Immunotoxic Effects of Mixtures of Endosulfan and Permethrin Via Caspase Dependent Thymocyte Apoptosis

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Abstract

Altered immune responses have been observed following occupational, inadvertent, or therapeutic exposure to xenobiotics. Many pesticides are known to cause immunotoxicity. Exposure to mixtures of pesticides, either concurrently or sequentially, may result in potentiating this effect partly because one can effect the metabolism of the other. The objective of this study was to determine the effect of the insecticides endosulfan, permethrin and their mixtures on C57/BL6 male mice thymocytes *in vitro* and to ascertain the mechanism by which these effects take place. Permethrin, a broad-spectrum synthetic pyrethroid, is a widely used insecticide in agriculture and public health. Endosulfan is a highly toxic chlorinated hydrocarbon insecticide used worldwide. We examined the immunotoxic potential of these pesticides using a flow cytometric technique in combination with 7-Amino Actinomycin D (7AAD) to distinguish live, early apoptotic, and late apoptotic/necrotic cells. DNA ladder assay, a hallmark of apoptosis, was also used to determine the occurrence of apoptosis. Both endosulfan and permethrin were found to cause significant apoptotic death of thymocytes in a dose- and time-dependent manner. Thus, permethrin at 50, 100 or 300 μM was found to cause 5.5, 11.5 and 26.1% increases in early apoptotic cell death relative to control, respectively. Endosulfan at 25, 50 or 250 μM was found to cause 11.9, 15.7 and 68.0% early apoptotic cell death, respectively. For the mixture study, concentrations of 100 μM permethrin and 50 μM endosulfan were selected and found to cause 27.1% apoptosis. Thus, these pesticides in mixture have an additive immunotoxic effect. Increases in late-apoptotic/necrotic cells were found at these concentrations for either pesticide when exposed for 12 hours. DNA ladder assay confirmed the presence of DNA fragments and therefore the presence of significant apoptotic cell death.

Apoptosis is a morphologically distinct form of cell death that can be mediated by a variety of pathological and physiological stimuli. Because permethrin and endosulfan were found to induce apoptosis in C57/BL6 mice thymocytes *in vitro*, the objective of the second half of this study was to elucidate the potential mechanism by which these pesticides regulate apoptosis in immune cells. Caspases are a family of cystine-dependent, aspartate-directed proteases that have an integral role in apoptotic cell death. Caspases, which are normally inactive in healthy cells, are activated during apoptosis and form an irreversible cascade. There are two subsets of caspases, initiator caspases (i.e. caspase 8 and 9) and effector caspases (i.e. caspases 3 and 6). Caspase 3, a downstream effector of apoptosis, is activated by many different pathways and is an apoptotic marker in cells. Caspase 8 is the apical caspase in the extrinsic pathway. Caspase 9 is the apical caspase in the intrinsic pathway, therefore we investigated mechanisms of pesticide induced apoptosis involving the thymocyte caspase system. Thymocytes from C57/BL6 mice were incubated with varying concentrations of pesticides for varying amounts of time. Active caspase 3 was then measured using EnzCheck Caspase 3 Assay Kit. Relative fluorescence for permethrin exposed cells after 12 hours incubation in the presence of pesticides at 150, 100, and 50 μM and 40 minutes in the presence of AFC-

substrate was found to be 387, 386, and 297, respectively. Relative fluorescence for endosulfan exposed cells at 150, 100 and 50 μM was 188, 177, and 294. Caspase 3 activity increased as permethrin concentrations increased and decreased as endosulfan concentrations were increased. Then the extrinsic and intrinsic pathways of apoptosis were further investigated. Active caspase 8 was measured using the ApoAlert Caspase Fluorescent Assay Kit. Relative fluorescence for permethrin exposed cells after 7 hours incubation in the presence of pesticides at 100, 150, and 200 μM was found to be 35.5, 10.5, and 0, respectively. Relative fluorescence for endosulfan exposed cells after 7 hours incubation at 25, 50, 100 and 150 μM was found to be 32.8, 63.8, 69.5, and 55.5, respectively. A mixture study was then performed using endosulfan (50, 100, 150 μM) combined with permethrin (100 μM). All combinations were found to have more than an additive effect, therefore the extrinsic pathway seems to be involved. Caspase 9 activity was measured using Caspase 9/Mch6 Fluorometric Protease Assay Kit. Relative fluorescence for endosulfan exposed cells after 7 hours incubation at 25, 50, 100 and 150 μM was found to be 43, 73, 78.9, and 5.12, respectively. Relative Fluorescence for permethrin exposed cells at 100, 150 and 200 μM was found to be 34.5, 39, and 55.5, respectively. A mixture study was then performed using endosulfan (25, 50 μM) combined with permethrin (100 μM). Both combinations were found to have less than an additive effect. These results suggest that apoptosis caused by both endosulfan and permethrin exert their effects via the caspase pathway. The results also show that mixtures of pesticides have a less than additive effect on caspase 9 activation and more than an additive effect on caspase 8 activation, therefore the extrinsic pathway is predominantly involved in thymocyte apoptosis caused by mixtures of permethrin and endosulfan.

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List of Abbreviations

7AAD- 7-Aminoactinomycin-D
AFC- 7-Amino-4-Trifluoromethyl Coumarin
CARD- Caspase Recruitment Domain
CO_2- Carbon Dioxide
DED- Death Effector Domain
DEET- N,N Diethyl-Meta-Toluamide
DEX- Dexamethasone
DD- Death Domain
DNA- Deoxyribonucleic Acid
Endo- Endosulfan
EtOH- Ethanol
FADD- Fas Associated Death Domain
H_20_2- Hydrogen Peroxide
LD- Lethal Dose
PARP- Poly (ADP-Ribose) Polymerase
PBO- Piperonyl Butoxide
PBS- Phosphate Buffered Saline
PDT- Photodymic Therapy
Perm- Permethrin
RPMI 1640- Roosevelt Park Memorial Institute Medium
SEM- Standard Error of Mean
T-cells- T Lymphoccytes
TBE- Tris-Borate-EDTA
TCR- T Cell receptor
TNF- Tumer Necrosis Factor
Unt- Untreated
USEPA- United States Environmental Protection Agency

Chapter 1

- **Study Goals**

To examine the effects of certain commonly used pesticides and their mixtures on the immune system and to elucidate the mechanisms of their action.

- **Study Hypothesis**

Endosulfan and permethrin cause activation of the caspase system in immune cells resulting in programmed cell death and mixtures of pesticides result in enhanced immunotoxic risk.

- **Study Rational**

We predicted that mixtures of pesticides may enhance or inhibit the immunotoxic risk. Caspases are involved in the apoptotic mechanism of action, and exposure to certain insecticides has resulted in apoptotic immunotoxicity. It is reasonable to assume that immune cell apoptosis caused by pesticides may, in part, be the result of certain caspase activation.

- **Study Objective**

To broaden our understanding of the mechanisms by which simultaneous exposure to multiple chemicals causes immune cell dysfunction in a mammalian model.

- **Specific Aims**

To investigate whether endosulfan and permethrin induce apoptosis in murine thymocytes *in vitro* and if so would mixtures of these pesticides enhance or inhibit the thymocyte cytotoxicity.

To examine if caspases are involved in the apoptotic pathways of thymocytes exposed to individual pesticides and pesticide mixtures and identify the specific caspase pathway involved in thymocyte apoptosis.

Chapter 2 - Literature Review

2.1 Pesticide Usage

A pesticide is any substance intended for preventing, repelling, or mitigating any pest. Pests can include insects, mice, rodents, unwanted plants, fungi, bacteria, and viruses (USEPA 2001). It has been estimated that food and fiber crop losses between 37 and 100% would occur without the use of pesticides (Pimental 1991). These numbers translate into 16 billion-dollars worth of crops saved each year after an estimated 4 billion-dollar investment in pesticide products. The economic benefits seem to support the use of pesticides, however, these numbers do not evaluate the human health and environmental costs. There are an estimated 20,000 deaths and 1 million poisonings each year worldwide. Each year in the United States a minimum of 67,000 poisonings and 27 accidental deaths are caused by pesticides (Pimental et al. 1992). Along with human casualties, an even greater impact occurs on other animals, most notably domestic animals. Another costly effect of widespread pesticide usage is the effects on other crops. Pesticide-induced death of honeybees and wild bees results in approximatly 320 million dollars a year in damages. An estimated loss of 8 billion dollars per year can be attributed to pesticide use. With an investment of 4 billion dollars, and 16 billion dollars worth of crops saved, pesticide use prevails in the financial court (Pimental et al. 1992).

Pesticides are used worldwide, but in 1995, close to 62 percent of the 30 billion-dollar industry was in the United States, Western Europe, and Japan. While most of the market for pesticides is in advanced countries, most of the poisonings and adverse environmental effects occur in developing countries, where pesticides such as DDT and other organochlorine pesticides, including endosulfan, are still in widespread use. These pesticides were harshly regulated as early as 1960 in more advanced countries.

Pesticides, based on cost and profit alone, are a successful industry, but there are many other factors that need consideration. Each year in the United States, an estimated 17 pounds of pesticides per citizen will be incorporated into our environment for a total of 4.6 billion pounds (Aspelin 1998). Of these 4.6 billion pounds, an estimated 85-90% will not reach their target organisms, thus, will enter the air, water, and soil (Repetto and Baliga 1996). Although many pesticides have been evaluated for their acute and chronic toxicity, their effect on the immune system has yet to be thoroughly investigated (Repetto and Baliga 1996). Lower doses of xenobiotics have been found to affect the immune system than would affect target organs (Burns et al. 1996).

2.2 Specific Pesticides: Permethrin

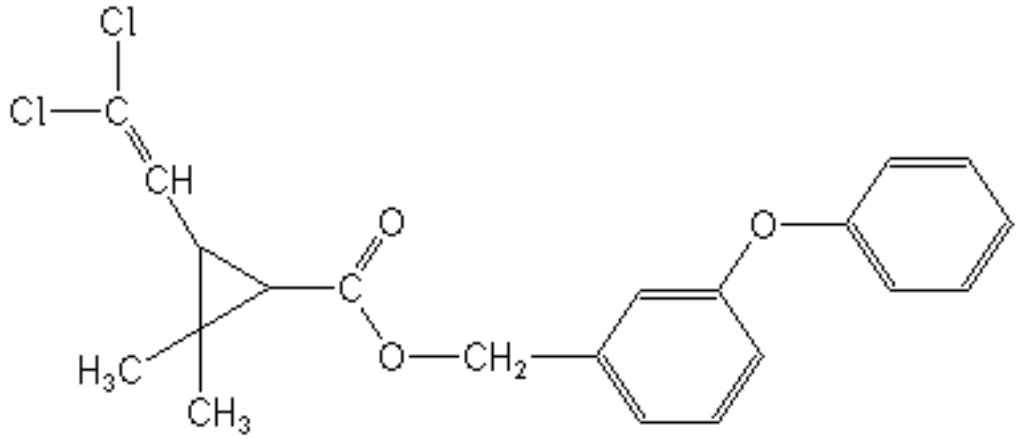

Figure 2.1. Chemical structure of permethrin, a synthetic pyrethroid insecticide.

Permethrin is a synthetic pyrethroid insecticide (Figure 2.1). Pyrethroids became stable agricultural pest control agents in the early 1970's (Casida and Quistad 1998). Pyrethroid use has grown to represent approximately 25% of the insecticide market worldwide. Among pyrethroids used in California, permethrin contributes greater than 53% of the total amount (Pesticide Use Report 1997). Permethrin and other such pyrethroids are widely perceived as safe insecticides. After introduction, synthetic pyrethroid insecticides achieved great success and set new standards for insecticides. Some of the major improvements seen in pyrethroid insecticides were photostability without compromising biodegradability and selective toxicity by target site and metabolic degradation. Pyrethroids also reduced fish toxicity and environmental impact while maintaining insecticidal potency.

Pyrethroids are divided into two distinct groups, type 1 and type 2. Permethrin, and other type 1 pyrethroids affect sodium channels in nerve membranes, causing repetitive (sensory and motor) neuronal discharge and a prolonged negative afterpotential. Although the repetitive discharges can occur in any region of the nervous system, those of the presynaptic nerve terminals would have the greatest effect on synaptic transmission. Permethrin has other sites of action such as inhibiting Ca2+, Mg2+-ATPase and also Na+, K+ -ATPase. This would increase neurotransmitter release and post- synaptic depolarization (Clark and Matsumura 1982)(Kakko et al. 2003). Signs of exposure to permethrin in rats are hyperexcitation, sparring, aggressiveness, enhanced startle response, whole body tremors, and prostration (Ecobichon 1996).

