Immunotoxicity of Pesticide Mixtures and the Role of Oxidative Stress

by
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ABSTRACT

The immunotoxic effects of multiple pesticide exposure were evaluated. C57BL/6 mouse thymocytes were exposed to lindane, malathion, and permethrin, either separately or in mixtures of two pesticides, in concentrations ranging from 37.5 μM to 1mM. These exposures caused both apoptotic and necrotic cell death in thymocytes as evaluated by 7-aminoactinomycin-D, Annexin-V/PI, and lactate dehydrogenase release assays. When cells were exposed to lindane+malathion, or lindane+permethrin, a significantly greater-than-additive cytotoxicity was observed. The pesticide exposure caused DNA ladder formation with increased laddering in mixtures. Further, the effect of these pesticides on thymocyte oxidative stress was investigated. Thymocytes treated with any of these pesticides generated superoxide and H_2O_2. The lindane + malathion caused more-than-additive increase in superoxide production compared to single treatments of these pesticides. However, the effect of the lindane + permethrin was not significantly different from individual components of this mixture. The effects of pesticides on antioxidant enzymes were also investigated and only mixtures were found to have significant effects. Alteration in transcription factor NFκB level was measured as an indicator of oxidative stress in thymocytes following 12 h pesticide exposure, *in vitro*. Only lindane + malathion was found to increase the protein level. Furthermore, the effects of pesticides and their mixtures on immune functions of mice were studied *in vivo*. Animals (8-12 week old, male mice) were randomly divided into groups of six and injected intraperitoneally with three different doses (one-half, one-third, one-fourth, or one-eight of LD_{50}) of individual pesticides. Exposure to individual pesticides did not alter the thymus/body or spleen/body weight ratios, thymic or splenic cell counts, or CD4/CD8 or CD45/CD90 ratios. However, anti-sRBC plaque forming cell (PFC) counts were significantly lowered with all treatments. Two other groups of animals were injected with lindane + malathion or lindane + permethrin at one-third of the LD_{50} of each pesticide.

Exposure to pesticide mixtures did not alter the CD4/CD8 or CD45/CD90 ratios. However, the thymus/ and spleen/body weight ratios, thymic and splenic cell counts, and PFC counts were significantly lowered. These data indicate that lindane, malathion, and permethrin are immunotoxic and their mixtures can cause higher toxicity compared to individual exposures. In addition, these data support the hypothesis that oxidative stress were induced in thymocytes by exposure to these pesticides *in vitro*.

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To Celal Guney and Deniz Guney…

DECLARATION OF WORK PERFORMED

I declare that I, Selen Olgun, performed all the work described herein, except for the portions of flow cytometric analysis.

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7AAD- 7-aminoactinomycin-D

AhR – Aryl hydrocarbon receptor

AT – 3-Amino-2,3,4,-Triazole

ATPase – Adenosine triphosphatase

β-ME – β-mercaptoethanol

BSA – Bovine Serum Albumin

Con A – Concanavalin A

CTL – Cytotoxic T lymphocytes

Cu – Copper

Cyt P450 – Cytochrome P450

dH_2O – Distilled water

DBTC – Di-n-butyltindichloride

DCF – 2',7'-Dichlorofluorescein

DCF-H – 2',7'-Dichlorofluorescene

DCFH-DA – 2',7'-Dichlorofluoroscin Diacetate

DDA – 2,2-bis (p-chlorophenyl)acetic acid

DDE – Dichloro diphenyl dichloro ethylene

DDT – Dichloro-diphenyl-trichloroethane

DHT – Delayed type hypersensitivity

DMPO – 5,5-dimethylpyrroline-N-oxide

DNA – Deoxyribonucleic acid

EDTA – Ethylenediamminotetraacetic acid

EtOH – Ethanol

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

FFDCA – The Federal Food, Drug, and Cosmetic Act

FIFRA – The Federal Insecticide, Fungicide, and Rodenticide Act

FITC – Fluorescein Isothiocyanate

FQPA – Food Quality Protection Act

G-Px – Glutathione Peroxidase

G-R – Glutathione Reductase

GSH – Reduced Glutathione

GSSG – Oxidized Glutathione

H_2O_2- Hydrogen Peroxide

HCH – Hexachlorocyclohexane

HEPES- N-[2-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5' bi-1H-benzimidazol

Hg_2Cl_2 – Mercuric chloride

IFN-γ – Interferon gamma

IgM – Immunoglobulin M

IgG – Immunoglobulin G

IL-4 – Interleukin-4

I.P. (i.p.) – Intraperitoneal

LC – Lethal Concentration

LD_{50} – Lethal Dose that kills 50% of the population

MALT – Mucosa associated lymphoid tissues

MDA – Malondialdehyde

Mg^{2+} – Magnesium

$MgCl_2$ – Magnesium chloride

NaCl – Sodium chloride

NADH – Nicotinamide adenine dinucleotide

NADPH– Nicotinamide adenine dinucleotide phosphate-Reduced form

NK – Natural killer cell

NaN3 – Sodium Azide

NRC – National Research Council

$O_2^{\uparrow\downarrow}$ – Singlet oxygen

$O_2^{-\bullet}$ – Superoxide Anion

$OH^{\bullet}$ – Hydroxyl radical

oh8dG – 8-hydroxy-2-deoxyguanosine

p – Statistical probability

PBB – Polybrominated biphenyls

PCB – Polychlorinated biphenyls

PBS – Phosphate Buffered Saline

PCR – Polymerase chain reaction

PHH – Polyhalogenated hydrocarbons

ppm – Parts per million

$R^{\bullet}$ – Organic radicals

RNA – Ribonucleic acid

ROS – Reactive Oxygen Species

R-PE – R-Phycoerythrin

RPMI 1640 – Roosevelt Park Memorial Institute Medium

SE – Standard Error of Mean

SOD – Superoxide Dismutase

SRBC – Sheep red blood cells

TBE – Tris-Borate-EDTA

TCDD – 2,3,7,8-tetrachlorodibenzo-p-dioxin

TEMPO – 2,2,6,6-tetramethylpiperidine-*N*-oxyl

Th1 – T helper lymphocytes type 1

Th2 – T helper lymphocytes type 2

TNF – Tumor necrosis factor

USDA – The United States Department of Agriculture

U.S.EPA – The United States Environmental Protection Agency

UV – Ultraviolet

Zn – Zinc

Chapter 1. INTRODUCTION

1.1. Study Hypothesis

The hypothesis tested was that the concurrent exposure to more than one pesticide could cause additive, synergistic, or antagonistic toxic effects on immune parameters. It was expected that the alterations could occur due to the possible interaction between these chemicals, and the generation of reactive oxygen species (ROS) during exposure to these pesticides might, in part, be responsible for the cellular injury or cell dysfunction. Support of the hypothesis would improve our understanding on cumulative effects of these chemicals for risk assessment, and provide insight on pesticide mixture effects on immune cells.

1.2. Study Rationale

It is accepted that human environmental exposures are often not to just single chemicals (Simmons, 1995). Instead, humans and animals are exposed to multiple chemicals sequentially or concurrently (Simmons, 1995). In contrast to this reality, most toxicity testing and mechanistic research on environmental chemicals has been done on single chemicals (Yang, 1994). Although the research on single chemical toxicity provided valuable information for risk assessment, the data are not sufficient to explain chemical mixture toxicity. Pesticides, toxic chemicals that are developed to destroy pests, are also traditionally studied as single compounds. This research was initiated to improve understanding of toxic effects of multiple pesticide exposures. The experiments were performed on immune cells, because the immune system is considered to be sensitive to chemicals at low dose levels when no other system toxicity is evident (Sharma et al., 1987). Additionally, several drugs and environmental chemicals were shown to have the potential to unintentionally impair different parts of the immune system (Galloway and Depledge, 2001; Ahmed, 2000; Vial et al., 1996).

This research examined three compounds from three different classes of pesticides. Lindane (γ-isomer of hexachlorocyclohexane) is an organochlorine insecticide and an inducer of the mixed function oxidase enzyme system (Junqueira et al., 1997; Barros et al., 1991). Malathion, an organophosphate insecticide, is an acetylcholinesterase inhibitor (Rodgers, 2001). Permethrin, a synthetic pyrethroid, is a moderately toxic insecticide (Rodgers, 2001). The underlying principle of this study was that the reactive oxygen species (ROS) generated during exposure to these pesticides may, in part, be responsible for the cellular injury or cell dysfunction.

In mammalian cells, ROS are produced during autoxidation of certain biomolecules and several enzyme-catalyzed reactions and are vital for several biological processes. They are produced during arachidonic acid metabolism leading to prostaglandin and leukotriene synthesis, and are used therapeutically for relaxation of smooth muscle cells to control blood pressure. Also, during phagocytosis, neutrophils and macrophages generate superoxide anion and other ROS to destroy foreign molecules (Knight, 2000). Reactive species causing these effects are superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($O_2^{\uparrow\downarrow}$), and organic radicals ($R^{\bullet}$) (Marks et al., 1996).

Under normal physiological conditions, endogenous ROS sources do not place biological tissues and cells at risk because there are adequate functioning protective systems (Reed, 1995). The antioxidant enzyme system, including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase enzymes, as well as antioxidant molecules such as glutathione, and vitamins C and E, keep cells in balance against the accumulation of ROS. However, protective systems can be overwhelmed by xenobiotics (foreign substances). These chemicals, or their metabolites, could be chemically unstable and capable of interacting with cell macromolecules, and lead to oxidative events in cells (Reed, 1992). This is known as *oxidative stress*, and it may contribute to overall toxicity of a particular chemical. This interaction could cause damage in cellular structures and functions. For example, cell membranes are usually involved in toxic reactions, because the reactive metabolites are often produced at the endoplasmic reticulum, where the

cytochrome P-450 enzyme system is located. ROS formation from the modification of a parent molecule by cytochrome P-450 leads to alkylation of cellular proteins, extensive conjugation and depletion of intracellular glutathione, protein thiol oxidation, and lipid peroxidation (Comporti, 1987).

It has been suggested that certain pesticides may be able to alter the oxidative balance of cells. For example, an organochlorine insecticide dieldrin induced hepatotoxicity via oxidative stress in mice (Bachowski et al., 1998). Toxaphene, a persistent organochlorine pesticide, induced superoxide production in human neutrophils (Gauthier et al., 2001). Different proportions of LD_{50} doses of TCDD, endrin, naphthalene, and sodium dichromate (VI) increased hepatic lipid peroxidation and DNA fragmentation (Bagchi et al., 2000). Deltamethrin, a pyrethroid insecticide, administration to rats resulted in DNA fragmentation in the testicular cells with increased plasma levels of nitric oxide and lipid peroxides (El-Gohary et al., 1999). Toxicity of cypermethrin, a pyrethroid insecticide, was correlated with increase in reduced-glutathione content in rat erythrocytes as an initial response to increased oxidative stress (Kale et al., 1999).

Previous studies suggest that injury to the immune system may also be caused by increased ROS production (Del Rio et al., 1998; Frei B., 1991; Hernanz et al., 1990). Optimal immune response requires proper elimination of these oxygen radicals. Despite their obvious vulnerability to oxidants, immune cells regularly use ROS in several functions. Activated neutrophils and macrophages produce free radicals via respiratory burst during phagocytosis (Laurent et al., 1991). Activated B and T lymphocytes also produce increased amounts of ROS (Dorseuil et al., 1992; Hunt et al., 1991). The increase in ROS formation creates a signal transduction pathway to elicit cellular events such as increased expression of cell surface adhesion molecules, changes in protein phosphorylation, and activation of nuclear transcription factors (Bradley et al., 1993; Marui et al., 1993; Schreck et al., 1991a, b). Chemotactic response and random migration of macrophages can be enhanced with supplementation of antioxidants (Ball et al., 1996; Johnston et al., 1992).

There are several studies indicating that the organochlorine insecticide lindane causes toxicity to liver cells through oxidative stress (Junge et al., 2001; Giavarotti et al., 1998; Junqueira et al., 1997; Videla et al., 1991). However, there are very few studies related to lindane toxicity via oxidative stress in immune cells. In a study by Koner et al. (1998) oral administration of lindane markedly suppressed the IgM production, and simultaneous treatments with antioxidant ascorbic acid attenuated the effects of lindane on lipid peroxidation and humoral immune suppression. There is only one recent study with the organophosphate insecticide malathion that suggested it caused toxicity in the immune system via oxidative stress (Galloway and Handy, 2003). The authors recommended that immunotoxicity was likely to occur through oxidative damage to immune organs, or via chronic effects of altered metabolism/nutrition on immune organs. The contribution of oxidative stress to the toxicity of the third insecticide used in the present research, permethrin, was suggested in two recent studies (Abu-Qare and Abou-Donia, 2003; Abu-Qare et al., 2001). Neither of these reports, however, investigated oxidative stress on immune parameters. Furthermore, the combined toxicity of these three pesticides on immune cells has not been studied before.

Although immune cells normally control intracellular ROS formation, multiple pesticide exposure may increase the extra-cellular ROS, and this may alter the control of ROS levels inside the immune cells. This shift in oxidative status of cells may influence the overall immune response. The uncontrolled change in ROS levels has been shown to differentiate certain families of genes in cells (Curutti and Trump, 1991). Reactive oxidants can induce the transcription of several genes such as c-fos, c-jun, c-myc and b-actin (Puri et al., 1995a). Also, two transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), were shown to be regulated by the intracellular redox status (Sen et al., 1996). These findings suggest that ROS are being used as second messengers in the activation of signal transduction pathways to lead to cytotoxicity. In T lymphocytes, ROS activate many signaling systems. The resultant increase in tyrosine phosphorylation activates protein kinase C, the Ras pathway and the NF-κB transcription factor (Schulze-Osthoff et al., 1995). NF-κB is a eukaryotic transcription factor and plays an important role in regulation of several genes involved in response to pathogens and

cellular defenses. Many immunologically relevant genes, mainly those encoding cytokines and cytokine receptors, growth factors, and cell adhesion molecules, contain functional NF-κB binding sites in their promoter and enhancer regions (Baeuerle et al., 1994; Grilli et al., 1993). The inactive form of NF-κB is present in the cytoplasm and it is a heterodimer of 50 kDa DNA-binding (p50) and 65 kDa DNA-binding (p65) subunits bound with a inhibitory (IκB, RelA) unit. In activated T lymphocytes, for example, NF-κB complexes are composed of a p50/c-Rel heterodimer. In unstimulated T lymphocytes, a transcriptionally inactive p50 homodimer occupies κB sites and inhibits access of active complexes to DNA (Kang et al., 1992). Activation of cells results in the rapid release of IκB, which allows subunits to translocate to the nucleus and to bind to their DNA sequences (Schulze-Osthoff et al., 1995).

In addition to the primary role of NF-κB to activate defense genes in immune cells during inflammatory response, it has also been shown to function during programmed cell death (apoptosis) in normal and transformed murine B cells (Wu et al., 1996). Several studies have shown that certain pesticides can induce apoptosis. For example, organochlorine pesticides heptachlor, chlordane and toxaphene induce apoptosis in monkey leukocytes (Miyagi et al., 1998). Another organochlorine pesticide, endosulfan, causes dose- and time-dependent apoptosis in a human T-cell leukemic cell line (Kannan et al., 2000). N-nitrosocarbofuran, the major metabolite of the carbamate pesticide carbofuran, induces apoptosis of Chinese hamster lung fibroblasts (Yoon et al., 2001).

Because of the involvement of NF-κB both in response to oxidative stress and in apoptotic cell death, it is reasonable to expect that expression of this transcription factor may play a role in the pathogenesis of pesticide induced cell injury. This was investigated as part of the present dissertation research.

1.3. Specific Aims

The overall goal of this research was to increase understanding of the immunotoxic effects of multiple pesticide exposure, and the role of oxidative stress in pathogenesis. These data are needed for future risk assessment studies of pesticide mixtures.

Aim 1: *The ability of pesticides from different classes to induce apoptosis and/or necrotic cell death on thymus cells, in vitro*:

Freshly isolated lymphocytes from thymus of male C57Bl/6, 8-12 week old mice were exposed to varying concentrations of three different pesticides to assess concentration-response relationships for apoptotic and necrotic cell death. The pesticides were lindane, an organochlorine insecticide; malathion, an organophosphate insecticide and acetylcholinesterase inhibitor; and permethrin, a synthetic pyrethroid. Flow cytometric 7-aminoactinomycin D and colorimetric lactate dehydrogenase enzyme release assays were used for the evaluation of apoptotic and necrotic cell death.

Aim 2: *The immunotoxic potential of pesticides mixtures, in vitro*:

Concentrations that cause minimum cell death ($\leq LC_{25}$) were chosen from the concentration-response curves of each pesticide for mixture studies. The effects of malathion and permethrin were evaluated with the combination of lindane. Cytotoxicity of lindane + malathion and lindane + permethrin mixtures on thymocytes were evaluated with multiple assays, including 7-aminoactinomycin D (7-AAD), Annexin-V/ Propidium iodide, DNA ladder, and lactate dehydrogenase (LDH) enzyme release assays. The use of more than one assay strengthens the evaluation of apoptosis and necrosis.

Aim 3: *The effects of pesticide mixtures on the immune system, in vivo*:

In addition to *in vitro* studies, the mode of toxicity with pesticide mixtures was also studied *in vivo*. Animals were randomly divided into groups of six and injected intraperitoneally with different doses (one-half, one-third, one-fourth, or one-eight of LD_{50}) of individual pesticides. Thymus/body and spleen/body weight ratios, thymic and splenic cell counts, anti-sheep red blood plaque forming cell assay, CD4/CD8 and

CD45R220/CD90.2 phenotyping of cells were performed to evaluate immune parameters. From individual exposure data, one-third of LD_{50} of each pesticide was selected for the mixtures studies. Animals were randomly divided into groups of six and injected intraperitoneally with the mixture of one-third of LD_{50} of two pesticides (lindane + malathion and lindane + permethrin). Tests were repeated to evaluate immune parameters. The effects of individual and mixture exposures of pesticides on drug metabolizing liver enzyme system cytochrome P-450 were also studied to evaluate whether these compounds alter the enzyme levels.

Aim 4: *The role of oxidative stress in pesticide induced immunotoxicity:*

a) Reactive oxygen species (ROS) production: To investigate the role of ROS in the immunotoxic responses, pro-oxidant and anti-oxidant status of thymocytes were examined, *in vitro*. H_2O_2 production in cells following pesticide exposure was monitored using dichlorofluorescein diacetate (DCFH-DA) assay. Superoxide anion ($O_2^{-\bullet}$) production was measured via flow cytometry using hydroethidine-ethidium bromide assay.

b) Specific activities of antioxidant enzymes: Levels of antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase were measured spectrophotometrically prior to and following pesticide exposure in thymocytes, *in vitro*.

c) Regulatory proteins: Possible induction of transcription factor NFκB (induced by oxidative stress) in thymocytes was studied prior to and after pesticide exposure, *in vitro*. NFκB protein levels in thymocytes were detected by western blotting analysis.

Chapter 2. LITERATURE REVIEW

2.1. Pesticide Usage

The registry and use of toxic chemicals are controlled by four federal agencies in the United States. These are the Environmental Protection Agency, the Food and Drug Administration, the Occupational Safety and Health Administration, and the Consumer Product Safety Commission (Beck et al., 1994). The United States Environmental Protection Agency (U.S.EPA), in cooperation with the Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA), is responsible for regulating the production and use of *pesticides* (U.S.EPA-2).

The U.S.EPA defines *pesticide* as "any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest" (U.S.EPA-1). Although, the term pesticide is often misunderstood to refer only to insecticides, it also applies to herbicides, fungicides, and various other substances used to control pests. *Pests* are "living organisms that occur where they are not wanted or that cause damage to crops or humans or other animals". Insects, mice, unwanted plants (weeds), fungi, and microorganisms such as bacteria and viruses are some examples of pests.

The U.S.EPA regulates pesticides under two laws, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). Briefly, FIFRA mandates that all pesticides sold in the United States (including imported pesticides) have to be registered by the U.S.EPA. Under the FIFRA, the EPA has to balance the risks and benefits of a pesticide use. However, under the FFDCA, the EPA had to follow the regulation set by the Delaney Clause until the late 1990's. The Delaney Clause required that the acceptable risk from carcinogens had to be *zero* when they were used as food additives. This requirement proved to be very difficult to apply, because there were improvements in analytical chemistry after the introduction of the Delaney Clause in 1958 (Public Law, 1958). Recently developed techniques allow scientists to detect chemical concentrations hundred to thousand times lower than they could detect in

the 1950's. Also, several chemicals naturally found in food were shown to be carcinogenic in laboratory animals (U.S.FDA, 1986). All these developments made it clear that the *absolute safety* was impossible to reach. To improve on these difficulties in regulation, a new law called the Food Quality Protection Act (FQPA) was signed in 1996 (U.S.EPA-3). The FQPA of 1996 was also an important response to public concern about increased chemical usage in food.

The FQPA replaced the Delaney Clause, and by that, the *zero tolerance* rule for potential carcinogens in food was replaced by "reasonable certainty of no harm". The EPA now must have data assuring that the compound will not cause unreasonable risk before registering it as a pesticide. Under the FQPA, FFDCA now requires the EPA "to set pesticide tolerance limits for all pesticides used in food". Tolerances (allowable limits) have to consider non-occupational exposure to pesticide, susceptibility of infants or children, and whether the pesticide produces a similar effect to that of naturally occurring estrogens (U.S.EPA-4). The FQPA also suggests that the risk assessments should be based on similar modes of action when pesticides are used in mixtures. The EPA is currently reviewing the allowable limits (tolerances) for pesticide residues in food. By August 2006, the EPA is expected to complete the review of all tolerances that were in effect in August 1996 when the Food Quality Protection Act of 1996 was passed (U.S.EPA-4).

According to the most recent reports published by the EPA, worldwide spending for pesticides was more than $33.5 billion in 1998 and 1999 (U.S.EPA-5). Herbicides accounted for the largest portion of total spending (more than 40%), followed by insecticides, fungicides, and other pesticides. In the U.S. alone, $11 billion was spent for pesticides during 1998 and 1999 (U.S.EPA-5).

Development of chemicals for use as pesticides is important in agriculture. About 70 percent of all pesticides sold in U.S. are for the agricultural use (Aspelin, 1997). According to a report published by Pimentel et al. (1992), for each dollar invested in pesticide control approximately 4 dollars in crops can be saved. Because of this high rate

of effective return, use of conventional pesticides on farms increased from about 400 million pounds (active ingredient) in the 1960s to over 800 million pounds in the late 1970s and early 1980s (NRCS, USDA).

During the 1960's, insecticides, accounting for about half of all pesticides used, dominated agricultural pesticide usage. The quantity of insecticides used on fields decreased in time, as the organochlorines (i.e., DDT, aldrin) were replaced by pyrethroids and other chemicals that were less persistent. Today, 70 percent of the pesticides used in agriculture are herbicides (NRCS, USDA).

Although increased use of chemicals in agriculture provides abundant inexpensive food, it has also created environmental problems. When the chemical revolution first started (1950's) there was little concern about environmental consequences. Early toxicity testing assumed that DDT and other agricultural chemicals were generally not harmful to humans. By the mid-1960's, however, there was a growing awareness that some agricultural chemicals were damaging the environment, and may have been affecting humans as well (NRCS, USDA). The development of sensitive chemical testing procedures helped us understand that agricultural chemicals were not remaining on the fields, but were being washed into streams and rivers and seeping into ground water. Today, pesticide levels in water are monitored routinely. The U.S.EPA began to utilize ground water monitoring for pesticides in 1979 following discovery of aldicarb in ground water in several states. In 1985, 38 states reported that agricultural activity was a known or suspected source of ground water contamination within their borders (ASIWPC, 1985). Monitoring for pesticides in surface water was frequent in the 1960s and 1970s as studies were conducted that led to the banning of organochlorine insecticides.

Most of the pesticides used presently and in the past have also been found in the atmosphere, including DDT, toxaphene, dieldrin, heptachlor, organophosphorous insecticides, triazine herbicides, alachlor and metalochlor (USGS, 1995).

Due to concerns about potential risks to human health and to the environment, the EPA first banned use of some organochlorine pesticides for agricultural purposes in the 1970's, and has since limited the use of many other pesticides.

2.2. Chemical Mixtures

It is generally accepted in today's world that human environmental exposures are not to single chemicals. Instead, humans, and animals, are exposed to multiple chemicals either concurrently or sequentially (Simmons, 1995). In contrast to this reality, the vast majority of the toxicology studies examine the cancer and non-cancer health effects of only single chemicals. A review by Yang (1994) reported that 94.3% of 122 technical reports published by the National Toxicology Program in recent years were on the chronic toxicity and carcinogenicity of single chemicals.

To address concerns over health risks from chemical mixtures, the U.S. EPA published guidelines for the "Health Risk Assessment of Chemical Mixtures" in 1986 (U.S.EPA, 1986). Subsequent to this, the EPA published another document to provide more information on toxicity of whole mixtures and on toxicological interactions between components in mixtures (U.S.EPA, 1990). Following these guidelines and the National Research Council's recommendations (NRC, 1993), the number of studies on chemical mixtures increased. In the 1993 report, NRC recommended that the EPA investigate the possibility of synergistic interactions following multiple chemical exposures (prior approach was towards additivity). In 1998, the EPA proposed another initiative, the *Healthy People 2000*, to emphasize the growing concern about chemical mixture exposures. The most recent document on mixtures by the EPA is *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S.EPA, 2000a).

The Food Quality Protection Act also requires consideration of the non-cancer health effects of multiple pesticides for proper risk assessment. According to the numbers published by the EPA in 2001, there were 19,533 pesticide products on the market, and

every year new chemicals are added to this list. Therefore, it is very difficult to do risk assessment on cumulative effect of these chemicals.

The main problem of studying the mixtures of these chemicals is that the mixtures can be extremely complex in their composition. It is difficult to test the dose-response without knowing the proportions of the chemicals in a mixture. The EPA recommends studying chemical mixtures in two ways; one way is to select certain high-priority mixtures and investigate them, the other way is to develop extrapolation methods and use them for mixtures on the available data from single chemicals. The EPA advises that the mixtures that pose the highest public health risk should be investigated primarily (U.S.EPA, 2000a).

The latest EPA guideline (2000a) suggests that risk assessment for chemical mixtures should be done by incorporating the data on modes of toxic action of chemicals. The EPA says that this effort requires the development of new statistical methods and mathematical models.

The second option, to include all the remaining mixtures, is to develop methods that can extrapolate toxicity from available data. Because mixtures can be unstable and result in chemical interactions, the use of extrapolation methods (e.g., cross-species, cross-route) should be studied carefully. Chemical interactions mainly include the toxicologic interactions that influence pharmacokinetics. Others are physiological interactions between affected tissues or organs, and the biochemical interactions affecting degradation and transport of the chemicals. Because there are a wide variety of mixtures, all relevant information has to be used to improve the understanding of the chemicals interaction in mixtures (U.S.EPA, 2000a).

2.3. Pesticides:

2.3.1. Lindane

Lindane (gamma-1,2,3,4,5,6-hexachlorocyclohexane) is an organochlorine insecticide and fumigant. Some of the trade names for lindane are Ambrocide, BoreKil, Gammex, Kwell, and Lorexane (Extoxnet-L).

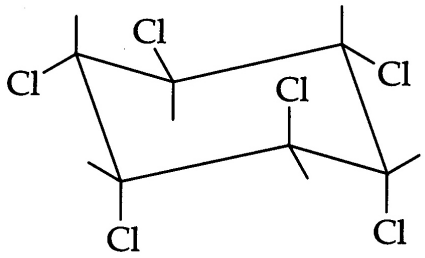

Figure 1. The chemical structure of lindane, the gamma isomer of hexachlorocyclohexane (Reprinted from Rose R.L., Hodgson E., and Roe R.M. Pesticides. In: Toxicology. Marquardt H., Schafer S.G., McClellan R.O., and Welsch F. (eds.). Academic Press, San Diego, CA, Chapter 17, pp. 663-698, Copyright (1999), with permission from Elsevier)

Technical lindane formulation contains the gamma-isomer of hexachlorocyclohexane (HCH). Five other isomers of HCH are commonly found in technical lindane, but the γ-isomer is the predominant one, including at least 99% of the mixture of isomers (Extoxnet-L). None of these isomers or technical-grade HCH is currently produced in the United States. The commercial lindane production was banned in the United States in 1977 (U.S. EPA, 1989). Also, most agricultural and dairy industry uses were cancelled by the EPA due to its potential to bioaccumulate and promote carcinogenesis (Extoxnet-L). Lindane is reported to be persistent in most soils with a field half-life of approximately 15 months (Wauchope et al., 1992). It is very stable in both fresh and salt-water environments, and is resistant to photodegradation (Kidd et al., 1991). According to the EPA, in 1977, over 900,000 pounds of lindane were used in the United States. However,

from 1996 to 2001, only 233,000 pounds of lindane were imported and used (U.S.EPA, 2002).

