymocyte Differentiation

cUCA (μg)	CD4-CD8- (x 10^6)	$CD4^+$ (x 10^6)	$CD8^+$ (x 10^6)	CD4+CD8+ (x 10^6)
0	**1.46 ± 0.44 (1.78%)**	**15.09 ± 0.97 (18.36%)**	**6.33 ± 0.52 (7.75%)**	**58.78 ± 1.49 (71.98%)**
50	**2.68 ± 0.43 (3.56%)**	**8.40 ± 0.89 ** (11.15%)**	**3.41 ± 0.48 ** 4.53%**	**60.80 ± 1.37 ** (80.75%)**
100	**0.76 ± 0.46 (0.91%)**	**6.92 ± 1.00 ** (8.24%)**	**2.61 ± 0.54 ** (3.11%)**	**73.66 ± 1.54 ** (87.74%)**

Table 3.1. Thymocyte surface antigen expression response to five-day exposure to cUCA in female C57BL/6N mice. Cis-urocanic acid was administered intradermally at 0, 50, or 100 μg daily for five consecutive days. The relative percentages of thymocyte subpopulations are expressed in parentheses, and the absolute numbers are above; the absolute numbers of double negative and both $CD4^+$ and $CD8^+$ SP thymocytes diminished in a dose-related fashion, and $CD4^+$ $CD8^+$ numbers increased in response to cUCA exposure 48 hours following the final cUCA dose. Each measurement is the mean ± SEM of absolute cell numbers of each phenotype (n=5 per treatment group; *p<0.05, **p<0.01).

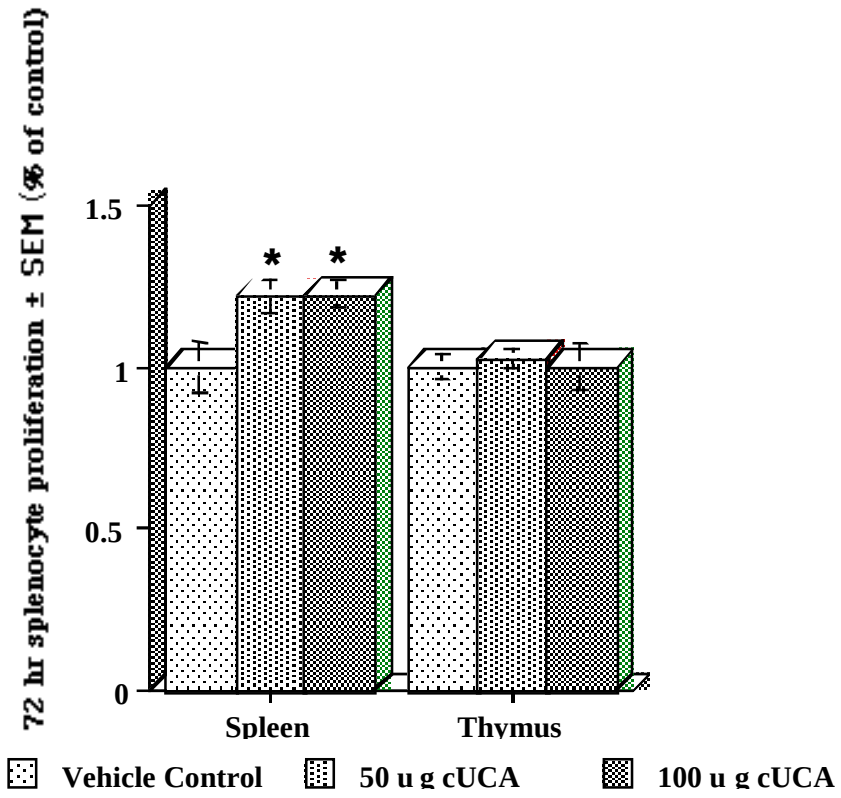

Figure 3.1. Spontaneous proliferation response of splenic and thymic lymphocytes to five-day cUCA exposure in female C57BL/6N mice. cUCA was administered intradermally at 0, 50, or 100 μg. Each measurement is the mean ± SEM (n=5 per treatment group; *$p<0.05$).

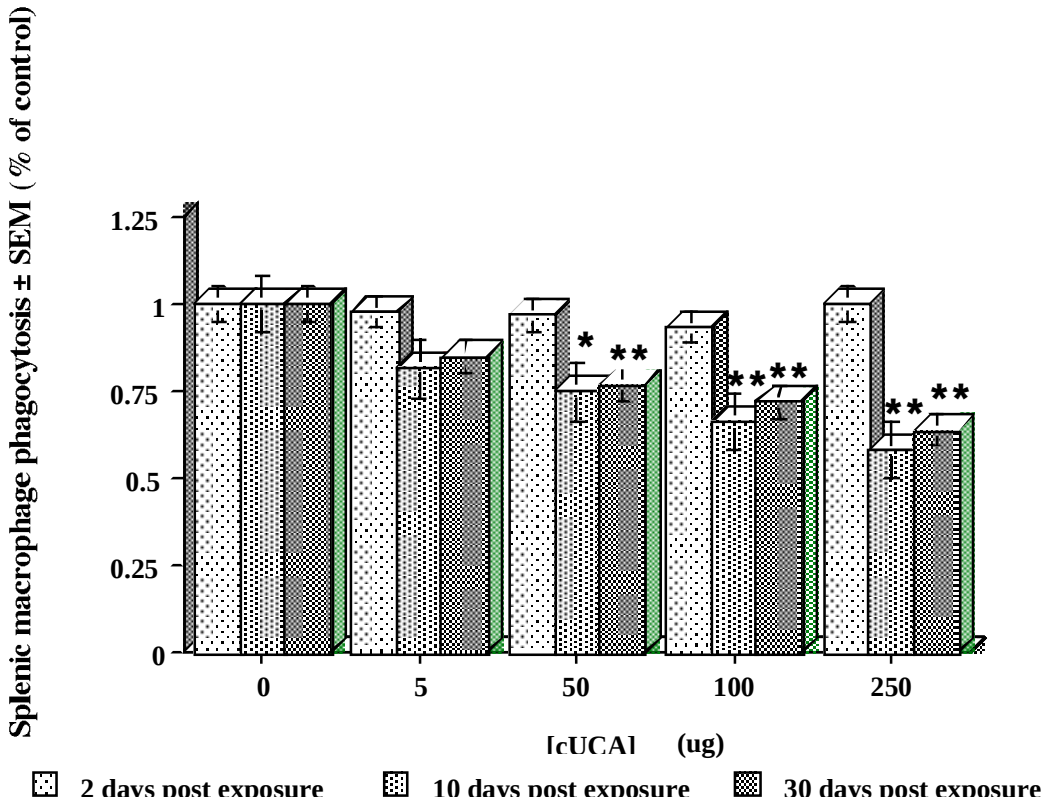

Figure 3.2. Splenic macrophage phagocytic response to single exposure cUCA in female C57BL/6N mice. cUCA was administered intradermally at 0-250 μg. Each measurement is the mean ± SEM (n=5 per treatment group; *p<0.05, **p<0.01).

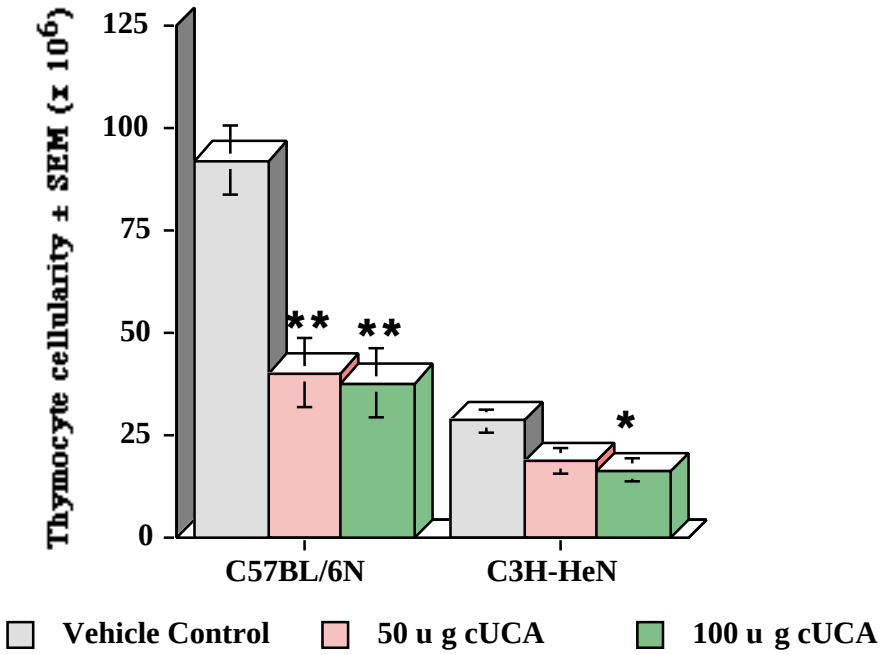

Figure 3.3. Thymic cellularity response to four-week exposure to cUCA in female C57BL/6N and C3H-HeN mice. cUCA was administered intradermally at 0, 50, or 100 μg three times per week for four weeks. Each measurement is the mean ± SEM (n=5 per treatment group; *p<0.05, **p<0.01).

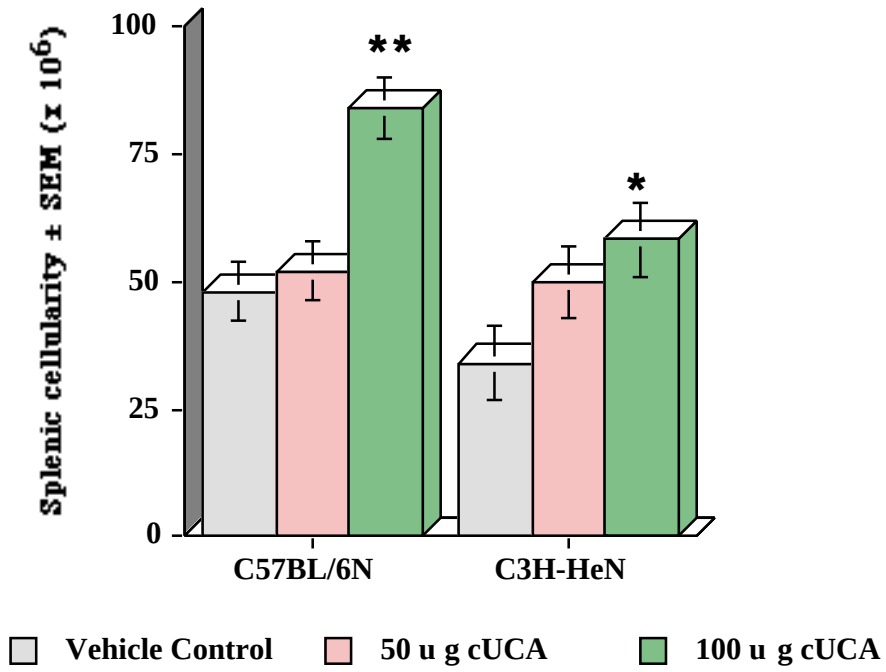

Figure 3.4. Splenic cellularity response to four-week exposure to cUCA in female C57BL/6N and C3H-HeN mice. cUCA was administered intradermally at 0, 50, or 100 µg thrice per week for four weeks. A dose-related increase in splenic cellularity of 74% for C57BL/6N mice and 71% for the C3H-HeN strain was present at the 100 µg dose 48 hours following the final cUCA dose. Each measurement is the mean ± SEM (n=5 per treatment group; *$p<0.05$, **$p<0.01$).

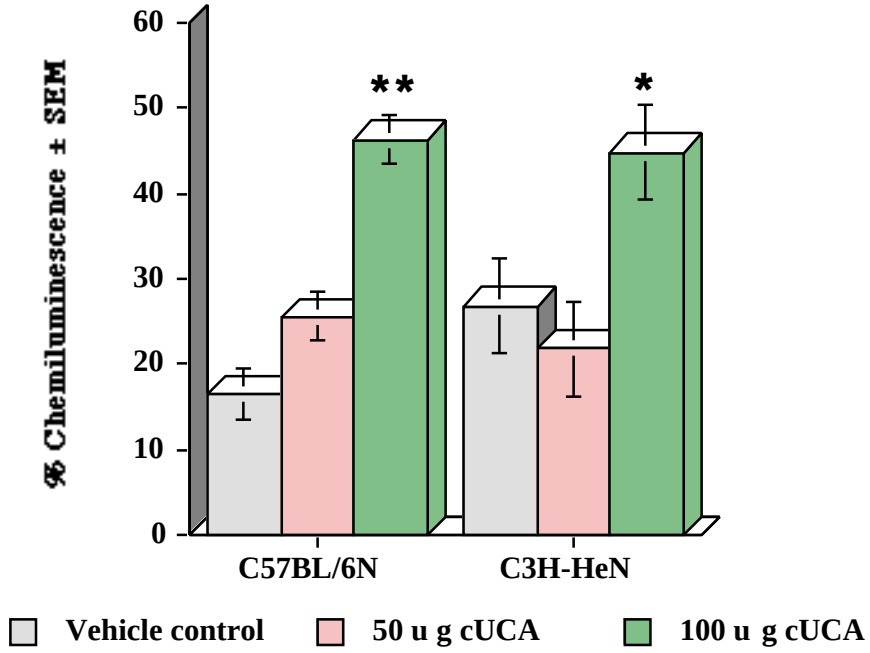

Figure 3.5. Splenic leukocyte production of active oxygen species (hydrogen peroxide, chemiluminescence response) to four-week exposure to cUCA in female C57BL/6N and C3H-HeN mice. cUCA was administered intradermally at 0, 50, or 100 μg three times per week for four weeks. A significant increase in splenic leukocyte production of hydrogen peroxide was present at the 100 μg dose level in both mouse strains 48 hours following the final cUCA dose. Each measurement is the mean ± SEM (n=5 per treatment group; *$p<0.05$, **$p<0.01$).

CHAPTER 4: CIS-UROCANIC ACID INCREASES BOTH IMMUNOTOXICITY AND LETHALITY OF DERMALLY-ADMINISTERED PERMETHRIN IN C57BL/6N MICE

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4A. ABSTRACT

Immunomodulatory effects of a single topical permethrin exposure, five-day exposure to cis-urocanic acid (cUCA) or a combination of the two chemicals were evaluated in four-to-five-week-old female C57Bl/6N mice. Permethrin alone decreased thymic weight and cellularity. Although cUCA alone did not affect thymic end points, co-exposure to topical permethrin and cUCA exacerbated the thymolytic effects of permethrin. The single topical dose of permethrin also depressed several immune responses in leukocytes isolated from the spleen. This included splenic T cell proliferative response to mitogen, splenic macrophage hydrogen peroxide production, and splenic B lymphocyte specific antibody production against sRBC. Unlike the effect of co-exposure to these agents on thymic endpoints, cUCA did not exacerbate permethrin's adverse effect on any of the splenic endpoints examined. These results appear to suggest divergent mechanisms by which these compounds affect precursor and functionally mature T cells. Topical permethrin did not cause signs of overt toxicity in experimental mice, with the exception of occasional mice that displayed neurotoxic effects that were ultimately lethal if mice were not euthanized. For undetermined reasons, cUCA significantly increased the rate of lethality caused by permethrin. These results suggest that sunlight, via cUCA, may increase the risk of adverse CNS and immune effects caused by permethrin alone.

4B. INTRODUCTION

Sunlight exposure has been shown to inhibit cutaneous and systemic immune responses and cause increased risk of infectious and neoplastic disease. This effect is thought to be mediated by an isomerization of the endogenous cutaneous chromophore, urocanic acid (Baadsgaard et al. 1990, Giesele et al. 1994, Jeevan et al. 1995, Bacci et al. 1996, El-Ghorr and Norval 1997, and Hart et al. 1997). Likewise, prolonged or acute exposure of mice to permethrin reduces thymic weight and cellularity, and inhibits the functional competency of immune cells *in-vivo* and *in-vitro* (Blaylock et al. 1995, Diel et al. 1998, Punareewattana et al. 2000, 2001). Subacute topical exposure to permethrin in mice was recently also reported to cause persistent inhibition of splenic macrophage phagocytic activity and chemiluminescent responses (Punareewattana et al. 2001). Similar subacute intradermal exposure of mice to the cis isomer of urocanic acid (cUCA) reduced thymic weight and cellularity (El-Ghorr and Norval 1997), and a single exposure to cUCA inhibited splenic macrophage phagocytosis (Prater et al. unpublished data). Preliminary studies in our laboratory further demonstrate that even a single topical application of permethrin in mice results in decreased thymic and splenic weight and cellularity, and decreased antibody production by splenic B cells (Prater et al., in review).