Permethrin insecticide is a mixture of four chiral isomers, a consequence of 2 chiral centers in the cyclopropane ring of the molecule. The insecticidal activity of the two isomers differs depending on the target insect (Elliot et al. 1978). Trans permethrin is metabolized more rapidly than cis in mammals, which is why trans isomers dominate the commercial product, comprising 60-75% of permethrin used (Gaughan et al 1977 and Casida and Ruzo 1980). Although between the years 1982 and 1988 there were 573 cases of acute pyrethroid poisonings, permethrin is classified as moderately to practically non-toxic, of a toxicity class 2 or 3 depending on formulation (Extoxnet 2000).

Subchronic dermal application of permethrin and DEET (N, N diethyl-m-toluamide) alone, or in combination can significantly reduce the density of healthy neurons in the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus, and the cerebellum. This could lead to motor deficits, behavioral abnormalities, learning and memory

dysfunction, and many physiological abnormalities (Abdel-Rahman et al. 2001). Permethrin in combination with DEET was found to cause release of cytochrome c in rat brain mitochondria, urinary excretion of 3-nitrotyrosine and 8-hydroxy-2-deoxyguanosine (markers of DNA damage), and also to cause oxidative stress (Abu-Qare and Abou-Donia 2001)(Abu-Qare and Abou-Donia 2003).

Studies have found permethrin to be toxic to the immune system. Permethrin can inhibit the mitogentic response of murine spleenic lymphocytes to concanavalin A and lipopolysaccharide (Stetzer and Gordan 1984). Topical exposure to permethrin was found to cause reduction of macrophage function and antibody production in the spleen, indicating that exposure may produce systemic immune effects (Punareewattana et al. 2001). Permethrin alone has been found to decrease thymic weight and cellularity. Cis-urocanic acid was not found to affect thymic weight and cellularity, but when combined with permethrin, was found to potentiate permethrins negative effect (Prater et al. 2003).

Humans are exposed to permethrin in several ways. In the United States and Great Britain, permethrin is the treatment of choice for lice and scabies (Roos et al. 2001). Recently, when outbreaks of West Nile Virus occurred in Washington D.C. and New York, permethrin was applied aerially, directly to affected neighborhoods. Although direct exposure does occur, secondary exposure is more likely. In a study of people that were not exposed to pyrethroids at home or work, pyrethroid metabolites were found in the urine of between 16 and 65 percent of the people, depending on the metabolite (Heudorf and Angerer 2001).

2.3 Endosulfan

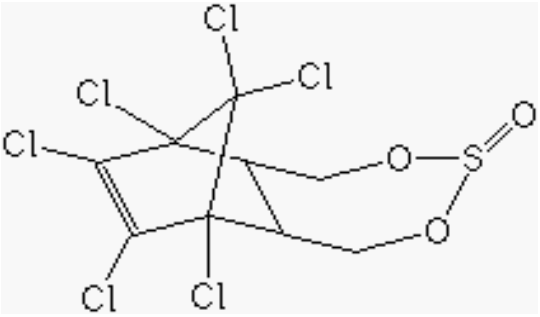

Figure 2.2. Chemical structure of endosulfan, an organochlorine insecticide.

Endosulfan is a member of the cyclodiene group of organochlorine pesticides used worldwide in agriculture (Figure 2.2). From the 1940's until the mid 60's, organochlorine pesticides were used extensively in all aspects of agriculture to control a wide variety of pests. The cyclodiene insecticides are among the most toxic and environmentally persistent insecticides known. Some of the physical and chemical properties of cyclodienes, such as low volatility, chemical stability, lipid solubility, and slow rate of biotransformation and degradation were originally seen as positive attributes for a successful pesticide. These attributes lead to bioconcentration, biomagnification in certain food chains, and persistence in the environment. Endosulfan is also a known environmental endocrine disrupter (Rose et al. 1999). Organochlorine insecticides are now harshly regulated in the United States but are still in

production and used extensively in developing countries because of their effectiveness and inexpensiveness.

Cyclodienes bind to the picrotoxin-binding site on the GABA receptor, preventing chloride-ion uptake and subsequently stimulating the mammalian nervous system (Rose et al. 1999). Along with picrotoxin-binding, cyclodienes inhibit NA+, K+-ATPase, but more importantly inhibit Ca2+, Mg2+-ATPase. This leads to an accumulation of intracellular free calcium ions, which promotes the release of neurotransmitters and subsequent depolarization of adjacent neurons (Ecobichon 1996). Endosulfan has an acute LD-50 of 30-79 mg/kg in rats (Matsumura 1985). Signs of cyclodiene poisoning include irritability, restlessness, muscular twitching, and convulsions that can end in death (Ribeiro et al. 2001).

Endosulfan is a highly toxic pesticide, toxicity class 1 in the EPA (ExToxNet 2000). Endosulfan has genotoxic effects on HeoG2 cells (Yuquan et al. 2000) and has been found to inhibit testicular function in pubertal rats (Chitra et al. 1999). Along with these negative effects, endosulfan is immunotoxic, inhibiting the metabolic activity of peripheral blood phagocytes in sheep (Pistl et al. 2001).

Although endosulfan is highly regulated in developed countries, exposure is still likely. For example, endosulfan has been detected in wine corks, 22% of virgin olive oils (Strandberg and Hites 2001, Lentza-Rizos et al. 2001), and oranges (Fernandez et al., 2001). Further evidence of human endosulfan exposure in regulated countries was confirmed by its presence in the blood of study participants in Spain (Arrebola et al. 2001). Endosulfan is stable in the environment and, because of its lipophilicy, can be stored in fat tissue and released later during sickness or starvation.

2.4 Immunity

Immunity requires a series of delicately balanced, complex, multicellular, and physiological mechanisms that allow an individual to distinguish foreign material from self and either neutralize or eliminate it (Burns et al. 1996). The immune system consists of two distinct but interrelated entities, known as humoral immunity and cell-mediated immunity. Humoral immunity is characterized by the production of antigen-specific blood proteins called antibodies. Cell-mediated immunity is mediated by antigen-specific cells called thymus-derived or T-lymphocytes, of which there are two populations, T helper cells and T cytotoxic cells. These cells, along with many other cells, organs, and the entire lymphatic system must work in unison to keep an individual's body healthy from foreign diseases.

The immune system plays a central role in the maintenance of health in an individual, so the study of the interactions of xenobiotics with the immune system has become an area of increased interest. In some cases, the immune system is compromised even when target organ toxicity is not observed. Immunosuppression can leave an individual vulnerable to opportunistic diseases. Because disrupting the delicate balance of the immune system can produce such profound effects, it seems necessary to elucidate mechanisms of xenobiotic-induced immunomodulation. With the advent of reproducible, sensitive, and predictable tests, immunotoxicity testing may alter safety evaluations for pharmaceutical, biological, and chemical agents.

Organs of the immune system can be divided into primary and secondary organs. Antigen-independent differentiation of lymphocytes occurs in the primary immune organs. Secondary immune organs such as the lymph nodes and the spleen are where the lymphocytes encounter antigens and undergo antigen-dependent differentiation. The thymus is a primary immune organ located above the heart, near or in the throat, resting on the pericardium. It has

two distinct lobes and is further divided into the cortex and the medulla. The larger of the two compartments is the cortex, which makes up 85-90% of the thymus. The murine thymus receives about 50 million immature lymphocytes each day. Immature lymphocytes, along with macrophages and endothelial cells, collectively called thymocytes, make up the cortex where thymocyte differentiation occurs. After thymocyte differentiation, the cells migrate to the medulla and undergo selection and maturation followed by release into the blood as mature T cells. In mice, roughly 1 million T-cells leave the thymus every day (Elgert 1996).

T lymphocytes (T-cells) are diverse. The defining characteristic of a T-cell is the expression of T cell receptor (TCR), which is unique to T-cells. T-Cells are required for the full expression of immunity. They regulate cellular immune reactions, antibody production, and kill altered cells. T- cells can mature in the thymus into two kinds of cells that will distribute themselves throughout the body, helper T- cells and cytotoxic T-cells (Elgert 1996).

Helper T-cells assist B-cells and other T-cells to multiply into clones and carry out their role in the immune response. These cells are usually identified by the presence of CD4 cell surface molecules. Cytotoxic T-cells are responsible for killing virus-infected cells, transplanted tissue, and cancer cells. These cells are usually identified by the presence of CD8 cell surface molecules. Cytotoxic T-cells can kill a target cell by two separate mechanisms - either injection of a membrane-disrupting enzyme called perforin or engagement of the Fas cell surface receptors and initiation of apoptosis (Elgert 1996).

2.5 Apoptosis

The death of a cell, until the last two decades, was thought to be an uncontrolled, degenerative, and catastrophic failure of homeostasis. Cells in multicellular organisms are now found to have the ability to activate an enzyme cascade, culminating in the programmed death of the cell. This process is called apoptosis. Apoptosis is the counterpart and counterbalance to mitosis in cell population and determination. Apoptosis plays a key role in the control of cellular populations in development, the immune system, and in carcinogenisis. It is now recognized that most, if not all, physiological cell death occurs by apoptosis. For example, in the immune system, immature thymocytes undergo apoptosis if their antigen receptors fail to rearrange correctly, if they are self reactive, or if they do not recognize foreign antigens (Cohen et al. 1995). Apoptosis is an efficient process; cells are disposed of by phagocytosis rapidly.

There are many morphological changes that are apparent in the latter stages of apoptosis. These changes are remarkably similar across a wide variety of tissues. An apoptotic body from the thymus is indistinguishable from an apoptotic body from the liver. In a cell undergoing apoptosis, the chromatin will condense to form dense compact masses, the cytoskeleton will break down, and the cell membrane will begin to bleb. Followed by the breakdown of the cell into membrane bound fragments called apoptotic bodies. Apoptotic bodies are found and eliminated quickly by phagocytosis. An important aspect of apoptosis is that it occurs without inflammation and facilitates the elimination of cells with minimal disruption to the surrounding cellular environment (Gill and Dive 2000).

Apoptosis is the natural form of cell death in all organs, but the thymus is the most studied due to its rapid turnover of cells. The induction of apoptosis in thymocytes was one of the first systems extensively studied (Williams 1995), and was the first place toxicologists looked for insight into chemically-induced apoptosis. Numerous agents, including glucocorticoids, etoposide, irradiation, tributyltin oxide, anti-CD3 antibodies, and thromboxane A2 agonists were shown to induce apoptosis in thymocytes. It has been suggested that cells, such as thymocytes, are primed to die and the trigger can be activated by any number of different

events because the stimuli that induce thymocytes are both physiological and pathological (Cohen et al. 1995). Apoptosis is the less invasive form of cell death, but this is not to say that apoptosis doesn't have serious negative effects on the body. Apoptosis can deplete the body of necessary cells, such as immune cells, which could result in decreased immune responses.

Necrosis is the second form of cell death. Necrosis is observed in toxicological studies when the toxicant is at high enough concentrations to cause gross cellular injury or a major perturbation of the cellular environment (Gill and Dive 2000). During necrosis, cell swelling and rupture of the membrane lead to release of proteases and intracellular toxic enzymes, possibly causing death of adjacent cells and inflammation (Williams 1995). Necrosis often leads to scarring and tissue destruction of the affected organ.

2.6 Chemical Mixtures

Humans and other animals are exposed to large numbers of chemical mixtures either concurrently or sequentially, from such sources as food, air, water, and consumer products. In food alone we are exposed to hundreds of thousands of chemicals. This makes it essential that chemical mixtures be studied in depth for their effects on the environment and humans.

There are three ways to characterize simple chemical mixture reactions. The chemicals can have an antagonistic, additive, or synergistic effects on each other. An additive effect is when the effect of two chemicals combined is equal to the sum of the two chemical effects when alone. An antagonistic interaction is when the chemical mixture effects are less than additive. A synergistic interaction is when the combined effect of the chemicals is greater than the sum of the two chemicals alone.

The study of chemical mixtures is both interesting and complex because of the scope of the endeavor. Each chemical has a unique toxic potential but there can be an interaction between the chemicals. The interactions may be physiochemical and/or biological, and then this reaction may occur in the toxicokintetic phase and/or in the toxicodynamic phase (Groten et al. 1999).

One of the most studied combinations of chemicals is permethrin and DEET. These chemicals may have contributed to the development of a syndrome that appears to have effected personnel who served in the gulf war. Mixtures of permethrin and DEET have behavioral effects (Haaren et al. 2001). The mixture also has neurotoxic effects (Abdel-Rahman et al. 2001) and has been found to cause release of cytochrome c from rat brain mitochondria (Aqel et al. 2001). Release of cytochrome c from mitochondria is known to induce apoptosis by activating the caspase pathway. Permethrin has been studied in mixtures with other chemicals such as PBO (piperonyl butoxide), arsenic, and mercury, and cis-urocanic acid. When permethrin was combined with mercury and arsenic, the substances modified the immunotoxic effects of the single compounds (Institoris et al. 2001). A study of subjects who were exposed to permethrin and PBO showed the combination having an immunotoxic effect (Diel et al. 1999). Cis-urocanic acid was found to potentiate permthrins immunotoxic effects (Prater et al. 2003).