Lindane is used as a therapeutic scabicide for humans (Budavari et al., 1989). Medically, lindane is used topically for the treatment of head and body lice and scabies. It is available in 1% preparations as a lotion, cream, or shampoo. As an insecticide, it has been used on fruit and vegetable crops, for seed treatment, and in forestry (Extoxnet-L).
The effects of lindane on the body have been extensively studied. It has been shown to have toxicity to liver, kidney, and immune cells. Hepatotoxicity of lindane was demonstrated with its effects on certain enzymes that indicate liver function. For example, rats given 72 mg/kg/day lindane for 2 weeks had elevation in serum aminotransferases and decrease in hepatic soluble enzymes (Srinivasan and Radhakrishnamurty, 1988). When the dose was decreased to 13.5 mg lindane/kg/day in the diet for 12 days, male Wistar rats still exhibited decreased activities of liver lipogenic enzymes and increased levels of serum triglycerides (Boll et al., 1995). Dietary exposure to lindane for 12 weeks increased liver microsomal mixed function oxidase activity and liver weights in female rats fed 32.3 mg/kg/day and male and female CF1 mice fed 21.1 mg/kg/day (Oesch et al., 1982).

Liver hypertrophy and degeneration was reported in several studies with lindane. For example, liver centrilobular hypertrophy increased in a dose-dependent manner beginning at 0.4 mg lindane/kg/day in Wistar rats exposed in their diet for 12 weeks (Suter, 1983). Another study showed that chronic exposure of rats to 8 mg lindane/kg/day in the diet for 104 weeks caused a periacinal hepatocytic hypertrophy (Amyes, 1990). In mice, chronic administration of 13.6–27.2 mg lindane/kg/day in the diet resulted in an increased rate of liver cancer (Wolff et al., 1987). Research done in rabbits demonstrated that 4.21-7 mg/kg/day lindane given by gavage for 4 weeks caused focal degeneration of hepatocytes (Grabarczyk et al., 1990; Kopec-Szlezak et al., 1989) and significant increase of plasma alkaline phosphatase and alanine aminotransferase activities (Cerón et al., 1995).

Oxidative stress has been suggested as a mechanism of lindane-induced hepatotoxicity (Azzalis et al., 1995; Puri and Kohli, 1995b; Junqueira et al., 1993; Videla et al., 1991; Barros et al., 1988 and 1991). Oxidative stress was characterized in the rat liver by reduction in hepatic glutathione levels and decrease in superoxide dismutase and catalase activities (Junqueira et al., 1997; Barros et al., 1991). This was accompanied by a decrease in lipid peroxidation, and an increase in microsomal superoxide anion generation coupled to cytochrome P-450 induction (Junqueira et al., 1997). However, species differences in lindane-induced effects on hepatic enzymes activities have been reported, with 10 mg/kg/day for 6 days increasing hepatic cytochrome P-450 in the rat, but not in the rabbit or monkey (Puri and Kohli, 1995). Inhibition of Mg^{2+}-ATPase activity has also been observed in rat liver, suggesting ATPase enzyme sensitivity to lindane (Gopalaswamy and Aiyar, 1984). These authors suggested that some toxic effects of lindane in mammals may occur from its influence on ATPase activity.

Lindane has also been reported to be toxic to the kidneys. For example, lindane has been shown to produce histopathological changes in the proximal tubule epithelial cells (Dietrich and Swenberg, 1990 and 1991). These changes included the accumulation of protein droplets, hypertrophy and necrosis, pyknotic nuclei, and epithelium regeneration, following gavage doses of 10 mg/kg/day of lindane for 4 days in Fischer-344 rats. Other changes such as significantly increased excretion of glucose in urine, and hypertrophy and degeneration of the renal tubular epithelia, were observed in Wistar rats exposed to 72 mg/kg/day of lindane for up to 2 weeks (Srinivasan and Radhakrishnamurty, 1988). Kidney toxicity was also seen in male rats exposed to 0.07-7 mg/kg/day lindane for 2 years, with hyaline droplets noted in renal proximal tubules at 0.07 mg/kg/day, and increased kidney weights, higher urinary protein excretions and tubular necrosis at 7 mg/kg/day (Amyes, 1990).

Lindane also has effects on immune parameters. Kashyap (1986) reported a statistically significant increase (approximately 18%) in IgM levels in individuals occupationally exposed to technical-grade hexachlorocyclohexane. In animal studies, rats showed depressed antibody response to *Salmonella* antigens after oral lindane exposure of 6.25

and 25 mg lindane/kg/day for 5 weeks (Dewan et al., 1980). Lindane exposure has also been shown to cause thymus cortex atrophy, suppression of bone marrow cellularity, and decreased granulocyte-macrophage progenitor cells in mice with doses as low as 10–20 mg/kg/day for 10 days. (Hong and Boorman, 1993). Humoral immune response, as shown by antibody response to injected sheep red blood cells (sRBC), was suppressed in rats that were exposed to lindane in dietary doses of 3.6 or 7 mg/kg/day for 8 weeks (Koner et al., 1998). The IgM antibody response to sRBC was also suppressed in albino mice after exposure to 9 mg/kg/day lindane in the diet for 12 weeks (Banerjee et al., 1996a). Meera et al. (1992) reported an initial immune-stimulation followed by immunosuppression in mice fed 0.012, 0.12, or 1.2 mg lindane/kg/day for 24 weeks, with decreased lymphocyte populations and necrosis in the thymus, and a reduction in overall cellularity in the spleen in the high dose group.

Although lindane can remain in the body for a relatively long period of time, it does not necessarily remain intact. Microsomal enzyme metabolism was shown to be important in detoxification in a study using DBA/2 and C57BL/6 mice (Liu and Morgan, 1986). The blood and brain concentrations and the toxicity of lindane were greater in DBA/2 mice, a strain of mice in which microsomal enzymes are not induced. Other investigators also demonstrated the importance of hepatic microsomal enzymes in the detoxification of lindane. For example, pretreatment of rats with hepatic enzyme inducers, such as Aroclor 1254, increased the excretion of lindane metabolites in urine nearly 4-fold (Chadwick et al., 1981).

2.3.2. Malathion

Malathion [0,0-dimethyl-S-(1,2-dicarbethoxyethyl) dithiophosphate] is a nonsystemic, wide-spectrum organophosphate insecticide (Kidd and James, 1991; Extoxnet-M). Trade names for products containing malathion include Celthion, Dielathion, Emmaton, and Maltox.

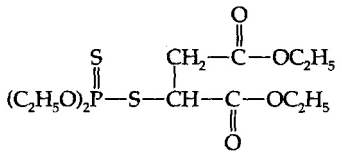

Figure 2. The chemical structure of malathion.
(Reprinted from Rose R.L., Hodgson E., and Roe R.M. Pesticides. In: Toxicology. Marquardt H., Schafer S.G., McClellan R.O., and Welsch F. (eds.). Academic Press, San Diego, CA, Chapter 17, pp. 663-698, Copyright (1999), with permission from Elsevier)

Malathion is used for the control of sucking and chewing insects on fruits and vegetables, and is also used to control mosquitoes, flies, household insects, animal parasites (ectoparasites), and head and body lice (Extoxnet-M).

Malathion is a protoxicant that must be oxidized to malaoxon before it is effective as an insecticide. This can be done in the environment, where it can be activated by atmospheric photooxidation. Malathion and malaoxon are degraded by microorganisms found in sediment and soils. Malaoxon is more toxic than malathion and it may also be present as an impurity in the parent compound (Extoxnet-M).

Malathion is applied in million pound quantities worldwide because of its potent insecticide activity and relatively low mammalian toxicity (Rodgers and Ellefson, 1992). The average annual use of malathion in the United States is 16.7 million pounds. Approximately 12.5 million pounds are applied to agricultural crops, and nearly 90% of that is applied to cotton. Another 0.3 million pounds are applied as post harvest grain treatment to corn, wheat, and oats. Roughly 3.4 million pounds are used for other purposes, such as control of medflies, and mosquito eradication in areas of malaria outbreak (U.S.EPA, 2000b).

Acute exposure to malathion produces clinical signs and symptoms of cholinergic poisoning, which is typical of organophosphate poisoning. Cholinergic poisoning occurs because neural acetylcholinesterase is inhibited by the active metabolite of malathion,

malaoxon. Malaoxon combines with the esteratic site of this enzyme in a reaction that is essentially irreversible. Acetylcholinesterase inhibition prevents the breakdown of the acetylcholine in the central and peripheral nervous systems. Continuous presence of acetylcholine at parasympathetic autonomic muscarinic receptors results in ocular effects (miosis, blurred vision), gastrointestinal effects (nausea, vomiting, abdominal cramps, diarrhea), respiratory effects (excessive bronchial secretions, bronchoconstriction), cardiovascular effects (bradycardia, decreased blood pressure), and effects on the bladder (incontinence) (Extoxnet-M).

The toxic effects of malathion have been studied on the nervous and immune systems. Many studies on malathion's effects on the nervous system appear in the literature that describes its inhibitory effect on acetylcholinesterase.

Effects on the nervous system were reported by Lamb (1994a) after 500–2,000 mg/kg/day malathion were fed to rats in short term feeding. Clinical signs of organophosphate intoxication were also observed in the 90 day feeding study in rats administered approximately 1,500 mg/kg/day malathion (Lamb, 1994b). Lower doses of malathion inhibited cholinesterase activities with 395 mg/kg/day malathion in the diet for 90 days causing 15–30% decrease in plasma cholinesterase activity, 49–53% decrease in red blood cell cholinesterase, and 12–20% decrease in brain cholinesterase levels. In another study, 411 mg/kg/day of malathion fed to rats for 7 days resulted in recurrent convulsions, and tremors. None of these effects were seen at the 163 mg/kg/day level (Ojha et al., 1992). In a study by Ehrich et al. (1993) a single gavage dose of 88% pure malathion was administered to rats at doses of 600, 1,000, or 2,000 mg/kg. The animals were evaluated at 7, 14 and 21 days after administration to test behavioral and central nervous system excitability, autonomic effects, and muscle tone and equilibrium. Significant changes were found mostly during the 21st day examination and were mostly indicative of increased excitability. The authors did not observe any pathologic lesions in segments of the medulla, cervical and lumbar spinal cord, or cerebellum 21 days after dosing (Ehrich et al., 1993).

Cholinergic poisoning was also noted in mice exposed to malathion. Casale et al. (1983) reported tremors, fasciculations, and excessive salivation in mice following a single 720 mg/kg malathion (95% pure) administration. However, Rodgers et al. (1986a) did not observe these signs after a single dose of 715 mg/kg or 14 doses of 143 mg/kg/day of recrystallized malathion (>99% pure). These studies suggest that malathion impurities may play a role in causing these toxicities.

The toxicity of malathion on the immune system has been examined mainly in rats and mice. Rodgers et al. (1986a) did not observe any clinical signs of cholinergic poisoning but reported alterations in several immune parameters at a 715 mg/kg dose level. In this study, the single dose of malathion had no effect on the thymocytes counts and on the cytotoxic T lymphocyte (CTL) response to alloantigen, but increased the splenic IgM production to sheep red blood cells (sRBC), and significantly increased the proliferative response to concanavalin A (Con A). In contrast, in vitro exposure of splenocytes to malathion resulted in a suppression of the proliferative response to Con A and lipopolysaccharide (Rodgers and Ellefson, 1990). In the same study, administration of single doses of 715 or 900 mg/kg malathion to mice significantly elevated respiratory burst activity, a measure of macrophage activation of peritoneal leukocytes. However, this elevation was not found after *in vitro* treatment of cells.

Rodgers and Xiong (1997a) suggested that malathion induced degranulation of mast cells followed by release of tumor necrosis factor or histamine, may increase macrophage function. The authors observed increased serum levels of histamine in both rats and mice after administration of malathion with a maximal effect 4 hours after a dose of 10 mg/kg (Rodgers and Xiong, 1997b). To further investigate the role of mast cell degranulation in malathion induced toxicity, Rodgers et al. (1996) used mast cell-deficient mice. In this study, administration of 300 mg/kg malathion reduced the macrophage function. However, when these animals were transplanted with bone marrow-derived mast cells from wild-type mice, macrophage function improved. This also enhanced the production of circulating IgM antibodies to sRBC on days 3 and 5 after immunization.

2.3.3. Permethrin

Permethrin (3-phenoxybenzyl-cis, trans-3- (2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) is a broad-spectrum synthetic pyrethroid insecticide (Kidd and James, 1991; Extoxnet-P).

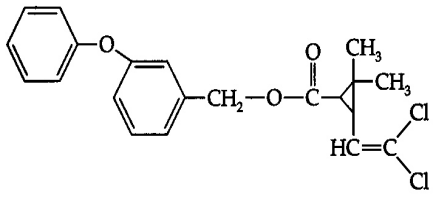

Figure 3. The chemical structure of permethrin.
(Reprinted from Rose R.L., Hodgson E., and Roe R.M. Pesticides. In: Toxicology. Marquardt H., Schafer S.G., McClellan R.O., and Welsch F. (eds.). Academic Press, San Diego, CA, Chapter 17, pp. 663-698, Copyright (1999), with permission from Elsevier)

Permethrin has been registered by the U.S.EPA since 1977. It is currently registered and sold in a number of products such as insect sprays for households and yards, flea dips and sprays for cats and dogs, products for agricultural and livestock use, and product for mosquito eradication (U.S.EPA-6). All products with agricultural uses (except livestock uses) are *Restricted Use Pesticides* because of their possible adverse effects on aquatic organisms (Extoxnet-P).

There are two different types of pyrethroids. They are grouped based on the differences in chemical structure and the poisoning symptoms seen in laboratory animals (Coats, 1990). Briefly, Type I pyrethroids (such as permethrin) do not include a cyano group, but Type II pyrethroids (such as cypermethrin) include a cyano group. Also, in Type I pyrethroid toxicity clinical symptoms include whole body tremors, elevated body temperature, and coma, while Type II pyrethroid toxicity is characterized by increased salivation, increased startle response and choreoathetosis (involuntary movements of the limbs, trunk, and facial muscles) (Coats, 1990).

Available data from animal studies show that the nervous system is the primary target of pyrethroid toxicity (pyrethroids work by paralyzing the nervous system of insects) (U.S.EPA-6). In addition, a few recent studies indicated its toxic effects on reproductive and immunological systems.

Due to its moderate to low toxicity, permethrin is used to treat military uniforms to prevent insect-borne diseases, such as leishmaniasis, (Schreck and Kline, 1989). It was used during the 1991 Gulf War, and there have been suggestions that this contributed to neurological health problems (Gulf War Illness) reported by about 30,000 veterans (Abou-Donia, 1996). However, these allegations have not yet been substantiated experimentally.

Topical exposure to permethrin may have some relation to some symptoms appearing in military personnel who served in the 1991 Gulf War. For example, to study the toxic effects following topical exposure to permethrin, Punareewattana et al. (2001) applied permethrin to the shaved dorsal interscapular region of C57Bl/6N mice at doses of 0.5, 1.5 or 5.0 μl /day (doses corresponded to 22–220 mg/kg/day topical insecticide). Animals were exposed to permethrin daily for 10 or 30 consecutive days, or every other day for 7 or 14 exposures. These doses did not inhibit the phagocytic ability of macrophages, but antibody production as shown by plaque-forming cell (PFC) assay was decreased significantly after 10 consecutive days of exposure to permethrin. This study did not evaluate neurotoxic actions of the permethrin. In another study related to insecticide exposure in the 1991 Gulf War, Karen et al. (2001) treated C57BL/6 mice with permethrin with three intraperitoneal doses of 0.2-200 mg/kg for 2 weeks. These treatments altered maximal [3H]dopamine uptake in striatal synaptosomes in treated mice along with a decrease in open field behavior at the highest dose tested.

In terms of the neurotoxic effects of permethrin, many acute and chronic toxicity studies provided information. Ishmael and Lithfield (1988) investigated the effects of permethrin in a chronic toxicity study on rats (2 years), and mice (lifetime). Alpk:AP (Wistar-

derived) rats were fed diets containing 0, 500, 1000 or 2500 ppm permethrin for 2 years and Swiss-derived mice were maintained for their lifetime on diets containing 0, 250, 1000, or 2500 ppm permethrin. Toxicologically significant changes were limited to the highest dose of 2500 ppm permethrin in both species. During the first 2 weeks of treatment, high-dose male and female rats exhibited slight whole body tremors, hypersensitivity to noise and other disturbances, and piloerection. These findings were not seen at lower dose levels, or in mice. Pathological examination of the central and peripheral nervous systems did not show any abnormalities related to permethrin exposure. The effect on mice at 2500 ppm permethrin was demonstrated by decreased body weight gain. Other studies also examined neurological effects of permethrin. For example, oral exposure to permethrin at 120 mg/kg level increased tremors in rats (Herr et al., 1986). A 7 day subacute dosing regimen with permethrin caused a transient functional impairment in rats (Rose and Dewar, 1983). This treatment also caused significant increases in beta-glucuronidase and beta-galactosidase in the distal portion of the sciatic/posterior tibial nerves 4 weeks after the start of dosing.

There are few publications on the immunotoxic effects of permethrin. Diel et al. (1998) examined the effects of permethrin by using the peripheral blood lymphocytes from three groups of dermatitis patients. These groups included 3 non-atopic permethrin exposed patients after 6 months indoor contamination with permethrin spray, 4 atopic patients showing history of allergy, and 4 nonatopic controls. Permethrin induced histamine release with a significant inter-individual variation but no significant differences among the three groups. Permethrin caused a decrease from 270 pg/ml to 186 pg/ml in IFN-γ levels and from 200 pg/ml to 60 pg/ml in IL-4 (interleukin-4) levels in the atopic group. In another *in vivo* study, Blaylock et al. (1995) evaluated the immunotoxic effects of permethrin in BALB/c mice splenocytes after 0–0.4 mg/kg/day oral administration for 10 days. The authors reported significantly reduced mixed lymphocyte responses, T-lymphocyte cytotoxic activity, and natural killer cell activity with the highest test dose. There were no significant changes in spleen weights with any of the treatment groups. In another study, Prater et al. (2002) studied the effects of 5-25 µl (equivalent to 220-1100 mg/kg body weight) single topical exposure to permethrin in C57BL/6N mice. The

authors observed 32% inhibition in splenic T cell proliferation, 80% decrease in thymic cellularity, and 72% inhibition in splenocyte proliferation at 25 μM. Permethrin also increased splenic hypocellularity by 31% at 15 μl, and by 50% at 25 μl doses (Prater et al., 2002). Stelzer and Gordon (1984) exposed murine splenocytes to permethrin *in vitro* in a concentration range of $1x\ 10^{-5}$ M to $5x10^{-5}$ M, and reported an inhibition in mitogenic response to concanavalin A.

Pyrethroids may also have effects on the reproductive system. The estrogenic potential of permethrin was studied by Go et al. (1999) using the MCF-7 human breast carcinoma cell line. In this study, permethrin did not affect pS2 expression, but it had a noticeable effect on cell proliferation at 100 μM. In another study by Garey and Wolff (1998), cells from the Ishikawa Var-I human endometrial cancer cell line and the T47D human breast cancer cell line were exposed to 30 μM permethrin for 48 hours. Permethrin did not produce a significant estrogen antagonist activity or acted as progestin in these cells.

2.4. Immune System

The *Immune System* consists of organs, cells, and molecules whose complex interactions form an efficient system to protect an individual from both outside invaders and its own altered internal cells. Our environment contains several different types of infectious agents, such as viruses, bacteria, fungi, or protozoa. All these pathogens can cause disease, and may eventually kill the host if the immune system that does not function well (Male, 2001). The ability of an organism to respond to foreign invaders is phylogenetically ancient, and can be traced back to the protozoa (Gleichmann, 1989).

There are two types of immune responses, innate (natural) and adaptive immunity. The major difference between these is that an adaptive immune response is highly specific for a given pathogen. Cells of the vertebrate adaptive immune system basically remember the previous encounter with an infectious agent and fight with a stronger response on later re-infection (Lydyard and Grossi, 2001). The specificity, memory, and the capacity to distinguish between self and non-self are the main features of mammalian adaptive immune response (Male, 2001).

The organs of the immune system are divided as primary and secondary lymphoid organs. The primary lymphoid organs are the bone marrow and thymus, depending on the species. These organs support the production of mature T- and B-lymphocytes and myeloid cells, such as macrophages. The bone marrow is the origin of the pluripotent stem cells. All other hematopoietic cells are derived from these stem cells. The thymus is the site to which T-cell prolymphocytes migrate and undergo selection for recognition of self or nonself antigens (Male, 2001).

The secondary lymphoid organs are the spleen, lymph nodes and mucosa-associated lymphoid tissues (MALT), which include the tonsils and Peyer's patches (Lydyard and Grossi, 2001; Burns et al., 1996). These organs have a highly organized microenvironment in which lymphocytes come into contact with exogenously derived antigens. They also serve as biological sieves. For example, the spleen filters the blood

by removing foreign antigens and any circulating dead cells (i.e., erythrocytes, platelets, granulocytes and lymphocytes). The lymph nodes, on the other hand, filter antigens from the fluid surrounding tissues. Specific antigen recognition, clonal expansion of antigen-specific cells and differentiation of antigen stimulated lymphocytes occurs in the secondary lymphoid organs (Burns et al., 1996; Sharma and Reddy, 1987).

Leukocytes and the soluble molecules they secrete are the main component of the immune system. Leukocytes include 1) phagocytes: mononuclear phagocytes, neutrophils, and eosinophils; 2) lymphocytes: B cells, T cells, and large granular lymphocytes; and 3) auxiliary cells: basophils, mast cells and platelets (Lydyard and Grossi, 2001).

Monocytes, macrophages and polymorphonuclear neutrophils, also known as phagocytic cells, are the members of innate immune response. These cells bind to microorganisms directly, phagocytose and ultimately kill them. Phagocytic cells can bind to a wide range of pathogens due to their primitive, non-specific recognition systems (Gordon, 2001). Monocytes (mononuclear phagocytes) are the longest living phagocytic cells. Their job is to engulf and destroy pathogens. Blood monocytes derived from bone marrow pass through the endothelium and become macrophages in the tissues. These resident phagocytic cells are called microglial cells in brain, kuppfer cells in the liver, mesangial cells in kidney, alveolar macrophages in lungs, and A cells in synovial fluid. Lymph nodes, lung, spleen, and kidney have phagocytes of their own as well. These macrophages are very effective in antigen presentation to T lymphocytes. Neutrophils, similar to monocytes, migrate into tissues where there is inflammation. However, neutrophils have a much shorter life-span compared to macrophages. Another type of phagocyte, eosinophil leukocytes, has specific ability to damage large extracellular parasites (Male, 2001).

Lymphocytes are critically important cells contributing to the adaptive immune response. Lymphocytes can recognize individual pathogens either in the blood stream, in tissue fluids or inside the host cells. This is accomplished by two types of cells, B and T

lymphocytes (Lydyard and Grossi, 2001; Male, 2001). Lymphocytes are derived from the bone marrow stem cells, develop into T-cells within thymus, and into B-cells within the bursa of fabricius (in birds) or in the bone marrow (in adult mammals). B-cells fight against pathogens by releasing antibodies, molecules that specifically recognize and bind protein molecules called *antigens*. Each B-cell recognizes only one specific antigen, then multiplies and differentiates into *plasma cells*. These cells have the ability to produce large amounts of antibody. These antibodies can bind to the antigen that initially activated the B-cell (Male, 2001).

Recognition of antigens is different in B and T lymphocytes. T-cells can only recognize antigens if they are presented on the surface of other cells by major histocompatibility complex (MHC) molecules. T-cells function either by direct cell-cell interaction, or by releasing soluble proteins to signal other cells. There are two major groups of T-cells. One group is responsible for killing of the virus or internal pathogen infected cells. This group is called cytotoxic T cells (or, regulatory T cells, $CD8^+$ T cells). Another group is the T-helper cells ($CD4^+$ T cells). Type-1 T helper (TH1) cells communicate with mononuclear phagocytes and help them kill intracellular microorganisms. Type-2 T helper (TH2) cells interact with B cells and help them to divide, differentiate and produce antibody (Lydyard and Grossi, 2001).

Collectively, these cells of the immune system communicate with each other to enable an individual to effectively respond to an infection. For instance, certain phagocytes can take up a pathogen, process it and show it to T–cell in a form that they can recognize. Antibodies produced by B cells increase antigen recognition by phagocytes. Similarly, T-cells help activate phagocytes by releasing cytokines, so that these phagocytes can destroy their engulfed microorganisms.

Another special subset of lymphocyte is a large granular lymphocyte. They have the ability to recognize and damage virally infected or tumor cells without the MHC molecule presence on these cells (natural killer activity).

Auxiliary cells mediate inflammation, and their main purpose is to attract leukocytes and the soluble immune mediators towards the site of infection (Male, 2001). Basophils and mast cells synthesize and secrete mediators when they are triggered. Mast cells lie close to blood vessels, and contain certain mediators to act on endothelial cells. Basophils are functionally similar to mast cells but they are mobile, circulating cells (Male, 2001).

2.5. Immunotoxicity

There is an increasing awareness that a variety of chemicals have the potential to impair the immune system (Galloway and Depledge, 2001; Ahmed, 2000; Vial et al., 1996; Gleichmann, 1989). Certain xenobiotics (foreign substances), or their metabolites, can damage immuno-competence either by directly interacting with the immune cells, or by affecting the other systems (i.e., neuroendocrine system) that will in turn affect immune functioning (Friedman and Lawrence, 2002; Fuchs and Sanders, 1994). Due to its importance in assessing the toxicity of drugs and non-drug chemicals, immunotoxicity testing has become one of the requirements of regulatory agencies (i.e., the FDA) (Holsapple, 2003).

Data obtained from numerous rodent studies have shown that xenobiotic exposure can produce immune dysfunction and alter host resistance following acute or chronic exposure (Dean and Murray, 1991; Luster et al., 1987; Dean et al., 1982). Three main groups of chemicals studied intensively are the polyhalogenated hydrocarbons, the organotins, and heavy metals.

Polyhalogenated hydrocarbons (PHH) have low biodegradability, and, therefore, have become persistent environmental contaminants. Their resistance to decomposition in the environment and their lipophilic properties allow these chemicals to accumulate in fatty tissues and to bioconcentrate at higher levels of the food chain (Safe, 1994). Research with PHHs, such as polychlorinated biphenyls (PCB) (Jeon et al., 2002; Stack et al., 1999; Yoo et al. 1997; Harper et al., 1995), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

(Camacho et al., 2001; Thurmond and Gasiewicz, 2000; Blaylock et al., 1992; Holladay et al., 1991) and the polybrominated biphenyls (PBB) (Damstra et al., 1982; Luster et al., 1978) demonstrated that they are, at certain doses and exposure routes, immunotoxic to the experimental animals.

The PHHs that are PCBs have been detected in human blood, milk, and other tissues (Giesy and Kannan, 1998). They were widely used for a variety of industrial purposes for several decades before their production was banned in the 1970s. Many of PCBs toxic effects, including their immunotoxicities, are mediated by the activation of aryl hydrocarbon receptor (AhR), (Jeon et al., 2002). Voie et al. (1998) reported that some PCB congeners bind to the AhR with low affinity, and alter neutrophil function by both stimulating respiratory burst and inducing degranulation. Stack et al. (1999) have reported that in two strains of mice, C57Bl/6 (high affinity AhR complex) and DBA/J (low affinity AhR complex), PCB mixtures (Aroclors) or individual PCB congeners significantly decreased the in vitro proliferation of murine splenocytes.

TCDD is a member of the PHH class of chemicals. Most of the described effects of TCDD and its related congeners are also mediated by the AhR, which upon TCDD binding acts as a transcription factor and binds to dioxin responsive elements in target genes (Gu et al., 2000). One of the genes that is regulated by TCDD, for instance, is adseverin, and its expression resulted in an effect restricted to the immune system, mostly in the thymus (Svensson and Lundberg, 2001). Inappropriate activation of thymocytes via the AhR induces immune suppression. The improper activation of cells leads to anergy or death, and premature termination of the immune response. Other immune cells are also affected. Bone marrow derived cells are the critical AhR-expressing targets for TCDD. In addition, TCDD enhances the activation of B-cells, dendritic cells and $CD4^+$ T-cells and also causes earlier disappearance of the $CD4^+$ T-cell populations from peripheral lymphoid organs (reviewed in Kerkvliet, 2002).