It is recognized that human beings are exposed simultaneously to many chemicals in their work and home environments (Feron et al. 1998, Haddad and Krishman 1998, Groten 2000, Wilkinson et al. 2000, Conolly 2001, Groten et al. 2001). Little is known however, about the toxic interactions of multiple exposures on human health (Seed et al. 1995). The Food Quality Protection Act of 1996 provided for evaluation of the cumulative effects of pesticides and other substances to establish whether there are

common (i.e., synergistic or antagonistic) or divergent (additive) mechanisms of toxicity (Wilkerson et al. 2000, Feron et al. 1998) by quantifying a hazard index, margin of exposure, and cumulative risk index based on *in-vivo* toxicologic endpoints. Most such assessments that employ *in-vivo* exposure in animal models have attempted to evaluate interactions of multiple toxicants by using a top-down approach (whole-mixture analysis) or a bottom-up approach (component-interaction analysis) (Yang et al. 1995a). With either approach, a limitation of these studies has often been that doses of one or more of the studied chemicals were by routes of exposure that were often divergent from human exposure (Haddad and Krishnan 1998).

A realistic example of concerns centering around the effects of chemical mixtures was recently experienced in human subjects, following reports of a variety of neurologic, gastrointestinal, dermal, and musculoskeletal somatic signs in soldiers returning from the Persian Gulf War. In response to these complaints, there was considerable effort placed upon examination of the potential toxic effects of military-related pesticide mixtures on human health (Abou-Donia et al. 2001). Conflicting reports arose, without firm consensus as to whether concurrent exposure to permethrin, DEET, pyridostigmine, and other pesticides may result in additive, more than additive, or less than additive effects (Buchholz et al. 1997, Abou-Donia et al. 1996, 2001, Abu-Qare et al. 2001, Ortiz et al. 1995, McCain et al. 1997, Hoy et al. 2000a and b). McCain et al. (1997) reported a synergistic, not additive, increased lethality of oral permethrin in rats when administered concurrently with pyridostigmine and DEET. These authors also reported increased lethality of permethrin in corn oil or propylene glycol vehicle, as compared to pure technical grade permethrin (LD_{50} 380 mg/kg vs. 4 g/kg, respectively). Potential

mechanisms include increased absorption or inhibition of liver and plasma esterase detoxification enzymes (Casida et al. 1983, Abou-Donia et al. 1996). Interestingly, other authors reported increase in permethrin's lethality with concurrent exposure to parathion, but xylene vehicle had no influence on increased lethality, suggesting that enhanced absorption may not contribute significantly to increased permethrin toxicity with concurrent chemical exposure (Ortiz et al. 1995). Other researchers examined the combined effects of permethrin, pyridostigmine, and DEET in combination, all at physiologically relevant doses, with similar (i.e., greater than additive) neurotoxic effects in rats (Abou-Donia et al. 2001). And further, when dosed alone in rats, these physiologically relevant doses produced no effect, whereas these chemicals administered in combination caused significant alterations in neurotoxicity (Hoy et al. 2000a and b). Clearly, further investigations are needed to clarify the toxic effects of chemical mixtures.

The similar spectrum of immunologic effects described in experimental animals subsequent to UV light or topical permethrin exposure has raised questions regarding increased risk of harmful immune system effects with combined exposure to these agents. Evaluating such effects is important, since humans using topical insecticides would be expected to often be co-exposed in outdoor situations to UV light. Such insecticide and sunlight co-exposure may be particularly important in children who have high surface area to body weight ratios (as compared to adults), and who receive relatively high doses of topical permethrin from permethrin-based shampoos to kill head lice or permethrin-based creams to kill scabies mites (Punareewattana et al. 2000). The present report therefore evaluated established systemic immune targets of permethrin and cUCA in co-exposed mice.

4C. MATERIALS AND METHODS

1. MICE. Five-week-old female C57Bl/6N mice were purchased from Charles River Laboratories (Portage, MI). Mice were acclimated one week and maintained under controlled conditions of temperature (22±1˚C), humidity (40-60%), and light (12/12-hour light/dark cycle). Food and water were provided *ad libitum*. Mice in all experiments were humanely treated, in accordance with the guidelines of the Virginia Tech Institutional Animal Care and Use Committee (VT IACUC). All procedures were reviewed and approved by the VT IACUC prior to the experiments.

2. PERMETHRIN AND CIS-UROCANIC ACID PREPARATION AND TREATMENT PROTOCOLS. Permethrin was provided by the US Army Center for Health Promotion and Preventive Medicine (Aberdeen Proving Ground, MD) from stock purchased immediately before by the Army from Coulston Industries (Coulston Products, Easton, PA). The permethrin was a 91.6% pure mixture of 57.7% trans and 42.3% cis permethrin, respectively. Mice were exposed to a single dose of 25 μL permethrin on the shaved interscapular skin, to mimic the most common route of human exposure (Snodgrass 1992). Control groups received identical volumes of vegetable oil (vehicle) in the interscapular space, 48 hours prior to sacrifice. Mice were sacrificed by cervical dislocation 48 hours following dosing.

Cis-urocanic acid was prepared from tUCA (Sigma, St. Louis, MO) and purified as previously described (Takahashi et al. 1997), and then evaluated for purity by mass spectroscopy prior to use. Treatment with cUCA consisted of five consecutive daily intradermal doses of either 100 μL PBS or 100 μg cUCA in PBS (equivalent to

approximately 6.6 mg/kg) in mice arbitrarily assigned to treatment or control groups. This exposure to cUCA was previously determined to be a suberythremic exposure (Prater et al., unpublished data).

3. ORGAN WEIGHTS, CELL PREPARATION, AND CELLULARITY. The thymus and spleen from each mouse were collected by dissection and placed, individually, into pre-weighed 60 x15 mm culture dishes (Fisher Scientific, Norcross, GA). Wet organ weights were immediately obtained using an Ohaus analytical scale (TS 120S, Florham Park, NJ). Eight mL cold incomplete culture medium (RPMI-1640, Fisher) were added to petri dishes with organs, after which thymocytes and splenic cells were gently dissociated into the culture medium using a 60 μm wire sieve screen (Sigma) and curved forceps. Suspended cells were washed twice in incomplete culture medium, resuspended in 2 mL culture medium, and counted using a Scharfe CASY-1 electronic cell counter (Scharfe System, GmbH, Germany). The accuracy of the cell counter was verified in each experiment by counting 2-3 samples on a hemocytometer using Natt Herrick's vital stain.

4. PROLIFERATION OF SPLENOCYTES AND THYMOCYTES. Splenocytes and thymocytes from mice treated with topical permethrin, intradermal cUCA, or both of these agents, were aseptically isolated and dissociated 48 hours after treatment, as described above. Following resuspension in complete medium, 100 μL aliquots (5×10^5 cells/100 μL) were added to triplicate wells containing the T cell mitogen concanavalin A (ConA) at an optimal concentration of 1.0 μg/well. Blastogenesis of splenocytes and

thymocytes was quantified in the presence and absence of ConA using the nonradioactive colorimetric/fluorometric assay originally described by Ahmed et al. (1994) and Gogal et al. (1997). Briefly, after 24 hours of culture for splenocytes or 48 hours of culture for thymocytes at 37˚C and 5% CO_2, 20 µL of Alamar blue dye (Accumed International, Inc., Westlake, OH) were added to each well of the plate and plates were returned to the incubator. The dye, when added, is in an oxidized (blue color) form that is reduced (red color) as the cells proliferate. After 72 hours of culture, the absorbance at 570 nm and 600 nm was measured with a kinetic microplate reader (Molecular Devices, Menlo Park, CA). The 570 nm absorbance measures the reduced form and 600 nm measures the oxidized form. Because there is some degree of overlap between the two absorbances, it is necessary to subtract the 600 nm absorbance from the 570 nm absorbance to obtain the specific absorbance, which reflects the specific level of proliferation. The specific absorbance of unstimulated cells (in media alone) was compared to the specific absorbance of cells incubated with the mitogens, and was expressed as percent of PBS-treated controls.

5. SPLENIC MACROPHAGE PHAGOCYTOSIS. Splenic macrophages were dissociated and suspended as above in culture medium. Erythrocytes were removed from splenocyte cell suspensions in ACK lysing solution (0.015 M NH_4Cl, 1.0 mM $NaHCO_3$, 0.1 mM EDTA) for five minutes at room temperature. Cells were washed twice in culture medium, resuspended in 2 mL standard buffer (Hank's balanced salt solution, HBSS, Fisher), and counted electronically as described above. The phagocytic capacity of splenic macrophages was determined by a modification of the method of Dunn and

Tylor (1981). Briefly, 10 µL of PBS-washed and disaggregated (Ultrasonic Cell Disrupter, 30 sec at 35%, Misonix, Inc., Farmingdale, NY) Fluoresbrite microspheres (1.16 µm; Polysciences, Inc., Warrington, PA) were added to 5 x 10^5 splenic cells/well to result in approximately 50 microspheres/cell per well. Following coincubation of microspheres and splenic macrophage suspensions at 37˚ C and 5% CO_2 for 18 hours, cells were washed twice and resuspended in PBS prior to flow cytometric analysis. For each sample, 5,000 events were collected and the number of cells ingesting fluorescent particles was expressed as a percentage of 5,000, as previously described (Hart et al. 1997).

6. CHEMILUMINESCENCE RESPONSE IN SPLENIC MACROPHAGES. The production of H_2O_2 in phorbol-12-myristate 13-acetate (PMA) – stimulated splenic macrophages was determined by the method of Bass et al. (1983). Briefly, splenic cell suspensions that were prepared as above and diluted to 5 x 10^5 cells in complete culture medium were incubated with 5 µL of 5 mM dichlorofluorescein-diacetate (DCF-DA; Molecular Probes, Eugene, OR) for 15 minutes at 37˚C, 5% CO_2. Following incubation with DCF-DA, cells were stimulated by the addition of 10 µL of 100 ng/mL PMA (Sigma) in a subsequent 30-minute incubation period at 37˚C. 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.

7. B LYMPHOCYTE ANTIBODY PRODUCTION: PLAQUE-FORMING CELL ASSAY. The plaque forming cell assay (PFC) was used to quantify the ability of

splenic lymphocytes to mount an antibody-mediated immune response to the T-dependent antigen, sheep red blood cells (sRBC) (Roitt and Delves 1992). Four days prior to sacrifice, mice received by intraperitoneal injection 0.5 mL 10% sRBC in PBS. Four days after sRBC administration, mice were euthanized and spleens were isolated and dissociated as described above. After two washings, splenocytes were counted and suspended to a concentration of 2 x 10^7 cells/mL in RPMI. Agar was prepared from 500 mg agar dissolved in 110 mL HBSS, with pH adjusted to 7.3, and 1.6 mL DEAE-dextran solution obtained from a stock of 150 mg DEAE-dextran dissolved in 5 mL saline. Agar was kept in a 47°C water bath, and 0.8 mL was used per sample, into which the following was added: 30 µL 30% sRBC, 100 µL splenic cell suspension, and 20 µL guinea pig complement (Sigma). This solution was mixed and poured into a 35 x 10 mm petri dish top, and immediately the lower part of the same dish was placed on top of the agar to ensure even and thin spreading of the agar. Samples were performed in duplicate, and the solidified plates were incubated (37°C, 5% CO_2) for a minimum of 4 hours. Lysed red cells appeared as foci of clearing in the agar. These areas of clearing (plaques) indicated the location of splenocytes that were producing IgM against the foreign red cell antigens. The plaques were enumerated microscopically with a low magnification light microscope and expressed as a ratio of number of IgM-producing splenocytes per 1,000 splenocytes as an indicator of the animal's ability to respond to foreign antigen with an antibody-mediated immune response.

8. STATISTICAL ANALYSIS. Data were expressed as arithmetic mean ± SEM. A two-way analysis of variance was completed to determine interactive effects of

permethrin and cUCA, with a simple, arbitrary sampling structure. A randomized complete block design was used for error control. Dunnett's post-hoc t-test was used to establish significant differences in treatment groups versus controls. Results described as different in this paper indicate significantly different at $p < 0.05$.

4D. RESULTS

1. THYMIC AND SPLENIC WEIGHT AND CELLULARITY. Five consecutive days of exposure to 100 μg ID cUCA resulted in no effect on thymic weight or cellularity (Fig. 4.1 a, b). A single administration of 25 μL topical permethrin resulted in decreased thymic weight. Mice treated with both cUCA and permethrin displayed a significant drop in thymic weight and cellularity, as compared to either chemical alone. Splenic weight and cellularity were not affected by permethrin, cUCA or a combination of these treatments (Fig. 4.2 a, b).

2. SPLENOCYTE AND THYMOCYTE PROLIFERATION. In repeated experiments, cUCA caused a modest numeric increase in splenocyte T cell proliferation that approached but never attained significance, whereas permethrin or cUCA + permethrin caused a dramatic and approximately equal drop in 72 hr splenocyte blastogenesis (Fig. 4.3 a). Thymocyte proliferation was not affected by permethrin, cUCA, or a combination of these treatments (Fig. 4.3 b).

3. SPLENOCYTE FUNCTIONAL ASSAYS

a. SPLENIC MACROPHAGE PHAGOCYTOSIS. There was no change in splenic macrophage phagocytic ability in mice exposed to cUCA, permethrin, or cUCA + permethrin (Appendix XV).

b. CHEMILUMINESCENCE RESPONSE IN SPLENIC MACROPHAGES**.** Administration of ID cUCA for five consecutive days produced no chane in splenocyte

ability to produce H_2O_2 (chemiluminescence assay). A single exposure to permethrin or permethrin + cUCA resulted in marginal but significant diminished splenocyte chemiluminescence (Fig. 4.4).

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c. B LYMPHOCYTE ANTIBODY PRODUCTION: PLAQUE-FORMING CELL ASSAY. Exposure to ID cUCA for five consecutive days did not alter the number of antibody-producing splenic B cells (PFC assay). A single exposure to topical permethrin, or permethrin + cUCA resulted in profound and approximately equal diminishment in the ability of splenocytes to mount an IgM response against xenogeneic (sheep) red cell surface antigens (Fig. 4.5).

4. ACUTE TOXICITY IN MICE EXPOSED TO PERMETHRIN AND cUCA. No signs of overt toxicity were observed in any experimental mice dosed with 100 μg cUCA. Mice dosed with permethrin also typically showed no adverse effects from the topical chemical exposure. For unknown reasons, in some but not all experiments, a permethrin-exposed mouse would show signs of central nervous system toxicity, e.g., tremors, ataxia. The first few such mice observed did not recover but died within hours of the development of ataxia. Therefore, in subsequent experiments any mouse showing signs of CNS toxicity was immediately removed and euthanized. Lowering the dose of topical permethrin from 25 μL to 15 μL did not greatly change the outcome of having an occasional ataxic mouse. An unexpected observation from these experiments was that co-exposure to cUCA caused a significant increase in the number of permethrin-treated mice that displayed CNS effects (Fig. 4.6). These data have been substantiated in a

separate laboratory that is conducting similar studies in mice (Blaylock, personal communication).

4E. DISCUSSION

Sunlight exposure has been shown to inhibit cutaneous and systemic immunity, resulting in increased risk of infectious and neoplastic disease. This effect is thought to be mediated by an isomerization of the endogenous cutaneous chromophore, urocanic acid (Baadsgaard et al. 1990, Giesele et al. 1994, Jeevan et al. 1995, Bacci et al. 1996, El-Ghorr and Norval 1997, Hart et al. 1997). Likewise, prolonged or acute exposure to permethrin depressed the functional competency of immune cells *in-vivo* and *in-vitro* (Blaylock et al. 1995, Diel et al. 1998, Punareewattana et al. 2000, 2001, Prater et al., in review). These data raised questions about adverse immune effects that may be associated with co-exposure to sunlight and permethrin, as may occur in individuals using topical insecticide and working outdoors.