Although the immunotoxicity of permethrin and its activity in mixtures is well documented, more research on the subject must be performed. The immunotoxic effects of endosulfan and its possible interactions with other chemicals are not well documented.

2.7 Caspases

Apoptosis is a form of cell death observed throughout nature. This form of cellular suicide can be triggered by a variety of stimuli, including cytokines, hormones, viruses, and toxic attacks (Earnshaw et al. 1999). In the past decade, studies have found that proteases play a

critical role in initiation and execution of this process. Cysteine-dependent aspartate-directed proteases, called caspases, are the most recognized of the cell death proteases. It is now widely believed that caspase activation, more than any other biochemical event defines cell death as apoptosis (Earnshaw et al. 1999).

Caspases exist in the cell as inactive zymogens. Caspases, when inactive, are composed of a prodomain, a large subunit, a linker segment and a small subunit. Caspase prodomains range in length from 23 amino acids in caspases 6 and 7 to 219 amino acids in caspase 10. The length of the prodomain appears to have an impact on the activity that this caspase will perform when activated. A long prodomain is associated with the initiation of the apoptotic response, commonly termed initiator caspases. A short prodomain usually indicates an effector caspase, which is activated by the initiator caspases. There are 2 main segments found in this section of the inactive caspase. The first is the DED, or death effector domain. This domain is found in caspases 8 and 10 and is most likely involved in interaction with signaling proteins such as MORT1/FAD (Chiannaiyan et al. 1995) and TRADD (Hsu et al. 1995). Another domain found in this segment of caspases 1, 2, 4, and 9 is the caspase recruitment domain, or CARD. CARD is thought to promote interaction with other caspases (Hofmann et al. 1997).

Phylogenicaly, caspases are divided into two groups. The first group is the caspase 1 family, composed of caspase 1, 4, 5, and 13. This subfamily is involved primarily in the control of inflammation and is not thought to be involved in the process of apoptosis. The second group is the caspase 3 family, including caspase 3 and 6-10, involved primarily in apoptosis.

As described before, inactive caspases or procaspases are composed of 4 main sections. Each active caspase is derived from the combination of 2 inactive caspases. Conversion of the inactive caspase to the active caspase requires a minimum of 2 cleavages. The first cleavage separates the prodomain from the large subunit and the second cleavage separates the large and small subunit. Activation of caspases involves hetero- or auto-proteolytic processing of pro-caspases, most often at aspartate residues (Marti et al. 2001). Two large subunits and 2 small subunits then combine to form the active caspase.

2.8 Caspase activation

Although many ways of activating caspases are evident, fundamentally, the biochemical mechanisms appear to be remarkably similar and can be explained by one model. The induced proximity model is based on the empirical observation that the zymogen forms of caspases are not fully inactive but posses a weak protease activity. This activity in some caspases has been measured at less than 1% of the fully active enzymes. When the zymogens are brought close together through protein interactions, the zymogens can trans-process each other, producing fully active proteases. (Salvesen and Dixit 1999)

2.9 Caspase 3

Caspase 3 has been implicated as a key mediator of apoptosis. A downstream caspase activated by both caspase 9 and caspase 8, caspase 3 like proteases were the first caspases linked to apoptosis. This is not to say that these caspases are the only way caspase 3 can be activated. Caspase 6, another downstream caspase can activate caspase 3. Caspase 3 can also activate other caspases such as caspase 9 (Kuida et al. 1996).

It is nearly impossible today to distinguish which caspases are responsible for which events during apoptosis. The only real glimpse to date of which caspase causes which proteolytic cleavages is through the use of knockout mice. Caspase 3 knockout mice cause some

of the most serious phenotypes. These mice can survive to birth but die soon after because of problems associated with brain development (Kuida et al. 1996 and Woo et al. 1998). At birth, the thymus, heart, lung, liver, kidney, spleen, and testis appear normal. Caspase 3 negative mice thymocytes can also undergo apoptosis in response to dexamethasone, but caspase 3 is required for apoptosis in neutrophils and activated T cell development. In cells where apoptosis occurred, caspase 3 was required for DNA degradation and chromatin condensation but not for PARP cleavage (Kuida et al. 1996 and Woo et al. 1998). Caspase 3 has also been shown to cleave DNA-dependent protein kinase, and actin. Caspase 3 has been found to cleave many polypeptides in the cytoplasm, for example, cleavage of gelsolin and fodrin lead to calcium insensitive actin cleavage and plasma membrane blebbing, respectively. Important proteins involved in gene expression and signal transduction such as Pro-interleukin-16 and calpastatin are cleaved by caspase 3, leading to T lymphocyte chemotaxis and a decrease in inhibition of calpain. In addition to the actions of caspase 3 described above, it cleaves key proteins involved in the cell cycle, proliferation, and apoptotic regulatory proteins. Of the effector caspases, caspase 3 is the most active in the apoptotic cell death process, but it is not necessary in all cell types or in all kinds of apoptotic stimulation.

Stimulation of caspase 3 is a key indicator of apoptosis, and many cellular insults activate caspase 3. For example, caspase 3 activation is observed with high concentrations of heptachlor, an organochlorine insecticide (Rought et al. 2000). Many chemotherapeutic agents target caspase 3 in order to promote apoptosis is tumors, examples of this are doxirubicon and *N*- (4-Hydroxyphenyl) retinomide (4HPR) (Rought et al 2000 and Sazuki et al. 1999). Other cellular insults such as reactive oxygen species activate caspase 3-like caspases and caspase 3 itself, while also facilitating the release of cytochrome c. Cytochrome c can activate caspase 9 (Masahiro et al. 1998).

2.10 Caspase 9

Caspase 9 is the apical caspase in the mitochondrial or intrinsic pathway making it an important initiator caspase. Caspase 9 activates when combined with cytochrome c from the mitochondria, and the caspase activating protein Apaf-1. This complex, called an apoptosome plays a key role in apoptosis. The apoptosome will activate caspases 3 and 7. Caspase 3 can then activate caspase 6 and the caspase cascade leading to apoptosis begins (Earnshaw et al. 1999).

Caspase 9 is an initiator caspase, its only known function is to initiate downstream caspases such as caspase 3. Caspase 9 is important, as has been proven by the use of knockout mice (caspase 9 negative mice). Caspase 9 negative mice have very similar brain abnormalities to the caspase 3 negative mice, only more severe. Caspase 9 negative mice display a complex range of abnormalities in the apoptotic response. This response varies in both cell type and stimulation manners. Caspase 3 can still be activated in caspase 9 negative mice in certain tissues but not in the brain (Kuida et al. 1998 B). This effect is also observed during *in vitro* experiments with caspase 9 negative ES cells and embryonic fibroblasts. In these cells cytochrome c is released from the mitochondria but caspase 3 is not processed. This effect can also be observed in thymocytes and brain cells from caspase 9 negative animals where caspase 3 is not activated when cytochrome c and dATP were added.

As the apical caspase in the intrinsic pathway, caspase 9 plays a key role in apoptosis. This role makes it an important caspase to study in toxicology as well as in pharmacology. Examples of toxic agents that stimulate caspase 9 are the potent immunotoxic agents cadmium and T-2 toxin (Kondoh et al. 2002 and Nagase et al. 2001). Deltamethrin, a pyrethroid

insecticide, has been found to induce apoptosis in cultured neurons by stimulating Bax and inhibiting Bcl-2 (Wu et al 2003). Certain bacterial interactions have also been found to cause apoptosis via caspase 9, an example of this is by group A streptococcus pyogenes (Nakagawa et al. 2001). Photodymic therapy (PDT) is under investigation as a possible treatment for restinosis and atherosclerosis. PDT increases cytochrome c release and apoptosis-inducing factor, which in turn activates caspase 9, followed by caspase 3 and apoptosis (Granville et al. 2001).

2.11 Caspase 8

Caspase 8 is the apical caspase in the extrinsic or death receptor pathway. This pathway centers on the tumor necrosis factor family of receptors (TNF). This connects ligand binding on the cell surface to caspase activation and subsequent apoptotic initiation (Salvesen and Dixit 1997 and Wallach et al. 1997). Activation of caspase 8 involves a network of protein interactions. Fas is indirectly linked to caspase 8 through adapter proteins such as Fadd. Fadd activates pro-caspase 8 through death receptor complexes (Chinnaiyan et al. 1996 and Muzio et al. 1996). Caspase 8 can then activate caspase 3 of the effector caspases. Caspase 8 can cleave pro-caspases 4,7, and 9 *in vitro* (Srinivascula et al. 1996, Muzio et al. 1997, Stennicke et al. 1998).

Caspase 8 negative mice develop normally until embryonic day 11.5 and then begin to die. Examination reveals an undeveloped heart musculature, accumulation of erythrocytes in the liver, lung, mesenchymal spaces, and the lens (Varfolomeev et al. 1998). When caspase 8 negative embryonic fibroblasts are exposed to cytotoxic signaling through FAS, TNR, and DR3, the cells are resistant and do not undergo apoptosis. Apoptosis does still occur in these cells when exposed to serum withdrawal, ultraviolet irradiation, ceramide, and etopside (Varfolomeev et al. 1998). These results confirm caspase 8 as an essential component of apoptosis caused by ligand binding to cell surface death receptors such as TNF and further confirm the ability of apoptosis to occur without activation on the cell surface.

Caspase 8, being the apical caspase in the extrinsic pathway of apoptosis has been studied in both toxicology and pharmacology. Caspase 8 was activated in apoptosis caused by magnolol, a substance found to have anticancer activity (Shyr-Yi Lin et al. 2001). Influenza virus induced apoptosis also occurs through the activation of FADD and caspase 8 (Balachandran et al. 2000). One final example of caspase 8 activation was found in lymphocytes exposed to low-doses of morphine. Apoptosis via caspase 8 activation is common in many cell types and through many cellular insults. Apoptosis can occur without caspases 8 and in many situations the intrinsic pathway is not the only apoptotic pathway activated.

2.12 Extrinsic Pathway

There are many pathways by which apoptosis can occur and there are many ways caspases can be activated, but two have been studied in detail. One begins with the tumor necrosis factor family of receptors and the other involves participation of the mitochondria. Though these pathways are viewed as separate pathways that act independently, a degree of crosstalk is possible at several points.

The intrinsic pathway begins at the mitochondria with the release of cytochrome c. Cytochrome c is then free to bind with the caspase activating protein Apaf-1 (Zou et al. 1999). Apaf -1 can normally be found as an inactive protein in the cytosol. The oligomerized complex that is formed with the combination of cytochrome c and Apaf-1 can then bind pro-caspase 9

(Figure 2.3). This new complex, called an apoptosome can then trans-process caspase 9 zymogens via the induced proximity mechanism (Li et al. 1997). The caspase 9 enzyme must then remain bound to Apaf-1 for full activity.

Several proteins are thought to cause release of cytochrome c from the mitochondria. One example of how cytochrome c is released from the mitochondria is that the membrane ruptures. This is presumed to be caused by an overexpression of the pro-apoptotic protein Bax (Inohara et al. 1999 and Willis et al. 1999). Also thought to induce release of cytochrome c from mitochondria is the pro-apoptotic protein of the Bcl-2 family Bid (Li et al. 1998 and Luo et al. 1998).

Activation of procaspase 9 by cytochrome c/Apaf-1 is regulated by the anti-apoptotic Bcl family members, Bcl-2 and Bcl-Xl. These proteins are located in the outer mitochondrial membrane and can inhibit the release of cytochrome c (Figure 2.3)(Ng et al. 1997 and Rasper et al. 1998). Bcl-2 and Bcl-Xl can form heterodimers with the proapoptotic Bcl-2 family members mentioned before and act as decoys, binding to the proapoptotic Bcl-2 members and restricting the release of cytochrome c (Kooseki et al. 1999 and Yuan et al. 1999).

2.13 Intrinsic Pathway

Caspase 8 is the apical caspase in the TNF family death receptor pathway. This is demonstrated by a wide variety of experimental evidence including gene ablation (knockout) experiments in mice. Caspase 8 activation is first initiated by Fas (Apo-1/CD95), the tumor necrosis factor receptor and death receptor-3 (Bolden et al. 1996, Chinnaiyan et al. 1996, Muzio et al. 1997). These three members of the tumor necrosis factor family then pass on this death signal through a cytoplasmic sequence called the DD or death domain. After receptor activation, the DD rapidly associates with adapter molecules that contain a similar DD motif (Nagata 1997 and Wallach et al 1997). The Fas-associated DD (FADD) exhibits significant homology to a similar region on the caspase 8 prodomain. These regions of the caspases are called DED or Death effector domains, and permit heterodimerization of procaspase 8 with FADD (Boldin et al 1996, Fernandex-alnemri et al 1996, Muzio et al. 1996, Vincenz and Dixit 1997). Fas aggregation rapidly results in FADD-mediated recruitment in the apoptosis inducing signaling complex (Muzio et al. 1996 and Medema et al. 1997). When two procaspase 8 are associated with this receptor complex, it becomes an active caspase 8 protein. Caspase 8 can then activate other caspases such as caspase 3 and apoptosis occurs (Figure 2.3).