Prenatal exposure to TCDD has been reported to affect the immune system differently. It was proposed that exposure to TCDD prenatally may cause postnatal autoimmune

diseases. Holladay (1999) reported that TCDD was able to cross the placenta and inhibit thymocyte maturation and reduce thymic major histocompatability complex class II molecule expression. It was also suggested that gestational exposure to TCDD may interfere with normal development of self-tolerance in rodent offspring (Holladay, 1999).

A third type of PHH is polybrominated biphenyls (PBBs). In 1973, accidental contamination of PBBs occurred in a Michigan farm, resulting in serious harm to farm families and animals. When PBBs effect on the immune parameters were studied, abnormalities in the contaminated group included hypergammaglobulinemia, exaggerated hypersensitive response to streptococci, and significant decrease in percentages of T and B lymphocytes (Bekesi et al., 1987).

Metals have also been recognized as immunotoxicants. Organotins, the long chain dialkyltin compounds, have been widely used in industry and agriculture, mainly as biocides, catalysts and plastic stabilizers. Depending on the length of the alkyl chains, these organotins show toxic effects on the immune system, the bile duct, liver or pancreas (Merkord et al., 2000). For example, organotins, such as di-n-butyltin dichloride (DBTC) or tri-n-butyltin chloride (TBTC), given in the diets of rats caused thymic atrophy. A single oral dose of DBTC or TBTC (dose range of 5-60 mg/kg) given to rats induced a dose-related reduction of relative thymus weight (Snoeij et al., 1988). Penninks et al. (1985) suggested that the dialkyltin-induced (single i.p. injection of 1 mg di-n-octyltin dichloride /kg) thymus involution was caused by a direct anti-proliferative activity of this metal on lymphocytes.

Heavy metals including mercury, lead, and cadmium are present throughout the environment, especially in rivers and lakes (Bernier et al., 1995a). The main route of exposure of humans to these metals is via the ingestion of contaminated food, especially fish.

Several animal and human studies demonstrated that lead induces immune dysfunction, (Lee et al., 2002; Bunn et al., 2001; McCabe 1994 and 1999; Razani-Boroujerdi et al.,

1999; Miller et al., 1998; Sata et al., 1998). These reports suggested that the lead causes immunotoxicity by affecting T cell functioning. Lead specifically disturbs the balance between type-1 T helper (Th1) and type-2 T helper (Th2) cells. McCabe and Lawrence (1991) showed using $CD4^+$ T helper cell clones, *in vitro*, that lead exposure enhanced Th2 cell activity and proliferation but diminished Th1 cell proliferation. In an *in vivo* study published by Heo et al. (1998), lead enhanced the development of Th2 cells and impaired Th1-mediated immune functions.

Another immunotoxic metal is mercury. Mercury is a widely used industrial chemical (i.e., in wood preservatives, or seed-dressings). The risk of human exposure by repeated low doses of mercury, therefore, is fairly high (Institoris et al., 2001). Administration of 3, 15 and 75 ppm mercury to mice in drinking water for 7 weeks resulted in decreased proliferation of splenic lymphocytes, reduced concanavalin A induced T-cell proliferation, and decreased plaque forming cell numbers in the spleen (Dieter et al., 1983). The effect of mercury has also been shown to be gender and genetics dependent. When mice with different H-2 haplotypes were treated with mercuric chloride ($HgCl_2$) at a dose of 0.5-16 mg Hg/L drinking water for 10 weeks, mouse strains without the susceptible haplotype did not develop any autoimmune reaction. In the susceptible H-2 haplotype, males and females had different thresholds for induction of autoimmune reactions (Nielsen and Hultman, 2002).

2.6. Immunotoxicity of Pesticides

Starting about 25 years ago with Vos, Moore, Dean and few others, effects of environmental chemicals on immunological parameters were studied in experimental animals (Neubert and Neubert, 1999). These studies revealed that any xenobiotics at doses high enough to cause acute poisoning may indirectly affect immune parameters (Rodgers, 2001). However, studies evaluating immunotoxicity of a pesticide have usually been done with doses below those causing obvious clinical signs. Administration of such a dose of pesticide helps diminish the confounding effects of stress and other organ

system toxicities on the immune system (Rodgers, 2001). The data collected from these animal studies show that pesticides often cause immunotoxicity. However, due to the lack of well-designed longitudinal studies to investigate this effect of pesticides in humans, these findings cannot be taken as evidence for the general population (Rodgers, 2001). To completely understand the effects of pesticides on the immune response, functional tests are recommended to be done on persons occupationally exposed to known levels of pesticides. These tests should take day-to-day and person-to-person variabilities into account (Rodgers, 2001).

The immunotoxicity of pesticides was studied primarily in four classes of insecticides: organochlorines, organophosphates, carbamates, and pyrethroids.

Frequently studied organochlorine pesticides include chlorinated ethane derivatives (i.e., DDT), cyclodienes (i.e., chlordane, heptachlor), and hexachlorocyclohexanes (i.e., lindane). In an early study with dichlorodiphenyltrichloroethane (DDT), 100-400 ppm in the diet for 5 weeks resulted in decreased humoral immune response to bovine serum albumin (Glick, 1974). Administration of 200 ppm DDT or DDT metabolites (DDE and DDA) in the diet for 5 weeks suppressed the generation of both humoral and cellular immune responses to ovalbumin in rats (Banerjee et al., 1996b). Oral administration of up to 150 ppm DDT to mice for 4 weeks did not affect the IgM production to sheep red blood cells (Andre et al., 1983). Only 150 ppm DDT changed the delayed type hypersensitivity (DTH) response to tuberculin in this study. In case of cyclodiene organochlorines, 0.1-8 mg/kg/day chlordane administration to adult mice for 14 days did not affect the humoral immune responses (Johnson et al., 1986). In contrast, when BALB/c mice were given chlordane at 0.16 or 8 mg/kg/day during gestation, the offspring had depressed DTH response to oxazolone (Menna et al., 1985). Lindane, when given 150 ppm per day orally for 1 month did not change IgA, IgG1, and IgM levels in the serum in mice (Andre et al., 1983). Lindane administration of 3 mg/kg/day for 5 weeks decreased the humoral immune response to bacterial antigen in the rabbit (Desi et al., 1978). In summary, most of the tested organochlorine pesticides suppressed the immune response. The alteration in membrane fluidity and modulation of membrane

mediated changes in cells were suggested to partly explain the mechanism of action for the immunotoxicity of organochlorines (Rodgers, 2001).

The effects of organophosphate pesticides on the immune system have been extensively studied. Parathion, malathion, dichlorvos and fenthion are some examples of these widely studied compounds. 1-125 μg/ml parathion and methyl parathion exposure to splenocytes *in vitro* inhibited the cell mediated immune responses (Rodgers et al., 1986b). In a study with rabbits, 3 mg/kg/day methyl parathion in the diet decreased the animal's resistance to *Salmonella typhimurium* infection (Fan et al., 1981). Administration of high doses of malathion (up to 715 mg/kg) to mice elevated the IgM generation and increased proliferative responses to mitogen (Rodgers and Ellefson, 1990). The same study also indicated that macrophages were the main cell type affected by malathion. Rodgers and Xiong (1997c, d) also reported that 14 or 90 day administration of malathion caused macrophage activation and systemic degranulation of basophilic cells. Another organophosphate pesticide, dichlorvos, slightly decreased spleen weight but did not affect humoral immune response in mice following acute administration (Casale et al., 1983). Cholinergic doses of dichlorvos (up to LD_{50} doses) caused mobilization of bone marrow cells and suppression of cellular and humoral responses (Zabrodski, 1993). Fenthion, when applied to murine splenocytes in vitro (125 μg/ml) blocked their ability to elicit a cell mediated immune response (Rodgers et al., 1986b). Most of the studies with organophosphates reported their effects as decrease in immune function, and suggested that these compounds probably act through inhibition of serine esterases (Rodgers, 2001).

Carbamates are another group of pesticides that were extensively studied for effects on the immune system. Toxic effects of carbamates occur through acetylcholinesterase inhibition so symptoms of exposure are similar to those of organophosphates (Rodgers, 2001). Carbamates are not broad spectrum pesticides, but they have been widely used because of their low persistence in the environment. Carbaryl, carbofuran, aminocarb, and aldicarb are examples of generally used chemicals within this group. Andre et al. (1983) showed that carbaryl selectively increased serum IgG1 and IgG2b levels in mice following oral exposure of one month. In contrast, Ladics et al (1994) reported that the

humoral immune response was not affected by oral or dermal exposure of carbaryl, but it was suppressed by inhalation exposure with doses of up to 335 mg/kg given to rats. In an *in vitro* study by Rodgers et al (1986b), carbaryl exposure inhibited the cytotoxic T-cell responses. Carbofuran, another carbamate pesticide, decreased the humoral immune response to neutral and pathogenic antigens but did not affect the generation of a DTH response in vivo (Fournier et al., 1988). The same study also investigated the toxicity of aminocarb, and reported that aminocarb also decreased the humoral immune response to neutral and pathogenic antigens. Additionally, it increased the cytolysis of pathogens by macrophages (Fournier et al., 1988). Another study by Bernier et al. (1995b) showed that oral and dermal administration of low doses of aminocarb to mice elevated humoral responses, whereas inhalation exposure had no effect. Studies with aldicarb performed by Olson et al. (1987), and Shirazi et al. (1990) reported that low levels (up to 100 ppb) decreased IgM production. However, in a chronic toxicity (90 day) study, administration of 0.1-10 ppb aldicarb in drinking water did not affect any immune parameters (Hajoui et al., 1992). In conclusion, carbamate pesticides elevated, suppressed, or did not affect immune parameters, depending upon the dose and route the carbamate was administered.

For pyrethroids, there is little information available on effect on the immune system. Desi et al. (1986) showed that with up to 40 mg/kg cypermethrin administration, IgM production was depressed in response to sRBC and ovalbumin in rats. Stelzer and Gordon (1984) exposed murine splenocytes to permethrin in a concentration range of $1x\ 10^{-5}$ M to $5x10^{-5}$ M, and reported an inhibition in mitogenic response to concanavalin A. In a 28-day study with F344 male rats, deltametrin was given at 0, 1, 5, or 10 mg/kg body wt/day (Madsen et al., 1996). The effects seen in the groups receiving 5 or 10 mg/kg doses included increased weight of mesenterial lymph nodes, decreased thymus weight in immunized animals and an increase in numbers of sRBC-plaque forming cell and splenic NK cell activity. The authors did not observe any severe effects on the immune system. In another study, Prater et al. (2002) studied the effects of single topical exposure to permethrin in C57BL/6N mice. Permethrin caused inhibition in splenic T-cell proliferation, thymic cellularity, and splenocyte proliferation. Punareewattana et al. (2001) applied permethrin topically to C57Bl/6N mice at doses of 0.5, 1.5 or 5.0 µl /day.

These doses did not inhibit the phagocytic ability of macrophages, but antibody production as shown by plaque-forming cell (PFC) assay was decreased after 10 consecutive days of exposure to permethrin.

2.7. The Reactive Oxygen Species and Oxidative Stress

Free radicals are molecules which contain one or more unpaired electrons, and are capable of reacting randomly with any molecules with which they come in contact (Younes, 1999; Halliwell, 1996). They extract an electron from a neighboring molecule to complete their own orbital, and generate new free radicals in cytotoxic oxidative chain reactions (Marks et al., 1996). There are more than one form of free radical species, such as oxygen-centered, carbon-centered (R·, RCOO·) or nitrogen-centered (NO·, ONOO-) radicals (Halliwell, 1996). Many of these free radical species are formed when chemicals are metabolized to one or more reactive intermediates (Comporti and Pompella, 1994). The major oxygen metabolites are superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($O_2^{\uparrow\downarrow}$), and the partially reduced form of oxygen, hydrogen peroxide (H_2O_2) (Marks et al., 1996).

The superoxide anion is formed from free O_2 by donation of an electron to another free radical. It is highly reactive but has limited lipid solubility and cannot diffuse far in tissues. However, superoxide anion can also generate the more reactive hydroxyl and hydroperoxy radicals by reacting with hydrogen peroxide in the Haber-Weiss reaction (Marks et al., 1996). The Haber-Weiss Reaction is:

$$\xrightarrow[H^+]{Fe^{2+}}$$

$$O_2^{-\cdot} + H_2O_2 \rightarrow O_2 + H_2O + OH\cdot$$

Superoxide anion can attack iron sulfur centers in enzymes such as aconitase, succinate dehydrogenase, and mitochondrial NADH-ubiquinone oxidoreductase, releasing iron and destroying the enzyme's catalytic function (Gardner et al., 1995; Flint et al., 1993). Superoxide, therefore, has to be removed rapidly by conversion to hydrogen peroxide in a

reaction catalysed by superoxide dismutase. There are three superoxide dismutases in mammalian systems: [1)] cytosolic CuZn superoxide dismutase (SOD1), [2)] mitochondrial manganese superoxide dismutase (SOD2), and [3)] extracellular CuZn superoxide dismutase (SOD3) (Fridovich, 1995). Dismutation of superoxide is catalyzed by SOD with the following reaction:

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

The most potent of the reactive oxygen species (ROS) is the hydroxyl radical (Raha and Robinson, 2000). Hydroxyl radical is the possible initiator of the chain reactions which form lipid peroxides and organic radicals. It causes peroxidative damage to proteins, lipids, and DNA (Raha and Robinson, 2001). It is generated from hydrogen peroxide in the presence of Fe^{2+} or another transition metal by the Fenton reaction (Marks et al., 1996).

The Fenton reaction is:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$$

Because hydrogen peroxide is lipid soluble, it can create damage at localized Fe^{2+} -containing membranes far from its site of formation. The hydrogen peroxide generated by either MnSOD or CuZnSOD is processed by catalase and glutathione peroxidase to water (Esworthy et al., 1997; Fridovich, 1995; Beyer, 1991). Catalase is an antioxidant enzyme that scavenges hydrogen peroxide and converts it to water and molecular oxygen:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Glutathione peroxidase is another antioxidant enzyme that catalyzes the reduction of hydroperoxides by using reduced glutathione (GSH) as a reductant:

$$ROOH + 2\ GSH \rightarrow ROH + GSSG + H_2O$$

In mammalian cells, reactive oxygen species (ROS) are produced during several enzyme-catalyzed reactions and are vital for several biological processes. They are produced during arachidonic acid metabolism leading to prostaglandin and leukotriene synthesis, and are used therapeutically for relaxation of smooth muscle cells to control blood pressure. Also, during phagocytosis, neutrophils and macrophages generate superoxide anion and other ROS to destroy foreign molecules (Knight, 2000).

The cells can battle against oxidative stress by either repairing the oxidative damage (removing the damaged nucleotides and lipid peroxidation byproducts) or by directly scavenging oxygen radicals via enzymatic and nonenzymatic antioxidants. For example, the free radical scavenger and immunomodulator melatonin has been shown to protect against the genotoxic effects of the herbicide paraquat (Ortiz et al., 2000). In a study by Forrest et al. (1994), hydrogen peroxide treatment of murine thymocytes caused apoptosis, and when cells were treated with trolox, a vitamin E analog and antioxidant, DNA fragmentation returned to control levels. In another study, spin trapping reagents (to bind to ROS), such as 5,5-di-methyl-1-pyroline-N-oxide (DMPO) and 2,2,6,6-tetra-methyl-1-piperidinyloxy (TEMPO) protected cells from apoptosis against different chemical agents (Slater et al., 1995). Another study demonstrated the role of ROS related lipid peroxidation in the toxicity of quinalphos, an estrogenic organophosphate insecticide (Debnath and Mandal, 2000). This study showed that at low doses quinalphos caused damage and degeneration of the testicular tissues due to ROS mediated lipid peroxidation. This study also showed that at the highest dose of treatment an antioxidant enzyme defense system became operative as a response to the damage.

The free radicals produced during exposure to certain pesticides and the resulting oxidative stress have been shown to be involved in cytotoxicity. For example, the organochlorine insecticide dieldrin has been shown to induce hepatotoxicity via oxidative stress in mice (Bachowski et al., 1998). In this study, biomarkers of oxidative damage to lipids [malondialdehyde (MDA)], and to DNA [8-hydroxy-2-deoxyguanosine (oh8dG)] were evaluated. Results showed that dieldrin-induced hepatic DNA synthesis was linked with increased urinary oh8dG and increased hepatic MDA. Hsu et al. (1998) reported that

the fumigant phosphine induced cytotoxic and mutagenic effects by increasing ROS levels, particularly hydroxyl radicals, and initiating oxidative damage. In another study, toxaphene, an organic pesticide, with concentrations ranging from 0.1 to50 μg/ml at 24 h induced superoxide production in human neutrophils (Gauthier et al., 2001). In a study by Bagchi et al. (2000), 0.01, 0.10, and 0.50 of LD_{50} doses of TCDD, endrin, naphthalene, and sodium dichromate (VI) increased hepatic lipid peroxidation and DNA fragmentation. Administration of deltamethrin, a pyrethroid insecticide, at 1 mg/kg daily for 21 days to rats resulted in DNA laddering, characteristic of apoptosis, in the testicular tissues (El-Gohary et al., 1999). In the same study the plasma levels of both nitric oxide and lipid peroxides measured as malondialdehyde were significantly increased. Melchiorri et al. (1998) reported that when adult male Wistar rats were injected a single dose of paraquat (25 nmol/0.5 μl), DNA fragmentation, nuclear chromatin marginalization and compaction were detected in all hippocampal subsectors. Pre-treatment with the free radical scavenger lazaroid U74389G (30 mg/kg, i.p.) significantly reduced paraquat-induced apoptosis.

2.8. Apoptotic and Necrotic Cell Death

Chemical induced acute cell death is a very complex combination of events. This lethal injury to the cell can occur via two specific mechanisms or modes of cell death, either apoptosis or necrosis (Corcoran et al., 1994; Wyllie et al., 1980).

In order to maintain a constant size, an organism has to equalize the rates of cell production and cell death. Any cells that have been produced in excess, or have developed improperly, or cells with genetic damage have to be removed from the system. Controlled, normal physiological death of unwanted cells occurs via *apoptosis* (Kerr et al., 1972; Searle et al., 1982; Wyllie and Duvall, 1992; Corcoran et al., 1994). Apoptosis plays a balancing but opposite role to mitosis in the regulation of a cell population (Kerr et al., 1972; Bengt et al., 1999). The most important feature of this programmed cell death is that it eliminates the dying cells without induction of an inflammatory response.

The pathologic form of cell death, necrosis, causes an inflammatory response due to leakage of cytoplasmic contents to the near environment (Corcoran et al., 1994; Schwartzman and Cidlowski, 1993). Although the terms *programmed cell death* and *apoptosis* are used interchangeably, apoptosis is actually one form of programmed cell death (Schulte-Hermann et al., 2000; Clarke, 1990). There are at least two types of physiological cell death; *apoptosis*, in which most of the early morphological changes occur in the nucleus, and a *lysosomal or cytoplasmic cell death* in which the early changes occur in the cytoplasm. The term *programmed cell death* appeared in the 1960's, and *apoptosis* in the 1970' when Kerr et al. (1972) described apoptosis (meaning 'falling off' petals from trees, in Greek) as a distinct way of cell death.

Cells undergoing apoptosis usually exhibit characteristic morphology, including fragmentation of the cell into membrane-bound apoptotic bodies, nuclear and cytoplasmic condensation and endolytic cleavage of the DNA into small oligonucleosomal fragments (Bosman et al., 1996; Wyllie, 1987). Apoptosis occurs during embryogenesis, organogenesis, during the course of normal tissue turnover and after withdrawal of a trophic hormone from its target tissue. In adult somatic cells apoptosis occurs during formation of keratinocytes, shedding of intestinal lining, atrophy of prostate and adrenal cortex following reduction in trophic hormone stimulation, and also in elimination of T and B-cells at the end of immunological reactions (Bengt, 1999; Desmouliere et al., 1997; Bosman, 1996; Schwartzman and Cidlowski, 1993). Positive and negative selection of thymocytes in the thymus also occurs via apoptosis (Perandones et al., 1993).

Dysregulation of apoptosis has been found to be related to autoimmune diseases, acquired immune deficiency syndrome, neurodegenerative disorders and cancers, and to several other viral and bacterial infections (Bengt, 1999; Desmouliere et al., 1997). Certain diseases occur due to increased apoptosis while others occur via decrease in apoptosis. For example, autoimmunity may arise from a lack of apoptotic cell deletion or by unscheduled apoptotic destruction of cells. Cancer may be seen as a result of ineffective apoptosis and a net gain of cells due to unrestrained proliferation, but also

cancer cells themselves may evade immune surveillance by triggering apoptosis of immune cells (Igney and Krammer, 2002; Barkett and Gilmore, 1999). In cells, disease causing apoptotic processes involve several pathways. Certain hormones, such as glucocorticoids, bind to their intracellular receptors and this receptor-ligand complex move into the nucleus and binds to DNA, altering expression of certain genes. According to specific gene expression, the effect can be pro-apoptotic (bax, bik, bak, bcl-x, bad, p53, c-jun) or anti-apoptotic (bcl-2, bcl-x, Rb, Mcl-1) (Granville et al., 1998; Robertson et al., 2000). UV irradiation, drugs for chemotherapy, and certain pesticides can also promote apoptosis by causing DNA fragmentation. Another pathway for the induction of apoptosis is the activation of caspase enzymes. Perforin or TNF (tumor necrosis factor) mediated pathways promote apoptosis in effector cytotoxic T-cells and natural killer (NK) cells. Effector cytotoxic T-cells (CTL), but not natural killer cells, also cause apoptosis by CD95 ligation (Jacobson et al, 1994; Schulze-Osthoff et al., 1994). In the perforin mediated pathway, CTL or NK cells release perforin molecules and granzyme B. TNF-TNF receptor ligation on the target cell's membrane activates death domains and causes activation of different caspases. CTL mediated CD95-CD95 ligand interaction also causes activation of different death domains and activates caspases (Perandones et al., 1993; Robertson et al., 2000).

Several studies have shown that certain pesticides can induce apoptosis. For example, the organochlorine pesticides heptachlor, chlordane and toxaphene induce apoptosis in monkey leukocytes (Miyagi et al., 1998). Another organochlorine pesticide, endosulfan, causes dose- and time-dependent apoptosis in a human T-cell leukemic cell line (Kannan et al., 2000). N-nitrosocarbofuran, the major metabolite of the carbamate pesticide carbofuran, induces apoptosis of Chinese hamster lung fibroblasts (Yoon et al., 2001).

Examination of the type of cell death caused by lindane, malathion, permethrin, or by their mixtures may help improve our understanding of circumstances surrounding toxicity at the cellular level, and it may improve our ability to interfere with the toxicity.

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Chapter 3. EXPERIMENTAL METHODS AND PROTOCOLS

Methods for both in vitro and in vivo experiments were used in this dissertation. These methods tested the hypothesis that lindane, malathion, permethrin, and their combinations were immunotoxic to mouse thymocytes. The secondary hypothesis suggested that reactive oxygen species produced during exposure to these chemicals contributed to the immunotoxicity. These hypotheses were tested in the following studies:

Chapter 4: This study established the mode of cell death (apoptosis versus necrosis) following single and multiple pesticide exposure in C57BL/6 mice thymus cells (thymocytes). Three endpoints were assessed: proportion of apoptotic and late apoptotic/necrotic cells, membrane integrity, and DNA fragmentation.

1. Percentage of cells undergoing apoptosis was assessed by flow cytometric techniques. These included 7-aminoactinomycin D staining, and Annexin-V/propidium iodide staining of cells.
2. Membrane integrity was assessed by lactate dehydrogenase enzyme leakage into the cell supernatant.
3. DNA fragmentation in apoptotic cells was evaluated by using the DNA laddering technique and visualized by electrophoresis on agarose gels.

Chapter 5: This study established the extent of single and multiple pesticide administration on five selected immune parameters of C57BL/6 mice. Evaluating the effects of multiple pesticides *in vivo* was done to permit comparisons with previous *in vitro* investigations. Endpoints evaluated were as follows:

1. Thymus/body and spleen/body weight ratios
2. The thymus and spleen cell count
3. CD4/CD8 immunophenotyping of spleen cells
4. CD45RB220/CD90.2 immunophenotyping of spleen cells

5. Plaque forming cell assay to evaluate splenic B cells ability to produce IgM antibody

Chapter 6: This study measured pesticide-induced alterations in superoxide anion and hydrogen peroxide generation in mouse thymocytes and the changes in specific activity of antioxidant enzymes. Evaluations of these parameters were done to assess the pro-oxidant and anti-oxidant status of the thymocytes.

1. Alterations in the superoxide anion generation in thymocytes were assessed by using hydroethidine/ethidium bromide dye and flow cytometry.
2. Alterations in the hydrogen peroxide generation of cells were assessed by utilizing dichlorofluorescein diacetate dye and flow cytometry.
3. Changes in specific activities of the antioxidant enzymes were evaluated by using spectrophotometric techniques.

Chapter 7: This study measured pesticide-induced activation of transcription factor NFκB in C57BL/6 thymocytes. This parameter was evaluated following pesticide exposure to strengthen the observation that oxidative stress occurs. Methods used to assess cytosolic NFκB levels, and activation of transcription were as follows:

1. NFκB protein concentration was determined by using anti-NFκB monoclonal antibody staining combined with secondary antibody labeling and blotting on nitrocellulose membranes
2. Alteration in transcription of NFκB was assessed by polymerase chain reaction (PCR) analysis [following total RNA isolation, and cDNA synthesis with reverse transcriptase (RT-PCR)].

Statistics: Statistical validity of quantitative in vitro and in vivo data points ($p \leq 0.05$) was determined by utilizing analysis of variance (ANOVA) followed by Dunnett's t-test to compare pesticide treatments to controls. StatView (Berkeley, CA) software was used to

perform the analyses. Data are presented as means of multiplicates ± standard error. All differences of $p \leq 0.05$ were considered significant.

RESULTS

Chapter 4. PESTICIDE MIXTURES POTENTIATE THE TOXICITY IN MURINE THYMOCYTES

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4.1. Abstract

The immunotoxic effects of multiple pesticide exposure were evaluated. C57BL/6 mouse thymocytes were exposed to lindane, malathion, and permethrin, either separately or in mixtures of two pesticides, *in vitro*. These pesticide exposures caused both apoptotic and necrotic cell death in thymocytes as evaluated by flow cytometric analysis in combination with 7-aminoactinomycin-D (7-AAD), Annexin-V/propidium iodide staining assays and lactate dehydrogenase release assays. When cells were exposed to mixtures of two pesticides, a significantly greater-than-additive interaction was observed in both apoptotic and late apoptotic/necrotic populations of cells. The gel electrophoresis of DNA of cells showed DNA ladder formation with limited genomic DNA and increased laddering in mixture exposures. Based on these findings, it is suggested that these pesticides are potent immunotoxicants, *in vitro*, and that the mechanism of cytotoxicity observed upon exposure to these pesticides may, at least in part, be due to induction of apoptosis. We also provided evidence that induction of the drug metabolizing mixed function oxidase system with lindane may, in part, be responsible for the potentiation of cytotoxicity in the combined exposures. As more information is obtained on the potential immunotoxic effects of pesticides, further insights will be gained for the risk assessment of these environmental pollutants.

4.2. Introduction

Pesticide applications have increased dramatically since the mid 1960s, and the related adverse health effects in humans as well as in wild/domestic animals have become a serious public concern (Crumpton, 2001). Although pesticide usage is beneficial for increasing agricultural productivity and reducing insect-borne diseases, human exposure to these toxic chemicals is virtually unavoidable due to contamination of air, water, ground, and food (Ecobichon, 2000). The potential toxicity of most of these chemicals has been studied extensively and several databases have been developed. However, more than 95% of all pesticide toxicity studies were conducted on individual environmental pollutants (Groten et al., 1999; Yang et al., 1994). Recently, more emphasis has been placed on chemical mixture studies because people are actually exposed to countless mixtures of chemicals daily.