Subacute intradermal dosing with cUCA, e.g., three days per week for four consecutive weeks, caused significant reduction in thymic weight in C3H-HeN (El-Ghorr and Norval 1997) and C57Bl/6N mice (Prater et al., in review). However, no reduction in thymic weight or cellularity was observed in the present mice dosed with cUCA for one week. The single topical exposure to permethrin caused a limited but significant depression of thymic weight, paralleled by a near significant (p=0.09) decrease in thymic cellularity. These data support previous observations in which subacute topical permethrin caused a severe reduction in thymic organ weight and cellularity (Punareewattana et al. 2000). Although cUCA alone did not affect thymic weight or cellularity in the present mice, co-exposure to topical permethrin and cUCA appeared to significantly exacerbate the thymolytic effects of permethrin alone.

A single topical exposure to permethrin also depressed several immune responses in leukocytes isolated from the spleen. This included a decrease in the proliferative response of splenic T cells to mitogen, a decrease in the ability of spleen macrophages to produce hydrogen peroxide, and a decreased ability of splenic B lymphocytes to produce specific antibody against sRBC. In each case, these observations agree with previous reports in which subacute topical permethrin diminished the same immune responses in the mouse spleen, an effect likely mediated by alterations in T cell-mediated immunity and antigen presentation (Punareewattana et al. 2001). Unlike the effect of co-exposure to these agents on thymic endpoints, cUCA did not exacerbate the adverse effect of permethrin on any splenic endpoint examined. These results may suggest different mechanisms by which these compounds affect precursor T cells (thymocytes) in the thymus, as compared to functionally mature T cells or other immune cell targets present in the spleen. The pyrethroid deltamethrin is structurally similar to permethrin and was reported to cause increased thymocyte apoptosis (Enan et al. 1996). It is not known if enhanced apoptosis may contribute to the thymic hypocellularity induced by permethrin, or if so, how co-exposure to cUCA may alter this effect. Further, limited information is available regarding mechanisms by which prolonged exposure to cUCA causes thymic atrophy. These areas represent gaps in the current database that need to be investigated to better understand immunotoxic risk that may be associated with pyrethroid insecticide and sunlight co-exposure.

The effects of cUCA and permethrin on cutaneous immunity have been investigated, and suggest that this arm of the immune system may be particularly sensitive to both of these agents. In particular, contact hypersensitivity has been shown to

suffer severe and persistent inhibition with exposure to cUCA (Hart et al. 1997) or permethrin (Punareewattana et al. 2000). It remains largely unknown if such inhibition is the result of altered immune cell trafficking, impaired antigen presentation, or other mechanisms. The present studies did not evaluate skin immune responses including contact hypersensitivity in mice co-exposed to cUCA and permethrin, however these recent studies suggest that such may be warranted. There is also growing evidence that pyrethroid exposure may be related to allergic-like phenomena, including asthma and possibly anaphylaxis (Flannigan et al. 1985, Fuortes 1999). Recent reports further suggest that UV light may result in cutaneous immune aberration by shifting the Th1/Th2 balance (El-Ghorr et al. 1995). These reports suggest future studies that examine hyper-immune endpoints (allergy; autoimmunity) may also be warranted.

Finally, permethrin is a well-characterized neurotoxicant that has been widely used because of its combination of selective efficacy as an insecticide and minimal described adverse effects in mammals. The observation that permethrin caused neurotoxicity in some of the present mice is not novel; however the finding that a chromophore induced by UV light (cUCA) increased the lethality of permethrin, apparently by increasing its neurotoxic effects, is new and potentially important information. These data are clearly preliminary but may suggest that humans, especially children, treated with relatively high doses of topical permethrin for mites or lice, should avoid excessive sunlight exposure during the immediate post-treatment period.

4F. ACKNOWLEDGEMENT

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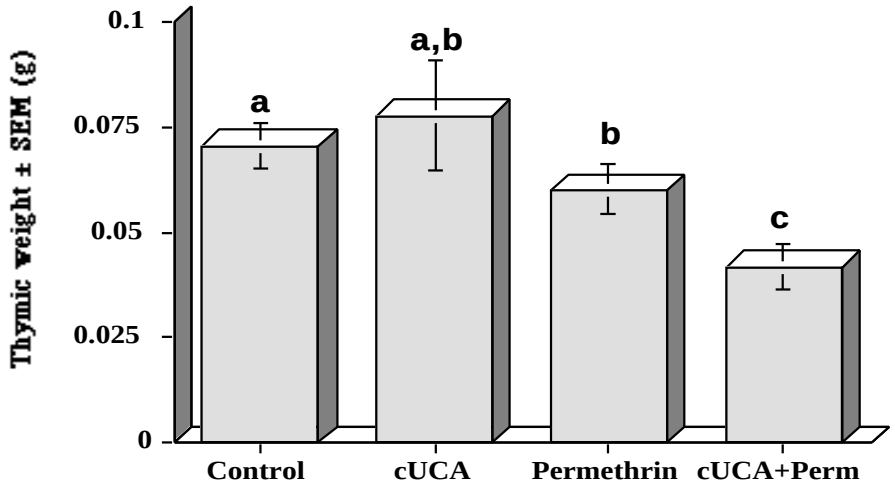

Figure 4.1a. Effect of cUCA and permethrin on thymic weight in female C57BL/6N mice. Results represent a dose-related decrease in thymic weight at 48 hr following permethrin (p=0.012) or permethrin + cUCA exposure (p=0.005). Each measurement is the mean ± SEM (n=5 per treatment group). Two-way ANOVA demonstrates an interactive effect of the two chemicals (p=0.006).

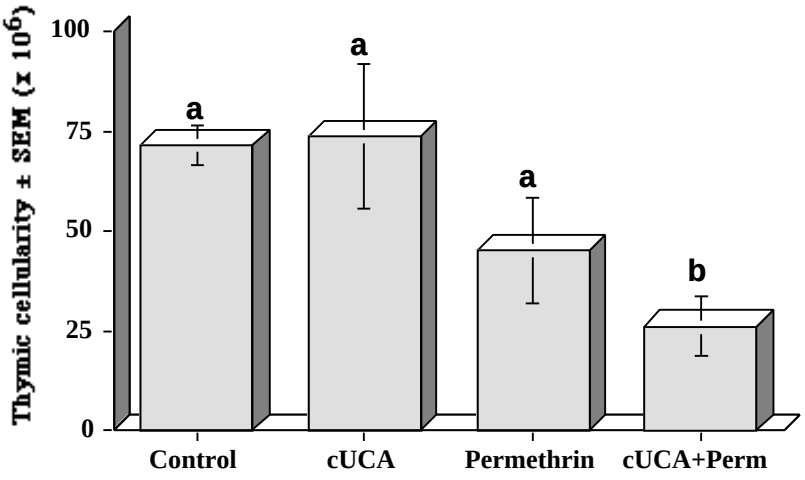

Figure 4.1b. Effect of cUCA and permethrin on thymic cellularity in female C57BL/6N mice. Results represent a dose-related and interactive chemical effect on decreasing thymic cellularity with combined exposure to permethrin and cUCA (p=0.013). Each measurement is the mean ± SEM (n=5 per treatment group).

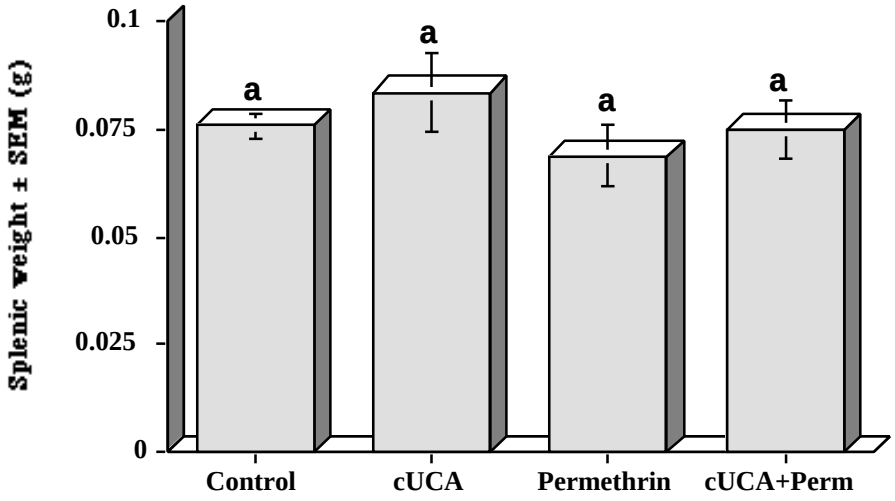

Figure 4.2a. Effect of cUCA and permethrin on splenic weight. Results represent no alteration in splenic weight following exposure to cUCA or permethrin alone or in combination. Each measurement is the mean ± SEM (n=5 per treatment group).

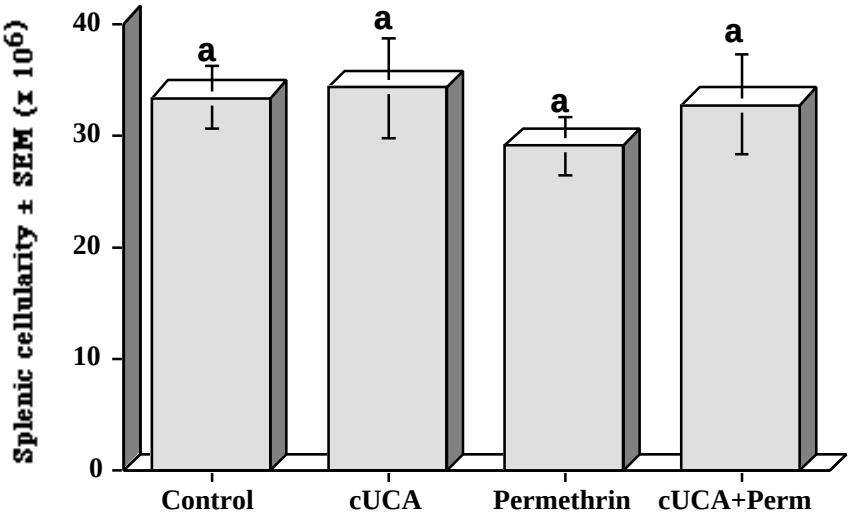

Figure 4.2b. Effect of cUCA and permethrin on splenic cellularity. Results represent no alteration in splenic cellularity following exposure to cUCA or permethrin alone or in combination. Each measurement is the mean ± SEM (n=5 per treatment group).

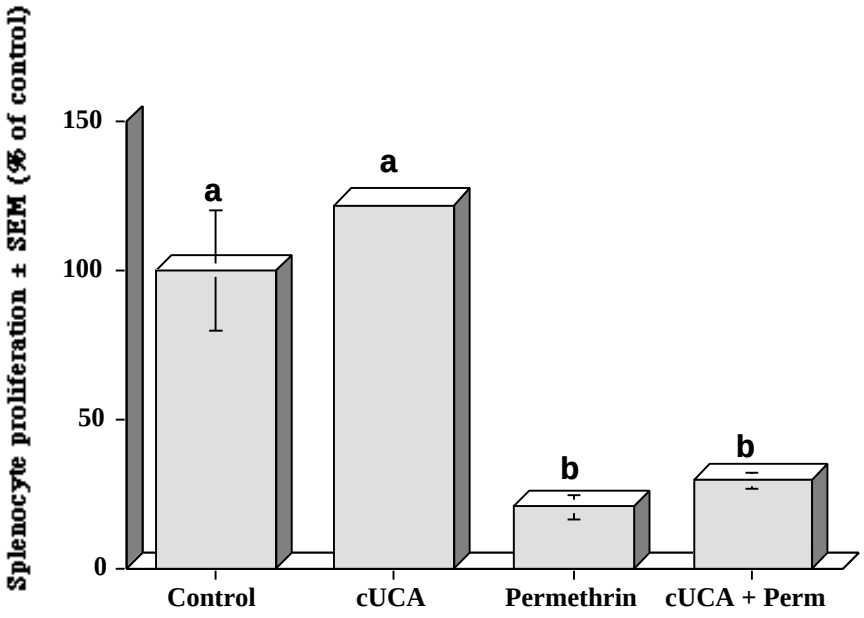

Figure 4.3a. Effect of cUCA and permethrin on splenocyte proliferation in female C57BL/6N mice. Results represent markedly diminished splenocyte proliferation following exposure to permethrin alone (p=0.001) or in combination with cUCA (p=0.001). Each measurement is the mean ± SEM (n=5 per treatment group).

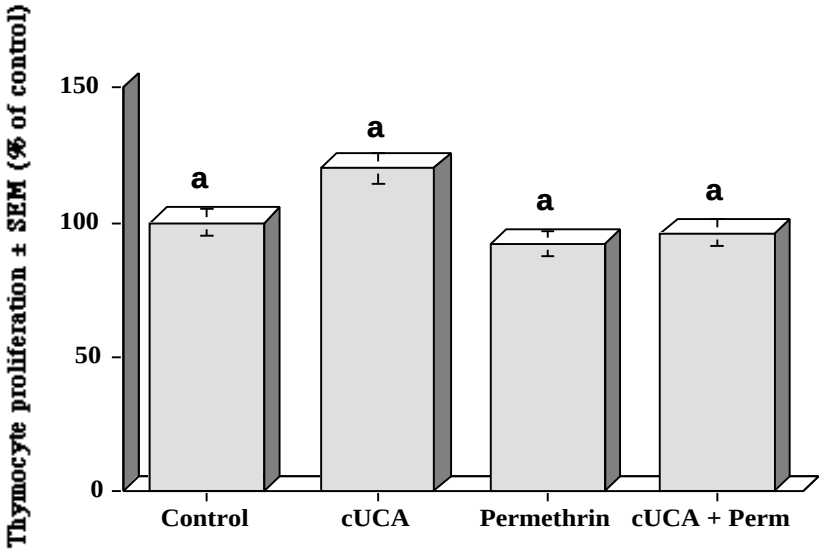

Figure 4.3b. Effect of cUCA and permethrin on thymocyte proliferation in female C57BL/6N mice. Results represent no effect of permethrin or cUCA either alone or in combination. Each measurement is the mean ± SEM (n=5 per treatment group).

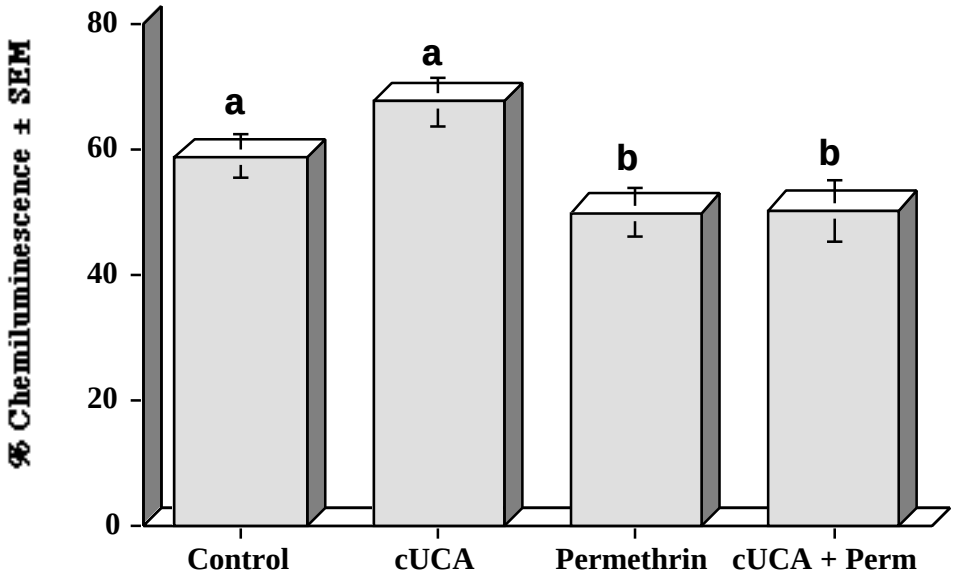

Figure 4.4. Effect of cUCA and permethrin on splenic macrophage production of active oxygen species (hydrogen peroxide, chemiluminescence assay) in female C57BL/6N mice. Results represent decreased ability of splenic macrophage H_2O_2 production following single exposure to permethrin (p=0.005), or permethrin + cUCA in combination (p=0.04). Each measurement is the mean ± SEM (n=5 per treatment group).