Activation of caspases by death receptor-associated scaffolds can be regulated by the polypeptide FLIP (Irmler et al. 1997 and Hu et al. 1997). FLIP contains a DED, which binds the prodomain of procaspase 8 (Goltsev et al. 1997 and Srinivascula et al. 1997). This inhibits the recruitment of procaspase 8 to the CD95/Fas activation complexes. FLIP's anti-apoptotic activity is illustrated in its role of regulating T-cell sensitivity to CD95-mediated apoptosis after interleukin-2 treatments (Irmler et al. 1997). A similar strategy is used by viruses to prevent cells from undergoing apoptosis that could abort the viral replication cycle. For example, the equine herpes type II virus E8 protein contains a DED domain homologous to the DED found on procaspase 8. This allows for the inhibition of procaspase recruitment to death receptor associated signaling complexes (Bertin et al. 1997 and Thome et al. 1997).

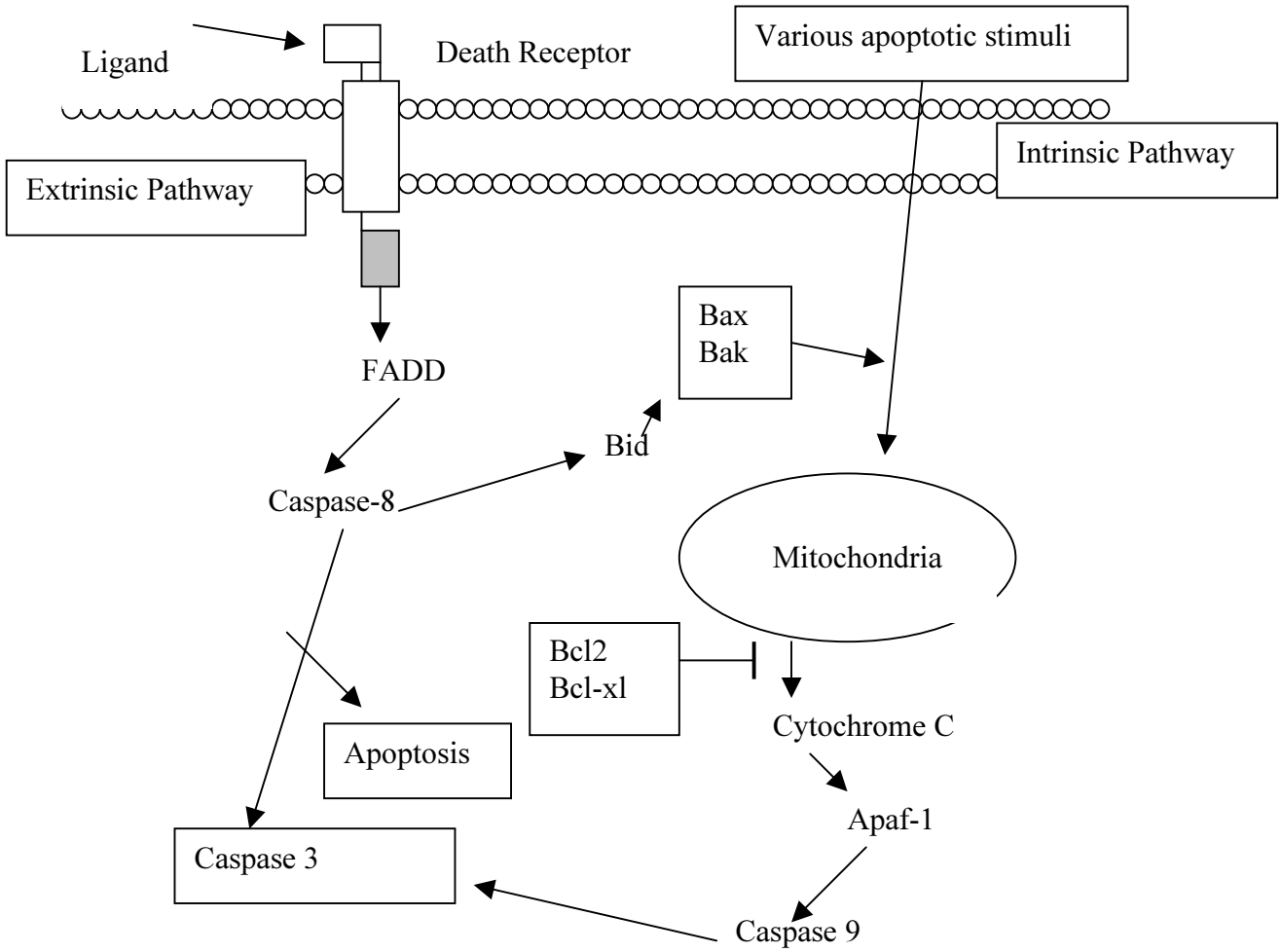

Figure 2.3 Intrinsic and Extrinsic pathways of apoptosis. The extrinsic pathway is triggered by death receptor engagement, which initiates a signaling cascade mediated by caspase 8 activation. Caspase 8 can both feed directly into caspase 3 activation and stimulate release of cytochrome c by the mitochondria. The intrinsic pathway occurs when various apoptotic stimuli trigger release of cytochrome c from the mitochondria. Cytochrome c interacts with Apaf-1 and caspase 9 to promote activation of caspase 3. Bak and Bax, Bcl-2- associated proteins block release of cytochrome c from the mitochondria. Bid, a proapoptotic Bcl-2 member triggers the release of cytochrome-c from the mitochondria.

Chapter 3

EFFECTS OF ENDOSULFAN, PERMETHRIN, AND THEIR MIXTURES THYMOCYTE APOPTOSIS.

(Toxicology, in Preparation)

Abstract

Altered immune responses have been observed following occupational, inadvertent, or therapeutic exposure to xenobiotics. Many pesticides are known to cause immunotoxicity. Exposure to mixtures of pesticides, either concurrently or sequentially, may result in potentiating this effect partly because one can effect the metabolism of the other. We tested the hypothesis that endosulfan and permethrin might induce apoptosis in C57BL/6 male mice thymocytes *in vitro* and mixtures of these pesticides may potentiate the immunotoxic risk. We examined the immunotoxic potential of these pesticides using a flow cytometric technique in combination with 7-Amino Actinomycin D (7AAD) to distinguish live, early apoptotic, and late apoptotic/necrotic cells. DNA ladder assay, a hallmark of apoptosis, was also used to determine the occurrence of apoptosis. Both endosulfan and permethrin were found to cause significant apoptotic death of thymocytes in a dose- and time- dependent manner. Pesticides in mixture were found to have an additive immunotoxic effect. DNA ladder assay confirmed the presence of DNA fragments, a characteristic of apoptosis. The results of this study suggest that the mixtures of endosulfan and permethrin have additive immunotoxic effects on C57BL/6 mice thymocytes, *in vitro*.

Keywords: apoptosis, immunotoxicity, thymocytes, endosulfan, permethrin, chemical mixtures

1. Introduction

Pesticides are designed to kill or adversely effect living organisms, so they can effect non-target organisms such as humans. It has been estimated that 85 to 90 % of pesticides applied in agriculture will not reach their target organisms and will enter the air, water, and soil (Repetto and Baliga 1996). Average use per American citizen was 17 pounds of pesticides in the United States in 1997, with a total of 4.63 billion pounds used (Aspelin 1998). Very low doses of xenobiotic can effect the immune system, often at much lower doses than those necessary to achieve target organ toxicity (Burns et al. 1996).

Endosulfan is a member of the cyclodiene group of organochlorine pesticides used worldwide in agriculture. From the 1940's until the 1960's organochlorine pesticides were used extensively in all aspects of agriculture to control a wide variety of pests. The cyclodiene insecticides are among the most toxic and environmentally persistent insecticides known. Endosulfan, a known environmental endocrine disrupter is not only toxic to insects but to non-target species such as fish and humans (Kiran et al. 1998, Boereboom et al. 1998).

Permethrin is a synthetic pyrethroid insecticide. Pyrethroids became agricultural pest control agents in the early 1970's. After introduction, the synthetic pyrethroid insecticides achieved great success and set new standards for contact insecticides (Casida et al. 1998). Studies have found permethrin to be toxic to the immune system. Permethrin can inhibit the mitogentic response of murine spleenic lymphocytes to concanavalin A and lipopolysaccharide (Stetzer and Gordan 1984). Topical exposure to permethrin was found to cause reduction of

macrophage function and antibody production in the spleen and also to decrease thymic weight and cellularity, indicating that exposure may produce systemic immune effects (Punareewattana et al. 2001)(Prater et al. 2003).

Numerous agents, including glucocorticoids, etoposide, irradiation, tributyltin oxide, anti-CD3 antibodies, and thromboxane A2 agonists were shown to induce apoptosis in thymocytes. It has been suggested that cells, such as thymocytes, are primed to undergo apoptosis and the trigger can be activated by any number of different events because the stimuli that induce thymocytes are both physiological and pathological (Cohen et al. 1995, Pistl et al. 2001). There are many morphological changes that are apparent in the latter stages of apoptosis. These changes are remarkably similar across a wide variety of tissues. An apoptotic body from the thymus is indistinguishable from an apoptotic body from the liver. In a cell undergoing apoptosis, the chromatin will condense to form dense compact masses, the cytoskelaton will break down, and the cell membrane will begin to bleb. This is followed by the breakdown of the cell into membrane bound fragments called apoptotic bodies. Apoptotic bodies are found and eliminated quickly by phagocytosis. An important aspect of apoptosis is that it occurs without inflammation and facilitates the elimination of cells with minimal disruption to the surrounding cellular environment (Gill and Dive 2000).

Necrosis, on the other hand is a form of cell death seen often in toxicological studies when the toxicant is at a high concentration as to cause gross cellular injury or a major perturbation of the cellular environment (Gill and Dive 2000). During necrosis, cell swelling and rupture of the cell membrane leads to release of proteases and intracellular toxic enzymes, leading to death of adjacent cells and inflammation (Williams 1995). We studied the effects of endosulfan, permethrin, and their mixtures on murine thymocytes *in vitro.* Here we present evidence that both endosulfan and permethrin cause apoptosis in thymocytes and mixtures of insecticides have an additive effect.

2. Materials and Methods

2.1. Animals

Male C57BL/6 mice aged 8 to 12 weeks were obtained from Charles River Laboratories (Wilmington, MA). The animals were kept in accordance with U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals (Nationals Research Council, 1996). Animals were housed with wood chip bedding in polycarbomate cages. Room temperature (21 ± 2°C), humidity (50 ± 10%), and light cycles (12 H on and 12 H off) were controlled.

2.2. Chemicals

Permethrin (99% purity) and endosulfan (99% purity) were obtained from Chem Services (West Chester, PA). Stock solutions of 50mM were prepared using 100% ethanol. Working solutions of pesticides were prepared using RPMI-1640 media (Gibco BRL, MD) with concentrations of pesticides ranging from 25μM to 200μM for each pesticide.

2.3. Isolation of Thymocytes

Mice were sacrificed using cervical dislocation and weighed (23g ± 2). The thymus was then removed and weighed (0.02g ± 0.001) and quickly ground against a metallic 60-mesh wire sieve (Sigma). Cells were pellated at 250 x g for 7 minutes at 4°C (IEC Centra GP8R, International Equipment, Needham Heights, MA). The Cells were then resuspended in 10 ml RPMI-1640 media and spun again at 250 x g for 7 minutes at 4°C. The Cells were resuspended in RPMI – 1640 Media and counted on a CASY I Cell Counter and Analyzer System (Scharfe Systems Gmblt, Reutlinger Germany). Cells were diluted to 1x 10^6 or 2x 10^6 cells/ml and suspended in RPMI complete media (10% FBS, 1% 100x L-glut, 1% non-essential amino acids, 1% Pen/Strp, 1% HEPES Buffer). Thymocytes were incubated at 37˚C with 5% CO_2 and 95% humidity in the presence and absence of pesticides for varying amounts of time (4,8,12,16 hours).

2.4. 7AAD Assay

Thymocytes were isolated and treated with pesticides as described above. After 12 hours incubation cells were rinsed with 37ºC PBS and centrifuged at 250 x g for 7 minutes at 4ºC. The medium was removed and 100 µl of 10 µg/ml 7-AAD DNA binding dye (Molecular Probes, Eugene OR) in a supplemented buffer (0.15% sodium azide and 2% BSA in PBS) were added to the wells. The plate was incubated on ice, in the dark for 30 minutes. These cells were then analyzed for their ability to take up 7-AAD by flow cytometry. 7-AAD "dull" are live cells, 7-AAD "moderate" are early apoptotic cells, and 7-AAD "bright" are late apoptotic or necrotic cells.