It is well recognized that many compounds are not directly cytotoxic. Rather they cause sublethal damages, which may trigger an innate suicidal sequence of activities in the cell (Hampton et al., 1998). This programmed cell death, or apoptosis, is important during embryo development, maintaining tissue homeostasis, and for removing damaged or infected cells. Apoptosis has been shown to be triggered by several factors, including exposure to pesticides (Carlson et al., 2000; Kannan et al., 2000; Warren et al., 2000).
There is an increasing awareness that a variety of drugs and environmental chemicals have the potential to impair different components of the immune system (Galloway and Depledge, 2001; Ahmed 2000; Vial et al., 1996). Moreover, immune system is considered to be sensitive to chemicals at low dose levels when no other system toxicity is evident (Sharma et al., 1987). Alteration of immune system functioning by different pesticides has been suggested to be the basis for hypersensitivity, increased allergy and diminished resistance against tumor formation (Gleichmann et al., 1989).

Because if certain chemicals may influence the metabolism of others, we examined the following three different classes of pesticides on thymocytes, *in vitro*, in this study. Lindane (γ-isomer of hexachlorocyclohexane) is an organochlorine insecticide and an

inducer of mixed function oxidase (MFO) system (Junqueira et al., 1997; Barros et al., 1991). Lindane is known to induce DNA fragmentation, a hallmark of apoptosis (Kang et al., 1998). It is also an immunosuppressant at moderate to high doses (Raszyk et al., 1997; Meera et al., 1992). Malathion, an organophosphate insecticide, is an acetylcholinesterase inhibitor (Barber et al., 1999). It is known to modulate or suppress immune responses at specific dose-levels (Johnson et al., 2002; Beaman et al., 1999; Rodgers et al., 1997d). Permethrin (20% cis-78% trans), synthetic pyrethroid, is a moderately toxic insecticide. Permethrin, reportedly, has no teratogenic, mutagenic or carcinogenic effects; however, it is shown to suppress cellular immune responses (Blaylock et al., 1995).

4.3. Materials and Methods

4.3.1. Animals

Male C57BL/6 mice, 8-12 weeks old, were purchased from Charles River Laboratories (Wilmington, MA) and quarantined 7 days prior to use. Mice were housed in polycarbonate cages with hard wood chip bedding in rooms in which the temperature (21^0 C), humidity (50 %), and light cycle (14 h dark/10 h light) were controlled. The food and water were available ad libitum. All animal care and animal protocols were in compliance with the Virginia Polytechnic Institute and State University Animal Welfare Committee guidelines.

4.3.2. Thymus Collection and Isolation

Mice were euthanized by cervical dislocation, body weights were recorded; the thymus was removed from each mouse, weighed and individually placed in a 60 x 15 mm Petri dish containing cold, phenol red-depleted RPMI-1640 media (Gibco BRL, Grand Island, NY). Each thymus was dissociated over a sterile 60 µm mesh Sieves screen (Sigma, St. Louis, MO). The cell suspension was washed once with RPMI-1640 media and centrifuged at 300 x g, 4^0C, for 7 min. The cells were resuspended in cold RPMI-1640, 10 µl of cells mixed with 10 ml of 1xPBS and enumerated with a CASY 1 model TTC cell counter plus analyzer system (Scharfe System GMbH, Germany). This system analyzes three 400-µl aliquots of sample per run and the data from the three aliquots are presented as a histogram based on the relative diameter or volume of the cells counted (Gogal Jr. et al., 2001).

4.3.3. Preparation of Pesticides

The 100 mM stock solutions of lindane (purity 99.5%, Chem Service, West Chester, PA) and malathion (purity 99.0%, Chem Service, West Chester, PA) were prepared in absolute ethanol. A 100 mM permethrin (purity 20% cis-78% trans, Chem Service, West Chester, PA) stock solution was prepared in acetone. These stock solutions were used in

preparation of 4x working solutions by diluting with incomplete media. Final concentrations were 37.5, 75, 150, 200 µM for lindane; 37.5, 75, 150, 300 µM for malathion, and 150, 300, 600 µM and 1mM for permethrin.

4.3.4. Cell Culture

Thymocytes were diluted to $5x10^6$ cells/ml in supplemented media [10% Fetal bovine serum (FBS; Atlanta Biol., Norcross, GA), 1% MEM Non-essential amino acids, 1% Penicillin/ Streptomycin, 1% L-glutamine 100x, 200mM, and 1% HEPES (GibcoBRL, Grand Island, NY)] and aliquoted ($5x10^5$ cells/100 µl/well) into 96 well tissue culture plates (Costar, Corning, NY). Fifty microliter aliquots of 4x pesticide solutions were combined for mixture exposure, or the volume compensated with supplemented media for individual exposures. Hundred microliter of 1 µg/ml dexamethasone (Sigma, St. Louis, MO) (Zhi-Jun et al., 1997) and 10 mM hydrogen peroxide (Sigma, St. Louis, MO) were used as apoptosis and necrosis control, respectively. These two controls were used every time to ensure that the assays worked properly. Solvent control samples consisted of 100 µl of 0.6% ethanol and 100 µl of 0.6% acetone. Cells were cultured at 37^0 C, 5% CO_2 in a Nuaire water-jacketed CO_2 incubator (Plymouth, MN).

4.3.5. Flow Cytometric 7-AAD Staining Assay

Following incubation with pesticides, cells were rinsed with PBS and centrifuged at 300 x g, 4^0 C, for 7 min. Cells were then resuspended in 100 µl of the DNA binding dye 7-aminoactinomycin D (7-AAD), (10 µg/ml; Molecular Probes, Eugene, OR) in FACS buffer (2% BSA, 0.15% sodium azide in PBS). The cells were incubated on ice, in the dark, for 30 min and analyzed on a Coulter Epics XL/MXL flow cytometer (Hialeah, FL). One untreated group of cells was kept unstained (PBS only) as a background fluorescence control. Apoptotic cells were identified based on the method previously described by Schmid et al. (1994a) and Donner et al. (1999). These methods evaluate cells in three different populations: live cells ($7AAD^{dull}$), apoptotic cells ($7AAD^{intermediate}$), and late apoptotic/necrotic cells ($7AAD^{bright}$). Relative fluorescence was recorded at an

excitation wavelength of 488 nm and emission wavelength of 670 nm with 5,000 cells counted per sample. The values were reported as percentages of total cells counted.

4.3.6. FITC-Annexin-V/Propidium Iodide Staining

Following incubation with pesticides, cells were rinsed with PBS and were centrifuged at 300 x g, 4^0 C, for 7 min. After centrifugation the supernatant was removed and cells were resuspended in 50 µl Annexin-V-Fluos binding buffer containing 1 µl Annexin-V-Fluorescein and 1µl propidium iodide (PI), (Annexin-V-FLUOS Staining Kit, Roche Diagnostics Co., Indianapolis, IN). The cells were analyzed on a Coulter Epics XL/MXL flow cytometer (Hialeah, FL). Readings were taken at 488 nm excitation, and 525 nm (PI: 625 nm) emission wavelengths with 5,000 cells counted per sample. The values were reported as percentages of total cells counted.

4.3.7. Lactate Dehydrogenase (LDH) Release Assay

This assay was modified from O'Brien et al., (2000) to evaluate cytotoxicity. The reaction is based on enzymatic conversion of tetrazolium salt (pale yellow) to formazan salt (dark pink) in the presence of LDH and diaphorase. The color formation during the reaction is proportional to LDH amount, which has an absorption maximum at 490 nm. Cells were centrifuged at 300 x g, 4^0 C, for 7 min. The supernatant was removed from samples and placed in flat-bottom, 96 well tissue culture plates (Costar, Corning, NY). Working solution-A [25 mg/ml NAD in 1x PBS mixed with DL-lactic acid 60%/carbonate-bicarbonate buffer], and Working solution-B [INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride)/ diaphorase mixture (all chemicals; Sigma, St. Louis, MO)] were added onto supernatants. The reaction was allowed to proceed for 15 min at room temperature, and then stopped by 2N HCl. The absorbance of the samples was measured at 490 nm using a microplate reader (VERSAmax tunable, Molecular Devices Corp., Sunnyvale, CA). Medium-only background control was included and the value obtained was subtracted from all other samples. Release of LDH into the medium was expressed as the percent of total LDH activity that was present in an equivalent number of cells lysed with Triton X-100 (Sigma, St. Louis, MO) with the formula of:

LDH Release (%) = (pesticide exposed samples / maximum release) x 100.

4.3.8. DNA Ladder Assay

Following incubation with pesticides, cells were centrifuged at 300 x g, 4^0 C, for 7 min. The supernatant was removed, and the cell pellets washed once with PBS. The DNA was immediately purified and analyzed as instructed in Boehringer Mannheim's Apoptotic DNA Ladder Kit (Boehringer Mannheim, GmbH, Mannheim). Briefly, cells were incubated with binding/lysis buffer for 10 min, and then mixed with isopropanol. This mixture was filtered through a glass fibered fleece, allowing nucleic acid binding to the surface, then washed twice to clean the cellular impurities off the fleece. Subsequently the purified DNA was released from the fleece using elution buffer at 70^0 C. DNA cleavage into oligonucleosomal fragments induced by pesticide treatment was visualized with agarose/ethidium bromide gel electrophoresis.

4.3.9. Immunophenotyping of Cells using Monoclonal Antibody/7AAD Double Staining

The dye 7-AAD has been shown to be compatible for double staining with fluorescein-isothiocyanate (FITC) and R-phycoerythrin (R-PE) cell surface labels (Schmid et al., 1994b; Donner et al., 1999). Double staining of cells allows evaluation of the sub-groups and quantitating apoptosis/necrosis in these populations. The monoclonal antibodies specific for $CD4^+$ cells [FITC conjugated rat anti-mouse CD4 (L3T4)] and for $CD8^+$ cells [R-PE conjugated rat anti-mouse CD8a (ly-2)] were used for staining. Cells ($5x10^5$ cells/100 μl) were rinsed with 1x PBS and centrifuged. After centrifugation, cell pellets were resuspended in 100 μl 1x PBS including monoclonal antibody concentration equivalent to 1μg/$1x10^6$ cells. Isotype-matching negative control samples were included. The cells were incubated, on ice, in the dark, for 30 min, gently shaking on an orbital shaker. After 30 min, cells were rinsed with 1x PBS and stained with 7-AAD as described in the 7-AAD Staining Assay. Cells then were analyzed with flow cytometer (Coulter Epics XL/MXL). The data were presented for the FITC positive CD4 cells, R-

PE positive CD8 cells. Early apoptotic and late apoptotic/necrotic populations were expressed within each group.

4.3.10. Statistical Analysis

All assays were repeated at least three times and all data are presented as means ± S.E. Analysis of variance (ANOVA) was used with Dunnett's t-test (StatView, Berkeley, CA) to statistically evaluate the data by comparing the results of control groups to treatment groups. All differences of $p \leq 0.05$ were considered as significant.

4.4. Results

4.4.1. Effects of Pesticides on Cytotoxicity

Concentration-dependent increase in cell death with exposure of individual pesticides is illustrated in Figures 4, 5, and 6.

Thymocytes were treated with 37.5, 75, 150, or 200 μM concentrations of lindane for 12h, and analyzed with 7AAD assay (Fig 4). The results indicate that 75 μM and higher concentrations of lindane caused significant increase in both apoptotic and late apoptotic/necrotic cell death compared to 0.6 % ethanol control ($p \leq 0.05$).

The treatment of cells with malathion at concentrations of 37.5, 75, 150, or 300 μM caused an increase in both apoptotic and late apoptotic/necrotic cell death (Fig 5).

Permethrin was found to be non-toxic at 37.5 or 75 μM concentrations (data not shown). When thymocytes were exposed to 150, 300, 600 μM and 1mM concentrations of permethrin, both apoptotic and late apoptotic/necrotic cell death were observed (Fig 6). The increase in cytotoxicity was concentration-dependent and statistically significant compared to 0.6 % acetone control ($p \leq 0.05$).

Concentrations that caused minimum cell death ($\leq LC_{25}$) were chosen from these curves of each pesticide for mixture studies. Then, the effects of malathion and permethrin were evaluated with the combination of lindane, mixed function oxidase (MFO) enzyme system inducer.

Tables 1 and 2 show the results of 7AAD analysis with exposure to pesticide mixtures. As shown in these tables, mixtures of lindane and malathion caused more-than-additive increase in both apoptotic and late apoptotic/necrotic cell populations. The percentage of late apoptotic/necrotic cell death from exposure to lindane-permethrin mixture was also significantly higher than the total toxicity of the individual pesticides ($p \leq 0.05$). It should

be noted that when the results of solvent controls were subtracted from pesticide exposed groups, the mixtures of lindane and permethrin were found to cause as much as two-times higher cell death (both apoptotic and necrotic) than the total cell death caused by individual pesticide exposures (Table 2). Whereas, when mixtures of lindane and malathion were used, almost five-times higher cell death was observed when compared to the total cell death caused by individual pesticide exposures (Table 1). These results are consistent with the potentiation of cytotoxicity when cells are exposed to mixtures of pesticides.

Figures 7 and 8 show representative flow cytometry charts of pesticide mixture exposures. As shown in Figure 7, a mixture of 50 μM lindane and 75 μM malathion exposure caused an intense shift in cells towards late apoptotic/ necrotic gate compared to individual 50 μM lindane or 75 μM malathion exposures. Dead cells with decreased cell size (forward scatter) and increased granularity (side scatter) shifted downward and to right, are more prominent in the mixture exposures.

Similarly, in Figure 8, 50 μM lindane and 150 μM permethrin mixture exposure caused an intense shift towards late apoptotic/necrotic cell population in comparison to individual exposures. In addition, the forward scatter, side scatter analysis of cells shows a comparative increase in dead cell population for the lindane-permethrin mixture.

4.4.2. Effects of Pesticides on LDH Release

To evaluate the cytotoxic effects of pesticides, cells were exposed to varying concentrations of each pesticide for 12 h. Table 3 shows the concentration-dependent LDH release from pesticide-exposed thymocytes. As shown in Table 3, concentrations as low as 37.5 μM of lindane and malathion caused significant increase in LDH release over the ethanol control group ($p \leq 0.05$). A significant increase was demonstrated by permethrin only at higher concentrations (150 μM) over the acetone control group ($p \leq 0.05$). These data indicate that pesticide exposures cause significant damage to plasma membrane of the cells.

4.4.3. Effects of Pesticides on Cell Morphology

Cells that are in earlier stages of apoptosis can be stained with Annexin-V but they exclude PI. However, cells in late stages of apoptosis or necrosis cannot exclude PI due to damaged cellular membrane. Based on this principle, cytotoxicity can be evaluated with Annexin-V/PI double staining. A mixture of 50 μM lindane and 75 μM malathion treatment was found to cause significantly ($p \leq 0.05$) higher toxicity in apoptotic and necrotic populations compared to individual pesticides (Table 4).

As shown in Table 4, the mixture of 50 μM lindane and 150 μM permethrin caused significantly higher toxicity ($p \leq 0.05$) compared to that of individual pesticides.

4.4.4. Effects of Pesticides on DNA Fragmentation

To further evaluate the effects of pesticides on the induction of apoptosis in thymic immune cells, fragmentation of DNA (a hallmark of apoptosis) was investigated by gel electrophoresis. The results showed DNA ladder formation in all pesticide treated cells which is indicative of apoptotic cell death (Figure 9). Exposure of cells to pesticide mixtures (lanes 5 and 7) caused intensive laddering and increased smearing that are indicative of random DNA cleavage, and necrotic cell death. Solvent control cells (lane 2) have a bright band on top of the lane showing intact DNA, and less visible apoptotic laddering.

4.4.5. Effects of Pesticides on Thymocyte Subpopulations

Pesticide exposures in thymocytes did not alter the percent of $CD4^-8^-$, $CD4^+8^-$, $CD4^+8^+$, or $CD4^-8^+$ subpopulations. There was no significant difference between the proportions of subpopulation counts among untreated, solvent control and any pesticide treated group (data not shown). However, pesticides caused significantly higher toxicity within $CD4^-8^+$ labeled cells. The double staining of cells with 7AAD showed that $CD4^-8^+$ cells

underwent both apoptosis (Figure 10) and necrosis (Figure 11) at a much higher rate than the other subgroups of thymocytes.

4.5. Discussion

In this study, we investigated the *in vitro* effects of individual pesticides and pesticide mixtures on the induction of apoptosis and necrosis in C57Bl/6 mouse thymocytes. All of the three selected pesticides --lindane, malathion and permethrin-- caused concentration-dependent increase in both apoptotic and necrotic cell death in thymocytes. The concentrations that caused minimum cell death ($\leq LC_{25}$) were selected from the curves of each pesticide for the mixtures. These low doses were selected because human exposure to low concentrations of different pesticides is more likely to occur in the environment. Moreover, when pesticides are applied to cells in mixtures, the cytotoxicity window should be less than 100% to enable the study of the combined effects without losing the cultured cells.

The 7-AAD staining method has been shown as a reliable assay in the quantification of apoptosis (Donner et al., 1999; Lecoeur et al., 1997; Schmid et al., 1994a & 1994b). In our present study, the results of 7-AAD assay showed a significantly greater induction of apoptosis/ necrosis in murine thymocytes exposed to pesticide mixtures compared to individual pesticides ($p \leq 0.05$). The mode of toxicity appears to be greater-than-additive interaction with pesticide mixtures.

Cells undergoing apoptosis decrease in cell size (forward-scatter), and increase in granularity (side-scatter) (Nicoletti et al., 1991; Swat et al., 1991). In this study, flow cytometric analyses of cells following pesticide treatment showed a similar pattern. Specifically, when cells were exposed to low concentrations of individual pesticides, they were mainly visible in the upper-left quadrant of the flow charts (Figures 7 and 8). However, the exposure of cells to a mixture of pesticides at the same low concentrations resulted in a dramatic downward and right shift of the cells. This is indicative of an increase in the apoptotic/necrotic populations.

Another method that we used to evaluate cytotoxicity was the LDH release assay. The measurement of LDH release from cells is a sensitive marker of the cytotoxicity for

different chemicals *in vitro* (Bonfoco et al., 1995; Lappalainen et al., 1994). In the current investigation, colorimetric analysis of supernatants after 12h exposure showed significant elevation in LDH release compared to control group even with very low lindane and malathion concentrations ($p \leq 0.05$). In case of permethrin, similar levels of LDH release were evident only at higher concentrations of this pesticide. Thus, it is not surprising that low concentrations of permethrin had trivial effects in release of this intracellular enzyme because permethrin is known to have less toxic effects compared to lindane or malathion. (LD_{50} for lindane, malathion and permethrin in male rats has been reported to be 88, 1000, and 1500 mg/kg, respectively).

To further examine the potential of individual pesticides and pesticide mixtures to induce apoptotic and/or necrotic conditions of thymocytes, FITC-Annexin-V/ Propidium Iodide (PI) assay was conducted. Vermes et al., (1995) have reported that this methodology allows the detection of phosphatidylserine (PS) translocation from the inner to the outer surface of the cell membrane in apoptotic cells. PI is a fluorescent vital dye that stains DNA and used to evaluate plasma membrane integrity. Cells that are in the late stages of apoptosis or cells that are necrotic lose plasma membrane integrity and are permeable to PI. Hence, PI is used to distinguish cells that are in the earlier stages of apoptosis (Annexin-V^+/PI^-) from those that are in the later stages of apoptosis or dead (Annexin-V^+/PI^+). The percentages of cell death are higher with Annexin-V labeling compared to 7AAD staining because Annexin-V technique allows labeling of cells in earlier stages of apoptosis. The translocation of phosphatidylserine from inner to the outer leaflet of cell membrane (detected with Annexin-V) occurs earlier than the loss of membrane integrity (detected with 7AAD). The Annexin-V/PI labeling of cells in this study showed that the toxicity caused by pesticide mixtures was significantly higher ($p \leq 0.05$) than that toxicity caused by individual pesticide exposures, both in early apoptotic and necrotic cell populations (Table 4).

The fragmentation of DNA, a hallmark of apoptosis, provided further evidence of cells undergoing apoptosis. The apoptotic cells are known to break-up the genomic DNA by the activation of endonucleases (Khodarev et al., 1998; Wyllie A.H., 1980). These

oligonucleosome-sized DNA fragments and their multiples appear as DNA laddering. In addition, the band at the top of the lane is indicative of the remains of genomic DNA after treatment.

Although several reports have highlighted the need for chemical-mixtures toxicity data for conducting risk assessment (Calabrese, 1995; Simmons, 1995), to date there is a paucity of data on the effects of pesticide mixtures on mammalian immune system. In addition, mechanistic information on the interactions of most pesticides and chemicals are equally unavailable. Marinovich et al. (1996) showed within nervous system that pesticide mixtures cause an increase in toxicity compared to the single compounds. In one recent study on immune system, Institoris et al., (2002) suggested that the exposure limits of chemicals should be reconsidered when the risk of combined exposure is high. The present paper presents an initial effort to elucidate the mechanism of toxicity (apoptosis) of pesticide mixtures to the immune system. Furthermore, it appears from our findings that the initial induction of drug metabolizing mixed function oxidase (MFO) system enzymes with lindane may, in part, be responsible for the metabolic activation of the second pesticide leading to the augmentation of the overall toxicity. However, the mechanistic basis for the toxicodynamic interactions of these different classes of pesticides needs further investigation.

In conclusion, the results of the present study indicate that pesticides cause concentration-dependent cell death in murine thymocytes and the toxicity becomes significantly higher for exposure to pesticide mixtures compared to individual pesticides.

Figure 4. C57BL/6 thymocytes were treated with 0.6 % ethanol, 37.5 μM, 75μM, 150 μM, and 200 μM lindane for 12h. 7AAD assay was employed to measure the percentage of apoptotic (A), and late apoptotic/necrotic (B) cells. Samples were examined via flow cytometry for the intensities of staining, $7AAD^{moderate}$ (apoptotic cells) and $7AAD^{bright}$ (dead/late apoptotic). Treatments with similar letters were *not* significantly different. All other treatment comparisons *were* statistically different ($p \leq 0.05$). Data are presented as the mean $\pm$ SE.

Figure 5. C57BL/6 thymocytes were treated with 0.6 % ethanol, 37.5 μM, 75μM, 150 μM, and 300 μM malathion for 12h 7AAD assay was employed to measure the percentage of apoptotic (A), and late apoptotic/necrotic (B) cells. Samples were examined via flow cytometry for the intensities of staining, $7AAD^{moderate}$ (apoptotic cells) and $7AAD^{bright}$ (dead/late apoptotic). Treatments with similar letters were *not* significantly different. All other treatment comparisons *were* statistically different ($p \leq 0.05$). Data are presented as the mean $\pm$ SE.

Figure 6. C57BL/6 thymocytes were treated with 0.6 % acetone, 150 μM, 300μM, 600 μM, and 1 mM permethrin for 12h. 7AAD assay was employed to measure the percentage of apoptotic (A), and late apoptotic/necrotic (B) cells. Samples were examined via flow cytometry for the intensities of staining, $7AAD^{moderate}$ (apoptotic cells) and $7AAD^{bright}$ (dead/late apoptotic). Treatments with similar letters were *not* significantly different. All other treatment comparisons *were* statistically different ($p \leq 0.05$). Data are presented as the mean $\pm$ SE.

Figure 7. Effect of pesticide mixtures on thymocyte apoptosis in C57Bl/6 mice. Cells were treated with lindane, malathion or their mixtures for 12h then stained with 7AAD and analyzed with flow cytometry. B= live cells ($7AAD^{dull}$), C= apoptotic cells ($7AAD^{intermediate}$), D= Late apoptotic/necrotic cells ($7AAD^{bright}$). Mixture exposure of pesticides caused decrease in forward scatter and increase in side scatter. Cells shifted through D (late apoptosis/necrosis) with mixture treatment of pesticides.

Figure 8. Effect of pesticide mixture on thymocyte apoptosis in C57Bl/6 mice. Cells were treated with lindane, permethrin or their mixtures for 12h then stained with 7AAD and analyzed with flow cytometry. B= live cells ($7AAD^{dull}$), C= apoptotic cells ($7AAD^{intermediate}$), D= Late apoptotic/necrotic cells ($7AAD^{bright}$). Mixture exposure of pesticides caused decrease in forward scatter and increase in side scatter. Cells shifted through D (late apoptosis/necrosis) with mixture treatment of pesticides.

Figure 9. DNA gel electrophoresis. Thymocytes were incubated with pesticides and pesticide mixtures for 12h, and then subjected to DNA gel electrophoresis. Electrophoresis showed DNA ladder formation, which is characteristic for apoptosis. Solvent control cells had intact DNA on top of the lane, and less visible apoptosis laddering. However, all pesticide treated groups showed intense laddering. Increased smearing in lanes 5 and 7 with pesticide mixtures also shows the necrotic death in cells.

Figure 10. The percentages of apoptosis within $CD4^+8^-$, $CD4^+8^+$ and $CD4^-8^+$ subgroups following treatment with pesticides and pesticide mixtures measured by monoclonal antibody & 7-AAD double staining. The monoclonal antibody specific for $CD4^+$ cells, FITC conjugated rat anti-mouse CD4 (L3T4), and for $CD8^+$ cells, R-PE conjugated rat anti-mouse CD8a (ly-2), were used for thymocytes.

Figure 11. The percentages of late apoptosis/necrosis within $CD4^+8^-$, $CD4^+8^+$ and $CD4^-8^+$ subgroups following treatment with pesticides and pesticide mixtures measured by monoclonal antibody & 7-AAD double staining. The monoclonal antibody specific for $CD4^+$ cells, FITC conjugated rat anti-mouse CD4 (L3T4), and for $CD8^+$ cells, R-PE conjugated rat anti-mouse CD8a (ly-2), were used for thymocytes.

Table 1. Evaluation of cytotoxic effects of lindane and malathion with 7AAD staining.

EXPOSURE	Live Cells (%)	Apoptotic Cells (%)	Late Apoptotic/ Necrotic Cells (%)
Ethanol control	85.5 ± 1.0	9.3 ± 0.8	5.0 ± 0.3
Lindane 50μM	83.3 ± 1.0	10.4 ± 0.8	6.2 ± 0.4
Malathion 75 μM	77.9 ± 1.5 [a]	13.0 ± 1.3 [a]	8.5 ± 0.3 [a]
Lindane 50 μM + Malathion 75 μM	39.4 ± 2.6 [a, b]	30.1 ± 1.3 [a, b]	29.8 ± 1.8 [a, b]

TABLE 1. The percent of live, apoptotic or necrotic cells following treatment with pesticides or pesticide mixtures as assessed by flow cytometer in combination with 7AAD Staining assay. C57Bl/6 murine thymocytes were treated with 0.6% ethanol, 50 μM lindane, 75 μM malathion, or lindane+malathion mixture for 12h. The percent of cells stained as $7AAD^{dull}$ (live), $7AAD^{moderate}$ (apoptotic) and $7AAD^{bright}$ (dead) is presented. Data shown are the mean ± SE, n = 5. **a** Statistically significant difference ($p \leq 0.05$) when compared to control. **b** Statistically significant difference ($p \leq 0.05$) when compared to single pesticide treatments.

Table 2. Evaluation of cytotoxic effects of lindane and permethrin with 7AAD staining.

EXPOSURE	Live Cells (%)	Apoptotic Cells (%)	Late Apoptotic/ Necrotic Cells (%)
Acetone control	86.8 ± 0.5	7.8 ± 0.5	5.1 ± 0.3
Lindane 50μM	82.2 ± 0.7	8.2 ± 0.8	9.4 ± 0.6
Permethrin 150 μM	70.6 ± 1.8[a]	13.1 ± 1.0 [a]	15.8 ± 1.2 [a]
Lindane 50 μM + Permethrin 150 μM	46.0 ± 5.4 [a, b]	17.1 ± 2.2 [a, b]	36.2 ± 3.3 [a, b]

TABLE 2. The percent of live, apoptotic or necrotic cells following treatment with pesticides or pesticide mixtures as assessed by flow cytometer in combination with 7-AAD Staining assay. C57Bl/6 murine thymocytes were treated with 0.6% acetone, 50 μM lindane, 150 μM permethrin, or lindane+permethrin mixture for 12h. The percent of cells stained as $7AAD^{dull}$ (live), $7AAD^{moderate}$ (apoptotic) and $7AAD^{bright}$ (dead) is presented. Data shown are the mean ± SE, n = 5. **a** Statistically significant difference ($p \leq 0.05$) when compared to control. **b** Statistically significant difference ($p \leq 0.05$) when compared to single pesticide treatments.