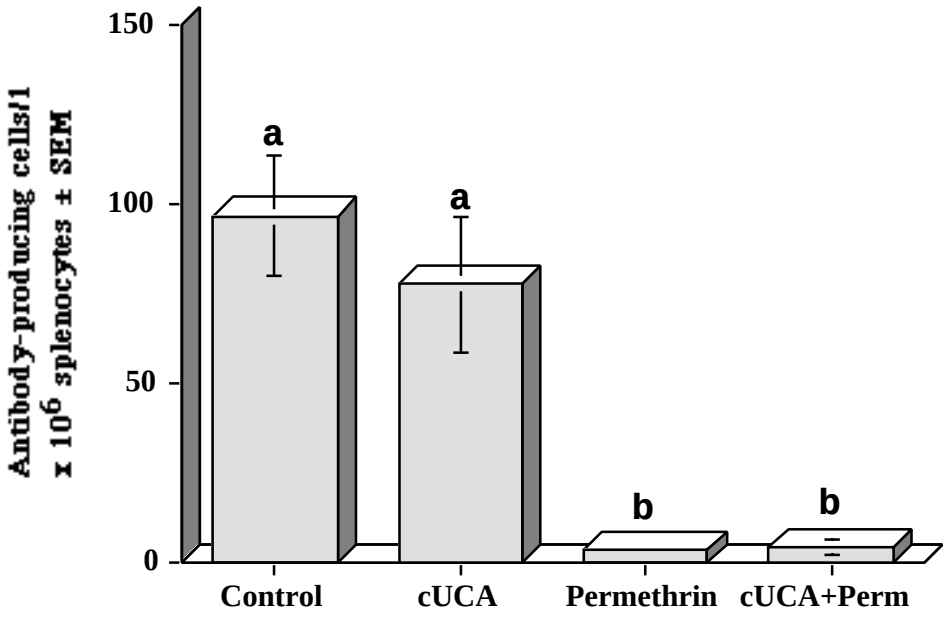

Figure 4.5. Effect of cUCA and permethrin on splenic B lymphocyte antibody production (PFC assay). Results represent decreased splenocyte antibody production following exposure to permethrin (p=0.0001) or permethrin and cUCA in combination (p=0.0001). Each measurement is the mean ± SEM (n=5 per treatment group).

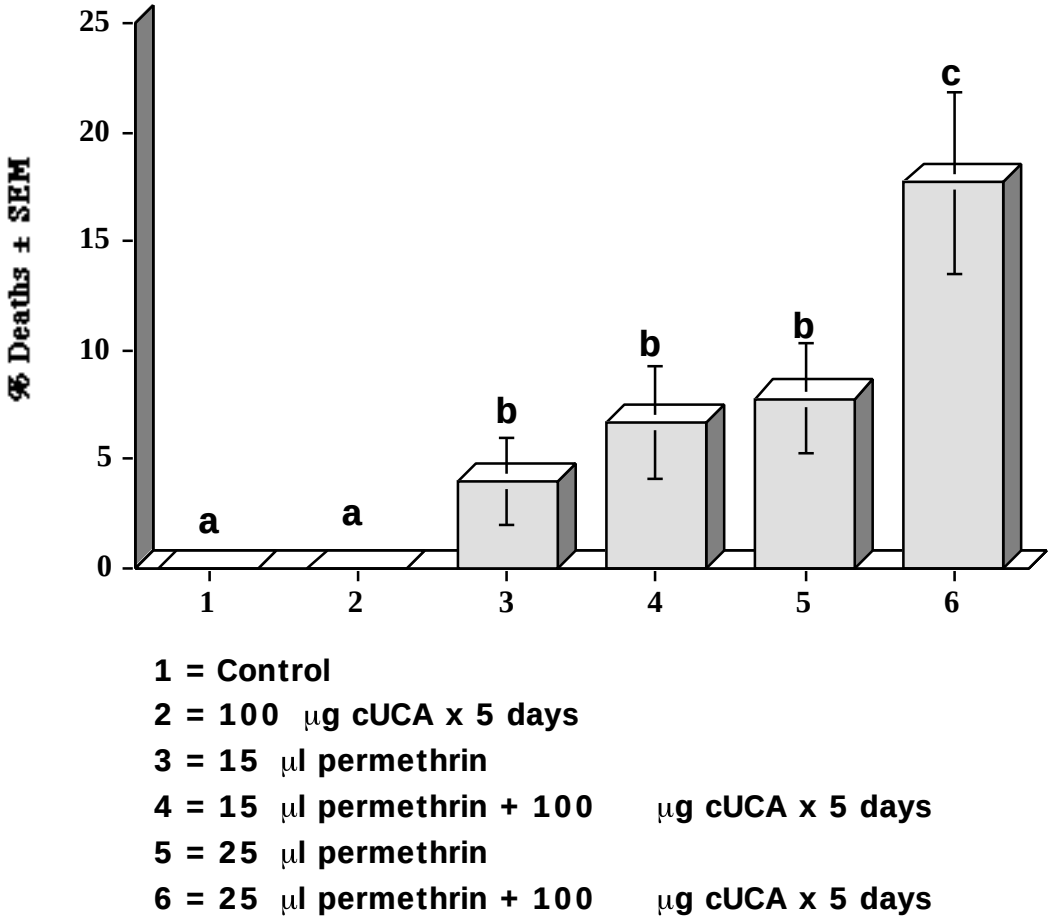

Figure 4.6. Effect of cUCA and permethrin on neurotoxic deaths. Results represent increased incidence of neurotoxic deaths following exposure to 15 μL permethrin (p=0.0001), 25 μL permethrin (p=0.001), or cUCA + permethrin at 15 or 25 μL, respectively (p=0.0001, p=0.001). Each measurement is the mean ± SEM (n=5 per treatment group).

CHAPTER 5: MOLECULAR MECHANISMS OF SUNLIGHT AND PERMETHRIN-INDUCED ALTERATIONS IN CUTANEOUS IMMUNITY

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5A. ABSTRACT

cUCA or UVB exposure has been shown to cause diminished cutaneous contact hypersensitivity (CH) and induces systemic tolerance (increased suppressor T cells) in mice. Permethrin is also a known CH inhibitor, but the molecular mechanisms are currently poorly understood. CH was evaluated in an immunosensitive strain (C57BL/6N), an immunoresistant strain (SvImJ), a strain developed from C57BL/6N mice but genetically altered at both the TNFαp55R and p75R, a strain developed from C57BL/6N but genetically deleted at the IFNγ locus, and a strain genetically equivalent to SvImJ strain genetically deleted at the CD1a locus. C57BL/6N had diminished CH following 5-day exposure to ID cUCA or single exposure to topical permethrin, and this effect was exacerbated with concurrent exposure to both chemicals. CH in SvImJ was both UVB- and permethrin-resistant relative to C57BL/6N mice, as 5-day cUCA or single exposure to permethrin did not diminish CH, nor did concurrent exposure to cUCA and permethrin. Mice deleted at both TNFαR loci displayed similar but somewhat blunted diminished CH responses to cUCA or permethrin. This trend became significant with combined chemical exposure, suggesting that TNFα may be involved in the immunomodulatory effects of cUCA and permethrin. IFNγ knockout mice displayed similar diminished CH responses to cUCA or permethrin alone. Unlike C57BL/6N mice, the IFNγ knockout mice did not show further reduction in CH with combined chemical exposure (Yamawaki et al. 1997). These results suggest that this cytokine may be required for the more than additive depression of CH caused by cUCA + permethrin. CD1a knockout mice were affected similarly to the SvImJ background strain, except that permethrin caused a greater decrease in CH in the knockout mouse. These results

suggest that permethrin may affect antigen processing/presentation of events required for CH.

5B. INTRODUCTION

Modulation of skin immunity, as may occur following exposure to sunlight or topical insecticides, may result in a variety of persistent advantageous or deleterious cutaneous effects such as eliciting or moderating cutaneous allergic disease, diminishing allograft rejection or lessening graft-versus-host disease. Decreasing contact hypersensitivity (CH) responses via chemical modulation of the immune system has been utilized for improvement or treatment of systemic immune-mediated disorders such as inflammatory bowel disease, where there is an imbalance between proinflammatory (Th1) and antiinflammatory (Th2) cytokines. The process of oral tolerance induction of orally-administered inflammatory bowel proteins results in increased transforming growth factor –beta1 and 2 (TGFβ1 and 2), and decreased interferon-gamma (IFNγ) serum levels by suppressor T lymphocytes, which results in diminished clinical signs (Han et al. 2000).

Decreased skin immunity may more commonly have deleterious effects, such as contributing to increased risk of infectious or neoplastic disease. Tumor-derived factors that are produced by some skin cancers (such as human melanoma) actively suppress the cutaneous immune environment by inhibiting differentiation and function of dendritic cells (DC). As a result, transformed cells may escape immunosurveillance, promoting development and spread of the tumor (Berthier-Vergnes et al. 2001). Several immunosensitive tumors have been seen following exposure to UVB or cUCA exposure, including melanoma, squamous cell carcinoma, basal cell carcinoma, and fibrosarcoma (DeFabo and Kripke, 1980), which are likely due to decreased presentation of tumor-

associated antigens by blunted and immobilized Langerhans cells (LC) in the epidermis (Geissert et al. 1997).

Chemical exposure is also thought to contribute to depressed CH responses and lead to increased incidence of development of infectious or neoplastic skin disease. The effects of the pyrethroid insecticide permethrin on systemic immunity include thymic atrophy and hypocellularity, inhibition of natural killer cell activity, and inhibition of lymphocyte proliferative responses to T cell mitogens (Santoni 1997, Diel et al. 1998, Punareewattanna et al. 2000, 2001). However, though previous exposure to topical permethrin has been shown to result in highly persistent reduction of the CH response, the molecular mechanisms are currently poorly characterized.

There is considerable evidence suggesting that sunlight exposure also suppresses cutaneous immunity, an effect initiated by isomerization of trans to cis urocanic acid in the stratum corneum. Cutaneous exposure to UVB irradiation or cUCA has been shown to depress various types of cellular immune responses, including delayed-type hypersensitivity and antiviral and antitumor immunity (Araneo et al. 1989). Administration of cUCA to mice 3-5 days prior to hapten sensitization may suppress LC function and thus CH response to challenge. A cascade of secondary immune mediators such as prostanoids, TNFα, IFNγ, and a shift from Th1 to Th2 cytokine profile are thought to contribute to altered cell growth, natural killer cell and cytotoxic T lymphocyte activation, and cutaneous immune competence (Jaksic et al. 1995, Phipps et al. 1991, Streilein, 1993, Skov et al. 1998). Consequently cUCA-induced diminished CH is thought to be an indirect rather than a direct effect, as *in-vitro* culture of normal human epidermal keratinocytes with physiologic doses of cUCA (100 μg/ml) does not affect

cytokine production of TNFα, IL-1α or β, IL-6, IL-8, or TGFβ1 and does not affect development of murine dendritic cells (Redondo et al. 1996, Lappin et al. 1997). Further evidence suggests that there may be strain variations amongst laboratory rodents, as some strains such as BALB/c have been shown to be relatively resistant to the deleterious effects of UVB or cUCA. Other strains such as C3H-HeN and C57BL/6N have markedly depressed CH following similar levels of UVB or cUCA exposure.

While some of the molecular mechanisms by which cUCA affects cutaneous immunity have been determined, very little is currently known about potential mechanisms of action by which permethrin inhibits skin immunity. The effects of concurrent exposure to sunlight and topical permethrin have not been previously considered. The present report examines mechanisms involved in CH suppression following single exposure to permethrin or 5-day exposure to cUCA, or concurrent exposure to both chemicals simultaneously.

5C. MATERIALS AND METHODS

1. MICE. Five-week-old female mice of the following strains were purchased: C57BL/6N, SvImJ, B6.129S7-IFNγ^{tm1Ts}, C;129S-CD1^{tm1Gru} and B6.129S-TNFRsf1a^{tm1MAK}TNFRsf1n^{tm1MAK}. C57BL/6N mice were obtained from Charles River Laboratories (Portage, MI). All knockout mice and the SvImJ controls for CD1 knockouts were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were acclimated for one week and maintained in microisolator cages under controlled conditions of temperature (22±1˚C), humidity (40-60%), and light (12/12-hour light/dark cycle). Food (irradiated Teklad Global 18% protein rodent diet 2918, Harlan Teklad, Madison, WI) and water were provided ad libitum. Mice in all experiments were humanely treated, in accordance with the guidelines of the Virginia Tech Institutional Animal Care and Use Committee (VT IACUC).

2. PERMETHRIN TREATMENT PROTOCOL. Permethrin was provided by the US Army Center for Health Promotion and Preventive Medicine (Aberdeen Proving Ground, MD) from stock purchased immediately before by the Army from Coulston Industries (Coulston Products, Easton, PA). The permethrin was a 91.6% pure mixture of 57.7% trans and 42.3% cis permethrin, respectively. Mice were exposed to a single dose of 15 µL permethrin (approximately 660 mg/kg body weight) on the shaved interscapular skin, to mimic the most common route of human exposure (Snodgrass 1992). This dose was previously found to cause persistent inhibition of CH in mice (unpublished data). Control groups received an identical volume of vegetable oil (vehicle) in the interscapular space.

3. CIS-UROCANIC ACID. Cis-urocanic acid was isomerized under UVB light from trans-urocanic acid (Sigma, St. Louis, MO), purified as previously described (Takahashi et al. 1997), and then evaluated for purity by mass spectroscopy prior to use. Administration of cUCA consisted of a daily suberythremic intradermal dose of PBS vehicle or 100 μg cUCA dissolved in PBS (equivalent to approximately 6.6 mg/kg cUCA) for five consecutive days in mice arbitrarily assigned to treatment or control groups. This dosing schedule was previously found to cause persistent inhibition of CH in mice (unpublished data).

4. CONTACT HYPERSENSITIVITY. The contact hypersensitivity response was assayed by quantifying the ear swelling response to chemical exposure using the method originally published by Asherson and Ptak (1968). Briefly, mice in all treatment groups were sensitized by topical application in the interscapular region of 25 μL 2% (w/v) 4-ethoxymethylene-2-phenyl–2-oxazolin-5-one (oxazolone) dissolved in a 4:1 reagent-grade acetone/olive oil mixture 48 hours following permethrin application or administration of the final dose of cUCA. Five days after oxazolone sensitization, the mice were challenged with 10 μL of 0.5% oxazolone to both the dorsal and ventral surfaces of the right ear. The acetone:olive oil vehicle was applied to the left ear as a control for vehicle-induced irritancy. Immunological response to permethrin or cis urocanic acid were assessed at 24 hours after challenge. The response was quantified as the difference in the thickness of the challenged ear before and after challenge (Oditest 313 Series micrometer; The Dyer Company, Lancaster, PA). The thickness of the left ear (vehicle-treated)

was also determined before and after treatment with the acetone:olive oil vehicle to control for vehicle-induced irritation and swelling.

5. STATISTICAL ANALYSIS. Data were expressed as arithmetic mean ± SEM. A one-way analysis of variance was completed. A randomized complete block design was used for error control. Dunnett's post-hoc t-test was used to establish significant differences in treatment groups versus controls. Results described as different in this paper indicate significantly different at $p < 0.05$.

5D. RESULTS

1. CONTACT HYPERSENSITIVITY IN C57BL/6N AND SvImJ MICE EXPOSED TO cUCA AND PERMETHRIN.

Results of this study supported previous suggestions that C57BL/6N is a UVB immunologically sensitive strain of mice. Following 5-day intradermal cUCA exposure, C57BL/N mice had a significantly diminished ability to respond to oxazolone challenge in a CH assay (n=5 per treatment group, p=0.0018, Fig. 5.1). A single administration of topical permethrin caused a similar dramatic decrease in CH (n=5 per treatment group, p=0.0006, Fig. 5.1), and this effect was significantly increased as compared to either chemical alone when both chemicals were administered concurrently (n=5 per treatment group, p=0.0023, Fig. 5.1).