2.5. DNA Ladder Assay

Thymocytes were isolated and treated with pesticides as described above. Cells were then incubated as described above for 4, 8, and 12 hours. The procedures for the Apoptotic DNA Ladder Kit were then followed (Boehringer Mannheim, Mannheim Germany). Purified DNA was then eluted and run on a 1% agarose gel with ethidium bromide. When the gel was viewed under UV light, DNA fragments were observed.

2.6. Statistical Analysis

SAS JMP software was used for data analysis. Use of Tukey-Kramer HSD allowed for comparison of each treatment to other specified treatments to determine whether statistical difference was evident. Data are presented as means ± standard error of the mean (SEM). If $p > 0.05$, treatments were not statistically different.

3. Results

3.1. Effects of Permethrin and Endosulfan on the 7AAD Assay

7-AAD assay was used to differentiate between live, early, and late apoptotic/necrotic cells. As shown in Figure 3.1 cells were exposed to endosulfan at 25, 50, 100, 150, and 250 μM concentrations. Early apoptosis occurred at 9.3, 24.6, 44.2, 46.6, and 50.2%, respectively. Late apoptosis/necrosis occurred at 9.0, 27.5, 40.2, 46.4, and 42.5%, respectively. As shown in Figure 3.2 cells were exposed to permethrin at 50, 100, 200, and 300 μm concentrations. Early apoptosis occurred at 6.9, 13.0, 18.0, and 24.0%, respectively. Late apoptosis/necrosis occurred at 6.4, 11.4, 14.2, and 14.9%, respectively. A dosage of 100μM permethrin and 50μM endosulfan was then used to perform a time response study. Cells were exposed at the concentrations above and incubated for 8, 12, and 16 hour time periods. Early apoptosis occurred in 48, 52.5, and 50.8% and late apoptosis/necrosis occurred in 8.5, 14.2, and 32.5% of cells when exposed to 50μM endosulfan for 8, 12, and 16 hours, respectively. Early apoptosis occurred in 28, 35.2, and 38.3% and late apoptosis/necrosis occurred in 4.4, 5.8, and 19% when exposed to 100 μm permethrin for 8, 12, and 16 hours, respectively. These data are presented in Figure 3.3. A mixture study was performed with concentrations of 100 μM permethrin and 50 μM of endosulfan. Cells were incubated for 12 hours with these concentrations of pesticides individually and in combination. As shown in Figure 3.4 permethrin at 100 μM had early apoptosis occur in 16.7% of cells and late apoptosis/necrosis in 7.5%. Endosulfan at 50 μM had early apoptosis occur in 20.9% of cells and late apoptosis/necrosis in 15.2%. A mixture of 50μM endosulfan and 100μM permethrin had early apoptosis occur in 31.9% of cells and late apoptosis/necrosis in 21%. A dexamethasone positive control of 10 μg/ml was performed and found to cause 37% early apoptosis. An H_2O_2 negative control was performed and found to cause no significant apoptosis and 40% necrosis.

3.2 Effects of Permethrin and Endosulfan on the DNA Ladder Assay

DNA ladder assay was performed with concentrations of 50μM endosulfan and 100μM of permethrin alone and in combination. Cells were incubated with these concentrations for 4, 8, and 12 hours. As shown in Figure 3.5 a laddering effect was seen at all concentrations of pesticides and at all time points (4 hours is represented here).

4. Discussion

There are two modes of cell death, necrosis and apoptosis. Although both endosulfan and permethrin have been shown to have immunotoxic properties, the specific cell-death pathway they utilize and the effect of their mixtures on murine thymocytes has not been studied. Two different assays were therefore used in the present report to identify specific morphological, biochemical or physiological aspects of either apoptosis or necrosis.

Cells exposed to permethrin or endosulfan for 12 h contained significant populations of cells in early apoptosis as measured by 7-AAD staining assay. 7-AAD staining is a reliable, quantitative method for detecting apoptosis/necrosis in immune cells (Donner et al. 1999), based on apoptotic-induced membrane changes in cells. Measurement of 7-AAD, a DNA binding stain, enabled us to distinguish between live (7-AAD^{Dull}), early apoptotic ($7\text{-AAD}^{Moderate}$), and late apoptotic/necrotic (7-AAD^{Bright}) cells via flow cytometric analysis. A concentration curve was performed to determine the optimal concentrations of each chemical for subsequent mixture studies. Mixture studies showed the insecticides to have an additive effect on thymocyte apoptosis. Significant necrosis was also observed in thymocytes exposed to these pesticides alone, and exposure to a combination of endosulfan/permethrin resulted in less than an additive effect on necrosis. Dexamethasone, an apoptotic agent, was used as a positive control. H_2O_2

was used as a necrotic control. In untreated and ethanol controls, a small percentage of early apoptotic cells was observed, confirming previous reports that spontaneous apoptosis can occur in mature thymocytes in culture (Perandones et al. 1993).

DNA ladder assays were performed to confirm endosulfan- and permethrin-induced apoptosis. During apoptosis, nuclear DNA is non-randomly cleaved into 180 base pair units. The DNA ladder assay is based on this principle; this ladder can be visually detected when DNA from apoptotic cells are electrophoresed on an agarose gel. Cells incubated for 8 hours with or without pesticides demonstrated a laddering effect in all samples and controls (confirming spontaneous apoptosis in cultured cells), but more intense and clearer laddering patterns were observed in samples exposed to pesticide mixtures or dexamethasone (data not shown). Cells exposed to pesticides, alone or in combination, or to dexamethasone for 4 h had an intense laddering effect, with no laddering pattern observed in untreated or ethanol control. Our observation of DNA laddering patterns after short-term incubation with pesticides is consistent with previous reports that DNA fragmentation is one of the first irreversible events in mature immune cell apoptosis (Goldstein et al. 1991). Along with the distinct laddering patterns, DNA smearing (an indicator of necrosis) was observed in all samples and controls. Smearing became more intense as time of exposure increased (up to 12 h), suggesting that long-term pesticide exposure may upregulate both apoptotic and necrotic pathways in immune cells. Genomic DNA, observed in all samples, demonstrated the presence of live cells. Results obtained from DNA laddering assays confirmed the presence of live, apoptotic, and necrotic cell populations in control and pesticide-exposed immune cells observed with 7-AAD staining.

The immune system plays a central role in maintaining the health of an individual. Therefore, investigating the effect of xenobiotics on the immune system is of interest. In some cases the immune system is compromised even when there are little or no detectable effects on other organ systems. Decreased immune response, or immunosuppression, can leave an individual vulnerable to opportunistic diseases, resulting in severe, prolonged, or repeated infections, as well as to the development of cancer. Because disrupting the delicate balance of the immune system can produce such profound effects, it is important to elucidate the biochemical and molecular mechanisms of xenobiotic-induced immunomodulation.

Humans and other animals are exposed to a large number of chemical mixtures, concurrently or sequentially, from sources such as air, water, food, or consumer products. In food alone, we are exposed to hundreds of thousands of chemicals. Clearly, it appears that the environmental and health effects of chemical mixtures should be studied in depth. Data reported here are unique in that we investigated the effect of chemical mixtures, rather than a single chemical, on cellular immunity.

Permethrin is widely thought to be a safe, non-persistent insecticide. Permethrin is often used in populated areas to control outbreaks of mosquito-born diseases. An example of this is West Nile Virus in New York and Virginia in recent years. Permethrin and DEET (N,N diethyl-meta-toluamide) were applied to American troops in the Gulf War and have been widely blamed for causing Gulf War Syndrome. Permethrin, in combination with DEET, has been shown to release cytochrome c in rat brain mitochondria, leading to the induction of apoptosis via the caspase pathway (Abu-Qare and Abou-Donia 2001). Permethrin inhibits the mitogenic response of murine spleenic lymphocytes, causing a reduction in macrophage function and antibody production in the spleen. This suggests that permethrin exposure might play a role in immune suppression. Endosulfan is not as extensively used as permethrin in the United States because it is classified as highly toxic (toxicity class 1) by the EPA (ExToxNet 2000). Although highly restricted in most developed countries, endosulfan is still used extensively in developing countries because of its effectiveness and low cost. Endosulfan is stable in the environment,

lipophilic, and inhibits the metabolic activity of peripheral blood phagocytes in sheep (Pistl et al. 2001), suggesting that even low exposure to this compound could result in deleterious effects on the immune system over time. Based on the reported data regarding these compounds, it is likely that humans and other animals exposed to permethrin and endosulfan in low doses could become immunocompromised

Data reported here demonstrate that exposure to permethrin and endosulfan, alone or in combination, results in increased apoptosis and necrosis in murine thymocytes. This confirms previous reports of the immunotoxicity of these chemicals, and provides novel data regarding their effects in combination. Permethrin and endosulfan are potent immunotoxicants, and mixtures of these pesticides cause an additive effect on apoptotic induction in murine thymocytes *in vitro*. Future studies should asses the molecular mechanism(s) by which this pesticide-induced apoptosis occurs.

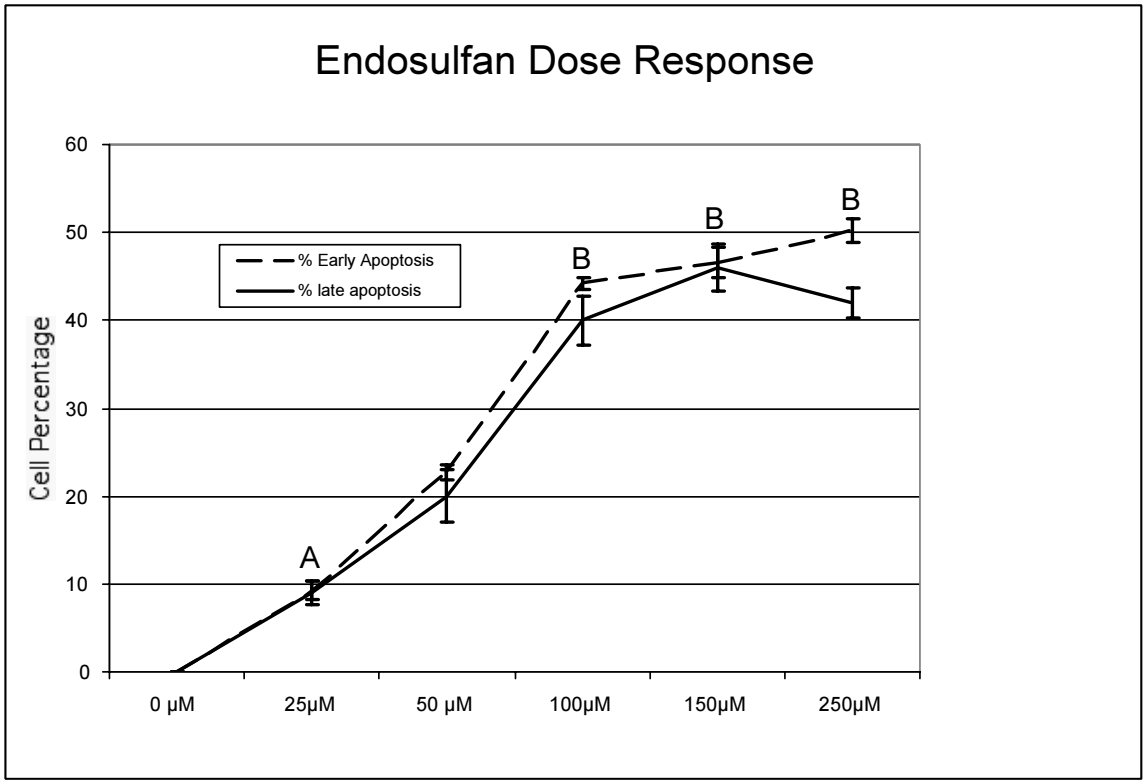

Figure 3.1. Effect of endosulfan on C57BL/6 murine thymocytes. Early apoptosis and late apoptosis/necrosis was measured using flow cytometry with the 7-AAD Staining assay. Thymocytes were treated with Endo 25, 50, 100, 150, and 250 µM for 12 hours. An Unt, Etoh, Dex 10 µg/ml, and H_2O_2 controls were also performed. Samples were examined via flow cytometry for three degrees of staining intensity, 7AAD- dull (live cells), 7AAD moderate (early apoptotic cells), and 7AAD bright (late apoptotic/necrotic). The percent of cells (n = 5000 events) stained 7AAD moderate and 7AAD bright is shown.