Table 3. Evaluation of cytotoxic effects of lindane, malathion and permethrin with LDH release assay.

Lindane Exposure	LDH Release (%)	Malathion Exposure	LDH Release (%)	Permethrin Exposure	LDH Release (%)
Ethanol control	8.9 ± 0.8	Ethanol control	8.9 ± 0.8	Acetone control	18.4 ± 1.2
37.5 µM	21.6 ± 1.7 [a]	37.5 µM	17.6 ± 0.6 [a]	150 µM	27.0 ± 1.6 [a]
75 µM	32.6 ± 1.7 [a]	75 µM	29.1 ± 2.0 [a]	300 µM	45.3 ± 2.1 [a]
150 µM	38.2 ± 1.1 [a]	150 µM	40.2 ± 3.6 [a]	600 µM	52.5 ± 1.7 [a]
200 µM	49.9 ± 1.7 [a]	300 µM	54.0 ± 3.4 [a]	1 mM	57.7 ± 2.2 [a]

TABLE 3. The percentages of LDH enzyme release from cells following treatment with 0.6% ethanol, 0.6% acetone as solvent controls, or different concentrations of lindane, malathion, and permethrin for 12h. Data shown are the mean ± SE. **a**: Statistically significant difference ($p \leq 0.05$) when compared to control.

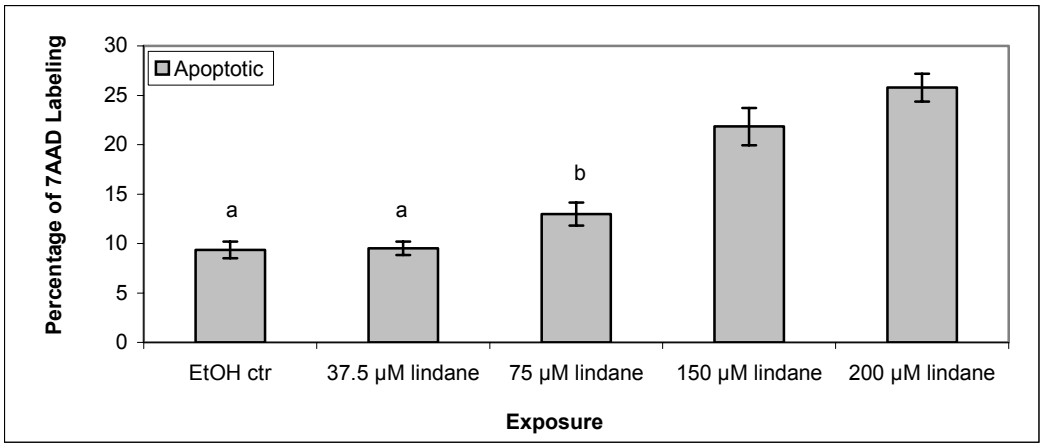
Table 4. Evaluation of cytotoxic effects of lindane, malathion, permethrin and their mixtures with the Annexin-V/PI assay.

Exposure (Ann-V Staining)	Live cells (%)	Early apoptotic cells (%)	Late apoptotic / Necrotic cells (%)
Ethanol control	68.3 ± 1.4	24.5 ± 1.3	6.9 ± 0.3
L -50 µM	63.1 ± 0.6 [a]	26.8 ± 0.5	8.7 ± 1.1
M -75 µM	59.5 ± 1.0 [a]	26.0 ± 1.0	14.2 ± 0.8 [a]
LM Mixture	42.8 ± 2.7 [a, b]	37.6 ± 2.6 [a, b]	19.2 ± 1.0 [a, b]
Acetone control	67.0 ± 1.2	25.8 ± 1.1	6.9 ± 0.3
L -50 µM	64.2 ± 0.6	26.6 ± 0.5	9.0 ± 0.3
P -150 µM	70.3 ± 0.9	19.3 ± 0.8	10.1 ± 0.7 [a]
LP Mixture	45.8 ± 2.9 [a, b]	30.0 ± 1.6 [a, b]	23.8 ± 2.0 [a, b]

TABLE 4. The percent of live, early apoptotic or necrotic cells following treatment with pesticides or pesticide mixtures as assessed by the Annexin-V Staining assay. Data shown are the mean ± SE, percentage of staining, n = 5. L = lindane, M = malathion, P = permethrin. The percent of cells stained as Ann-V^-/PI^- (live), Ann-V^+/PI^- (early apoptotic) and Ann-V^+/PI^+ (dead) is presented. **a** Statistically significant difference ($p \leq 0.05$) when compared to control. **b** Statistically significant difference ($p \leq 0.05$) when compared to single pesticide treatments.

Figure 4

A)


Apoptotic
Percentage of 7AAD Labeling
30
25
20
15
10
5
0
a
a
b
EtOH ctr
37.5 µM lindane
75 µM lindane
150 µM lindane
200 µM lindane
Exposure

B)

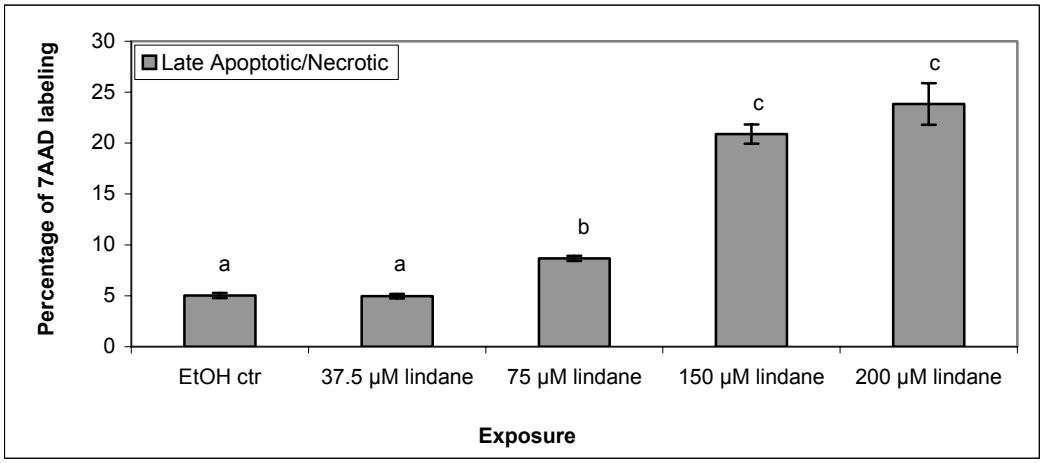
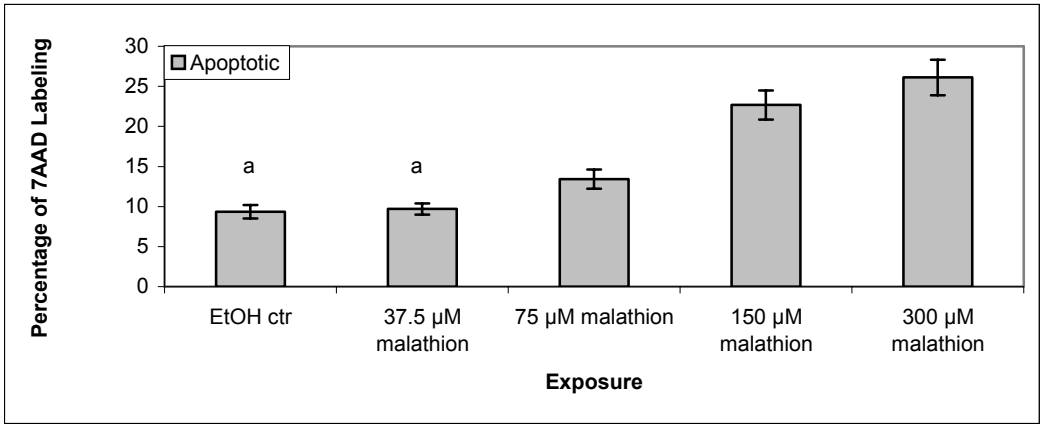

Late Apoptotic/Necrotic
Percentage of 7AAD labeling
30
25
20
15
10
5
0
a
a
b
c
c
EtOH ctr
37.5 µM lindane
75 µM lindane
150 µM lindane
200 µM lindane
Exposure

Figure 5

A)


Percentage of 7AAD Labeling
Apoptotic
30
25
20
15
10
5
0
a
a
EtOH ctr
37.5 µM malathion
75 µM malathion
150 µM malathion
300 µM malathion
Exposure

B)

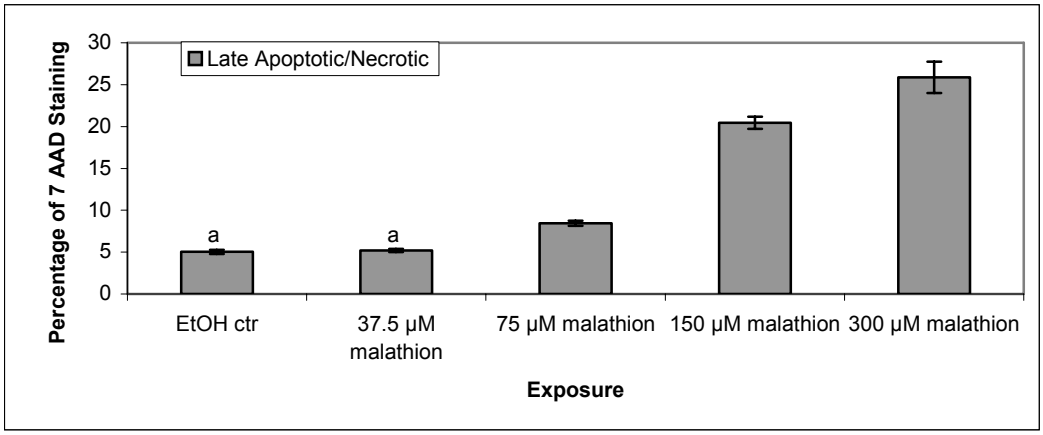
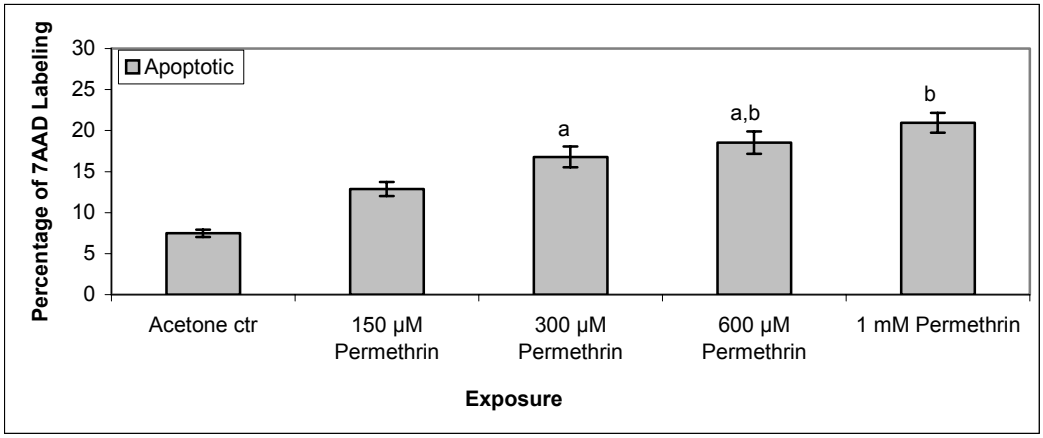

Percentage of 7 AAD Staining
Late Apoptotic/Necrotic
30
25
20
15
10
5
0
a
a
EtOH ctr
37.5 µM malathion
75 µM malathion
150 µM malathion
300 µM malathion
Exposure

Figure 6

A)


Percentage of 7AAD Labeling
30
25
20
15
10
5
0
Apoptotic
a
a,b
b
Acetone ctr
150 µM Permethrin
300 µM Permethrin
600 µM Permethrin
1 mM Permethrin
Exposure

B)

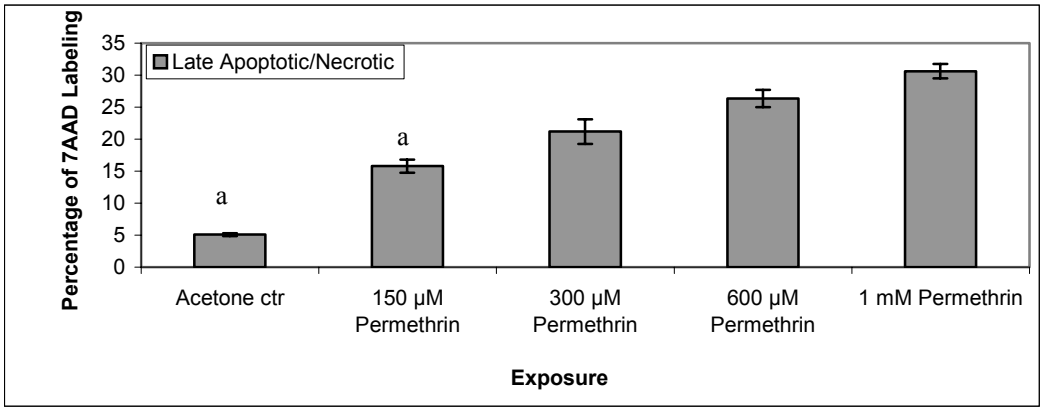

Percentage of 7AAD Labeling
35
30
25
20
15
10
5
0
Late Apoptotic/Necrotic
a
a
Acetone ctr
150 µM Permethrin
300 µM Permethrin
600 µM Permethrin
1 mM Permethrin
Exposure

Figure 7.

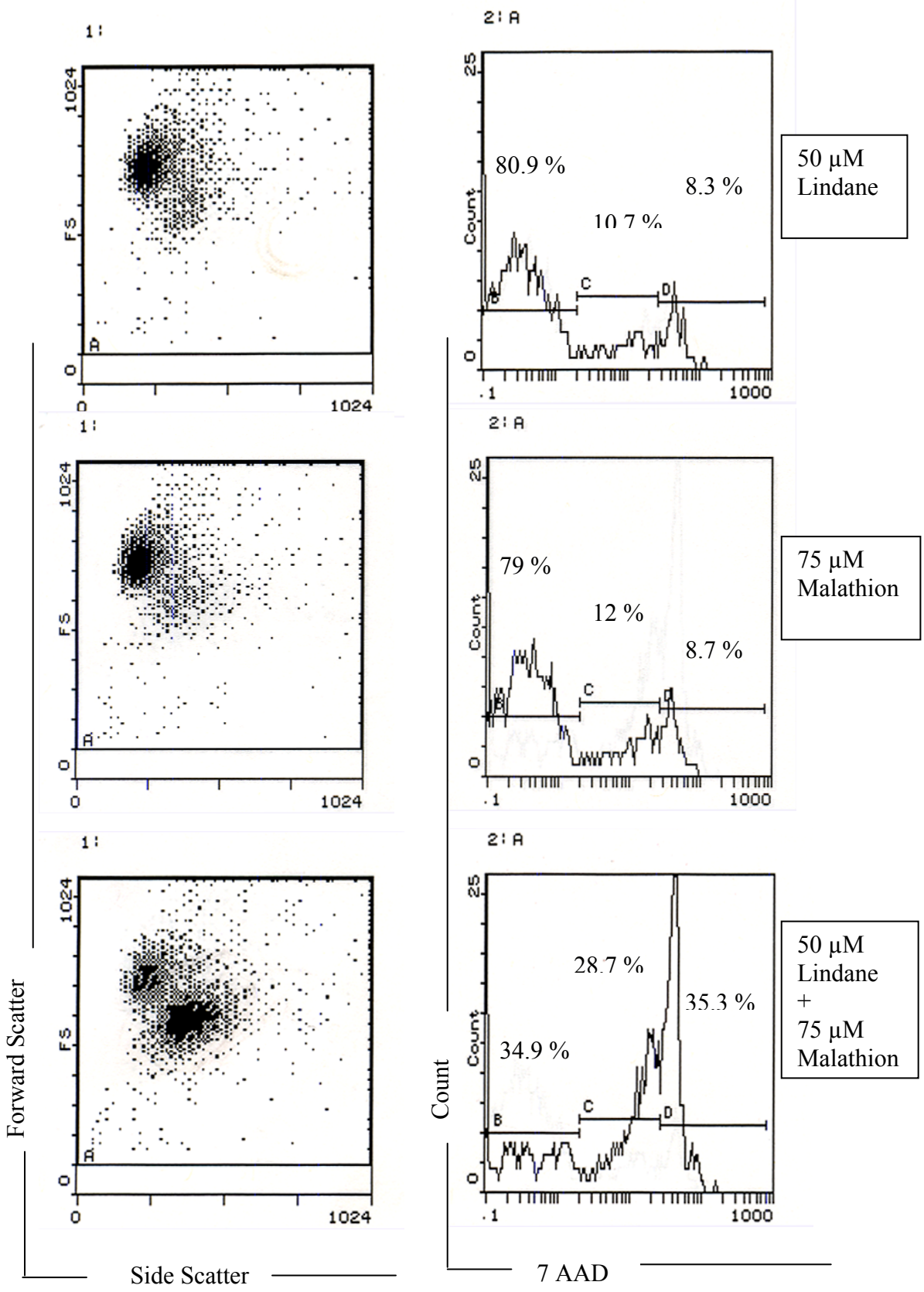

1:
1024
FS
0
A
0
1024
2: A
25
Count
80.9 %
8.3 %
10.7 %
B
C
D
0
.1
1000
50 µM
Lindane
1:
1024
FS
0
A
0
1024
2: A
25
Count
79 %
12 %
8.7 %
B
C
D
0
.1
1000
75 µM
Malathion
1:
1024
FS
0
A
0
1024
2: A
25
Count
28.7 %
35.3 %
34.9 %
B
C
D
0
.1
1000
50 µM
Lindane
+
75 µM
Malathion
Forward Scatter
Side Scatter
Count
7 AAD

Figure 8.

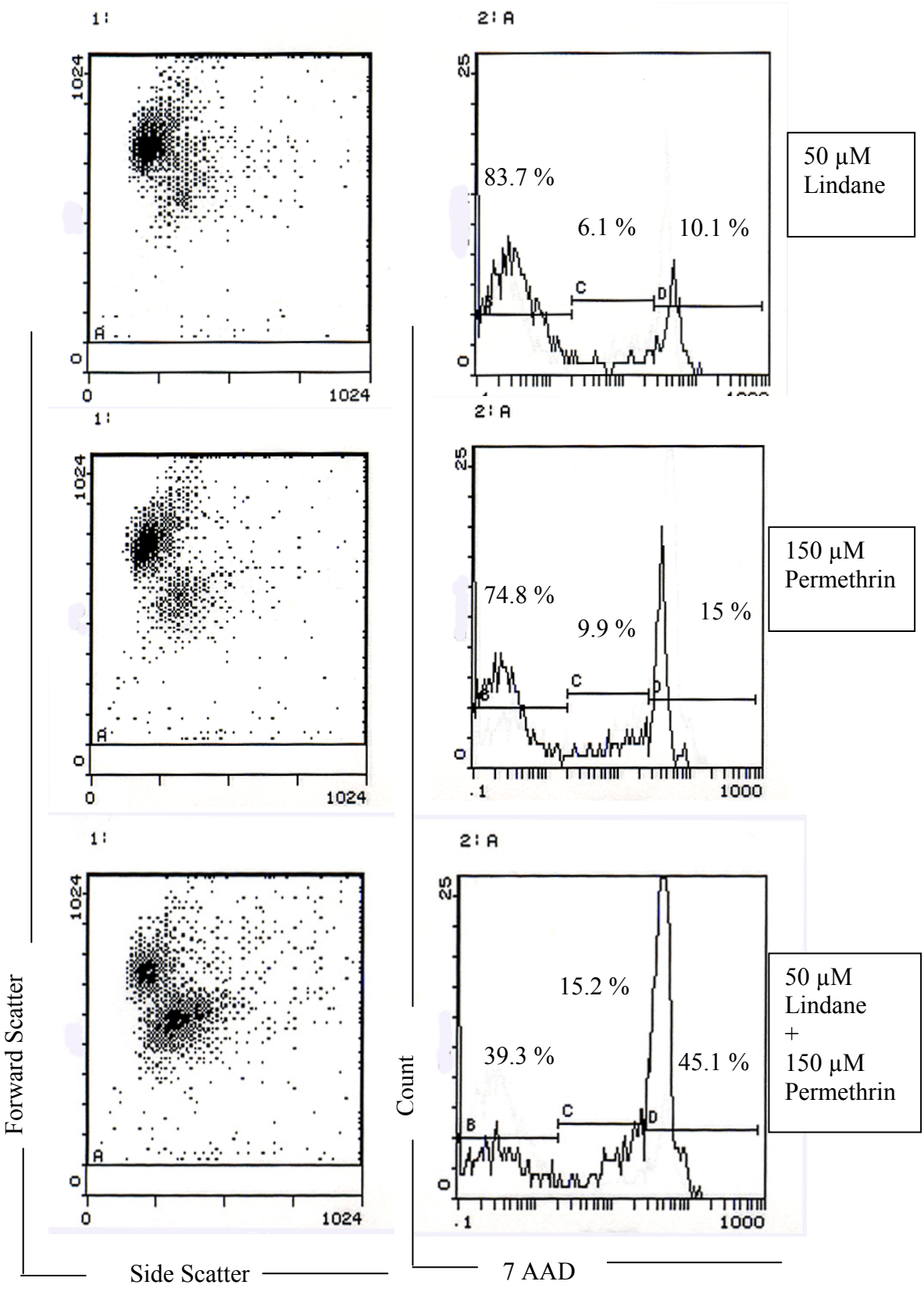
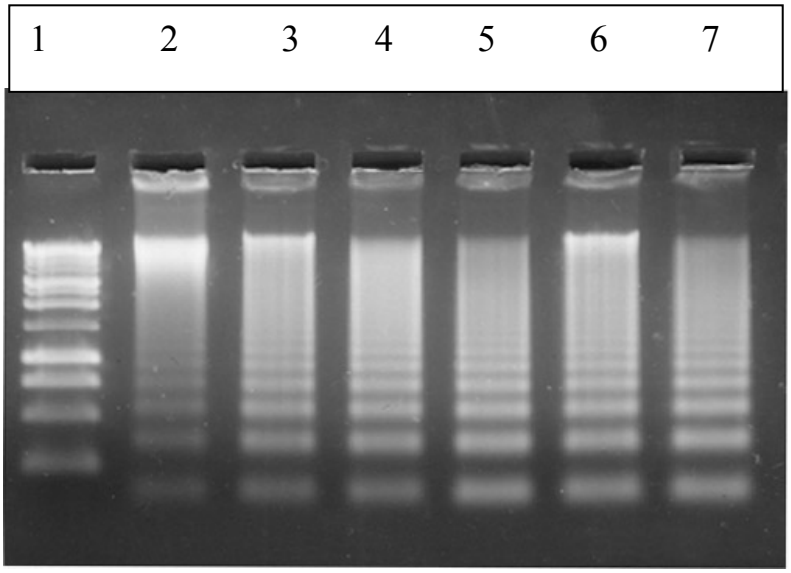

1:
1024
0
A
0
1024
2: A
25
0
83.7 %
6.1 %
10.1 %
C
D
50 µM
Lindane
1:
1024
0
A
0
1024
2: A
25
0
74.8 %
9.9 %
15 %
C
D
.1
1000
150 µM
Permethrin
1:
1024
0
A
0
1024
2: A
25
0
15.2 %
39.3 %
45.1 %
B
C
D
.1
1000
50 µM
Lindane
+
150 µM
Permethrin
Forward Scatter
Side Scatter
Count
7 AAD

Figure 9



Lanes:

1 = 1Kb ladder

2 = Control

3 = 50 μM Lindane

4 = 75 μM Malathion

5 = Lindane/Malathion Mixture

6 = 150 μM Permethrin

7 = Lindane/Permethrin Mixture

Figure 10

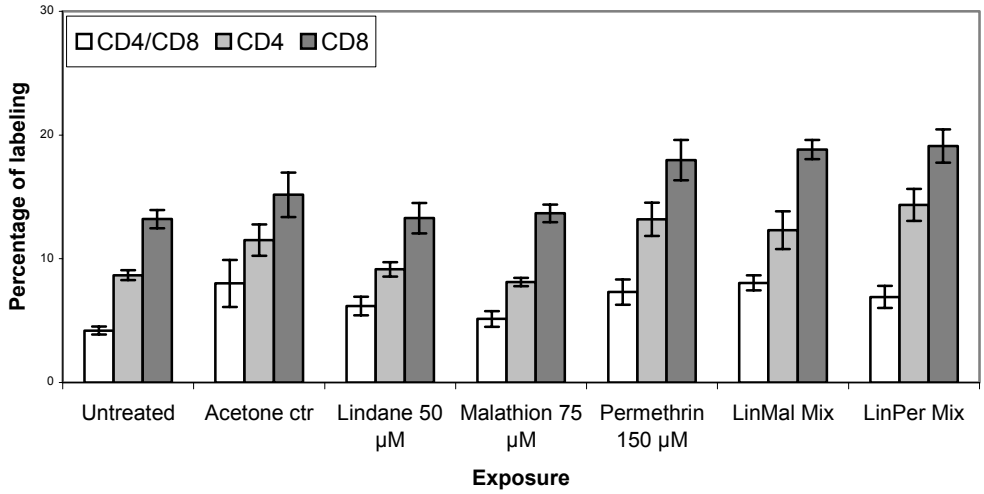
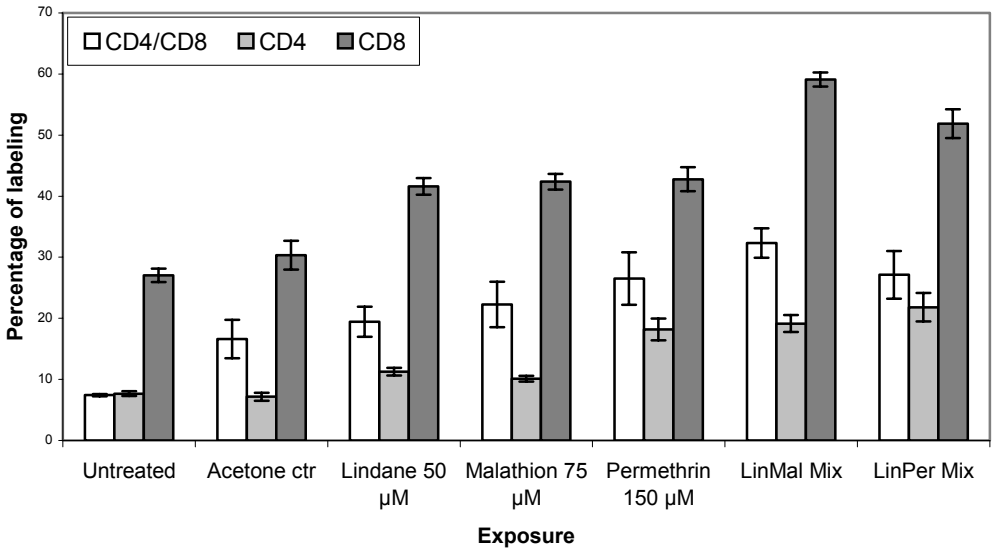

CD4/CD8
CD4
CD8
Percentage of labeling
30
20
10
0
Untreated
Acetone ctr
Lindane 50 µM
Malathion 75 µM
Permethrin 150 µM
LinMal Mix
LinPer Mix
Exposure

Figure 11


CD4/CD8
CD4
CD8
Percentage of labeling
70
60
50
40
30
20
10
0
Untreated
Acetone ctr
Lindane 50 µM
Malathion 75 µM
Permethrin 150 µM
LinMal Mix
LinPer Mix
Exposure

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Chapter 5. PESTICIDE MIXTURES INCREASED IMMUNOTOXICITY IN C57BL/6 MICE, in vivo

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5.1. Abstract

To increase information on the toxicity of mixtures, we studied the combined effects of lindane, malathion, and permethrin on the murine (C57BL/6) immune system, *in vivo*. Exposure to individual pesticides did not alter the thymus/body and spleen/body weight ratios, thymic and splenic cell counts, or CD4/CD8 and CD45R/B220/CD90.2 phenotyping of cells. However, anti-sRBC plaque forming cell (PFC) counts were significantly lowered even at the lowest doses of pesticide exposure. Thus, the mean PFC counts/10^6 splenocytes were found to be 58 for corn oil; 39, 37, 37 for high, medium and low doses of lindane; 36, 38, 46 for high, medium and low doses of malathion; and 41, 34, 42 for high, medium and low doses of permethrin, respectively. From individual exposure data, one-third of the LD_{50} of each pesticide was selected for the mixtures studies. Animals were injected intraperitoneally with the mixture of one-third of the LD_{50} of two pesticides (lindane + malathion and lindane + permethrin). Exposure to pesticide mixtures did not alter the percentage of $CD4^+CD8^-$ or $CD4^-CD8^+$ and CD45R/B220 or CD90.2 cell subpopulations. However, the thymus/body and spleen/body weight ratios, thymic and splenic cell counts, and PFC counts were significantly lowered in samples collected from mice exposed to pesticide mixtures. Lindane, malathion, and permethrin were immunotoxic and mixtures of these pesticides caused higher toxicity when compared to individual exposures.