Contact hypersensitivity responses in untreated mice from the SvImJ strain were not different from C57BL/6N controls, however the two strains differed dramatically in their CH response following exposure to cUCA or permethrin. SvImJ mice exposed to 5-day ID cUCA showed no decrease in CH (n=5 per treatment group, Fig. 5.1). Single dose permethrin (n=5 per treatment group, p=0.6784, Fig. 5.1), or concurrent administration of both chemicals (n=5 per treatment group, p=0.7294, Fig. 5.1) likewise caused no alteration in CH, suggesting that SvImJ mice may be relatively resistant to the cutaneous immunomodulatory effects of these chemicals.

2. CONTACT HYPERSENSITIVITY IN C57BL/6N CONTROL AND TNFαR KNOCKOUT MICE EXPOSED TO cUCA AND PERMETHRIN.

C57BL/6N mice that were genetically deleted at both the p55 and p75 TNFα receptor loci demonstrated control CH responses that were similar to C57BL/6N controls. The wild-type and knockout mice displayed approximately equal decreases in CH following cUCA or permethrin exposure (n= 5 per treatment group, p=0.0025 and p=0.003, respectively, Fig. 5.2). A non-significant trend toward reduced ear swelling was present in both groups of knockout mice that received a single chemical exposure. TNFαR knockout mice exposed to both cUCA and permethrin displayed significantly less reduced ear swelling as compared to wild-type mice, suggesting a role for TNFα in the severe inhibition of CH caused by the chemical mixture.

3. CONTACT HYPERSENSITIVITY IN C57BL/6N CONTROL AND IFNγ KNOCKOUT MICE EXPOSED TO cUCA AND PERMETHIRN. Vehicle-exposed C57BL/6N mice genetically deleted at the IFNγ locus demonstrated significantly diminished CH responses as compared to C57BL/6N controls (n=5 per treatment group, p=0.0001, Fig. 5.3). This observation indicates that IFNγ may be required to mount a control-level CH response. The wild-type and IFNγ knockout mice showed approximately equal depression of CH after treatment with either cUCA or permethrin alone, suggesting that IFNγ was not required for depression of CH to the level observed in these mice. When IFNγ knockout mice were co-exposed to cUCA + permethrin, no further depression in CH occurred than was observed after exposure to either chemical alone. In contrast, wild-type C57BL/6N mice exposed to the chemical mixture displayed considerable additional reduction in ear swelling which appears to require IFNγ as a mediator. Collectively, these data suggest that IFNγ knockout mice: 1) cannot mount a control-level CH response; 2) are able to

show diminished CH after exposure to cUCA or permethrin alone that is apparently IFNγ-independent, but 3) cannot achieve the maximal depression of CH that occurs in mixture-exposed mice and thus appears to require IFNγ.

4. CONTACT HYPERSENSITIVITY IN C57BL/6N CONTROL AND CD1a KNOCKOUT MICE EXPOSED TO cUCA AND PERMETHRIN. As previously observed, the vehicle- or chemical-exposed SvImJ mouse did not display reduced CH responses. The CD1a knockout mouse of otherwise similar genetic background to this strain was also largely refractory to the effects of treatment on CH, with the exception of mice exposed to permethrin only (n=5 per treatment group, p=0.0001, Fig. 5.4). The reduced ear swelling that occurred in these mice suggests that permethrin may affect antigen presentation to inhibit the ear swelling response. For unknown reasons, mice exposed to both cUCA and permethrin did not show the reduced ear swelling seen in permethrin-only mice.

5E. DISCUSSION

Cutaneous exposure to UVB irradiation or topical permethrin has been shown to depress cellular immune responses, including delayed-type hypersensitivity (Araneo et al. 1989, Punareewattana et al. 2000). Elicitation of CH is biphasic and involves two sets of antigen-specific Th1 cells: the first response is seen two hours following the antigenic challenge and results from activation of initiator cells, and the second follows initiator cell activation and is seen 24-48 hours later due to stimulation of effector cells. cUCA is thought to prevent the function or recruitment of the initiating cells, which in turn inhibits the effector phase (Norval et al. 1995).

It has been suggested that genetic variations among laboratory rodent strains may cause varied susceptibilities to the innate and adaptive cell-mediated immune suppressive effects of sunlight exposure. Immunosensitive strains include C57BL/6N and C3H-HeN, whereas immunoresistant strains include BALB/c mice (Wakeham et al. 2000, Streilein et al. 1993). Wakeham et al. observed that BALB/c developed an earlier response to proinflammatory cytokines (IL-12, IFNγ, TNFα, and macrophage chemoattractive protein-1) in response to bacterial infection and suggested that these cytokines were not as dramatically affected following UVB exposure. Streilein et al. (1993) identified genetic mutations at H-2 (TNFα) and LPS loci in the immunoresistant strains and suggested that UVB-sensitivity is a dominantly inherited trait such that homozygous mutations at these two loci are necessary to confer UVB resistance. Human studies have demonstrated similar molecular links to UVB-altered cell-mediated immunity (Streilein 1993). It is likely that these two loci are more important than skin pigmentation in the molecular mechanisms of cutaneous UVB sensitivity, and increased susceptibility to skin

cancer. However, additional study will be necessary to verify more specific mechanisms to explain the high UVB-susceptible trait (35-45% of individuals) in human beings (Streilein, 1993). A previous study demonstrated that C57BL/6N mice were highly sensitive to systemic immune effects caused by cUCA (unpublished data). The present report extends these findings to show that C57BL/6N mice are also highly sensitive to the cutaneous immune effects of UVB, and further suggested that SvImJ are a UVB-resistant strain.

Several molecular mediators have been implicated as possible contributors to the multifactorial effects of chemically-induced CH suppression, including prostaglandins, interleukin-10, and TNFα (Norval 1995). The prostanoid system is thought to be at least partially involved in this effect, as cUCA synergized with histamine causes increased prostaglandin E_2 (PGE_2) production by keratinocytes, which increases release of TNFα and causes diminishment of CH responses, activation of suppressor T lymphocytes and decreased Th1 production of IL-2 and IFNγ (Phipps et al. 1991, Jaksic et al. 1995). It is thought that TGFβ2 also causes antigen presenting cells to upregulate TNFα production, which biases the T cells in a direction that interferes with acquisition of antigen and hapten transfer to the draining lymph node, thus suppressing induction of CH (Hecker et al. 1999). This Th1 unresponsiveness is long-lasting (greater than 16 days) and not due to cell death, but rather the result of an interference in the capacity of LC to deliver the required activation costimulatory signal to the Th1 cells (Simon et al. 1991). Consequently, the Th1/Th2 ratio shifts in favor of a Th2 response, which diminishes cell-mediated immunity. Previous studies suggested that TNFα secretion by epidermal keratinocytes reaches maximal levels just 6 hr after UVB irradiation, confers locally

depressed CH response, (Streilein, 1993, Skov et al. 1998) which results in morphological changes in LC, migration of follicular dendritic cell precursors into splenic follicles, interference with antigen processing and presentation to Th1 cells, and altered activation of Th1 cell-driven inflammatory immune reactions in mice (Schmitt and Ullrich 2000, Pasparakis et al. 2000, Amerio et al. 2001). However, recent reports have suggested other molecular mediators besides TNFα may contribute to diminished CH responses to oxazolone challenges, and that the dramatic cutaneous immune suppression seen following 200 μg or less cUCA may be the result of multifactorial chemical mediator effects (Norval et al. 1995).

The present study evaluating changes in CH responses in mice that were genetically altered at both p55 and p75 TNFαR loci suggested that local immunomodulatory effects caused by cUCA and permethrin may at least in part be mediated by changes in TNFα production by epidermal cells. Both type I and type II TNFα receptors (p55TNFαR and p75TNFαR) are required for full expression of the delayed-type hypersensitivity reaction, as dendritic cells of mice lacking the p55TNFαR had a defect in antigen uptake due to blunting of cytoplasmic processes and disruption of vimentin microtubules in the cytoskeleton, but showed normal LC migration to local lymph nodes (Goettsch et al. 1993, Bacci et al. 1996). Conversely, those mice with dendritic cells demonstrating p75TNFαR deficiency had diminished LC migration to the local lymph nodes after exposure to contact allergen, without hindrance of antigen uptake itself (Becke et al. 2001).

IFNγ is thought to be a critical immunoprotective Th1 cytokine necessary for the production of normal UVB-induced erythema, perhaps due to IFNγ's effect of releasing

histamine from mast cells (Reeve et al. 1999). *In-vivo* UVB or cUCA suppresses IFNγ, which diminishes CH by interfering with antigen processing and presentation (Bacci et al. 1996, Laihia et al. 1996). IFNγ is thought to play a part in immunoprotection from the adverse effects of UVB or cUCA on CH responses (Reeve et al. 1999) by enhancing IL-12 production and diminishing IL-10 release by macrophages. IFNγ is an essential regulatory protein secreted by activated CD4+ T cells that acts as a negative feedback loop to eliminate by apoptosis excess activated CD4+ T cells in an attempt to maintain CD4+ T cell homeostasis during an immune response. Therefore, activated CD4+ T cells from IFNγ knockout mice that have clonally expanded following immune activation do not undergo IL-2-mediated apoptosis, thus predisposing these animals to the development of autoimmune disease (Dalton et al. 2000). The results of the present study seem to support the theory that IFNγ is an important contributor to the development of CH in murine skin, as the IFNγ knockout strain of mice demonstrated lower control (untreated) CH levels as compared to the genetically similar C57BL/6N mice.

Reports of development of cutaneous nodules rich in LC ($CD1a^+$, $S100^+$, $HLA\text{-}DR^+$) following cutaneous treatment with permethrin for scabies (Hashimoto et al. 2000) suggest that permethrin may inhibit the migration of LC to local lymph nodes. The present study found that the CD1a knockout strain of mice had diminished CH following exposure to permethrin. Following a single exposure of 4 minimal erythemal dose in human beings, contact hypersensitivity was significantly reduced. While numbers of $CD1a^+$ LC were significantly diminished and remaining LC were rendered ineffective functionally, numbers of $CD1a^-$ macrophages were increased in the skin (LeVee et al. 1997). Since the CD1a is involved in presentation of lipid and glycolipid antigens to a

variety of effector T cells (Sugita), deletion of this gene may contribute in small part to a defective CH response following exposure to permethrin. TGFβ1 is thought to work together with IL-4 in the development and differentiation of $CD1^+$ LC from hematopoietic progenitor cells in a normal inflammatory response (Caux et al. 1999). Human epidermal $CD1a^+$ Langerhans cells that have been previously exposed to UVB irradiation activate regulatory T lymphocytes, which may help to dampen responses to endogenous antigens that are pathogenic in autoimmune disorders. However, these same mechanisms may also predispose an individual to increased risk of development of UV-induced skin cancers (Baadsgaard et al. 1990). Exposure of experimental rodents to UVB results in significantly diminished release of IL-2 and IFNγ, with concomitant increased release of IL-4 (Araneo et al. 1989). Epidermal keratinocytes exposed to inflammatory mediators promote induction of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), which results in the retention of T lymphocytes in cutaneous immunohomeostasis (Mitra et al. 1993). However, exposure of epidermal (LC) cells to UVB irradiation inhibits their ability to activate T cells through decreased expression of ICAM-1 and resulting diminished T cell activation (De Luca et al. 1997). Cis-urocanic acid has also been shown to inhibit fibroblast synthesis of cAMP, an important second messenger involved in cutaneous immune responses such as IL-2 receptor expression (Johnson and Smith 1990, Bouscarel et al. 1998).

Repeated application of low-dose topical cUCA has also been shown to alter murine contact hypersensitivity by decreased IL-1α release and increased IL-10 production by keratinocytes and mast cells in the epidermis (Rasanen et al. 1987, Kurimoto et al. 2001). Increased IL-10 is thought to mediate systemic tolerance by

upregulation of suppressor T lymphocytes (Alard et al. 2001). Further support of IL-10-mediated effects on cutaneous immunity was demonstrated with retroviral-mediated gene transfer of viral IL-10, which mimics a switch from Th1 to Th2 cytokine profile and results in prolonged allograft survival by altering antigen presenting cell function (Qin et al. 2001). Murine recombinant IL-10 (200 ng) was injected intradermally on abdominal skin of a UVB-sensitive strain (C3H-HeN) and a UVB-resistant strain (BALB/c) of mice. This resulted in promotion of hapten-specific systemic tolerance to 2,4-dinitrofluorobenzene (DNFB) within 30 minutes to 3 days following IL-10 injection in both strains (Niizeki and Streilein 1997). Parallel experiments with UVB exposure in these strains of mice resulted in suppression of the cutaneous CH response only in the UVB-sensitive C3H-HeN strain (Niizeki and Streilein 1997). These studies suggest that tolerance induction is a systemic upregulation of regulatory T lymphocytes, mediated in part by IL-10, whereas the CH response is a local phenomenon, perhaps with divergent molecular mechanisms. Further experiments evaluating gene expression of cytokines potentially involved in the CH response in cultured murine keratinocytes may shed additional light on the molecular mechanisms behind diminished contact hypersensitivity following dermal cUCA or permethrin exposure. Additionally, further study of *in-vivo* interventions with cytokines involved in LC differentiation and lymphocyte activation, such as TCR/MHC II interaction and costimulatory signals involved in the CD28/CD80 or CD86 interactions would help to more clearly define the multifactorial effects of chemical exposure on skin immunity.

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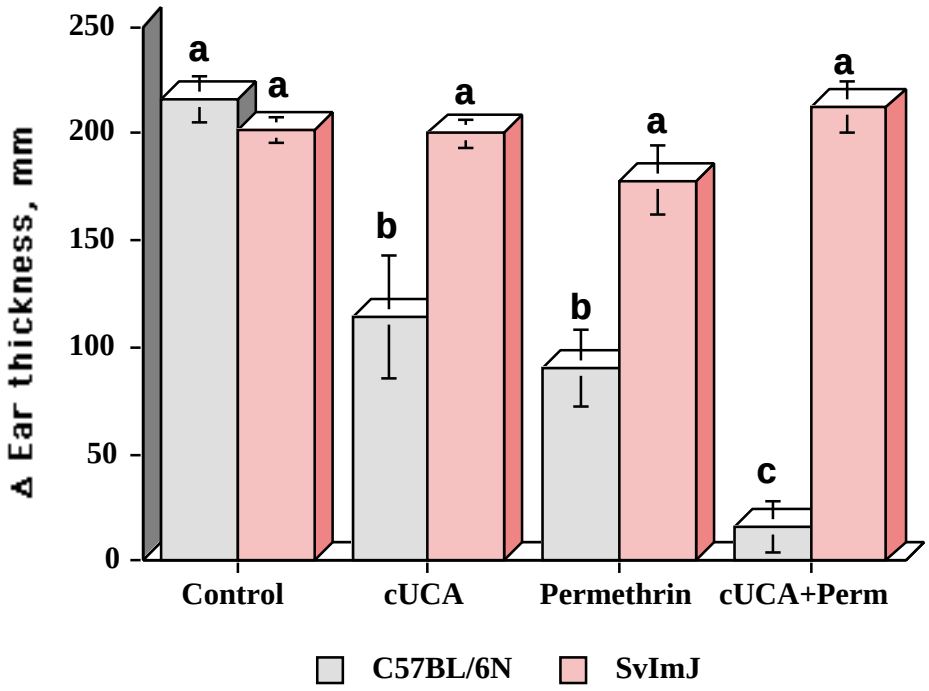


Figure 5.1. Contact hypersensitivity response to five-day ID cUCA, single topical administration of permethrin, or cUCA + permethrin in C57BL/6N and SvImJ mouse strains. Results represent change in ear thickness following oxazolone challenge 48 hr after final chemical exposure. Each measurement is the mean ± SEM (n=5 per treatment group); groups that display different letter are statistically significant, $p<0.05$.