Notes. **Treatments with similar letters were not significantly different ($p > 0.05$). All other treatment comparisons were significantly different. Values are the mean ± SEM of 4 experiments (Total of n = 8 per treatment)**

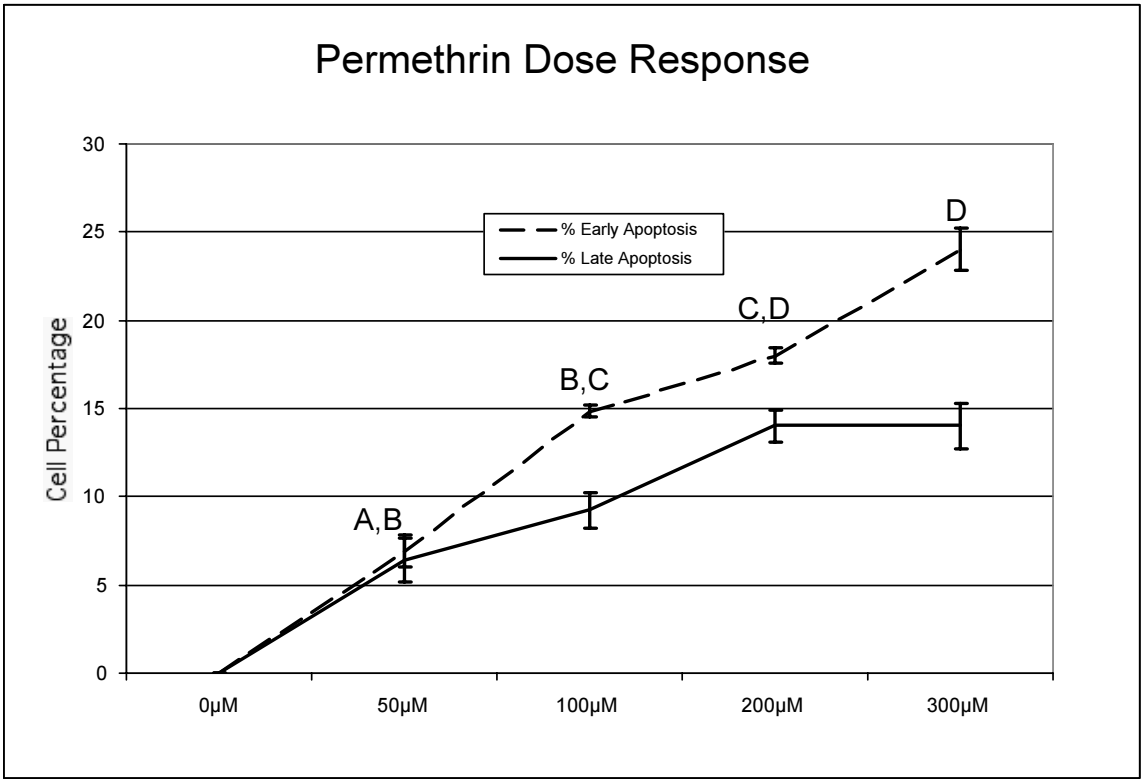

Figure 3.2. Effect of permethrin in C57BL/6 murine thymocytes. Early apoptosis and late apoptosis/necrosis were measured using flow cytometry and the 7-AAD Staining assay. Thymocytes were treated with Perm 50, 100, 200, and 300 µM for 12 hours. Unt, EtOH, Dex 10 µg/ml, and H_2O_2 controls were also performed. Samples were examined via flow cytometry for three degrees of staining intensity, 7AAD- dull (live cells), 7AAD moderate (early apoptotic cells), and 7AAD bright (late apoptotic/necrotic). The percent of cells (n =5 000 events) stained 7AAD moderate and 7AAD bright is shown.

Notes. **Treatments with similar letters were not significantly different ($p > 0.05$). All other treatment comparisons were significantly different. Values are the mean ± SEM of 4 experiments (Total of n = 8 per treatment)**

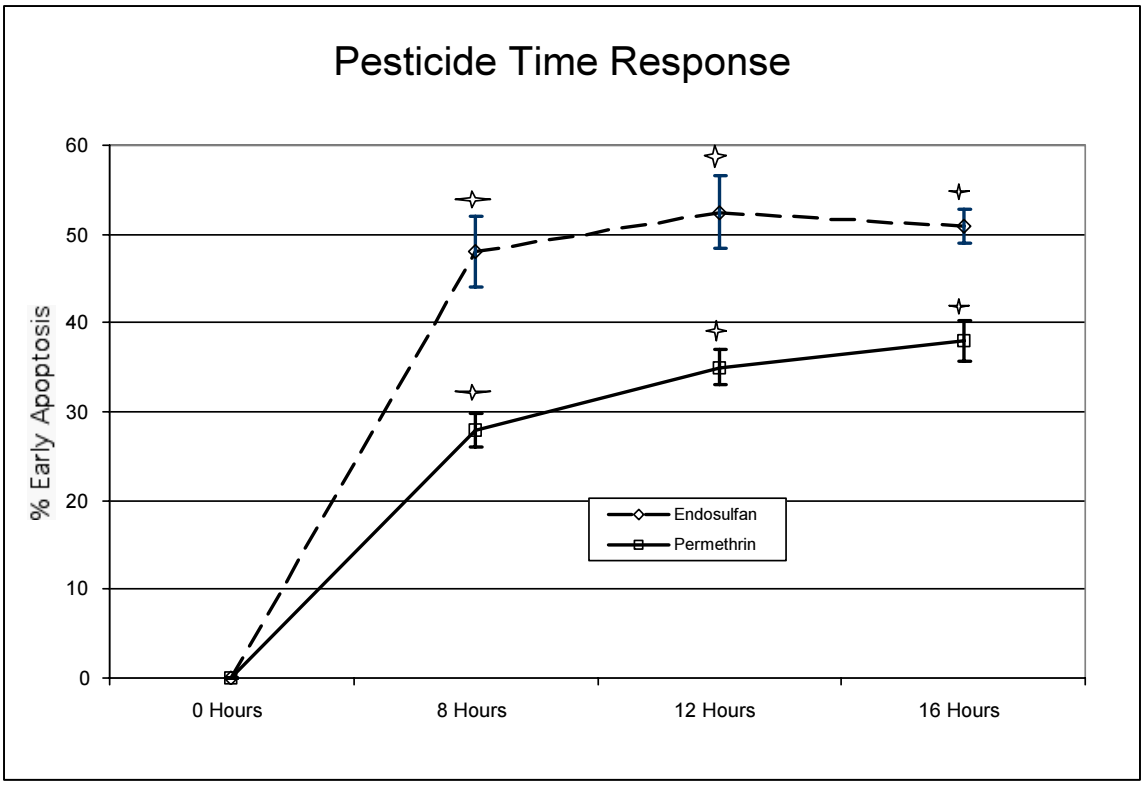

Figure 3.3. Effects of time on C57BL/6 murine thymocytes exposed to permethrin and endosulfan. Early apoptosis and late apoptosis/necrosis was measured using flow cytometry with the 7-AAD staining assay. Thymocytes were treated with Endo 50 µM, and Perm 100 µM for 8, 12, and 16 hours. Unt, EtOH, Dex 10 µg/ml, and H_2O_2 controls were also performed. Samples were examined via flow cytometry for three degrees of staining intensity, 7AAD- dull (live cells), 7AAD moderate (early apoptotic cells), and 7AAD bright (late apoptotic/necrotic). The percent of cells (n = 5000 events) stained 7AAD moderate is shown.

Notes. **Treatments with asterisks were significantly different from EtOH controls for that time point ($p > 0.05$). Values are the mean ± SEM of 4 experiments (Total of n = 8 per treatment)**

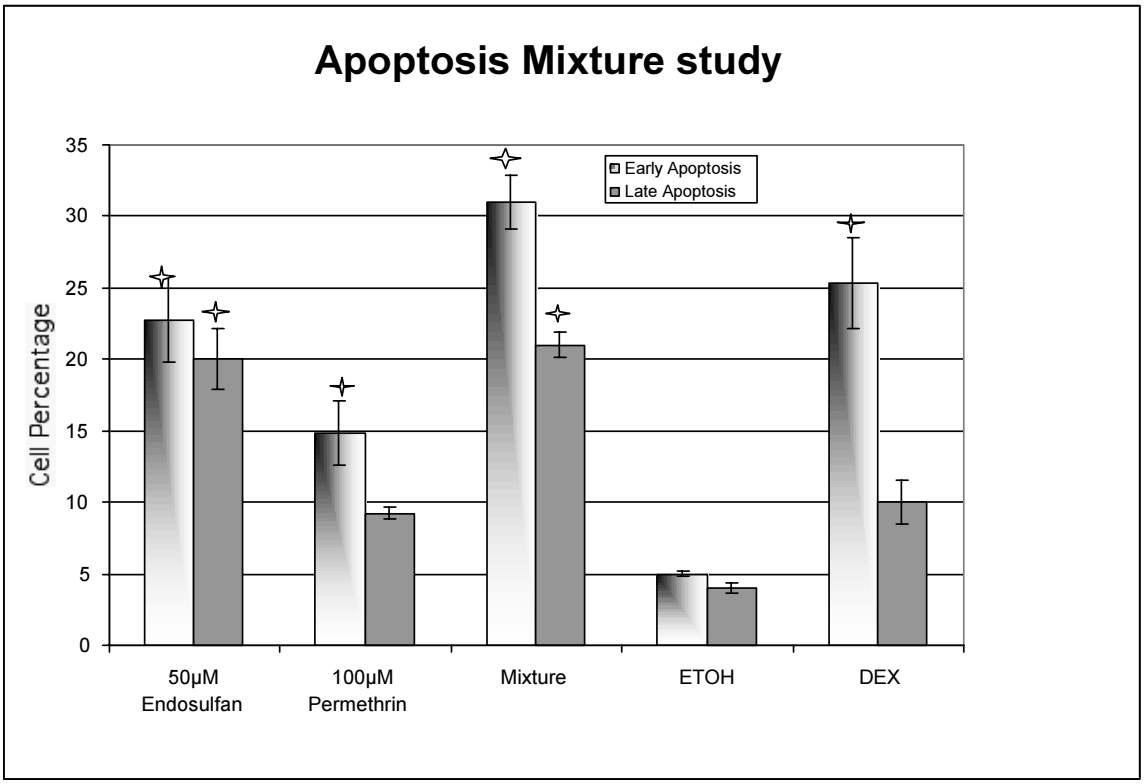

Figure 3.4. Effect of Permethrin and endosulfan alone or in combination on C57BL/6 murine thymocytes. Early apoptosis and late apoptosis/necrosis were measured using flow cytometry with the 7-AAD staining assay. Thymocytes were treated with Endo 50 µM, Perm 100 µM, and Endo 50 µM/Perm 100 µM, for 12 hours. Unt, EtOH, Dex 10 µg/ml, and H_2O_2 controls were also performed. Samples were examined via flow cytometry for three degrees of staining intensity, 7AAD- dull (live cells), 7AAD moderate (early apoptotic cells), and 7AAD bright (late apoptotic/necrotic). The percent of cells (n = 5000 events) stained 7AAD moderate and 7AAD bright is shown.

Notes. **Treatments with asterics were significantly different from EtOH ($p > 0.05$). All other treatment comparisons were significantly different. Values are the mean ± SEM of 4 experiments (Total of n = 8 per treatment).**