5.2. Introduction

Pesticides, which include insecticides, fungicides, herbicides, rodenticides, and fumigants, are the largest group of potentially toxic chemicals that are intentionally introduced into the environment. Despite public concern for their toxicity, pesticides contribute to human health through the control of insect vectors for diseases and by significantly improving crop production (Saunders et al., 1994). Toxic potentials of most of these chemicals have been studied and databases formed for risk assessment purposes. However, until recent years more than 95% of all these toxicity studies were carried out on single environmental pollutants (Groten et al., 1999; Simmons, 1995; Yang, 1994). Yet in industrialized societies, human exposure to chemicals is rarely limited to a single compound.

This study examined the combined effects of lindane (an organochlorine), malathion (an organophosphate), and permethrin (a synthetic pyrethroid) on the immune system of C57BL/6 mice, *in vivo*. Immunotoxicity of these pesticides has previously been reported in laboratory animals. For example, lindane exposure markedly suppressed the humoral immune response in rats as evaluated by reduced gamma glutamyl transpeptidase activity in different tissues of the lymphoid system (Koner et al., 1997), and by anti-sheep red blood cell antibody titers (Koner et al., 1998). Lindane exposure caused thymus cortex atrophy, suppression of bone marrow cellularity, and decreased granulocyte-macrophage progenitor cells in mice with doses as low as 10–20 mg/kg/day for 10 days. (Hong and Boorman, 1993). In addition, dose-related reduction in total white blood cell counts was found in spleen and pronephros of tilapian fish exposed to lindane (Hart et al., 1997). *In vitro* exposure of murine splenocytes to malathion inhibited the proliferative response to mitogen stimulation, while in vivo exposure of peritoneal cells suppressed the respiratory burst activity (Rodgers and Ellefson, 1990). Oral administration of malathion caused elevation in macrophage function (Rodgers and Xiong, 1997a and 1997b). Malathion has also been demonstrated to decrease serum immunoglobulin (IgM and IgG) concentrations, and inhibit leukocyte- and macrophage-migration (Banerjee et al., 1998). Topical permethrin application has been shown to inhibit splenic T cell proliferation and

cause a dose-related decrease in thymic cellularity (Prater et al., 2002). In an *in vivo* study, Blaylock et al. (1995) evaluated the immunotoxic effects of permethrin in BALB/c mice splenocytes after 0–0.4 mg/kg/day oral administration for 10 days. The authors reported significantly reduced mixed lymphocyte responses, T-lymphocyte cytotoxic activity, and natural killer cell activity with the highest test dose.

Studies evaluating the immunotoxicity of mixtures of these three chemicals are not available. We recently observed potentiation of toxicity in the form of increased apoptosis with the mixtures of these compounds, *in vitro* (Olgun et al., 2003). The present study extends these results to include immune parameters, such as humoral immune response, in mice after two days exposure to these pesticides.

5.3. Materials and Methods

5.3.1. Animals

Male C57BL/6 mice, aged 8-12 weeks, were purchased from Charles River Laboratories (Wilmington, MA) and quarantined 7 days prior to use. Mice were housed in polycarbonate cages with hard wood chip bedding in rooms in which the temperature (21^0 C), humidity (50 %), and light cycle (14 h dark/10 h light) were controlled. Food and water were available ad libitum. All animal care and animal protocols were in compliance with the Virginia Polytechnic Institute and State University Animal Welfare Committee guidelines. Each treatment group consisted of 6 mice.

5.3.2. Tissue Collection and Isolation

Mice were euthanized by cervical dislocation, and body weights were recorded. The thymus and spleen were removed from each mouse, weighed and individually placed in 60 x 15 mm Petri dishes containing cold, phenol red-depleted RPMI-1640 media (Gibco BRL, Grand Island, NY). Each organ was dissociated over a sterile 60 µm mesh Sieves screen (Sigma, St. Louis, MO). The cell suspension was washed once with RPMI-1640 media and centrifuged at 300x g, 4^0C, for 7 min. The cell pellet was resuspended in cold RPMI-1640, and 10 µl of this suspension was mixed with 10 ml of PBS before enumerating with a CASY 1 model TTC cell counter plus analyzer system (Scharfe System GMbH, Germany). This system analyzes three 400-µl aliquots of sample per run and the data from the three aliquots are presented as a histogram based on the relative diameter or volume of the cells counted (Gogal et al., 2001).

5.3.3. Pesticides Preparation and Treatment Protocols

Lindane (purity 99.5%, Chem Service, West Chester, PA), malathion (purity 99.0%, Chem Service, West Chester, PA) and permethrin (purity: 20% cis,-78% trans, Chem Service, West Chester, PA) were dissolved in corn oil. All dosing solutions were kept in

the dark at room temperature. One-half, one-third, one-fourth, or one-eight of LD_{50} dosages of each pesticides were prepared by adjusting to mouse body weight, and each mouse was given 50 µl of the pesticides by intraperitoneal (I.P.) injection. These values were based on an LD_{50} of 88mg/kg for lindane (Smith, 1991), an LD_{50} of 1000 mg/kg for malathion (Gallo and Lawryk, 1991), and an LD_{50} of 1500 mg/kg for permethrin (Cantalamessa, 1993). Controls were given an equivalent volume of corn oil.

For the single pesticide exposure experiments, animals were randomly divided into groups of six per treatment group (n = 6) and injected I.P. with individual pesticides on Day 1 and Day 3. One-third, one-fourth, and one-eight of the LD_{50} of lindane were 29.3, 22, and 11 mg/kg, respectively; one-half, one-third, and one-fourth of the LD_{50} of malathion were 500, 333.3, and 250 mg/kg of malathion, respectively; one-half, one-third, and one-fourth of the LD_{50} of permethrin were 750, 500, and 375 mg/kg, respectively. All animals were challenged with sheep red blood cells (sRBC; 500 µl, $2x10^8$ cells, I.P. injection) on Day 4, and sacrificed on Day 8. Six animals in the control group were injected with corn oil on Day 1 and Day 3, challenged with sRBC on Day 4, and sacrificed on Day 8.

For the pesticide mixture experiments, one-third of LD_{50} doses of lindane, malathion, and permethrin were used (29.3 mg/kg, 333 mg/kg, 500 mg/kg, respectively). Two groups of animals (n = 6) were injected I.P. with one-third of the LD_{50} of lindane on Day 1. On Day 3, one of these groups was injected with 29.3 mg/kg of lindane + 333 mg/kg malathion, and the other group was injected with 29.3 mg/kg of lindane + 500 mg/kg permethrin. All animals were injected I.P. with sheep red blood cells (sRBC; 500 µl, $2x10^8$ cells) on Day 4, and sacrificed on Day 8. Six animals in the control group were injected with corn oil on Day 1 and Day 3, challenged with sRBC on Day 4, and sacrificed on Day 8.

5.3.4. Immunophenotyping of Cells using Monoclonal Antibody Labeling

Percentages of T and B cell populations within the splenocytes were evaluated by fluorescein-isothiocyanate (FITC)-conjugated rat anti-mouse CD90.2 (thy1.2) (BD

Pharmingen, San Diego, CA) and R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD45R/B220 (BD Pharmingen) cell surface labels, respectively.

Splenic $CD4^+$ T cells and $CD8^+$ T cells subpopulations were labeled with the monoclonal antibodies specific for $CD4^+$ T cells [FITC-conjugated rat anti-mouse CD4 (L3T4)] and for $CD8^+$ T cells [R-PE-conjugated rat anti-mouse CD8a (ly-2)].

Cells ($5x10^5$ cells/100 µl) were rinsed with PBS and centrifuged. After centrifugation, the cell pellets were resuspended in 100 µl PBS including monoclonal antibody concentration equivalent to 1 µg/$1x10^6$ cells. Isotype-matching negative control samples were included (isotype FITC rat $IgG2_{a,\ kappa}$ and isotype R-PE rat $IgG2_{a,\ kappa}$). The cells were incubated, on ice, in the dark, for 30 min, gently shaking on an orbital shaker. After 30 min, cells were analyzed by flow cytometry (Coulter Epics XL/MXL).

5.3.5. Plaque-Forming Cell (PFC) Assay

Ability of splenocytes to produce specific antibody was measured by quantifying the plaque-forming cell (PFC) response to the T-dependent antigen, sheep red blood cells (sRBC) (Roitt and Delves, 1992). Mice were immunized by I.P. injection of 500 µl of 5% sRBC ($2x10^8$ sRBC), and IgM PFCs were enumerated in splenic cells 4 days later. Briefly, the spleen was collected under sterile conditions and immediately placed in 15 ml cold supplemented RPMI-1640 media in a 60 x 15 mm sterile petri dish. The spleen was then dissociated over a sterile 60 µm mesh Sieves screen, and cells were enumerated with a CASY 1 analyzer system. Splenic cell suspensions of $20x10^6$ cells/ml were prepared in sterile culture medium. An aliquot of each sample (50 µl) was mixed with 20 µl guinea pig complement (Sigma, St. Louis, MO), 30 µl of 30% sRBC ($12x10^8$ sRBC), and 400 µl of 47^0C warmed plaque-agar [Plaque-agar: 500 mg agar (Sigma, St. Louis, MO), mixed with 110 ml Earle's solution (Sigma, St. Louis, MO), and 1.6 ml DEAE-dextran, and adjusted to pH 7.3] in a 60 x 15 mm sterile petri dish, and covered with cover glass (Punareewattane et al., 2001). Dishes were allowed to sit at room temperature for 5 min to solidify the agar before they were incubated at 37^0 C, 90% humidity, 5% CO_2 for 4h (Punareewattane et al., 2001). After 4h incubation, the number of plaques was

counted under a light microscope. Results were expressed as number of PFC per 10^6 cells, and number of PFC per spleen.

5.3.6. Measurement of Cytochrome P-450 Enzymes

A published protocol for subfractionation of microsomes was adapted for use (Schneider, 1948). Minced and homogenized liver tissue (1:10) was mixed with 0.25 M sucrose/10mM Tris·Cl, pH 7.4 buffer. This homogenate was centrifuged for 10 min at 600x g, 4^0C, to sediment the unbroken cells, nuclei and cell debris. The supernatant from this step was then centrifuged for 15 min at 6500x g, 4^0C to sediment the mitochondria. The supernatant was further centrifuged for 10 min at 12,000x g, 4^0C. Sufficient volume of 80 mM $CaCl_2$ was added to the supernatant to prepare an 8 mM final concentration. This suspension was kept on ice for 15 min, and then centrifuged for 15 min at 25,000x g, 4^0C. The supernatant was discarded and 5 ml of 0.15 M KCl was layered on the pellet. This mixture was centrifuged for 20 min at 25,000x g, 4^0C, the supernatant was discarded, and the microsomal pellet was homogenized in enough buffered sucrose to provide a protein concentration of 20 mg/ml. The microsomal preparation was diluted (1:20) with 0.05 M Tris·Cl, pH 7.4. On the spectrophotometer, baseline was recorded (at 450 nm) with this dilution. After bubbling carbon monoxide for 30 seconds and adding a few crystals of dithionite, the spectrum was recorded again (at 500 nm). The concentration of cytochrome P-450 was then calculated using the absorption difference with the extinction coefficient as described by Omura and Sato (1964).

5.3.7. Statistical Analysis

One experiment was performed including six animals (n = 6) per treatment group, and data are presented as means ± standard error (S.E). Analysis of variance (ANOVA) was used with Dunnett's t-test (StatView, Berkeley, CA) to statistically evaluate the data by comparing the results of control groups to treatment groups. All differences of $p \leq 0.05$ were considered significant.

5.4. Results

5.4.1. Organ Body Weight Ratio

Exposure to individual pesticides did not significantly alter thymus/body weight or spleen/body weight ratios (Table 5). When animals were exposed to mixtures of two pesticides, the organ/body weight ratios were significantly decreased ($p \leq 0.05$). Lindane + malathion decreased thymus/body weight ratios, and lindane + permethrin decreased both thymus/body weight and spleen/body weight ratios compared to corn oil controls (Table 5).

5.4.2. Thymic and Splenic Cell Counts

None of the individual pesticide treatments caused a significant change in thymus or spleen cell counts (Table 6).

Mixtures of one-third of the LD_{50} of pesticides caused a significant decrease ($p \leq 0.05$) in both thymic and splenic cell counts (Table 6). This decrease was about 35% for lindane + malathion treated mice, and it was about 39% for lindane + permethrin treated mice.

5.4.3. Splenic B and T Cell Counts

The middle dose of lindane caused a significant decrease in both B ($CD45R/B220^+$ cells) and T ($CD90.2^+$ cells) cell subpopulations of splenocytes (Table 7). In addition, the lowest test dose of lindane significantly diminished B cell counts. None of the single treatments with malathion or permethrin altered the percentages of these subpopulations.

Lindane + malathion did not produce a significant difference when compared to corn oil control, however lindane + permethrin caused a slight but significant ($p \leq 0.05$) decrease within T cell populations of splenocytes.

5.4.4. Splenic $CD4^+$ and $CD8^+$ T Cell Counts

None of the single pesticide treatments altered the percentage of splenic $CD4^+$ T cells (Table 8). The middle doses of lindane and malathion caused a slight but significant decrease of splenocyte $CD8^+$ T cells.

Lindane + malathion and lindane + permethrin reduced the percentage of $CD4^+$ T cells by about 10% ($p \leq 0.05$).

5.4.5. Splenic Plaque Forming Cell (PFC) Counts

All doses of single pesticide treatments caused a significant suppression of antibody-specific IgM immune responses (plaque-forming cell, PFC) (Table 9). Although every test dose of each pesticide significantly reduced the PFC counts, the reduction did not appear to be dose related.

Both mixtures of pesticides significantly reduced the PFC counts of splenocytes. The decreases were about 20% compared to control group ($p \leq 0.05$).

5.4.6. Cytochrome P450 Measurement

Treatment of mice with permethrin 500 mg/kg caused a significant decrease in cytochrome P450 content of liver microsomes. Lindane treatment caused a slight (~ 15%) increase compared to the control group, but this effect was not statistically significant. Similarly, the lindane + malathion was 13% higher compared to control, but the effect was not statistically significant.

5.5. Discussion

We report in the current study that a single administration of lindane + malathion, or lindane + permethrin at one-third of LD_{50} doses caused higher toxicity on IgM PFC counts compared to individual applications of these compounds. However, this effect was not found to be additive or synergistic. In contrast, our earlier *in vitro* exposure studies with the same pesticides suggested that their toxicity to murine thymocytes could be more-than-additive when they were given in combination (Olgun et al., 2003).

Earlier studies demonstrated immunotoxicity in experimental animals treated with lindane (Koner, 1998; Hong and Boorman, 1993; Meera, 1992; Kashyap, 1986; Dewan et al., 1980), malathion (Rodgers and Xiong, 1997a, b; Rodgers et al., 1996; Rodgers and Ellefson, 1990; Rodgers et al., 1986), or permethrin (Prater et al., 2002; Punareewattana et al., 2001; Diel et al., 1995; Blaylock et al., 1995). The evaluation of toxicity of the pesticides in these studies was based on exposure to a single compound. While these and other studies on single pesticide toxicity provided valuable information for risk assessment, the data are not sufficient to explain pesticide mixture toxicity. The current approach assumes that there is little interaction between chemicals in their toxic effects or that any synergistic increase in toxicity will not exceed the safety factors applied (Wade et al., 2002). However, there are only few studies in the literature that tested these assumptions with combinations of chemicals and none that evaluated the immunotoxicity of mixtures of lindane, malathion, and permethrin are not available. It is important to study the effects of selected combinations of chemicals on the immune system, because alterations in immunological parameters may result in increased incidence of infections or the development of tumors (Galloway and Depledge, 2001; Ahmed 2000).

The enhanced effects of lindane, malathion, and permethrin mixtures on humoral immune function were suggested in this study. The individual administration of these pesticides did not alter any of the parameters tested, except the plaque forming cell (PFC) responses. The PFC counts were significantly lower at every dose tested; yet the decreases were not dose-dependent. When animals were given pesticide combinations,

however, all parameters tested were lowered, with the exception of splenic CD45R/B220 labeled B cells, and $CD8^+$ T cells numbers. While the administration of one-third of LD_{50} doses of lindane, malathion, or permethrin individually decreased the PFC/spleen counts by 30%, 38%, and 46%, respectively, lindane + malathion and lindane + permethrin mixtures at one-third of LD_{50} doses decreased the PFC/spleen counts by 49%, and 50%, respectively. Although the numbers presented here suggest less-than-additive effect with the combined exposure of these pesticides, the results were outcome of one experiment (n = 6), and replicates are needed to verify the data.

For this study, mixtures of two pesticides were administered subsequent to administration of lindane alone. This experimental design provided opportunity to evaluate the possible effects of lindane-induced cytochrome P450 induction on the combined pesticide toxicity. The results showed that two lindane administrations at one-third of the LD_{50} dose did not induce total cytochrome P450 levels. Secondly, the changes in total cytochrome P450 levels following mixture administrations did not appear to correlate with the toxicity observed. Lindane has been shown to increase cytochrome P450 content in rats when given at 20 mg/kg for 3 consecutive days (Junqueira et al., 1994), and after a single dose of 60 mg/kg (Videla et al., 2000; Junqueira et al., 1988). In the latter studies, the animals were sacrificed and liver cells were collected 4h after lindane administration, whereas in the present study liver cells were collected 5 days after lindane administration. The possibility that lindane-induced hepatic enzyme induction diminishes in time or that this specific strain of mice was less susceptible to induction may provide at least partial explanation of the results of our study.

The present study is an initial attempt to understand the biological activity of certain pesticide mixtures. Further understanding of the immunotoxic potential of these mixtures requires low-dose (for example, minimum risk level or tolerable daily intake doses), chronic toxicity studies. The findings also show that more research is needed to examine adverse effects of the combined exposure to lindane, malathion and permethrin on other physiological systems and to predict mechanistic pathways for these effects. The data are

needed particularly for mechanism of action at cellular and molecular levels and for possible alterations of gene transcription.

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Table 5. Effect of the Pesticide Administration on the Organ/Body Weight Ratios

Dose	(Spleen/BW) $x10^{-3}$	(Thymus/BW) $x10^{-3}$
Corn Oil	2.2 ± 0.4	1.1 ± 0.1
Lindane (mg/kg)		
29.3	2.4 ± 0.7	0.7 ± 0.1
22	3.5 ± 0.6	1.0 ± 0.1
11	3.3 ± 0.6	1.4 ± 0.2
Malathion (mg/kg)		
500	2.7 ± 0.2	1.3 ± 0.1
333.3	3.2 ± 0.4	1.3 ± 0.2
250	2.9 ± 0.5	1.2 ± 0.1
Permethrin (mg/kg)		
750	2.7 ± 0.5	0.9 ± 0.2
500	2.9 ± 0.2	0.6 ± 0.1
375	3.1 ± 0.7	1.3 ± 0.4

Dose	(Spleen/BW) $x10^{-3}$	(Thymus/BW) $x10^{-3}$
Corn Oil	3.1 ± 0.1	1.55 ± 0.1
Lindane-Malathion Mixture		
29.3 mg/kg Lindane + 333 mg/kg Malathion	**2.4 ± 0.1 ***	**1.0 ± 0.2 ***
Lindane-Permethrin Mixture		
29.3 mg/kg Lindane + 500 mg/kg Permethrin	**2.2 ± 0.3 ***	**1.1 ± 0.2 ***

Table 5. C57BL/6 mice were given 1/3, 1/4, and 1/8 of the LD_{50} of lindane, and 1/2, 1/3, and 1/4 of the LD_{50}'s of malathion and permethrin, respectively. Each pesticide was administered intraperitoneally twice, 2 days apart for single treatment studies. When combinations were used, 1/3 of the LD_{50} of lindane was given first and the mixtures administered 2 days later. Spleen/BW = spleen weight to body weight ratio, Thymus/BW = thymus weight to body weight ratio. Data are presented as the mean ± standard error, n = 6, * statistically different ($p \leq 0.05$) when compared to control.

Table 6. Effect of the Pesticide Administration on Thymic and Splenic Cell Counts

Dose	Splenocyte Count (x 10^6)	Thymocyte Count (x 10^6)
Corn Oil	67.9 ± 5.1	53.8 ± 5.9
Lindane (mg/kg)		
29.3	70.4 ± 8.3	42.9 ± 6.8
22	59.9 ± 10.6	44.5 ± 4.5
11	60.8 ± 5.2	52.0 ± 7.3
Malathion (mg/kg)		
500	61.2 ± 3.9	53.8 ± 5.8
333.3	59.4 ± 3.1	59.9 ± 9.4
250	72.5 ± 5.3	38.9 ± 5.2
Permethrin (mg/kg)		
750	70.3 ± 6.9	58.2 ± 10.9
500	62.7 ± 5.7	44.4 ± 6.1
375	56.7 ± 4.6	57.3 ± 3.7

Dose	Splenocyte Count (x 10^6)	Thymocyte Count (x 10^6)
Corn Oil	73.9 ± 4.6	66.5 ± 4.7
Lindane-Malathion Mixture 29.3 mg/kg Lindane + 333 mg/kg Malathion	**47.8 ± 5.8 ***	**44.0 ± 3.6 ***
Lindane-Permethrin Mixture 29.3 mg/kg Lindane + 500 mg/kg Permethrin	**45.4 ± 2.9 ***	**41.9 ± 2.9 ***

Table 6. C57BL/6 mice were given 1/3, 1/4, and 1/8 of the LD_{50} of lindane, and 1/2, 1/3, and 1/4 of the LD_{50}'s of malathion and permethrin, respectively. Each pesticide was administered intraperitoneally twice, 2 days apart for single treatment studies. When combinations were used, 1/3 of the LD_{50} of lindane was given first and the mixtures administered 2 days later. Data are presented as the mean ± standard error, n = 6, * statistically different ($p \leq 0.05$) when compared to control.

Table 7. Effect of the Pesticide Administration on Splenic B and T cell Counts

Dose	B cells ($CD45R/B220^+$ cells) (%)	T cells ($CD90.2^+$ cells) (%)
Corn Oil	60.2 ± 1.6	31.2 ± 1.6
Lindane (mg/kg)		
29.3	55.1 ± 3.3	29.5 ± 1.8
22	**47.7 ± 5.4 ***	**22.1 ± 3.9 ***
11	**42.3 ± 5.4 ***	25.12 ± 3.5
Malathion (mg/kg)		
500	53.7 ± 1.8	30.9 ± 1.0
333.3	51.8 ± 2.0	26.9 ± 3.4
250	53.5 ± 2.3	28.9 ± 1.7
Permethrin (mg/kg)		
750	54.4 ± 2.5	30.8 ± 1.7
500	54.8 ± 1.9	30.4 ± 0.9
375	56.1 ± 1.7	30.8 ± 2.4

Dose	B cells ($CD45R/B220^+$ cells) (%)	T cells ($CD90.2^+$cells) (%)
Corn Oil	56.3 ± 0.5	35.3 ± 0.5
Lindane-Malathion Mixture 29.3 mg/kg Lindane + 333 mg/kg Malathion	57.4 ± 0.6	33.7 ± 0.9
Lindane-Permethrin Mixture 29.3 mg/kg Lindane + 500 mg/kg Permethrin	55.7 ± 1.2	**32.7 ± 0.8 ***

Table 7. C57BL/6 mice were given 1/3, 1/4, and 1/8 of the LD_{50} of lindane, and 1/2, 1/3, and 1/4 of the LD_{50}'s of malathion and permethrin, respectively. Each pesticide was administered intraperitoneally twice, 2 days apart for single treatment studies. When combinations were used, 1/3 of the LD_{50} of lindane was given first and the mixtures administered 2 days later. Data are presented as the mean ± standard error, n = 6, * statistically different ($p \leq 0.05$) when compared to control.

Table 8. Effect of the Pesticide Administration on Splenic $CD4^+$ and $CD8^+$ T Cell Populations

Dose	$CD4^+$ cells (%)	$CD8^+$ cells (%)
Corn Oil	23.3 ± 1.3	13.4 ± 0.6
Lindane (mg/kg)		
29.3	21.5 ± 0.6	11.0 ± 0.2
22	19.8 ± 1.7	**9.8 ± 1.1 ***
11	19.5 ± 2.2	12.4 ± 0.9
Malathion (mg/kg)		
500	23.3 ± 0.4	11.8 ± 0.8
333.3	17.6 ± 2.6	**8.7 ± 2.0 ***
250	22.6 ± 0.8	12.5 ± 0.6
Permethrin (mg/kg)		
750	25.9 ± 2.4	11.8 ± 0.4
500	27.2 ± 1.8	12.0 ± 0.8
375	21.3 ± 0.7	12.8 ± 0.7

Dose	$CD4^+$ cells (%)	$CD8^+$ cells (%)
Corn Oil	23.0 ± 0.6	10.5 ± 0.1
Lindane-Malathion Mixture		
29.3 mg/kg Lindane 333 mg/kg Malathion	**21.2 ± 0.4 ***	10.7 ± 0.3
Lindane-Permethrin Mixture		
29.3 mg/kg Lindane + 500 mg/kg Permethrin	**20.2 ± 0.7 ***	9.6 ± 0.3

Table 8. C57BL/6 mice were given 1/3, 1/4, and 1/8 of the LD_{50} of lindane, and 1/2, 1/3, and 1/4 of the LD_{50}'s of malathion and permethrin, respectively. Each pesticide was administered intraperitoneally twice, 2 days apart for single treatment studies. When combinations were used, 1/3 of the LD_{50} of lindane was given first and the mixtures administered 2 days later. $CD4^+$ cells were $CD4^+CD8^-$ cells, and $CD8^+$ cells were $CD4^-$ $CD8^+$ cells. Data are presented as the mean ± standard error, n = 6, * statistically different ($p \leq 0.05$) when compared to control.

Table 9. Effect of the Pesticide Administration on Splenic PFC Numbers

Dose	PFC/10^6 cells	PFC/spleen
Corn Oil	58 ± 1.0	3939.4
Lindane (mg/kg)		
29.3	**39 ± 0.9 ***	2744.0
22	**37 ± 0.8 ***	2193.4
11	**37 ± 1.0 ***	2249.6
Malathion (mg/kg)		
500	**36 ± 0.7 ***	2203.6
333.3	**38 ± 1.0 ***	2415.7
250	**46 ± 1.2 ***	3334.5
Permethrin (mg/kg)		
750	**41 ± 1.0 ***	2881.5
500	**34 ± 0.8 ***	2130.8
375	**42 ± 0.8 ***	2379.7

Dose	PFC/10^6 cells	PFC/spleen
Corn Oil	60.2 ± 1.0	4457.9
Lindane-Malathion Mixture		
29.3 mg/kg Lindane + 333 mg/kg Malathion	**47 ± 1.1 ***	2246.1
Lindane-Permethrin Mixture		
29.3 mg/kg Lindane + 500 mg/kg Permethrin	**49.1 ± 0.8 ***	2226.7

Table 9. C57BL/6 mice were given 1/3, 1/4, and 1/8 of the LD_{50} of lindane, and 1/2, 1/3, and 1/4 of the LD_{50}'s of malathion and permethrin, respectively. Each pesticide was administered intraperitoneally twice, 2 days apart for single treatment studies. When combinations were used, 1/3 of the LD_{50} of lindane was given first and the mixtures administered 2 days later. PFC = plaque forming cell. Data are presented as the mean ± standard error, n = 6, * statistically different ($p \leq 0.05$) when compared to control.