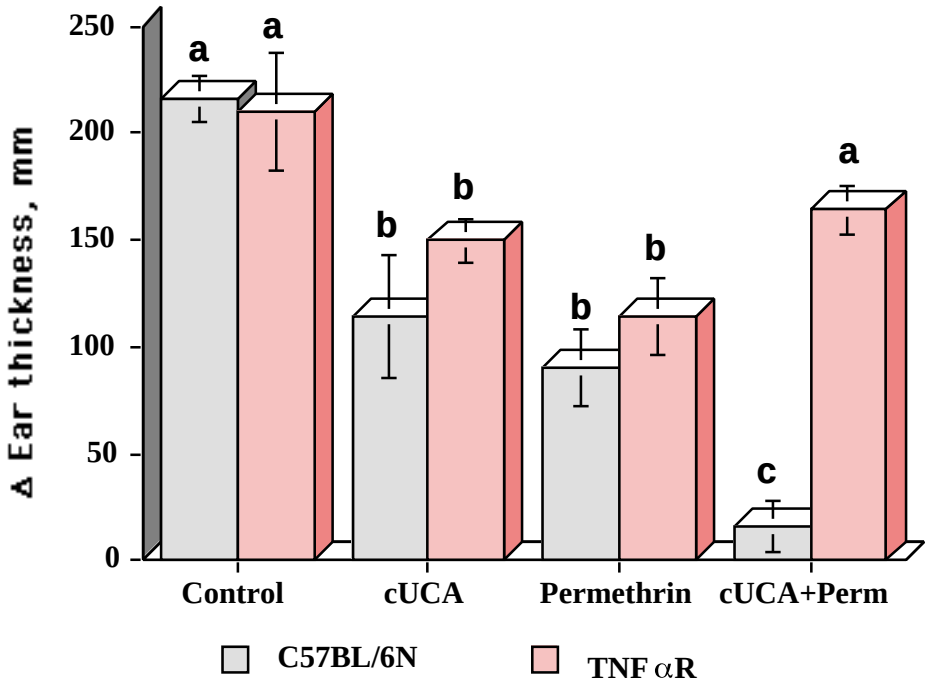


Figure 5.2. Contact hypersensitivity response to five-day ID cUCA, single topical administration of permethrin, or cUCA + permethrin in C57BL/6N mice and C57BL/6N mice that were genetically deleted at both the p55 and p75TNFα receptors. Results represent change in ear thickness following oxazolone challenge 48 hr after final chemical exposure. Each measurement is the mean ± SEM (n=5 per treatment group); groups that display different letter are statistically significant, $p<0.05$.

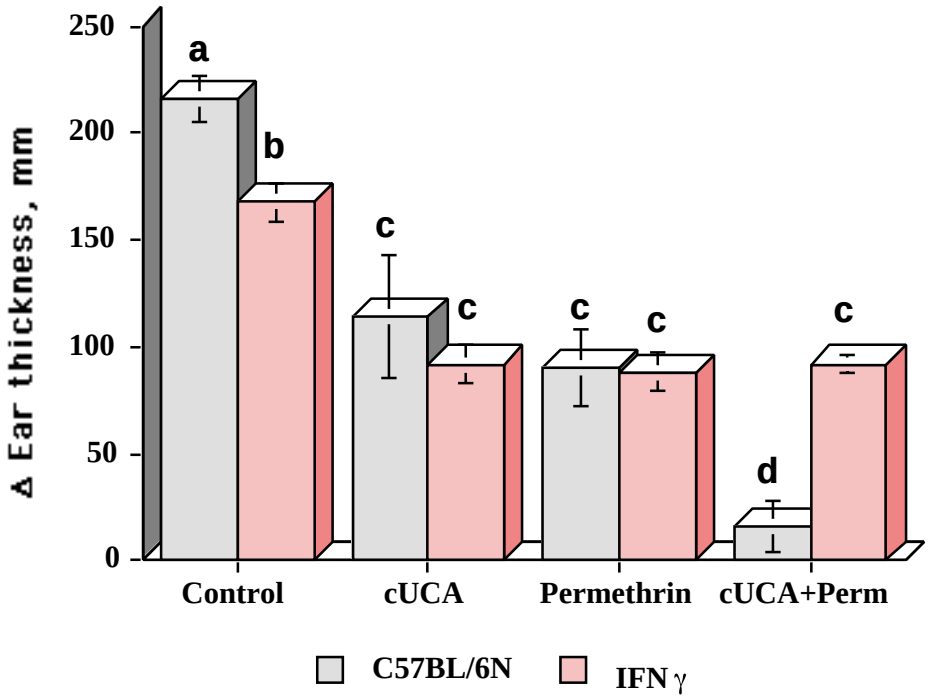


Figure 5.3. Contact hypersensitivity response to five-day ID cUCA, single topical administration of permethrin, or cUCA + permethrin in C57BL/6N mice and C57BL/6N mice that were genetically deleted at the IFNγ locus. Results represent change in ear thickness following oxazolone challenge 48 hr after final chemical exposure. Each measurement is the mean ± SEM (n=5 per treatment group); groups that display different letter are statistically significant, $p<0.05$. These results suggest that IFNγ release is needed for a control-level CH response, however the diminished CH caused by either cUCA or permethrin alone can be attained by IFNγ knockout mice, thus IFNγ is not required. The results of CH in combined-exposure mice suggest that these mice cannot respond at a level beyond the effect seen with exposure to either cUCA or permethrin alone.

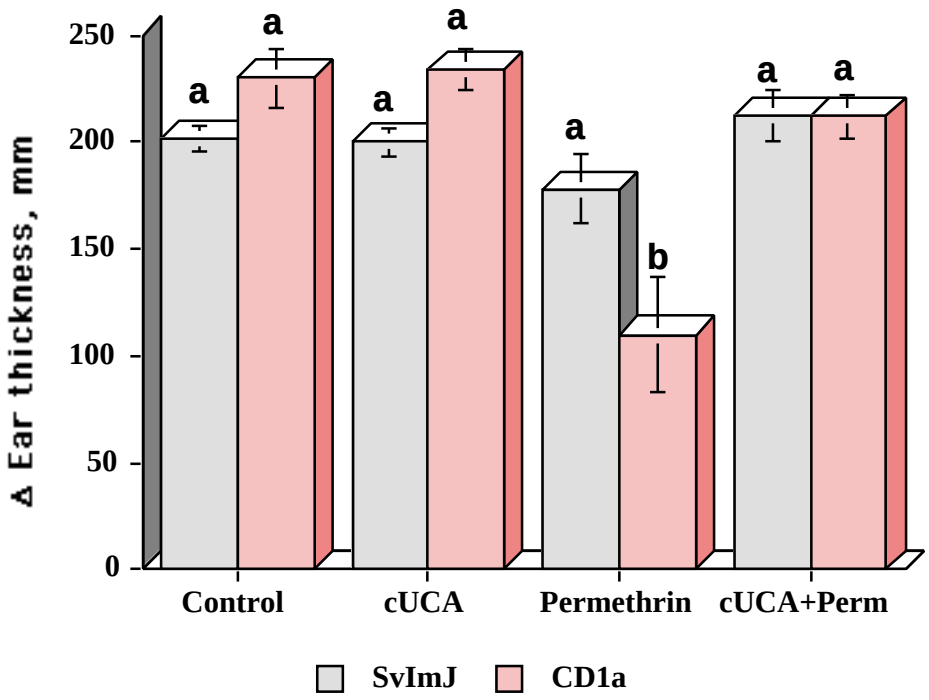


Figure 5.4. Contact hypersensitivity response to five-day ID cUCA, single topical administration of permethrin, or cUCA + permethrin in SvImJ mice and mice that were genetically deleted at CD1a locus. Results represent change in ear thickness following oxazolone challenge 48 hr after final chemical exposure. Each measurement is the mean ± SEM (n=5 per treatment group); groups that display different letter are statistically significant, $p<0.05$.

CHAPTER 6: CONCLUSIONS

Single dose exposure to topical permethrin caused a variety of immunological changes in mice, with the thymus being more profoundly affected than the spleen. Thymic atrophy and hypocellularity appeared to result, at least in part, from increased thymocyte apoptosis and from antiproliferative chemical effects on thymocytes. Antiproliferative effects in splenic T lymphocytes and reduced cell counts were also seen following a single exposure to permethrin, as well as diminished antibody production. Decreased ability to produce antibody has been demonstrated to have a high concordance (predictive values) for immunosuppression in mice. Beyond immunosuppression, recent data suggest possible increased incidence of allergic disease associated with chronic inhalation exposure to permethrin, resulting from increased histamine release by basophils and decreased IFNγ and IL-4 release by peripheral lymphocytes caused by permethrin. These collective reports verify the need for additional examination of the immunomodulatory effects of permethrin.

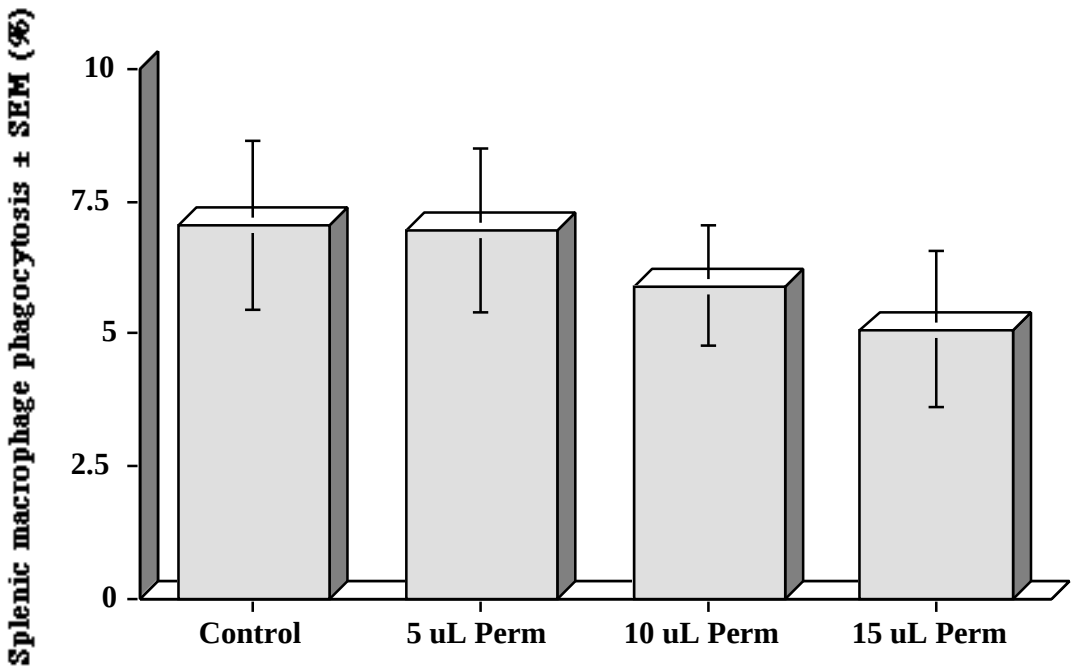
In contrast, a single exposure to intradermal cUCA produced few detectable changes in the selected immune parameters or immune function assays aside from a decrease in splenic macrophage phagocytosis. Thymic atrophy could not be produced after single dose cUCA or after daily dosing for 5 consecutive days, but was dramatic after four weeks dosing. This effect would not appear to be corticosteroid (stress) - mediated, in that the most sensitive $CD4^{+}8^{+}$ thymocytes were least affected by cUCA. Thymic atrophy was also more severe in C57Bl/6N mice than in C3H-HeN mice, suggesting underlying differences in sensitivity of these mouse strains to UVB irradiation. Finally, mice dosed daily for five days with cUCA did not show altered T

cell cytolytic ability or depressed antibody production, suggesting that these mice are likely to respond at a control level to immunologic challenge.

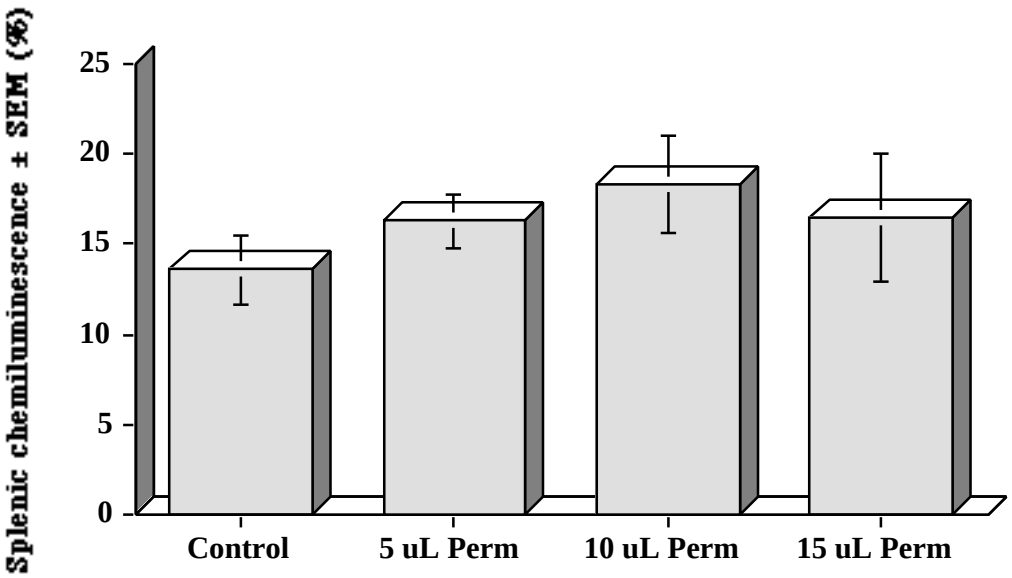
However, when administered concurrently, cUCA and permethrin resulted in alteration of several immune parameters, including ability of splenic B lymphocytes to produce specific antibody and thymic weight, in which an additive effect of the chemicals was evident. These results may suggest different mechanisms by which these compounds affect precursor T cells (thymocytes) in the thymus, as compared to functionally mature T cells or other immune cell targets present in the spleen. Cutaneous immunity was also affected by 5-day exposure to cUCA, permethrin, or concurrent exposure to both chemicals, and additive effects were seen in the immunosensitive C57BL/6N strain. Several potential molecular mediators involved in the development of contact hypersensitivity in mice were identified with the use of genetically altered strains of mice. These included TNFα, IFNγ, and CD1a. The present studies using gene knockout mice provided data that suggest TNFα is a required cytokine in the inhibition of contact hypersensitivity caused by either cUCA or permethrin. IFNγ was also identified as an important cytokine for mounting a control-level CH response, which appeared to be required for the severe depression of CH caused by co-exposure to cUCA and permethrin. Finally, permethrin is a well-characterized neurotoxicant that has been widely used because of its combination of selective efficacy as an insecticide and minimal described adverse effects in mammals. The observation that permethrin caused neurotoxicity in some of the present mice is not novel; however the finding that a chromophore induced by UV light (cUCA) increased the lethality of permethrin,

apparently by increasing its neurotoxic effects, is new and potentially important information.

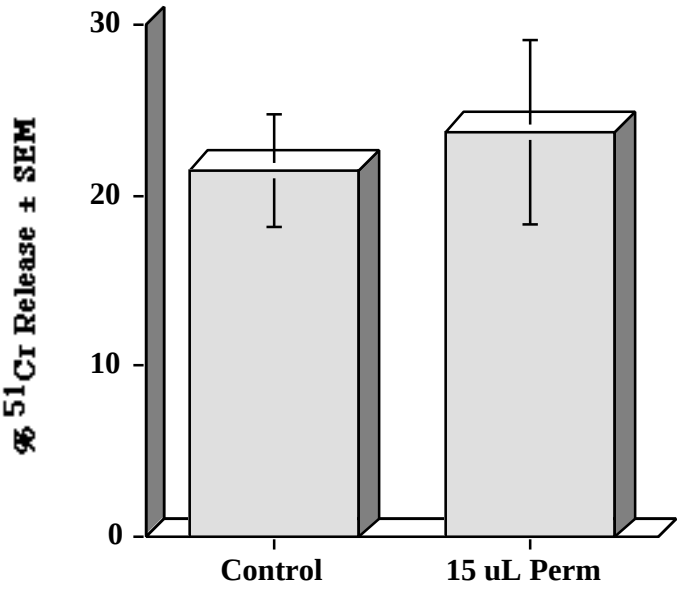
The present immunotoxocity and neurotoxicity data are clearly preliminary but may suggest that human beings, and especially children, treated with relatively high doses of topical permethrin for mites or lice, should avoid excessive sunlight exposure during the immediate post-treatment period.