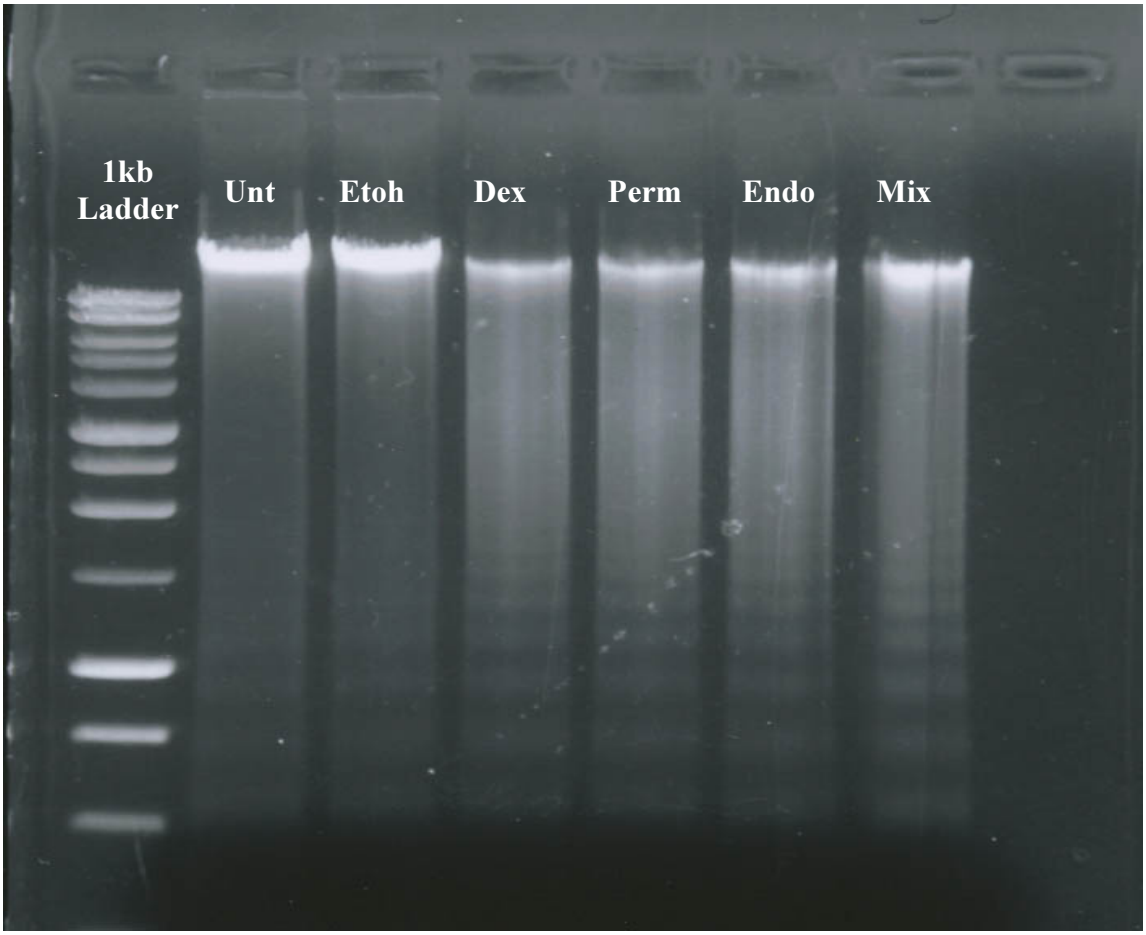

Figure 3.5. Detection of a DNA ladder in C57/BL6 thymocytes treated for 4 hours with pesticides or pesticide mixtures. The treatments included Unt, EtOH, Dex 10 µg/ml, Perm 100 µM, Endo 50 µM, and Endo 50 µM/Perm 100 µM. Samples were prepared using the Apoptotic DNA Ladder kit and run on a 1% agarose gel with TBE buffer, pH 8.0.

Chapter 4
THE ROLE OF CASPASES IN PERMETHRIN- AND ENDOSULFAN- INDUCED THYMOCYTE APOPTOSIS

Abstract

Caspases are a family of cystine-dependent, aspartate-directed proteases that have an integral role in apoptotic cell death. We have shown earlier that permethrin and endosulfan induce apoptosis in C57/BL6 mice thymocytes *in vitro*. The objective of this study was to elucidate the potential mechanism by which these pesticides regulate apoptosis in immune cells. We investigated the role of caspase 3 in thymocytes from C57/BL6 mice exposed to pesticides, using bisamide derivative of rhodamine as substrate. Thymocyte exposure to permethrin results in a dose-responsive increase, whereas endosulfan exposure results in a dose-dependent decrease in caspase 3 activity. Active caspase 8 was measured using IETD-AFC substrate. In a mixture study using endosulfan (50 μM, 100 μM, 150 μM) combined with permethrin (100 μM). The activity of caspase 8 in all combinations of pesticides was found to have more than an additive effect. Caspase 9 activity was measured using LEHD-AFC substrate. A mixture study using endosulfan (25 μM, 50 μM) combined with permethrin (100 μM) was performed. The combinations of pesticides were found to have less than an additive effect. These results suggest that apoptosis caused by both endosulfan and permethrin exert their effects via the caspase pathway. The results indicate the intrinsic pathway is inhibited while the extrinsic pathway is enhanced when murine thymocytes are exposed to mixtures of permethrin and endosulfan *in vitro*.

1. Introduction

A pesticide is any substance intended for preventing, repelling, or mitigating any pest. Pests can include insects, mice, other animals, unwanted plants, fungi, bacteria, and viruses (USEPA 2001). It has been estimated that 85 to 90 % of pesticides applied in agriculture will not reach their target organisms and will enter the air, water, and soil (Repello and Baliga 1996). Very low doses of xenobiotics can effect the immune system, often much lower doses than those necessary to achieve target organ toxicity (Burns et al. 1996). Endosulfan is a member of the cyclodiene group of organochlorine pesticides. Endosulfan was first used in 1956 as a general use insecticide. Endosulfan is a known environmental endocrine disrupter. Permethrin is a synthetic pyrethroid insecticide. Pyrethroids became stable agricultural pest control agents in the early 1970's.

There are two main types of cell death, apoptosis and necrosis. These two forms of cell death are easily distinguished from each other. In a cell undergoing apoptosis the chromatin will condense to form dense compact masses, the cytoskelaton will break down, and the cell membrane will begin to bleb. This is followed by the breakdown of the cell into membrane bound fragments called apoptotic bodies. These apoptotic bodies are quickly found and eliminated by phagocytosis. Apoptosis is a morphologically distinct form of cell death that can be mediated by a variety of pathological and physiological stimuli. These morphological changes can be attributed to a family of intracellular proteases, termed caspases.

Caspases can be divided into two main groups, initiator and effector. Initiator caspases such as caspase 8, 9 and 12 activate downstream caspases. Effector caspases such as caspase 3, 6 and 7 are responsible for the breakdown of cellular proteins. Caspases exist in the cell in the form of zymogens. Although many ways of activating caspases are evident, fundamentally, the biochemical mechanisms appear to be remarkably similar and can be explained by one model. The induced proximity model is based on the empirical observation that the zymogen forms of caspases are not fully inactive but posess a weak protease activity. This activity in some caspases has been measured at less than around 1% of the fully active enzymes. When the zymogens are brought close together through protein interactions, the zymogens can trans-process each other, producing fully active proteases. (Salvesen and Dixit 1999)

Caspase 8 is the apical caspase in the extrinsic pathway. Caspase 8 activation is first initiated by Fas (Apo-1/CD95), the tumor necrosis factor receptor and death receptor-3 (Bolden et al. 1996, Chinnaiyan et al. 1996, Muzio et al. 1996). These three members of the tumor necrosis factor family then pass on this death signal through a cytoplasmic sequence called the DD or death domain. After receptor activation, the DD rapidly associates with adapter molecules that contain a similar DD motif (Nagata 1997 and Wallach et al. 1997). The Fas-associated DD (FADD) exhibits significant homology to a similar region on the caspase 8 prodomain. These regions of the caspases are called DED or death effector domains, and permit heterodimerization of procaspase 8 with FADD (Boldin et al. 1996, Fernandex-alnemri et al. 1996, Muzio et al. 1997, Vincenz and Dixit 1997). Fas aggregation rapidly results in FADD-mediated recruitment in the apoptosis inducing signaling complex (Muzio et al. 1997 and Medema et al. 1997). When two procaspase 8 are associated with this receptor complex, the result is an active caspase 8 protein. Caspase 8 can then activate other caspases such as caspase 3, and apoptosis occurs.

Caspase 9 is the apical caspase in the intrinsic pathway. The intrinsic pathway begins at the mitochondria with the release of cytochrome c. Cytochrome c is then free to bind with the caspase activating protein Apaf-1 (Zou et al. 1999). Apaf –1 can normally be found as an inactive protein in the cytosol. The oligomerized complex that is formed with the combination of cytochrome c and Apaf-1 can then bind pro-caspase 9. This new complex called an apoptosome can then trans-process caspase 9 zymogens via the induced proximity mechanism (Li et al. 1997). The caspase 9 enzyme must then remain bound to Apaf-1 for full activity. Active caspase 9 can then activate effector caspases such as caspase 3 and apoptosis may occur.

Caspase 3 has been implicated as a key mediator of apoptosis. Caspase 3 is a downstream caspase activated by booth caspase 9 and caspase 8. Caspase 3 like proteases were one of the first caspases linked to apoptosis. Stimulation of caspase 3 is interpreted by many as a key indicator of apoptosis. Many cellular insults have been found that activate caspase 3. One example of caspase 3 activation can be seen with high concentrations of heptachlor, an organochlorine insecticide (Rought et al. 2000). Many chemotherapeutic agents target caspase 3 in order to promote apoptosis in tumors. Examples of this are doxirubicon and *N*- (4-Hydroxyphenyl) retinomide (4HPR) (Rought et al. 2000, Sazuki et al. 1999). Other cellular insults such as reactive oxygen species have been found to activate caspase 3 like caspases and

caspase 3 itself, while also facilitating the release of cytochrome c. Cytochrome c in turn can activate caspase 9.

In previous studies we have found that endosulfan and permethrin cause apoptosis in C57/BL6 thymocytes alone and in combination. To further investigate the mechanism of how these pesticides and their mixture cause apoptosis, we investigated whether apoptosis was accompanied by caspase activation and specifically we investigated whether apoptosis was accompanied by activation of caspases in the intrinsic and extrinsic pathway.

2. Materials and Methods

2.1. Animals

Male C57BL/6 mice aged 8 to 12 weeks were obtained from Charles River Laboratories (Wilmington, MA). The animals were kept in accordance with U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals (Nationals Research Council, 1996). Animals were housed with wood chip bedding in polycarbomate cages. Room temperature (21 ± 2°C), humidity (50 ± 10%), and light cycles (12 H on and 12 H off) were controlled.

2.2. Chemicals

Permethrin (99% purity) and endosulfan (99% purity) were obtained from Chem Services (West Chester, PA). Stock solutions of 50 mM were prepared in 100% ethanol. Working solutions of pesticides were then prepared using RPMI-1640 media (Gibco BRL, MD) with concentrations of pesticides ranging from 25 μM to 200 μM for each pesticide. Z-LEHD-FMK, an inhibitor of caspase 9 was purchased from MBL (Japan). Z-IETD-fmk, an inhibitor of caspase 8 was purchased from CLONTECH (Palo Alto, CA)

2.3. Cell culture and isolation

Mice were sacrificed using cervical dislocation. Mean body weight at time of death was 23 g ± 2. The thymus was then removed and weighed (0.02 g ± 0.001) and quickly ground against a metallic 60-mesh wire sieve (Sigma). Cells were then pellated at 250 x g for 7 minutes at 4°C (IEC Centra GP8R, International Equipment, Needham Heights, and MA). The Cells were then resuspended in 10 ml RPMI-1640 media and spun again at 250 x g for 7 minutes at 4°C. The Cells were then resuspended in RPMI – 1640 media and counted on a CASY I Cell Counter and Analyzer System (Scharfe Systems Gmblt, Reutlinger Germany). Cells were then diluted to 1x 10^6 or 2x 10^6 cells/ml and suspended in RPMI complete media (10% FBS, 1% 100x L-glut, 1% non-essential amino acids, 1% Pen/Strp, 1% HEPES Buffer). Thymocytes were then incubated at 37˚C with 5% CO_2 and 95% humidity in the presence and absence of pesticides.

2.4. Caspase 3 assay

Caspase 3 activity was observed using Enzcheck Caspase–3 Assay kit #2 (Molecular Probes). The basis for the assay is conversion of a nonfluorescent rhodamine 110 bis- (N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L aspartic acid amide)(Z-DEVD-R110) substrate (a bisamide derivative of rhodamine 110 containing DEVD peptides covalently bonded to each of R110's

amino groups) converted to the fluorescent monamide and then to the even more fluorescent R110 in the presence of active caspase 3. These products then exhibit spectral properties similar to those of fluorescien with excitation and emission wavelengths of 496nm and 520nm, respectively.

Thymocytes were first isolated as stated above and seeded at $1x10^6$ cells/well in a 96-well, U-bottomed tissue culture plate (Corning, Corning, NY). Treatments (100 ul/well) were added to the wells prior to seeding. Cells were then incubated for 12 hours. After incubation, cells were washed with PBS (Gibco BRL) and the 96 well plate was centrifuged at 250 x g for 7 minutes at 4°C. The media were then removed and cells were resuspended in 50 ul 1X reaction buffer and incubated on ice for 30 minutes. Cells were then centrifuged at 1000 x g for 5 minutes. 50 ul of the supernatant from each sample was then transferred to new individual 96 well microplates. 50 ul of 2X substrate was then added to each sample and control and incubated for 30 minutes at room temperature. Fluorescence was then measured on a fluorescence microplate reader.

2.5. Caspase 8 Assay

Caspase 8 activity was observed using the ApoAlert Caspase Fluorescent assay Kit (Clontech Labs, Ca). This kit detects the emission shift of 7-amino-4-trifluoromethyl coumarin (AFC). The AFC-substrate, IETD-AFC usually emits blue light (400nm) but upon proteolytic cleavage of the substrate by active caspase 8, free AFC fluoresces at 505nm.