Table 10. Effect of the Pesticide Administration on Liver Cytochrome P450 Enzymes

Dose	Concentration of Cyt- P450 /mg of microsomes (μM)
Corn Oil	0.658 ± 0.051
29.3 mg/kg Lindane	0.758 ± 0.068
333.3 mg/kg Malathion	0.593 ± 0.089
500 mg/kg Permethrin	0.437 ± 0.061 *
Lindane + Malathion	0.746 ± 0.066
Lindane + Permethrin	0.655 ± 0.065

Table 10. C57BL/6 mice were given 1/3 of LD_{50} of lindane first, and 1/3 of the LD_{50}'s of malathion and permethrin in combination with 1/3 of the LD_{50} of lindane 2 days later. Each pesticide was administered intraperitoneally. Total cytochrome P450 measurement of liver microsomes was performed spectrophotometrically. Cyt-P450 = cytochrome P450. Data are presented as the mean ± standard error, n = 6, * statistically different ($p \leq 0.05$) when compared to control.

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Chapter 6. IMMUNOTOXIC EFFECTS OF PESTICIDES VIA OXIDATIVE STRESS

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6.1. Abstract

The role of oxidative stress in immune cell toxicity caused by the pesticides lindane, malathion and permethrin was investigated in thymic cells from C57BL/6 mice. Thymocytes treated with any of these pesticides (concentrations ranging between 50-150 μM) were found to generate both superoxide ($O_2^{-\bullet}$) and H_2O_2. The production of $O_2^{-\bullet}$ was detected with hydroethidine-ethidium bromide assay. H_2O_2 production was monitored with a flow cytometric fluorescent (DCFH-DA) assay. All three pesticides stimulated $O_2^{-\bullet}$ release after 5 min exposure. Lindane and permethrin, but not malathion, continued to have significant ($p \leq 0.05$) effects on $O_2^{-\bullet}$ generation following 15 min of exposure. The lindane + malathion mixture was found to cause more-than-additive increase in $O_2^{-\bullet}$ production compared to single treatments of these pesticides (at both 5 and 15 min). However, the effect of the lindane + permethrin mixture was not significantly different than individual components of this mixture. The effects of these pesticides on levels of antioxidant enzymes were also investigated, and only mixtures were found to have significant ($p \leq 0.05$) effects. Lindane + malathion and lindane + permethrin mixtures increased superoxide dismutase (SOD) specific activity, had no effect on catalase levels and inhibited GSH-peroxidase and GSH-reductase specific activities. Although the results of these studies do not explain the mechanism of action of these pesticides on the generation of $O_2^{-\bullet}$ and H_2O_2, it is worthy of note that mixtures of these chemicals have oxidative responses greater than those of single chemicals.

6.2. Introduction

An imbalance between pro-oxidant (reactive oxygen species) and anti-oxidant mechanisms in cells causes oxidative stress. Increased quantities of reactive oxygen species (ROS) initiate lipid peroxidation in the cellular, mitochondrial and nuclear membranes, along with degradation of cytosolic proteins and damage to DNA (Marks et al., 1996). Antioxidant enzyme function to compensate for the elevated ROS levels. However, depletion of these defense elements further promotes oxidative stress (Banerjee et al., 2001).

We reported earlier (Olgun et al., 2003) that the pesticides lindane, malathion and permethrin caused apoptotic and necrotic cytotoxicity in murine thymocytes. Mechanisms associated with the cytotoxicities of these pesticides, however, were not determined. Development of oxidative stress conditions in different tissues following pesticide exposure has been suggested as a main cause of toxicity (Banerjee et al., 1999; Schweich et. al, 1994; Videla et al., 1990) and could contribute to the cytotoxicity. There are, however, very few reports on effects of pesticide mixtures, especially on immune cells. The immune system is considered to be sensitive to chemicals at dose levels lower than those causing other system toxicity (Sharma and Reddy, 1987). Alteration of immune system functioning by different pesticides has been suggested to be the basis for hypersensitivity, increased allergy and diminished resistance against tumor formation (Gleichmann et al., 1989).

The present study examined oxidative stress on murine thymocytes following exposure to lindane, malathion, permethrin or combinations of these pesticides, *in vitro*. Lindane (γ-isomer of hexachlorocyclohexane) is an organochlorine insecticide and an inducer of mixed function oxidase (MFO) enzymes (Junqueira et al., 1997; Barros et al., 1991). Lindane is an immunosuppressant at moderate to high doses (Raszyk et al., 1997; Meera et al., 1992). Malathion, an organophosphate insecticide, is an acetylcholinesterase inhibitor (Rose et al., 1999). It has been reported to modulate or suppress immune responses at specific dose-levels (Johnson et al., 2002; Beaman et al., 1999; Rodgers and

Xiong, 1997b). Permethrin (20% cis-78% trans) is a synthetic pyrethroid that has no reported teratogenic, mutagenic or carcinogenic effects. However, it has been demonstrated to suppress cellular immune responses (Blaylock et al., 1995).

In the present study, we examined the effects of lindane, malathion, and permethrin alone and in mixtures for their potential to stimulate oxidative stress in thymocytes, *in vitro*. The results of this study demonstrated that exposure of murine thymocytes to selected pesticides caused increase in superoxide ($O_2^{-\bullet}$) and H_2O_2, and also altered the levels of certain antioxidant enzymes. This suggests that certain pesticides, especially in mixtures, have potential to contribute to oxidative stress.

6.3. Materials and Methods

6.3.1. Animals

Male C57BL/6 mice, 8-12 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA) and quarantined 7 days prior to use. Mice were housed in polycarbonate cages with hard wood chip bedding in rooms in which the temperature (21^0 C), humidity (50%), and light cycle (12h dark/12h light) were controlled. The food and water were available ad libitum. All animal care and animal protocols were in compliance with the Virginia Polytechnic Institute and State University Animal Welfare Committee guidelines.

6.3.2. Thymus Collection and Isolation

Mice were euthanized by cervical dislocation, and body weights were recorded. The thymus was removed from each mouse, and weighed and individually placed in 60 x 15 mm Petri dishes containing cold, phenol red-depleted RPMI-1640 media (Gibco BRL, Grand Island, NY). Each thymus was dissociated over a sterile 60 µm mesh Sieves screen (Sigma, St. Louis, MO). The cell suspension was washed once with RPMI-1640 media and centrifuged at 300x g, 4^0C, for 7 min. The cells were resuspended in cold RPMI-1640, and 10 µl of cells were mixed with 10 ml of PBS before enumeration with a CASY 1 model TTC cell counter plus analyzer system (Scharfe System GMbH, Germany) (Gogal et al., 2001).

6.3.3. Cell Culture

Thymocytes were diluted to $5x10^6$ cells/ml in supplemented media [10% fetal bovine serum (FBS; Atlanta Biol., Norcross, GA), 1% MEM non-essential amino acids, 1% penicillin/streptomycin, 1% L-glutamine 100x, 200mM, and 1% HEPES (GibcoBRL, Grand Island, NY)]. Cells were aliquoted ($5x10^5$ cells/100 µl/well) into 96 well tissue culture plates (Costar, Corning, NY) for 12h incubation with pesticides. Cells were

cultured at 37^0 C, 90% humidity, 5% CO_2 in a Nuaire water-jacketed CO_2 incubator (Plymouth, MN).

6.3.4. Preparation of Pesticides

The 100 mM stock solutions of lindane (purity 99.5%, Chem Service, West Chester, PA), malathion (purity 99.0%, Chem Service, West Chester, PA) and permethrin (purity 20% cis-, 78% trans, Chem Service, West Chester, PA) were prepared in acetone (99.9%, ACS reagent). These 100 mM stock solutions were used in preparation of 4x working solutions by diluting with phenol red-depleted RPMI-1640 media. Final concentrations were 37.5, 75, 150, 200 μM for lindane, 37.5, 75, 150, 300 μM for malathion, and 150, 300, 600 μM and 1mM for permethrin. Vehicle control consisted of 0.6% acetone.

6.3.5. Flow Cytometric ROS Detection Assays:

6.3.5.a. Hyroethidine-ethidium bromide assay

Hyroethidine, a sodium borohydride-reduced derivative of ethidium bromide, was used to detect ROS, specifically superoxide anion ($O_2^{-\bullet}$). When hydroethidine is loaded in the cells, it binds to cellular macromolecules. Once $O_2^{-\bullet}$ is generated, it converts hydroethidine to ethidium bromide and increases fluorescence (620 nm), (Kitazawa et al., 2001). For this assay, murine thymocytes were suspended in Hanks balanced salt solution (HBSS) at a density of $1x10^6$ cells/ml. Cells were then incubated with 10 μM hydroethidine for 15 min at 37^0C in the dark to allow dye loading into the cells. After incubation with dye, excess dye was removed and the cells were resuspended with HBSS. Pesticide solutions were then added. At 5 and 15 minutes following the addition of pesticides, superoxide anion generation was measured by flow cytometry.

6.3.5.b. DCFH-DA Assay

The DCFH-DA probe was used to monitor pro-oxidant status of cells. 2', 7'-dichlorofluorescin diacetate (DCFH-DA) enters the cell, hydrolyzes to a nonfluorescent analog, 2'-7'-dichlorofluorescene (DCF-H), and is trapped in the cell. In the presence of certain ROS intermediates, such as H_2O_2 or low-molecular weight peroxyl radicals, DCF-H is oxidized to highly fluorescent 2'-7'-dichlorofluorescein (DCF), (Wang et al., 1999; Royal and Ischiropoulos, 1993).

Two sets of cells were prepared and one set was treated with 25 mM aminotriazole, a catalase inhibitor, for 15 minutes, to prevent catalase from scavenging H_2O_2. Then DCFH-DA (5 mM or 2.44 mg/ml in 100% EtOH) was added to all cell suspensions ($5x10^6$ cells/ml) for a final concentration of 5 μM (1 μl of 5 mM DCFH-DA/ml of cells). The incubation was allowed to proceed for 15 minutes at 37^0C, in the dark. In both sets of cells, a cell sample without DCFH-DA was included as a negative control, and a cell sample with DCFH-DA plus 0.6% acetone was included as a vehicle control. After 15 min incubation with DCFH-DA, cells were added into 24 well plate wells containing pesticide solutions. The cells were incubated with pesticides for 15 min at 37^0C, 90% humidity and 5% CO_2. The cells were removed from the wells after 15 min and observed with flow cytometry. The DCF fluorescence peak of the DCF only sample was subtracted from all other samples using the Coulter Epics Software v.1.5 Overton subtraction. The subtracted value was used to calculate the percent increase in DCF fluorescence caused by the pesticide.

6.3.6. Antioxidant Enzyme Assays

6.3.6.a. Superoxide dismutase assay

This assay was used to evaluate the levels of superoxide dismutase (SOD) enzyme in cells before and after the pesticide treatment. SOD enzyme catalyzes the dismutation of superoxide radicals ($O_2^{-\bullet}$) with the reaction:

$$2\ O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

Epinephrine is stable in acidic solutions but auto-oxidizes with increasing ease as the pH is increased. Therefore, at pH 10.2 and 30^0 C, the auto-oxidation of epinephrine is rapid, with a linear rate (following an initial short lag) of conversion to the oxidation product, adrenochrome (Misra, 1985). By measuring the rate at which SOD decreases this $O_2^{-\bullet}$ - dependent autoxidation reaction, the specific activity of SOD can be calculated (Misra and Fridovich, 1972). For these measurements, 485 µl of 0.05 M $NaHCO_3$ / Na_2CO_3 buffer (pH 10.2, with 10^{-4} M EDTA), and 15 µl of 10^{-2} M epinephrine (prepared fresh daily) were mixed in a 0.5 ml quartz cuvette and the kinetic rate was obtained on the UV spectrophotometer with a temperature controller unit (Shimadzu Model UV160, Kyoto, Japan). The amount of epinephrine was adjusted until the rate of 0.025 ΔA/min (± 0.002), 480 nm, at 30^0C was obtained.

Ten wells of cells ($5x10^5$ cells/100 µl/well) were incubated for 12h with each concentration of the test pesticides, and then pooled into a 1.5 ml tube to obtain a total of $5x10^6$ cells. These cells were centrifuged at 300x g, 4^0C, for 7 min, and then the cell pellet was resuspended with 100 µl of PBS. This suspension of cells was freeze-thawed twice at -20^0C, and centrifuged at 20,000x g, 4^0C, for 10 min. The supernatant was collected into a 0.5 ml tube. 20 µl of the supernatant was mixed with 485 µl of 0.05 M $NaHCO_3$ / Na_2CO_3 buffer and 15 µl of 10^{-2} M epinephrine, and the change in kinetic rate was recorded. By this reading, the percent inhibition in epinephrine auto-oxidation and the specific activity of the SOD were calculated. Each sample was measured in triplicate and the assay was repeated five times.

Percent inhibition = [1-(Sample ΔA/min / Epinephrine ΔA/min)] x 100
Units of SOD/ml = (1000 µl) / (Volume of sample (in µl) needed for 50% inhibition)
Specific Activity (Units of SOD/mg protein) = (Units of SOD/ml)/(mg protein/ml)
A unit of SOD is the quantity of SOD required to produce 50% inhibition in the rate of the conversion of epinephrine to adrenochrome.

Protein concentration of samples was measured with Bradford Protein Assay (Bradford, 1976). Briefly, the protein-dye (Bradford reagent; Sigma, St. Louis, MO) complex causes a shift in the dye absorption maximum from 465 to 595 nm. The amount of absorption produced is proportional to the protein concentration.

6.3.6.b. Catalase assay

Catalase is an enzyme that scavenges hydrogen peroxide and converts it to water and molecular oxygen:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

By monitoring the rate of breakdown of H_2O_2, the specific activity of catalase can be calculated. The procedure is adapted from Beers et al. (1952) and the Worthington Enzyme Manual (1972).

Cells from ten wells ($5x10^5$ cells/100 µl/well) of the same concentration of pesticide were pooled into a 1.5 ml tube following 12h incubation with pesticides to obtain a total of $5x10^6$ cells per pesticide treatment. These cells were centrifuged at 300x g, 4^0C, for 7 min, and then the cell pellet was resuspended with 100 µl of PBS. This suspension of cells was sonicated, on ice, for two 5-second intervals at setting 1.0 (Fisher Scientific Sonic Dismembrator F550, USA), and then it was centrifuged at 20,000x g, 4^0C, for 10 min. The supernatant was transferred into a 0.5 ml tube.
In 0.5 ml quartz cuvette, 20 µl of the supernatant was mixed with 167 µl of 0.05 M H_2O_2 (in 0.05 M potassium phosphate buffer, pH 7.0) and the total volume was brought up to 500 µl with 25^0C, 0.05 M potassium phosphate buffer, pH 7.0. The readings were taken to obtain the rate of decrease in absorbance at 240 nm. Each sample was measured in triplicate and the assay was repeated five times.

Units of catalase/ml = [ΔA/min at 240 nm x cuvette volume (ml)] / [43.6 M^{-1} cm^{-1} x sample volume (ml)]

43.6 M^{-1} cm^{-1} = Extinction coefficient of H_2O_2 (Beers et al., 1952).

Specific Activity (Units of catalase/mg protein) = (Units/ml)/(mg/ml protein)

One unit of catalase is equivalent to the amount of protein necessary to decompose 1 μmole of H_2O_2 per minute.

6.3.6.c. Glutathione peroxidase assay

Glutathione peroxidase (GSH-Px) is an enzyme that catalyzes the reduction of hydroperoxides by using reduced glutathione (GSH) as a reductant (reaction 1)

$$ROOH + 2\ GSH \rightarrow ROH + GSSG + H_2O \quad \ldots\ldots(1)$$

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+ \quad \ldots\ldots(2)$$

By coupling this reaction with glutathione reductase (GSH-R), the specific activity of GSH-Px can be calculated via monitoring the oxidation of NADPH. The procedure for the measurement of GSH-Rx was adapted from Tappel et al. (1978), with the rate of change in absorbance measured at 340 nm on a Shimadzu UV spectrophotometer.

The 100 μl of supernatant from the cells that were incubated with pesticides for 12h was prepared as described above for the catalase assay. In a 0.5 ml quartz cuvette, 25 μl of the supernatant was mixed with 452 μl of coupling cocktail (0.25 mM GSH, 0.12 mM NADPH, 1 Unit/ml G-R in Tris-Cl buffer, pH 7.6), 13 μl of cumene hydroperoxide (1 mg/ml by weight), and 10 μl of Tris-Cl buffer (pH 7.6, 37^0 C).

The mixtures were run in triplicate and the assay was repeated five times. One unit of GSH-Px was equivalent to the amount of enzyme necessary to reduce 1 μM of NADPH per minute under the conditions specified above (The extinction coefficient of NADPH is 6.22 x 10^{-3} μM^{-1} cm^{-1}).

Units of GSH-Px/ml = [ΔA/min at 340 nm x cuvette volume (ml)]/[6.22 x 10^{-3} $\mu M^{-1}cm^{-1}$ x sample volume (ml)]

Specific Activity (Units of GSH-Px/mg protein) = (Units/ml)/(mg/ml protein)

6.3.6.d. Glutathione reductase assay

Glutathione reductase (GSH-R) is an enzyme that reduces oxidized glutathione (GSSG) to restore intracellular concentrations of reduced glutathione (GSH). NADPH acts as a cofactor in this reaction.

$$\text{GSSG} \xrightarrow[\text{NADP}^+]{\text{NADPH} + \text{H}^+} 2\ \text{GSH}$$

By monitoring the oxidation of NADPH to $NADP^+$, the activity of GSH-R can be calculated. The protocol from Carlberg and Mannervik (1985) was used with slight modifications.

The 100 µl of supernatant from the cells that were incubated with pesticides for 12h was prepared as described above for the catalase assay. In a 0.5 ml quartz cuvette, 25 µl of the supernatant was mixed with 300 µl of coupling cocktail (1 ml of 1 M potassium phosphate buffer, pH 7.6, 2 ml of 0.05% bovine serum albumin in 0.1 M potassium phosphate buffer, 2 ml of 1mM NADPH in dH_2O), and 175 µl of dH_2O.
The rates of changes in absorbance at 25^0C and 340 nm with the mixtures were read on a Shimadzu spectrophotometer. The mixtures were run in triplicate and the assay was repeated five times.

One unit of GSH-R was equivalent to the amount of enzyme necessary to catalyze the oxidation of 1 µmole NADPH per minute under the conditions specified above.
Units of GSH-R/ml = [ΔA/min at 340 nm x cuvette volume (ml)]/[6.22 x 10^{-3} μM^{-1} cm^{-1} x sample volume (ml)]
Specific Activity (Units of GSH-R/mg protein) = (Units/ml)/(mg/ml protein)

6.3.7. Statistical Analysis

All experiments were repeated at least five times, and samples were prepared in triplicates for each treatment. Data are presented as means ± standard error. Analysis of variance (ANOVA) was used in combination with Dunnett's t-test (StatView, Berkeley, CA) to evaluate the data by comparing the results of treatment groups to control groups. Pesticide mixture treatment results were compared to individual pesticide treatments. Differences ($p \leq 0.05$) were considered statistically significant.

6.4. Results

6.4.1. Effects of Pesticides on Pro-oxidant Status of Cells

6.4.1.a. Hydroethidine-ethidium bromide assay

Superoxide anion ($O_2^{-\bullet}$) production in cells was measured using the hydroethidine-ethidium bromide assay. As shown in Table 11, 5 minutes exposure to 50 μM lindane and 75 μM malathion caused only a slight increase in superoxide production. However, 150 μM permethrin, 50 μM lindane + 75 μM malathion, and 50 μM lindane + 150 μM permethrin significantly increased the superoxide anion production in thymocytes. Furthermore, the effect of 50 μM lindane + 75 μM malathion, but not 50 μM lindane + 150 μM permethrin, was significantly higher when compared to 50 μM lindane or 75 μM malathion alone.

Results of 15 minutes exposures with the same concentrations of pesticides revealed a similar trend (Table 11).

6.4.1.b. DCFH-DA Assay

Hydrogen peroxide (H_2O_2) production in cells following pesticide exposure was monitored using the dichlorofluorescein diacetate (DCFH-DA) assay. As shown in Table 12**,** 15 minutes exposure to 50 μM lindane, 75 μM malathion, 150 μM permethrin, 50 μM lindane + 75 μM malathion, or 50 μM lindane + 150 μM permethrin augmented the H_2O_2 production in cells. Moreover, when cells were treated with aminotriazol (a catalase inhibitor) the DCF fluorescence improved further. However, the effects of pesticide mixtures were not significantly higher than individual pesticide exposures.

6.4.2. Effects of Pesticides on Antioxidant Enzyme Levels

The effects of individual and mixtures of pesticides on antioxidant enzyme levels in thymocytes were evaluated by spectrophotometric analysis. Results are illustrated in Figures 12, 13, 14a and 14b.

Pesticides in mixture, but not individually, increased superoxide dismutase (SOD) levels. Specific activity (Units/mg) of SOD was not changed with any of the single pesticide exposures compared to the vehicle control group. However, lindane + malathion and lindane + permethrin mixtures caused a significant increase in SOD specific activity compared to the vehicle control group (Figure 12).

The specific activity of another antioxidant enzyme, catalase, was not affected by pesticide exposures alone or in combination (Figure 13).

Glutathione peroxidase and glutathione reductase specific activities, which are the final steps of the antioxidant enzyme system, were significantly decreased following exposure to pesticide mixtures containing lindane. However, specific activities (Units/mg) of glutathione peroxidase (Figure 14a) or glutathione reductase (Figure 14b) were not changed with single pesticide exposures.

6.5 Discussion

We showed earlier that pesticides lindane, malathion and permethrin caused apoptotic and necrotic cell death in murine thymocytes after 12h incubation, in vitro (Olgun et al., 2003). In the present paper, we studied the role of oxidative stress in causing this toxicity. We exposed thymocytes to pesticides for 5 or 15 min and found increases in the generation of superoxide anion and hydrogen peroxide. When thymocytes were incubated with pesticides for 12h, there was an increase in SOD activity, and decreases in activities of GSH-peroxidase and GSH-reductase. This is the first study to examine the effects of these three pesticides on murine thymocytes.

Earlier studies with lindane demonstrated its capability to produce oxidative stress, but results varied greatly with time and tissue. For example, lindane, when given at a dose of 30 mg/kg body weight to C57BL/6J mice, produced increases of 1.6-3.0-fold in superoxide production in fetal and placental tissues 48 h after administration (Hassoun and Stohs, 1996). Junqueira et al. (1988) reported that when lindane was given orally, enhancement in microsomal cytochrome P-450 content and superoxide generation in rat hepatocytes occurred after 24 h. When treated for 12 h with pesticides, we did not find any superoxide or hydrogen peroxide present in thymocytes (data not shown). Effects were seen, however, at time points of 15 min or less. In an earlier study by Bagchi and Stohs (1993), lindane caused maximum increase in free radical generation within 5-15 min of incubation in rat peritoneal macrophages, hepatic mitochondria and microsomes. When we examined the effects of pesticides after 5 or 15 min exposure, lindane, malathion and permethrin caused slight increases in superoxide and hydrogen peroxide production in thymocytes. However, pesticide mixtures significantly increased the superoxide production in thymocytes. Our assay methods differed from the cited studies. We used flow cytometric assays to measure both superoxide and hydrogen peroxide production. The major advantage of flow cytometry over conventional fluorometry is that the flow cytometer measures fluorescence intensity only inside the cells and it does not include fluorescence in the extracellular medium.

When we examined the status of antioxidant enzymes in cells following pesticide treatment, we observed changes only in thymocytes exposed to the pesticide mixtures. Pesticides in mixtures produced increase in SOD, no alteration in catalase, and decreases in GSH-peroxidase and GSH-reductase specific activities of thymocytes. This contrasts with previous studies. In two different studies in rats by Junqueira et al. (1986 and 1988), the administration of lindane reduced the activity of liver superoxide dismutase and catalase, while it did not affect GSH-reductase or GSH-peroxidase levels (Junqueira et al.,1986). Malathion treatment caused an increase in the activities of SOD and catalase in erythrocytes in rats (John et al., 2001). Malathion was also shown to increase activities of GSH-peroxidase in erythrocytes and GSH-reductase in serum (Ahmed et al., 2000). The studies above did not examine the status of antioxidant enzymes in thymocytes. We expect that when thymocytes are exposed to mixtures of lindane, malathion and permethrin, the generation of free radicals increased. The cells strived to compensate for this by increasing superoxide dismutase enzyme levels. Thus the levels of this enzyme were found to be elevated. However, when the cells become progressively more damaged by the presence of pesticides, they could be overwhelmed by the excessive free radical formation and the levels of further step antioxidant enzymes diminished. The cytotoxic effects of these chemicals could be due to a rise in quantity of reactive oxygen species produced or to depressed functioning of antioxidant systems.

In the present study, mixtures of pesticides included lindane. This organochlorine insecticide has been reported to be a hepatic microsomal enzyme inducer (Kumar and Dwivedi, 1988; Junqueira et al., 1986) and an immunosuppressant (Raszyk et al., 1997; Meera et al., 1992). The ability to induce microsomal enzymes, however, would not be expected to contribute to the results presented here. Although not all cytochrome P450 isozyme proteins were examined, Pazirandeh et al. (1999) reported that BALB/c mice thymocytes did not express the cytochrome isozymes associated with corticosterone production. The mRNAs for these enzymes were found to be restricted to the thymic epithelial cell compartment of the thymus. This study suggests that other cytochrome P450 isozymes were unlikely to be present as well. Therefore, the induction of

cytochrome P450 enzymes with lindane cannot be suggested as the underlying mechanism for enhanced toxicity of mixtures seen in this study.

Predicting the interactions of these pesticide mixtures in humans on the basis of *in vitro* experiments is difficult. Simultaneous introduction of chemicals to a body system may cause decrease in clearance and increase in bio-availability (von Moltke et al., 1998). These chemicals may interact while crossing membranes (hepatic, renal), when binding to plasma proteins, or at the receptor level (Kremers, 2002). All of these possibilities contribute to the level of tissue damage that occurs. Therefore, these findings with *in vitro* exposure cannot be extended to entire body systems. However, the results presented here are still important because they demonstrated the possible sensitivity of the immune system to foreign compounds. If lindane, malathion or permethrin were to induce oxidative stress *in vivo*, the ability of liver antioxidant systems to overcome this stress before these compounds reach immune cells could provide a possible protective mechanism.

6.6. Acknowledgements

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Figure 12. Superoxide dismutase (SOD) specific activity in C57BL/6 thymocytes after 12 h treatment with pesticides and pesticide mixtures. Cells were treated with 50 μM lindane, 75 μM malathion, 150 μM permethrin, 50 μM lindane + 75 μM malathion (Lin+Mal), or 50 μM lindane + 150 μM permethrin (Lin+Perm). Data shown are the mean of 5 experiments ± standard error. * Statistically significant difference ($p \leq 0.05$) when compared to vehicle control.

Figure 13. Catalase specific activity in C57BL/6 thymocytes after 12 h treatment with pesticides and pesticide mixtures. Cells were treated with 50 μM lindane, 75 μM malathion, 150 μM permethrin, 50 μM lindane + 75 μM malathion (Lin+Mal), or 50 μM lindane + 150 μM permethrin (Lin+Perm). Data shown are the mean of 5 experiments ± standard error. There were no significant differences among the treatment groups.

Figure 14a. Glutathione peroxidase (GSH-Px) specific activity in C57BL/6 thymocytes after 12 h treatment with pesticides and pesticide mixtures. Cells were treated with 50 μM lindane, 75 μM malathion, 150 μM permethrin, 50 μM lindane + 75 μM malathion (Lin+Mal), or 50 μM lindane + 150 μM permethrin (Lin+Perm). Data shown are the mean of 5 experiments ± standard error. * Statistically significant difference ($p \leq 0.05$) when compared to vehicle control.

Figure 14b. Glutathione reductase (GSH-R) specific activity in C57BL/6 thymocytes after 12 h treatment with pesticides and pesticide mixtures. Cells were treated with 50 μM lindane, 75 μM malathion, 150 μM permethrin, 50 μM lindane + 75 μM malathion (Lin+Mal), or 50 μM lindane + 150 μM permethrin (Lin+Perm). Data shown are the mean of 5 experiments ± standard error. * Statistically significant difference ($p \leq 0.05$) when compared to vehicle control.