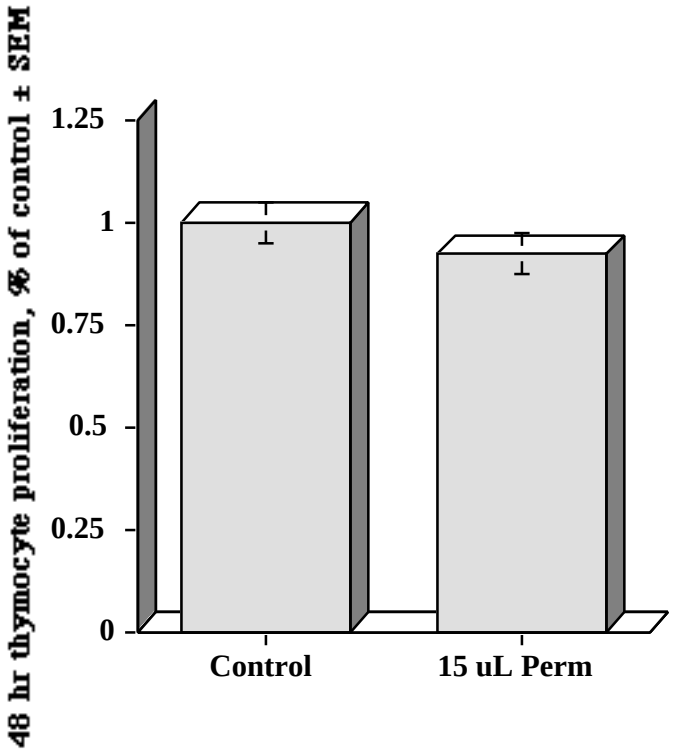
Appendix I. Effect of a single topical exposure to permethrin on splenic macrophage phagocytosis in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



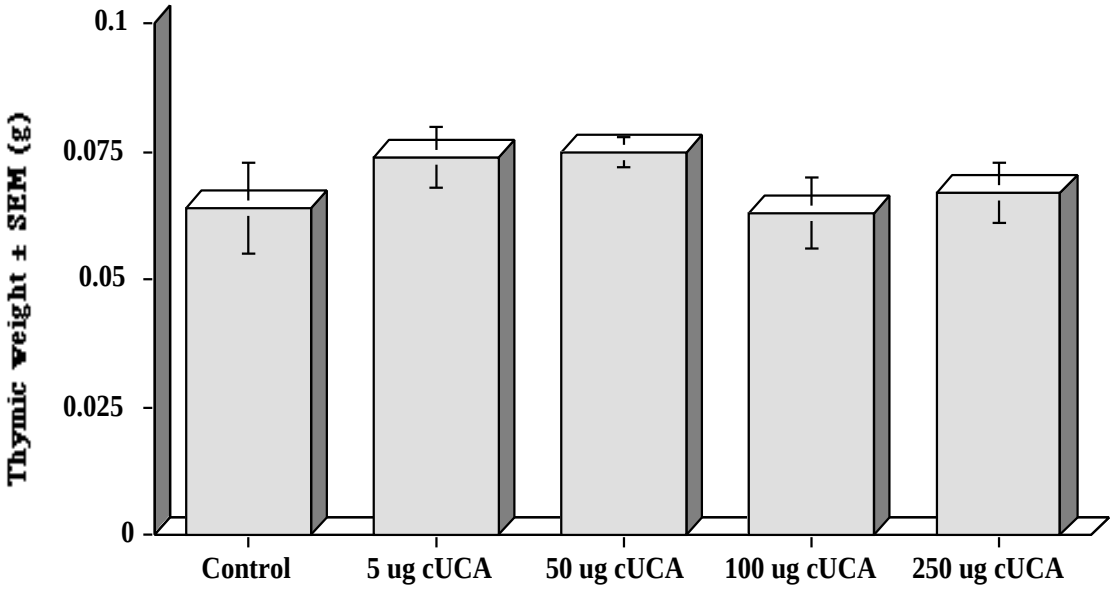
Appendix II. Effect of a single topical exposure to permethrin on splenic macrophage ability to produce active oxygen species (hydrogen peroxide, chemiluminescence assay) in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



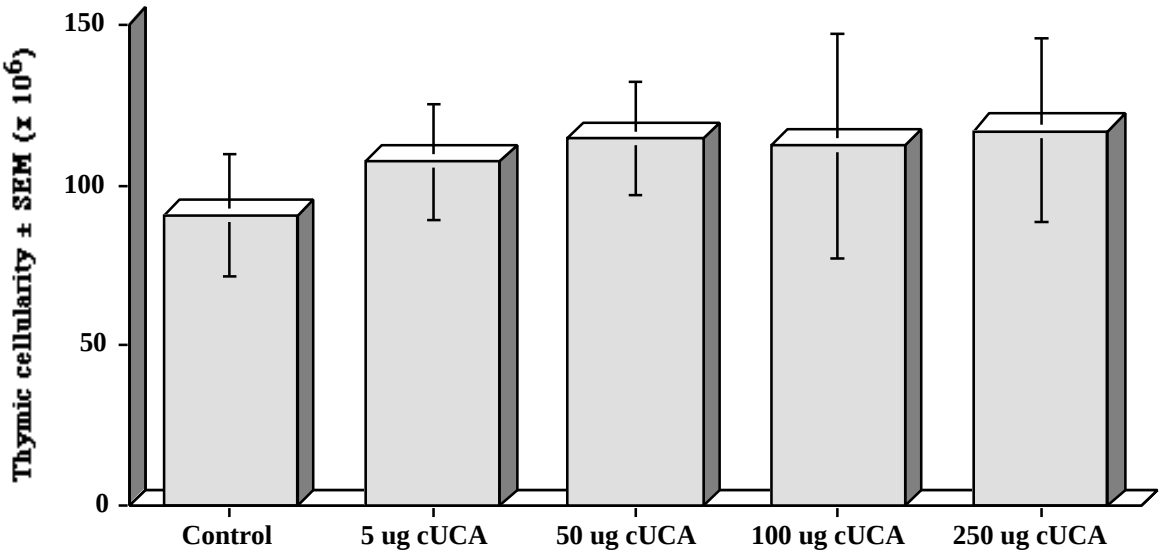
Appendix III. Effect of a single topical exposure to permethrin on splenic T lymphocyte cytolytic activity (^{51}Cr release assay) in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



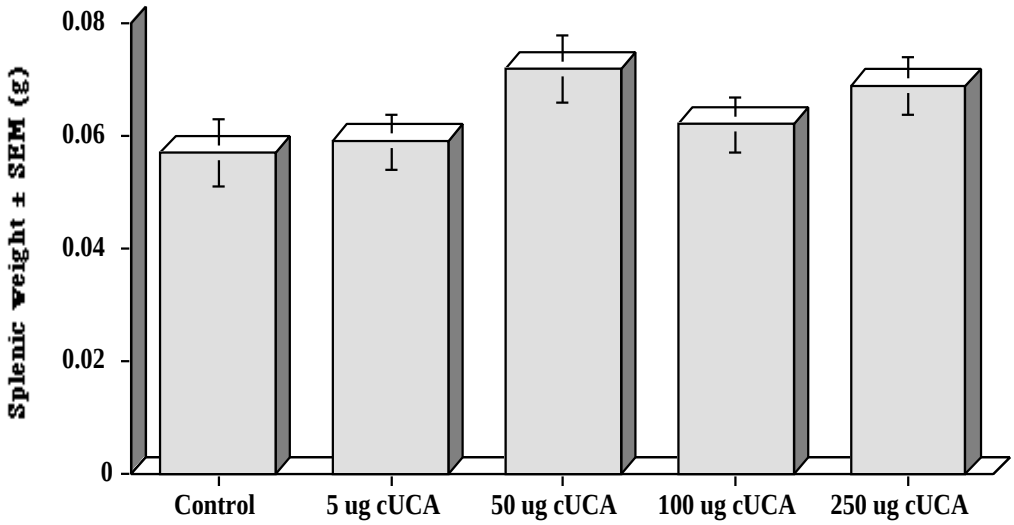
Appendix IV. Effect of a single topical exposure to permethrin on thymic proliferative response to Concanavalin A in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



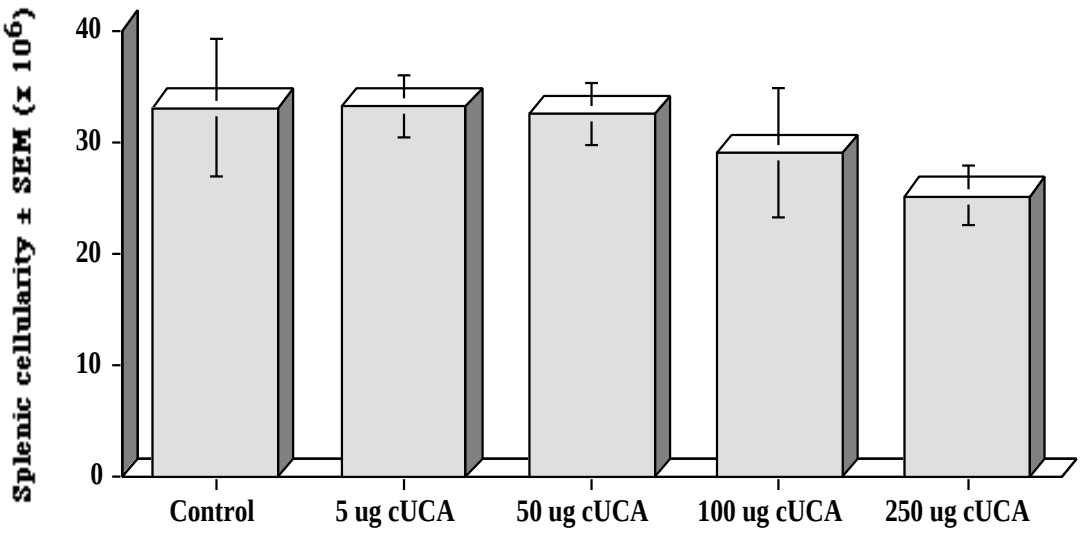
Appendix V. Effect of a single exposure to cUCA on thymic weight in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



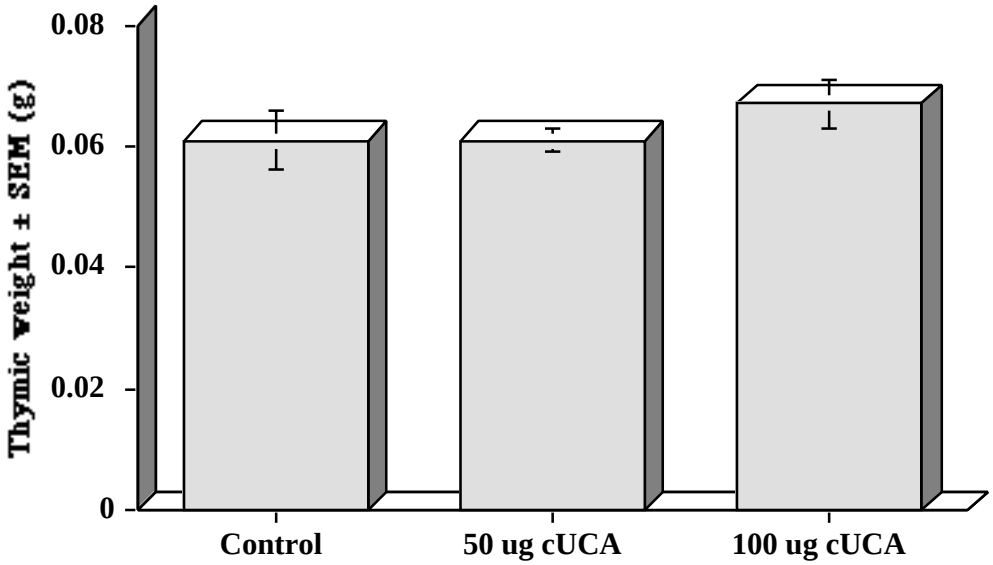
Appendix VI. Effect of a single topical exposure to cUCA on thymic cellularity in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



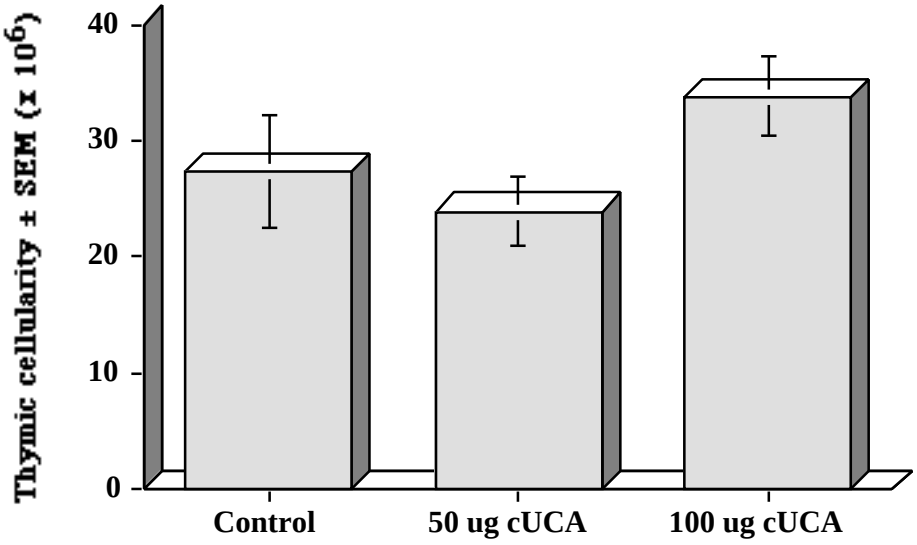
Appendix VII. Effect of a single exposure to cUCA on splenic weight in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



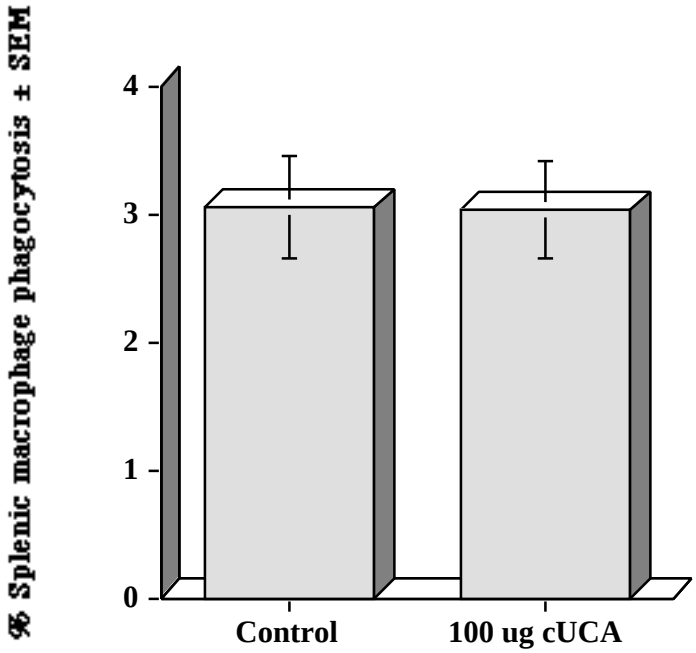
Appendix VIII. Effect of a single exposure to cUCA on splenic cellularity in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



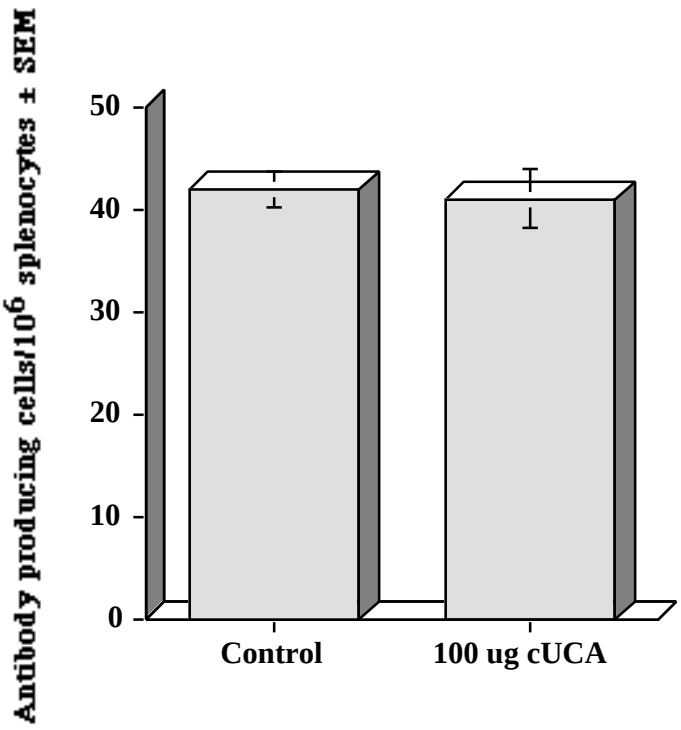
Appendix IX. Effect of five-day exposure to cUCA on thymic weight in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