Table 11. Superoxide anion ($O_2^{-\bullet}$) production in C57BL/6 mice thymocytes following pesticide exposure

Exposures	5 min	15 min
Vehicle control	3.61 ± 0.34	3.74 ± 0.46
50 µM Lindane	5.65 ± 0.81 [a]	5.64 ± 0.87 [a]
75 µM Malathion	5.90 ± 0.56 [a]	4.52 ± 0.63
150 µM Permethrin	12.03 ± 0.53 [a]	9.21 ± 1.02 [a]
Lindane + Malathion	25.12 ± 4.31 [a, b]	29.18 ± 2.65 [a, b]
Lindane + Permethrin	12.37 ± 0.69 [a]	12.74 ± 1.14 [a]

The percent increase in fluorescence is presented for cells exposed to 50 µM lindane, 75 µM malathion, 150 µM permethrin, 50 µM lindane + 75 µM malathion, or 50 µM lindane + 150 µM permethrin for 5 or 15 min. $O_2^{-\bullet}$ generation in cells was measured with flow cytometry monitoring hydroethidine reduction to ethidium bromide. Data shown are the mean of 5 experiments ± standard error. **a**. Statistically significant difference ($p \leq 0.05$) when compared to control. **b**. Statistically significant difference ($p \leq 0.05$) when compared to single pesticide treatments.

Table 12. Hydrogen peroxide (H_2O_2) production in C57BL/6 mice thymocytes following pesticide exposure

Exposures	15 min	15 min + AT
Vehicle control	0.36 ± 0.31	2.08 ± 0.89
50 µM Lindane	19.36 ± 1.59 [a]	24.78 ± 3.32 [a]
75 µM Malathion	22.34 ± 1.93 [a]	28.66 ± 3.86 [a]
150 µM Permethrin	7.62 ± 1.02 [a]	9.08 ± 1.69 [a]
Lindane + Malathion	16.98 ± 2.44 [a]	27.02 ± 3.66 [a]
Lindane + Permethrin	20.24 ± 2.04 [a]	25.96 ± 4.08 [a]

The percent increase in fluorescence is presented for cells exposed to 50 µM lindane, 75 µM malathion, 150 µM permethrin, 50 µM lindane + 75 µM malathion, or 50 µM lindane + 150 µM permethrin for 15 min. H_2O_2 production in cells was monitored with flow cytometry using dichlorofluorescein diacetate (DCFH-DA) assay. AT = aminotriazole, a catalase inhibitor, 25 mM, 15 min pre-incubation. Data shown are the mean of 5 experiments ± standard error. **a**. Statistically significant difference ($p \leq 0.05$) when compared to control. Mixtures did not cause significant increase compared to individual pesticide exposures.

Figure 12. Specific Activity of Superoxide Dismutase (Units/mg of protein)

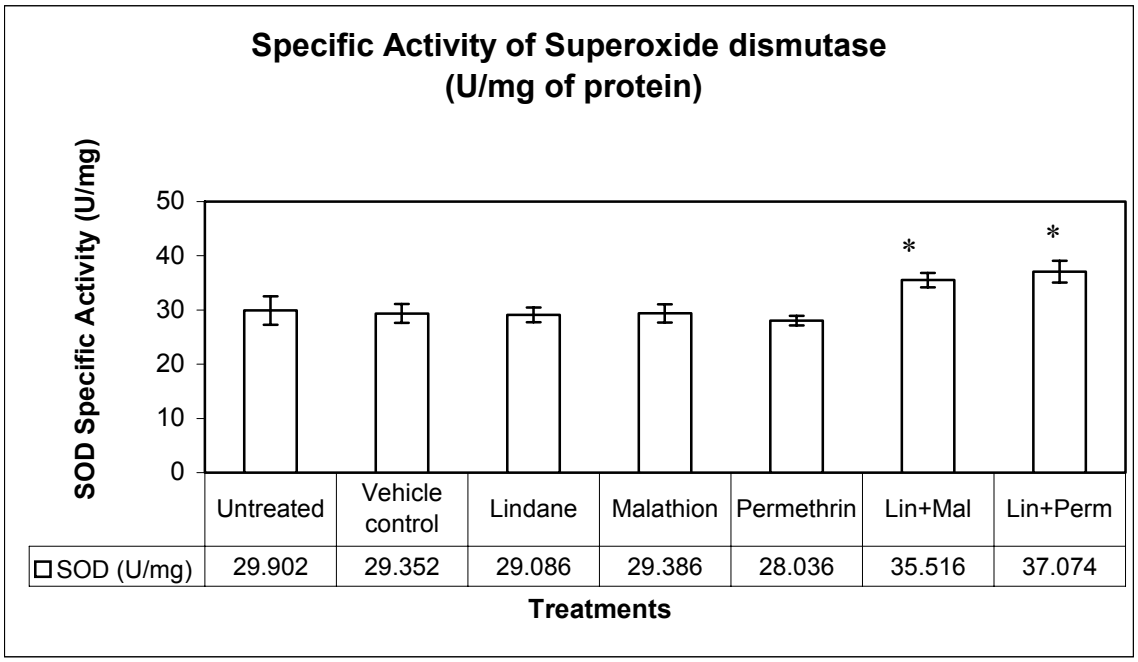


Figure 13. Specific Activity of Catalase (Units/g of protein)

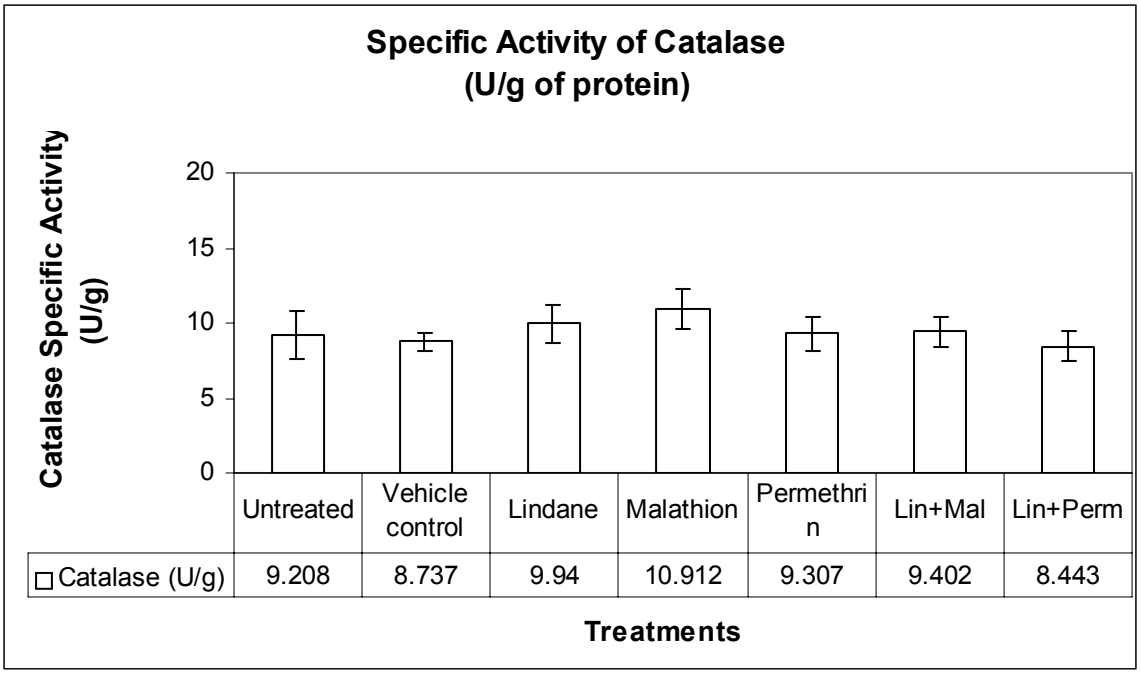


Figure 14a. Specific Activity of Glutathione Peroxidase (Units/mg of protein)

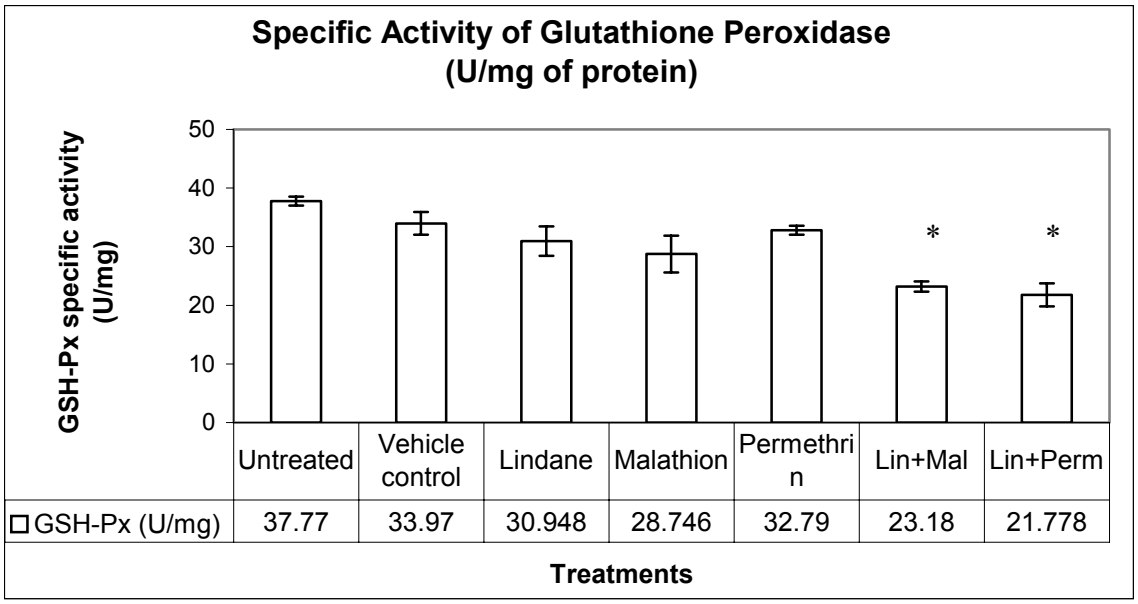
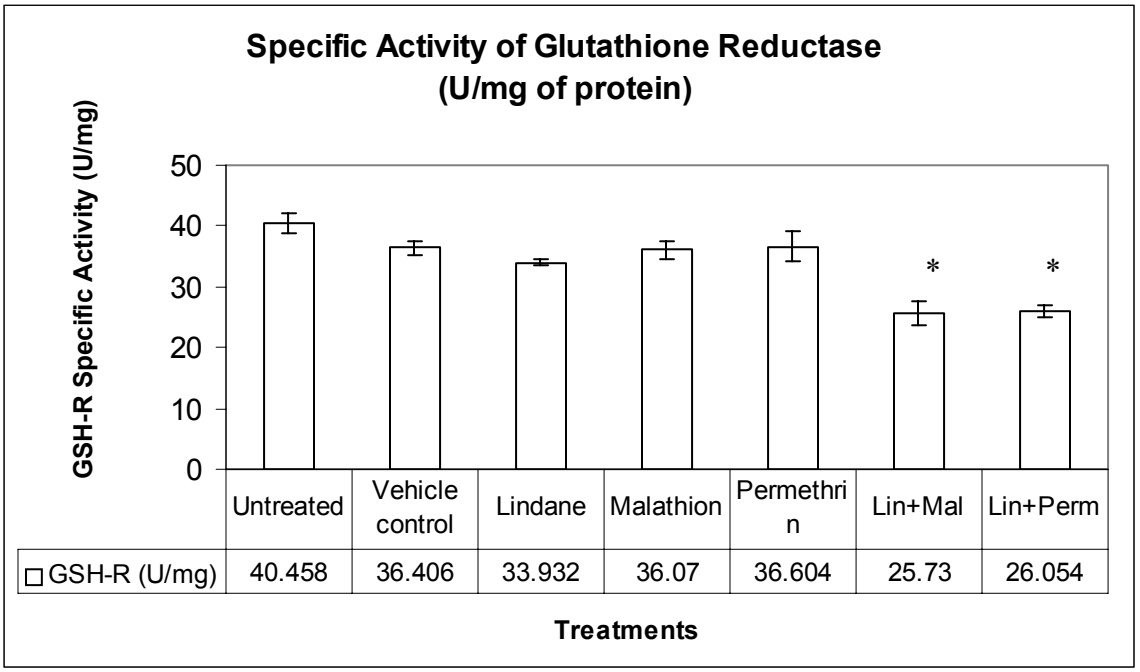


Figure 14b. Specific Activity of Glutathione Reductase (Units/mg of protein)



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Chapter 7. THE INVOLVEMENT OF NFκB TRANSCRIPTION FACTOR IN PESTICIDE INDUCED OXIDATIVE STRESS

7.1. Introduction

This part of the dissertation research was to investigate whether the reactive oxygen species (ROS) generated during exposure to pesticides (lindane, malathion, permethrin, or their mixtures) are responsible, even partially, for the cellular injury or cell dysfunction. To test this hypothesis that oxidative stress is involved in pesticide induced immunotoxicity, transcription factor nuclear factor-κB (NFκB) levels were examined following 12 h pesticide exposure.

Under normal physiological conditions, endogenous ROS sources do not create risk for the tissues and cells, since there are functioning protective systems (Reed, 1995). However, protective systems can be overwhelmed by the presence of pesticides, or their metabolites. These compounds can be chemically unstable and capable of interacting with cell macromolecules, and lead to disturbance of the control of oxidative events in cells (Reed, 1992). This is known as *oxidative stress*, and it may contribute to overall toxicity of the pesticides.

The exposure of immune cells to pesticides, and subsequent possible shift in oxidative status of cells may influence the overall immune response. It has been shown that the uncontrolled change in ROS levels differentiates the transcription of certain families of genes in cells (Puri et al., 1995a; Curutti and Trump, 1991), and regulates the transcription factor NFκB activation (Sen et al., 1996). These findings suggested that ROS were being used as second messengers in the activation of signal transduction pathways to lead to cytotoxicity. In T-cells, for example, ROS were shown to activate many signaling systems, including the NFκB transcription factor (Schulze-Osthoff et al., 1995). Furthermore, many conditions that are known to induce oxidative stress have been shown to activate NFκB (Bowie and O'Neill, 2000; Schmidt et al., 1995). Activation of NFκB was proposed based on four types of evidence: 1) Addition of hydrogen peroxide

to culture medium activated NFκB in some cell lines (Meyer et al., 1993; Schreck et al., 1991b); 2) In some cell types reactive oxygen species (ROS) have been shown to be increased in response to agents that also activate NFκB (Los et al., 1995; Schreck et al., 1992); 3) Compounds with antioxidant properties can inhibit pathways to NFκB activation (Schreck et al., 1992); and 4) Inhibition or overexpression of antioxidant enzymes that control the intracellular ROS levels have been shown to modulate the activation of NFκB (Bonizzi et al., 1999; Manna et al., 1998; Schmidt et al., 1995).

In unstimulated cells NFκB is found in a latent form in the cytoplasm, formed by a transcriptionally active dimer bound to an inhibitor protein, IκB (Bowie and O'Neill, 2000). The currently known subunits that form the dimer are 50 kD (p50), 65 kD (p65, or RelA), c-Rel, p52, and RelB polypeptides. The predominant form of NFκB is formed by the p50/p65 dimer. Upon stimulation with NFκB inducers, IκB is phosphorylated on two serine residues (S32 and S36), which targets the inhibitor protein for ubiquitination and degradation (Whiteside and Israel, 1997).

Many immunologically relevant genes, mainly those encoding cytokines and cytokine receptors, growth factors, and cell adhesion molecules, contain functional NF-κB binding sites in their promoter and enhancer regions (Baeuerle et al., 1994; Grilli et al., 1993). NFκB was also shown to have a key role in the regulation of many genes involved in immune and inflammatory responses (Bowie and O'Neill, 2000; Sha, 1998). NFκB was found to be activated in many cell types in response to different types of stimuli, such as T- and B-cell mitogens, IL-1 (interleukin-1), and TNF (tumor necrosis factor), and intracellular stresses such as endoplasmic reticulum protein overload or hydrogen peroxide overproduction (Pahl and Bauerle, 1995; Bauerle and Henkel, 1994).

In this part of the dissertation research, alterations in transcription factor nuclear factor-κB (NFκB) levels were measured as an indicator of oxidative stress in thymic cells following a 12 h pesticide exposure.

7.2. Materials and Methods

7.2.1. Animals

Male C57BL/6 mice, 8-12 weeks old, were purchased from Charles River Laboratories (Wilmington, MA) and quarantined 7 days prior to use. Mice were housed in polycarbonate cages with hard wood chip bedding in rooms in which the temperature (21^0 C), humidity (50 %), and light cycle (14 h dark/10 h light) controlled. The food and water were available ad libitum. All animal care and animal protocols were in compliance with the Virginia Polytechnic Institute and State University Animal Welfare Committee guidelines.

7.2.2. Thymus Collection and Isolation

Mice were euthanized by cervical dislocation, and body weights were recorded. The thymus was removed from each mouse, weighed and individually placed in a 60 x 15 mm Petri dish containing cold, phenol red-depleted RPMI-1640 media (Gibco BRL, Grand Island, NY). Each thymus was dissociated over a sterile 60 µm mesh Sieves screen (Sigma, St. Louis, MO). The cell suspension was washed once with RPMI-1640 media and centrifuged at 300 x g, 4^0C, for 7 min. The cells were resuspended in cold RPMI-1640, and 10 µl of cells mixed with 10 ml of 1xPBS and enumerated with a CASY 1 model TTC cell counter plus analyzer system (Scharfe System GMbH, Germany). This system analyzes three 400-µl aliquots of sample per run and the data from the three aliquots are presented as a histogram based on the relative diameter or volume of the cells counted (Gogal et al., 2001).

7.2.3. Preparation of Pesticides

The 100 mM stock solutions of lindane (purity 99.5%, Chem Service, West Chester, PA) and malathion (purity 99.0%, Chem Service, West Chester, PA) were prepared in absolute ethanol. A 100 mM Permethrin (purity 20% cis, 78% trans, Chem Service, West Chester, PA) stock solution was prepared in acetone. These stock solutions were used in

preparation of 4x working solutions by diluting with incomplete media. Final concentrations were 50 μM for lindane; 75 μM for malathion, and 150 μM for permethrin.

7.2.4. Cell Culture

Thymocytes were diluted to $5x10^6$ cells/ml in supplemented media [10% Fetal bovine serum (FBS; Atlanta Biol., Norcross, GA), 1% MEM non-essential amino acids, 1% penicillin/ streptomycin, 1% l-glutamine 100x, 200mM, and 1% HEPES (GibcoBRL, Grand Island, NY)] and aliquoted ($5x10^5$ cells/100 μl/well) into 96 well tissue culture plates (Costar, Corning, NY). To each well, 50 μl aliquots of 4x pesticide solutions were combined for mixture exposure, or the volume compensated with supplemented media for individual exposures. Solvent control samples consisted of 100 μl of 0.6% acetone. Cells were cultured at 37^0 C, 5% CO_2 in Nuaire water-jacketed CO_2 incubator for 12 h (Plymouth, MN).

7.2.5. Protocol for Performing SDS-Page and Western Blotting

SDS-PAGE and Western blotting was used to determine NFκB expression in pesticide exposed thymocytes.

SDS-Page: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10% gel (HPLC grade water, resolving gel buffer, 40% acrylamide solution, 10% sodium dodecyl sulfate, 1.5% ammonium persulafte, and TEMED) following the standard protocol of the Mini-Protean®II gel apparatus (Bio-Rad, Rockville, NY). Gels were run approximately for 60-90 minutes at 50 mA/gel in SDS-PAGE electrophoresis buffer (25mM Tris, 0.19M Glycine, 0.1%SDS, pH. 8.3).

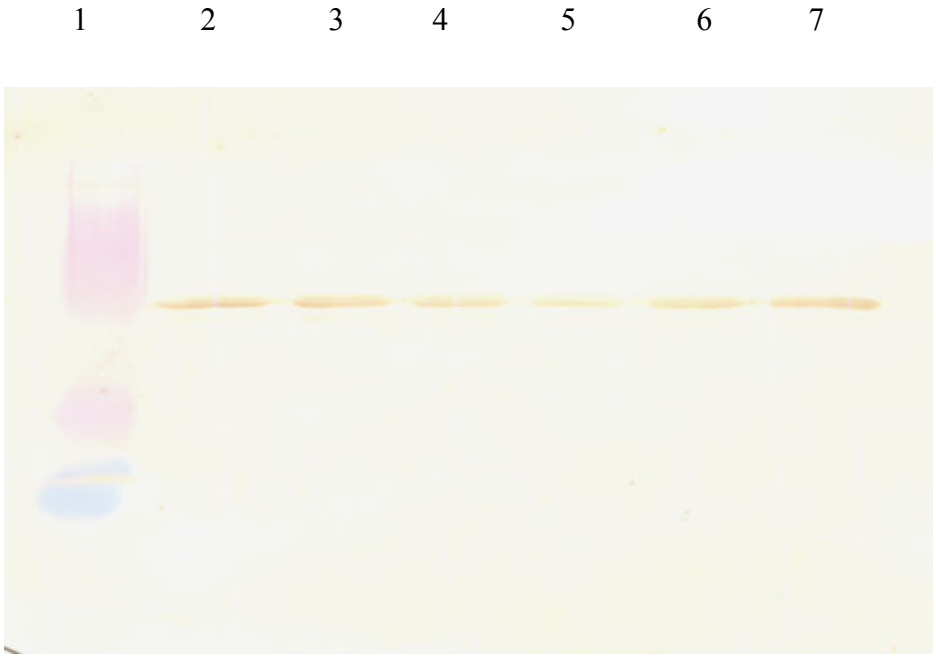
Western Blotting: All protein samples were separated on 15% SDS-PAGE and transferred to a nitrocellulose membrane (0.45 micron Nitropure membrane) (MSI Filters, Westboro, MA). The membranes were blocked with TBS containing 2% bovine serum

albumin (BSA) and 1.5% dry milk and reacted with tested sera as primary antibody and appropriate HRP-conjugated IgG as secondary antibody. The serological reactions were visualized by incubation in a developing solution consisting of 0.060g 4-chloro-1-napthol in 20ml methanol added to 100 ml of Tris buffered saline (TBS) (0.15M NaCl, 20mM Tris, pH 7.5) with 36 ml of 30% hydrogen peroxide. The development of the color reaction was stopped after 5 min by placing the membranes in distilled water.

7.3. Results

Western blotting analysis of pesticide exposed C57BL/6 thymocytes revealed that these cells contain NFκB. Exposure to 50 μM lindane and 75 μM malathion, and lindane + malathion caused significant increase in expression of p65 subunit of NFκB (Figure 15).

Figure 15. NFκB p65 subunit expression in pesticide exposed thymocytes.



1 : Size marker

2: Control = 21,740.43

3: 50 µM lindane = 25,616.03 *

4: 75 µM malathion = 25,202.64 *

5: 150 µM permethrin = 19,856.01

6: 50 µM lindane + 75 µM malathion = 29,379.4 *

7: 50 µM lindane + 150 µM permethrin = 20,802.7

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Chapter 8. GENERAL DISCUSSION

The present study is the first to demonstrate the effects of lindane, malathion, and permethrin mixtures on the C57BL/6 mouse immune cells (thymic and splenic) both *in vitro* and *in vivo*. The C57BL/6 mouse is suggested as one of the most reliable models for immunotoxicity studies (U.S.EPA, 2000c). The mouse is the usual animal of choice in immunotoxicity testing, and many reagents and *in vitro* systems are available for studying the immune system using mouse cells and tissues (Munson and Levier, 1995). In the present study, cells were collected for *in vitro* studies, or animals were injected for *in vivo* experiments from animals ranging in age of 8-12 weeks. This time frame was in compliance with the EPA standard evaluation procedures for immunotoxicity studies (U.S.EPA, 2000c).

The present study verifies the hypothesis that lindane, malathion and permethrin cause apoptotic and late apoptotic/necrotic cell death. This was observed *in vitro* (Chapter 4) when cells were incubated with increasing concentrations of pesticides for 12 h. Cellular membrane damage and fragmentation of DNA were determined in apoptotic cells. Furthermore, the combinations of two pesticides caused more-than-additive increase in both apoptotic and late apoptotic/necrotic cell populations. The more-than-additive outcome reported from *in vitro* experiments in this study supports (Calciu et al., 2002; Payne et al., 2001; Mills et al., 1991), as well as contradicts (Nellemann et al., 2003; Mumtaz et al., 2002; Moore and Lower, 2001; Oakes and Pollack, 1999; Stelzer and Chan, 1999) previous findings. Direct comparison of the effects of pesticides used in this study with effects of other compounds is not possible, because studies were done with different animals or organ systems under different conditions of exposure. Although the complex nature of combination studies makes comparison difficult, the present findings show the potentiation of risks with pesticide mixtures *in vitro*, and this deviates from the generally accepted simple additivity approach. One drawback of the *in vitro* parts of the present study is the use of cells from one immune organ (thymus) to represent the immune functioning.

The findings of the significantly increased cell death, both apoptotic and late apoptotic/necrotic, in CD4 (-)8(+) cells in this study (Chapter 4) supports previous observations (Prater et al., 2002), and suggest that thymocyte subgroups have different relative sensitivity to chemical induced toxicity.

The current study used several immunological endpoints to reveal the toxic effects of pesticide mixtures on thymic and splenic cells. For example, this study demonstrated altered immune responses after combined pesticide exposure, including thymus and spleen to body weight ratios, thymic and splenic cell viability, and specific antibody production. The combination of these parameters has high predictive value for immunosuppression in mice (Luster et al., 1992). These results supported our hypothesis that combined exposure to these three pesticides at their individual 1/3 of LD_{50} constituted an increased toxicity (Chapter 5). Although these findings indicated absence of synergistic interaction, and results were in contrast with the greater than additive effect noted after *in vitro* exposure (Chapter 4), they do support the previous reports of less-than-additive cytotoxicity in different organ systems *in vivo*. For example, combined chemical toxicity studies using different pesticides (i.e., organochlorines, organophosphates, or polyaromatic hydrocarbons) reported less-than-additive toxicity from *in vivo* experiments (Charles et al., 2002; Bailey et al., 2001; Peden-Adam et al., 2001; Flipo et al., 1992). One drawback for the *in vivo* part of the present study is that the concentrations used were higher than the concentrations would be expected from environmental exposures.

The two different outcomes noted when exposure to combinations of pesticides occurred *in vitro* and *in vivo*, support the need for validation of *in vitro* results with *in vivo* experiments.

Although we hypothesized that induction of drug metabolizing cytochrome P450 enzymes with lindane would enhance toxicity with concurrent pesticide administration, no increases were noted in mice treated with lindane in our experiments. Therefore, the

changes in total cytochrome P450 levels did not appear to correlate with the toxicity observed. This could be related to timing of liver cells collection or the use of relatively high doses (1/3 of LD_{50}) that may cause interfering effects on enzyme induction. While it was not further investigated in our studies, lindane induction of selective cytochrome P450 subgroups is possible and still remains a possible cause for the enhanced toxicity.

The findings reported in this study support other *in vitro* models that demonstrated the involvement of oxidative stress in toxicity of lindane (Cornejo et al., 2001; Criswell and Loch-Caruso, 1999; Junqueira et al., 1997; Videla et al., 1991), and malathion (Pedrajas et al., 1995). The alterations in oxidative status of cells following exposure to mixtures of these pesticides included the increase in reactive oxygen species generation, and decrease in certain antioxidant enzymes (Chapter 6), as proposed in the statement of hypothesis for this dissertation. The alterations observed in antioxidant enzymes did not correlate with previous reports (John et al., 2001; Ahmed et al., 2000; Junqueira et al., 1986, 1988), however none of these studies were performed using murine thymocytes. The present research is unique in that it investigates the oxidative potential of lindane, malathion and permethrin, or their combination, in murine thymocytes.

It is difficult to predict the changes in oxidant status of cells after pesticide exposures in human using these experiments done for this dissertation. Simultaneous introduction of chemicals to a body may cause decrease in clearance and increase in bio-availability (von Moltke et al., 1998). Also, these chemicals may interact while crossing membranes (hepatic, renal), when binding to plasma proteins, or at the receptor level (Kremers, 2002). All of these possibilities contribute to the level of tissue damage that occurs. Therefore, the findings from *in vitro* exposures cannot be extended to entire body systems. However, the results presented here are still important because they demonstrated the sensitivity of the immune system to foreign compounds, especially in mixtures.

This study reports the effects of combinations of lindane, malathion, and permethrin on murine thymocytes and splenocytes the first time. The outcome toxicity was more-than-

additive with mixture experiments *in vitro*, and less-than-additive but higher than the individual components of the mixture with *in vivo* experiments. These studies strongly suggest that alterations in oxidative status of cells were crucial for the enhanced toxicity observed with combined pesticide exposure. On the basis of all these results, we conclude that the toxicity of any pesticide mixture cannot be predicted with certainty from the effects of its components, due to possible but not yet predictable interactions between components.

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