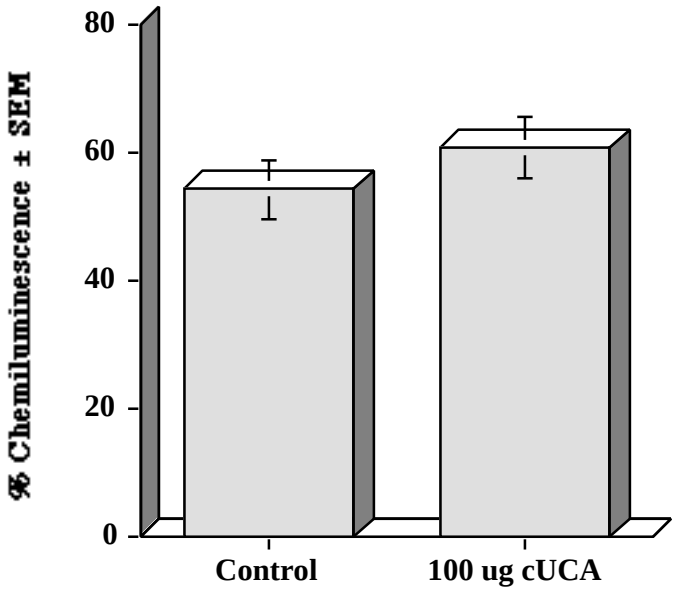
Appendix X. Effect five-day exposure to cUCA on thymic cellularity in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



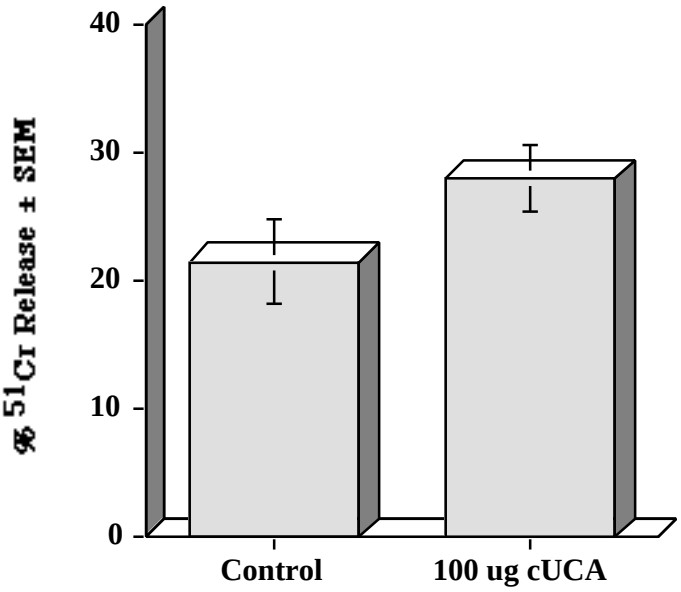
Appendix XI. Effect of five-day exposure to cUCA on splenic macrophage phagocytosis in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



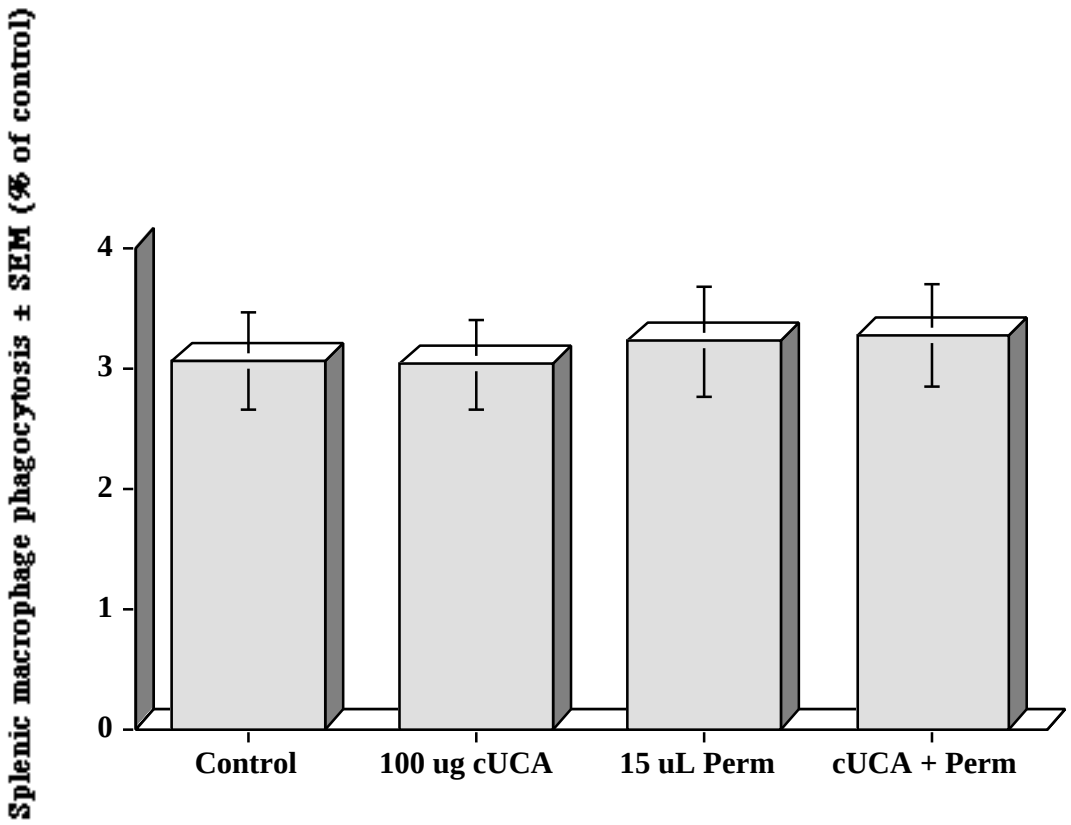
Appendix XII. Effect of five-day exposure to cUCA on splenic lymphocyte antibody production in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



Appendix XIII. Effect of five-day exposure to cUCA on splenic macrophage ability to produce active oxygen species (hydrogen peroxide, chemiluminescence assay) in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



Appendix XIV. Effect of five-day exposure to cUCA on splenic T lymphocyte cytolytic activity (^{51}Cr release assay) in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.



Appendix XV. Effect of a single topical exposure to permethrin, five-day exposure to cUCA, or combined exposure to both permethrin and cUCA on splenic macrophage phagocytosis in female C57BL/6N mice. Results are expressed as mean ± SEM, n=5 per treatment group.

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EDUCATION

Doctoral Candidate, Immunotoxicologic Pathology, 1999-2002
VA-MD Regional College of Veterinary Medicine, Blacksburg, VA, GPA: 3.9/4.0

Clinical Pathology Resident, 1996-1999
VA-MD Regional College of Veterinary Medicine, Blacksburg, VA, GPA: 3.9/4.0

Doctor of Veterinary Medicine with honors, 1992-1996
VA-MD Regional College of Veterinary Medicine, Blacksburg, VA, GPA: 3.8/4.0

Master of Science, Physiology, 1985-1987
University of Akron, Akron, OH, Magna cum laude GPA: 3.8/4.0

Bachelor of Science, Biology, 1981-1985
University of Akron, Akron, OH, Magna cum laude GPA: 3.5/4.0

WORK EXPERIENCE

Virginia-Maryland Regional College of Veterinary Medicine
Assistant Editor, Schalm's Veterinary Hematology Textbook 5th edition, 1998

Virginia-Maryland Regional College of Veterinary Medicine
Clinical Pathology Resident, 1996-1999
- diagnostic cytology, hematology, histopathology, necropsy
- teaching assistant: histology, applied laboratory medicine, clinical pathology, sheep week, clinical biochemistry, general pathology, immunology, and pathobiology journal club.

University of Virginia Department of Internal Medicine/Cardiology
Lab Specialist Advanced, 1989-92
- pharmacology, molecular biology research of adenosine A_1 and A_2 bovine brain receptors
- inventory management in support of laboratory research
- supervision and training of research personnel, technical liaison for collaborating investigators
- radiation safety training course

The Upjohn Company - Pharmaceutical Sales Representative, 1987-89
- human diabetes, CNS, and cardiovascular pharmaceutical products
- product information for private practitioners and regional hospitals
- continuing education seminars for area physicians
- inventory of controlled and noncontrolled physician samples

The University of Akron - Teaching Assistant, 1985-86
- histology laboratory teaching assistant
- natural science biology lecture discussion leader

Akron Children's Hospital - Lab Technician, 1982-85
- virology department-cell culture and virus isolation
- vivarium-care of laboratory animals and in vivo virus isolation
- pathology department-diener to assist pathologists in pediatric autopsies

RESEARCH

Immune Protection Against MNU-Induced Digital Defects. NIH-SERCA (K01) grant pending, priority score 167.

Virginia Tech, Center for Molecular Medicine and Infectious Disease (1998-2001) Immunotoxicity of Dermal Permethrin and Cis-urocanic Acid (Chemical Mixtures in Environmental Health).

Virginia Tech, Center for Molecular Medicine and Infectious Disease (1998-1999) Maternal Immune Protection Against Chemical-Induced Fetal Teratology.

Virginia Tech, Center for Molecular Medicine and Infectious Disease (1998-1999) Influence of Dietary Zinc Form and Level of Supplementation on Broiler Growth and Immune Function, Kemin Industries.

Virginia Tech, Center for Molecular Medicine and Infectious Disease (1998-1999) Utilization of the National Toxicology Program Approved Testing Battery for Chemical-induced Immunotoxicity.

Virginia Tech, College of Vet Medicine (1997) Graduate Research Development Project
-Characterization of a metastatic intestinal adenocarcinoma with Paneth cell, enteroendocrine cell, and goblet cell differentiation in a mature Virginia opossum, with the use of electron microscopy, histochemistry, and immunohistochemistry to characterize the neoplasm.

Chemical Industry Institute of Toxicology (1995) Veterinary Medicine Clerkship
-Physiologically based pharmacokinetic computer modeling, comparison of acetylcholinesterase and carboxylesterase enzyme activity in the presence and absence of various organophosphorous esters in cell culture, tissue homogenates, and in vivo. This clerkship was in partial fulfillment of the Honor's Program senior project at the Virginia-Maryland Regional College of Veterinary Medicine.
-fire safety training course.

Food and Drug Administration (1996) Veterinary Medicine Student Volunteer
-Study of the utilization of physiologically based pharmacokinetic computer modeling in the approval process of pharmaceuticals in the animal and human medical fields. This clerkship was in partial fulfillment of the Honor's Program senior project at the VMRCVM.

Virginia Tech, College of Vet Medicine (1995) Veterinary Medicine Clerkship
-Acetylcholinesterase and carboxylesterase time-response and dose-response assays on rat and mouse brain and SH-SY5Y human neuroblastoma cells and NB41A3 murine neuroblastoma cells in response to organophosphorous ester enzyme inhibition, in partial fulfillment of the senior Honor's Program project at the VMRCVM.
-Development of pharmacokinetic computer models using Stella program.
-Clinical pharmacology: appropriate drug therapy in large and small animal medicine.

The University of Virginia , Division of Cardiology (1989-1992) Lab Specialist Advanced
-Adenosine receptor research in pharmacology and molecular biology: gel electrophoresis, protein purification via column chromatography, HPLC, radiolabelling, receptor-ligand competition and inhibition assays.

The University of Akron, Department of Biology (1985-1987) master's degree candidate research

-dietary and drug effects on rat myocardial catecholamines and left ventricular function.
-Langendorf isolated heart technique, spontaneously hypertensive rats as an animal model.

The University of Akron, Department of Biology (1984) undergraduate research student
-neuroanatomy pilot research to isolate β-endorphin and G-6-PD activity in brain nuclei.

PRESENTATIONS

American College of Veterinary Pathologists, Amelia Island, FL 2000
American Society for Veterinary Clinical Pathology, Amelia Island, FL 2000
Society of Toxicology Poster Presentation, Philadelphia, PA 2000
Virginia Tech 16th Annual Graduate Research Symposium, Blacksburg, VA 2000
VMRCVM 12th Annual Research Symposium, Blacksburg, VA 2000
Virginia Veterinary Medical Association, Hot Springs, VA, invited speaker, 2000
American Society for Veterinary Clinical Pathology, Chicago IL 1999
Eli Lilly and Company, Greenfield, IN, invited speaker 1999
American College of Veterinary Pathologists, St. Louis, MO 1998
American Society for Veterinary Clinical Pathology, Albuquerque, NM 1997
American College of Veterinary Pathologists, Albuquerque, NM 1997
Chemical Industry Institute of Toxicology, Research Triangle Park NC, invited speaker 1996
Federation of American Societies for Experimental Biology, Washington DC 1991
Federation of American Societies for Experimental Biology, Washington DC 1990
Federation of American Societies for Experimental Biology, St. Louis, MO 1986

PUBLICATIONS

Papers

MR Prater, RM Gogal, Jr., J Longstreth, EC DeFabo, SD Holladay. Immunotoxic effects of cis-urocanic acid exposure in C57BL/6N mice, in preparation.

MR Prater, BL Blaylock, SD Holladay. Molecular mechanisms of sunlight and permethrin-induced alterations in cutaneous immunity, in preparation.

MR Prater, RM Gogal, Jr., BL Blaylock, J Longstreth, SD Holladay. Single-dose topical exposure to the pyrethroid insecticide, permethrin in C57BL/6N mice: effects on thymus and spleen, Food and Chemical Toxicology, submitted 11/28/01.

MR Prater, BL Blaylock, RM Gogal, Jr., SD Holladay. Sunlight exposure, mimicked by cis-urocanic acid, increases both immunotoxicity and lethality of dermal permethrin in C57BL/6N mice, in preparation.

RM Gogal Jr., CT Larsen, MS Johnson, MR Prater, RB Duncan, SD Holladay. Influence of orally-administered RDX on the Northern Bobwhite (*Colinus virginianus*). Environ Tox Chem submitted 4/2001.

KL Zimmerman, MR Prater, HS Bender, RB Duncan, GD Boon. A cervical mass from a cat with weight loss. Vet Clin Pathol submitted 5//2001.

RM Gogal Jr., MS Johnson, CT Larsen, MR Prater, RB Duncan, DL Ward, SD Holladay. Influence of dietary 2,4,6-trinitrotoluene exposure in the Northern Bobwhite (*Colinus virginianus*). Environ Tox Chem 21:81-86, 2002.

SD Holladay, LV Sharova, K Punareewattana, TC Hrubec, RM Gogal Jr., MR Prater, AA Sharov. Maternal immune stimulation in mice decreases fetal malformations caused by teratogens. International Immunopharmacology, 2:325-332, 2002.

MR Prater, KL Zimmerman, EG Besteman, GD Boon, GK Saunders. Splenic mass aspirate from an aged pekapoo, in preparation.

MR Prater, RB Conolly, M Ehrich. Comparison of computer modeled kinetic data with in-vitro data for inhibition of acetylcholinesterase activity induced by organophosphorous compounds, in preparation.

MR Prater. Cerebrospinal fluid cytology, USDA Higher Education Challenge Grant sponsored digital textbook, in press.

RM Gogal Jr., MR Prater, BJ Smith, MS Johnson, SD Holladay. Bilateral dissected spleens and thymus in rodents exhibit homogeneity in leukocyte markers. Toxicology 157:217-223, 2001.

K Punareewattana, BJ Smith, BL Blaylock, RM Gogal Jr., MR Prater, J Longstreth, SD Holladay. Topical permethrin exposure inhibits antibody production and macrophage function in C57Bl/6N mice. Food and Chemical Toxicology 39:133-139, 2001.

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HONORS AND AWARDS

2002 Outstanding Graduate Student Award, VMRCVM
2001 VMRCVM "hot team" interviewer
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CL Davis Senior Pathology Resident Award
Va Tech Graduate Student Assembly Travel Grants
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Thomas Moore Academic Scholarship
Top 500 Sales Award
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American Heart Association Summer Fellowship

PROFESSIONAL ASSOCIATIONS

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