Thymocytes were first isolated as stated above and seeded at $2x10^6$ cells/well in a 96-well, U-bottomed tissue culture plate (Corning, Corning, NY). Treatments (100 ul/well) were added to the wells prior to seeding. Cells were then incubated for 12 hours. After incubation, cells were washed with PBS (Gibco BRL) and the 96 well plate was centrifuged at 1000 x g for 5 minutes at 4°C. Cells were resuspended in 50ul-chilled lysis buffer and incubated on ice for 10 minutes. Samples were then transferred from microplate wells to 0.5ml microcentrifuge tubes and centrifuged at maximum speed for 3 minutes. Supernatants were then transferred to new microcentrifuge tubes. Select pesticide samples were then incubated for 30 minutes at 37°C in a water bath with 1ul of IETD-fmk caspase 8 inhibitor (Clontech). During this time the other samples were kept on ice. 50ul 2X reaction buffer and 5ul 1mM Caspase 8 substrate were then added to each sample and incubated in a hot water bath at 37°C for 1 hour. After incubation each sample was transferred to a separate well on a 96 well microplate. The reactions were then read on a fluorescence microplate reader with a 400 nm-excitation filter and 505 nm-emission filter.

2.6. Caspase 9

Caspase 9 activity was observed using the Caspase 9/Mch6 Fluorometric Protease Assay Kit (MBL). This kit detects the emission shift of 7-amino-4-trifluoromethyl coumarin (AFC). The AFC-substrate, LEHD-AFC, usually emits blue light (400nm) but upon proteolytic cleavage of the substrate by active caspase 8, free AFC fluoresces at 505nm.

Thymocytes were first isolated as stated above and seeded at $2x10^6$ cells/well in a 96-well, U-bottomed tissue culture plate (Corning, Corning, NY). Treatments (100ul/well) were added to the wells prior to seeding. Cells were then incubated for 12 hours. After incubation cells were washed with PBS (Gibco BRL) and the 96 well plate was centrifuged at 1000 x g, for 5 minutes at 4°C. Cells were resuspended in 50ul-chilled lysis buffer and incubated on ice for 10

minutes. Select pesticide samples were then incubated for 30 minutes at 37°C with 1ul of caspase 9 inhibitor. Other samples were kept on ice during this period. 50 ul of 2X reaction buffer and 5 ul 1mM LEHD-AFC substrate were added to each sample and samples were incubated for 1 hour at 37°C. Fluorescence was read on a fluorescence microplate reader with a 400nm-excitation filter and 505nm-emission filter.

2.7 Statistical Analysis

SAS JMP software was used for data analysis. Use of Tukey-Kramer HSD allowed for comparison of each treatment to other specified treatments to determine whether statistical difference was evident. Data are presented as means ± standard error of the mean (SEM). If $p > 0.05$, treatments were not statistically different.

3. Results

3.1. Effects of endosulfan and permethrin on thymocyte caspase 3 activity

Caspase 3 activation in thymocytes was measured on a fluorescence microplate reader and is shown here as relative fluorescence (Figure 4.1). Caspase 3 activity was measured after 12 hours incubation with varying concentrations of pesticides and after 40 minutes in the presence of caspase 3 substrate. Permethrin at 50, 100, or 150 µm was found to cause a relative fluorescence of 74, 233, and 236, respectively. Relative fluorescence in the presence of endosulfan at 50, 100, or 150 µm was 180, 64, and 93, respectively, thus, as the dosage of permethrin was increased, the caspase 3 activity also increased and appeared to reach a maximum of 236 with a permethrin concentration of 150 µM. However, as the dosage of endosulfan was increased, caspase 3 activity decreased. Controls of dexamethasone 10 µg/ml, ethanol, H_2O_2, and untreated were used. Dexamethasone was used as a positive apoptotic control, ethanol as a solvent control, and H_2O_2 as a necrotic control. Dexamethasone, H_2O_2, and untreated were found to cause a relative fluorescence of 180, 0, and 45, respectively.

3.2. Effects of endosulfan and permethrin on caspase 8 activity

Caspase 8 activity was measured on a fluorescence microplate reader and is reported here as relative fluorescence. A time response study was performed at doses of 50 µM endosulfan and 150 µM permethrin. These doses were used because they had the most caspase activity in the caspase 3 assay. Time points of 1, 4, 7, and 10 hours were chosen so that cells from the same animal could be used for each time point. Results are relative fluorescence caspase 8 products in thymocytes exposed to pesticides minus relative fluorescence of the ethanol treated controls at that time point. As shown in Figure 4.2 cells exposed to endosulfan at 1, 4, 7, and 10 hours were found to generate -36.0, 114.0, 77.0, and 36.5 fluorescent units, respectively. Permethrin at 1, 4, 7, and 10 hours had -13.0, 58.6, 25.0, and 21.5, respectively. Thus, in cells exposed to endosulfan, caspase 8 activity reached a maximum of 114. Where as cells exposed to permethrin activated caspase 8 activity that reached a maximum of 58.6 after 4 hours. Caspase 8 activity in cells exposed to both pesticides reached a maximum after 4 hours indicating caspase 8 activation is early in apoptotic cell death. Negative results are caused by the ethanol control having greater caspase 8 activity at the lower time points.

The second study performed on caspase 8 activity was a dose response study. A time point of 7 hours was used for further studies. As shown in Figure 4.3 endosulfan at 25, 50, 100 or 150 µm had a relative fluorescence of 32.8, 63.8, 69.5, and 55.5, respectively. Permethrin at 100, 150, or 200µM each had a relative fluorescence of 35.5, 10.5, and 0, respectively. Controls used were untreated, EtOH, and pesticides + caspase 9 inhibitor. Ethanol had a relative fluorescence that was not significantly different than the untreated control. Cells incubated with pesticides and caspase 9 inhibitor were not above background. Activation of caspase 8 increased as endosulfan concentrations increased until the 150 µM concentration where it began to decrease. Caspase 8 activity decreased as permethrin concentrations decreased.

The third study performed on caspase 8 activity was a mixture study. As shown in Figure 4.4 endosulfan at 50 100, or 150 µM had a relative fluorescence of 62.0, 69.5, and 55.5, respectively. Permethrin at 100 µM had a relative fluorescence of 35.5. Mixtures of 100 µM permethrin and 50, 100, and 150 µM endosulfan were assayed and were found to have a relative fluorescence of 115.0, 117.3, and 97.1 respectively. Thus the mixtures of endosulfan and permethrin were found to have a slightly more than additive effect on caspase 8 activity.

3.3. Effects of endosulfan and permethrin on caspase 9 activity

Caspase 9 activity was measured on a fluorescence microplate reader and is reported here as relative fluorescence. Results are relative fluorescence of the pesticide sample minus relative fluorescence of the ethanol control. A time response study on caspase 9 activation was conducted. Again, concentrations of 50 µM endosulfan and 150 µm permethrin at time points of 1, 4, 7, and 10 hours were used. As shown in Figure 4.5 endosulfan at 1, 4, 7, and 10 hours was found to exhibit a relative fluorescence of -8.7, 59.3, 74.5, and 99.0, respectively. Permethrin at 1, 4, 7, and 10 hours was found to have -4.7, -21.8, 25.0, and 47.3, respectively. Caspase 9 activity in cells exposed to endosulfan reached a maximum of 99 after 10 hours. Caspase 9 activity in cells exposed to permethrin reached a maximum of 47.3 after 10 hours. Thus, there was a time dependent activation of caspase 9 in cells observed when exposed to the pesticides up to 10 hours.

The second study performed on caspase 9 activity was a dose response study. As shown in figure 4.6 the cells were exposed to 25, 50, 100, or 150 µM endosulfan had relative fluorescence of 43.0, 73.0, 78.9, and 5.1 after 7 hours incubation, respectively. Concentrations of 100, 150, or 200 µM permethrin had 34.5, 39.5, and 55.5 relative fluorescence after 7 hours incubation. Controls used were untreated, EtOH, and pesticide + caspase 9 inhibitor.

The third study performed on caspase 9 activity was a mixture study. Endosulfan at 25 and 50 µM had 43.0 and 73.0 relative fluorescence, respectively. Permethrin at 100 µM had a relative fluorescence of 34.5. As shown in Figure 4.8, a mixture of 100 µM permethrin and 25 µM endosulfan had a relative fluorescence of 45.2 and a mixture of 100 µM permethrin and 50 µM endosulfan had a relative fluorescence of 63.0. Thus, mixtures of endosulfan and permethrin have less than an additive effect on caspase 9 activity under the above experimental conditions.

3.4 Effects of caspase inhibitor on DNA laddering

In a previous study, 50 µM endosulfan and 100 µM permethrin alone and in combination were found to cause a laddering effect on a 1% agarose gel after 4 hours incubation. This study ascertained the effects of specific caspase inhibitors on DNA fragmentation and subsequent laddering on an agarose gel after 4 hours incubation. Caspase 9 and caspase 8 inhibitors alone

and in combination were incubated with C57/BL6 thymocytes for 1 hour before the addition of pesticides alone and in combination. As shown in Figure 4.9, after 4 hours incubation with endosulfan and permethrin alone and in combination, a laddering effect was observed. DNA fragmentation and laddering was inhibited with the addition of both caspase inhibitors and the combination of the two. Controls of untreated, untreated + caspase 9 inhibitor, untreated + caspase 8 inhibitor, untreated + caspase 8/caspase 9 inhibitor, ETOH, and Dex 10µg/ml were also performed.

4. Discussion

Recent studies from our lab have reported both endosulfan and permethrin to be immunotoxicants, causing apoptosis in C57/BL6 mice thymocytes (unpublished data). Mixtures of these chemicals caused an additive increase in this observed apoptosis. Immunosuppression can leave an individual vulnerable to opportunistic diseases. Because disrupting the delicate balance of the immune system can produce such profound effects, it is important to elucidate mechanisms of xenobiotic-induced immunomodulation. With the advent of reproducible, sensitive, and predictable tests, immunotoxicity testing may alter safety evaluations for pharmaceutical, biological, and chemical agents. This is the first study to elucidate caspase activation as an immunotoxic mechanism of action for these pesticides.

There are three ways to characterize the effects of chemical mixtures. The chemicals can have an antagonistic, additive, or synergistic effect on each other. An additive effect occurs when the effect of two chemicals combined is equal to the sum of the two chemical effects when alone. An antagonistic interaction is when the chemical mixture effects are less than additive. A synergistic reaction occurs when the combined effects of the chemicals are greater than the sum of the two chemicals alone. One of the most studied combinations of chemicals is permethrin and DEET (N,N diethyl-meta-toluamide). These chemicals have been implicated as causative agents in Gulf War Syndrome. Mixtures of permethrin and DEET have behavioral, neurotoxic, and immunotoxic effects. The mixture causes release of cytochrome c from rat brain mitochondria (Aqel et al. 2001), which can induce apoptosis by activating the intrinsic caspase pathway. Permethrin has been studied in combination with other chemicals such as PBO (piperonyl butoxide), arsenic, and mercury. When permethrin was combined with mercury and arsenic, the substances modified the immunotoxic effects of the single compounds (Institoris et al. 2001).

To determine if the caspase pathways were involved in permethrin- and endosulfan-induced apoptosis of murine thymocytes, we performed a study of caspase 3 activity. Caspase 3 is a key mediator of apoptosis, it is a down-stream effector of caspase 8 and 9. Caspase 3 is required for DNA degradation and chromatin condensation. Exposure to permethrin and endosulfan resulted in DNA fragmentation on agarose gels, suggesting DNA degradation, and therefore caspase 3 activation was occurring in these thymocytes. Thymocyte exposure to permethrin resulted in a dose-dependent increase in caspase 3 activity, whereas exposure to endosulfan resulted in a dose-dependent decrease in caspase 3 activity. Dexamethasone, an apoptotic agent, was used as a positive control and H_2O_2 as a necrotic control. The dose-dependent decrease in caspase 3 activity suggests that pathways of cell death may change depending on concentration and type of pesticide that is present. Specifically it appears that as endosulfan concentrations increase, necrosis replaces apoptosis as the dominant form of cell death in murine thymocytes.

In order to determine which caspase pathway was responsible for permethrin- and endosulfan-induced caspase 3 activation, we investigated the effects of these insecticides, alone

or in combination, on caspase 8 and 9 activity. Caspase 8 is the apical caspase in the TNF (tumor necrosis factor) family death receptor or extrinsic pathway. Caspase 9 is the apical caspase in the intrinsic or mitochondrial pathway (Varfolomeev et al. 1998, Kuida et al. 1998). The intrinsic and extrinsic pathways are often viewed as separate pathways, however they can operate in conjunction with each other.

Murine thymocyte exposure to both permethrin and endosulfan increased caspase 9 activity in a time- and dose-dependent manner. This suggests that caspase 9, and subsequently the intrinsic pathway, are activated by permethrin or endosulfan. However, the highest concentration of endosulfan (150μM) decreased caspase activity almost completely, suggesting that this concentration of endosulfan results in the majority of cell death occurring via necrosis, rather than apoptosis. Murine thymocyte exposure to permethrin resulted in a dose-dependent decrease in caspase 8 activity, whereas endosulfan exposure resulted in a dose-dependent increase in caspase 8 activity. This indicates that caspase 8, the extrinsic pathway, is activated by either permethrin or endosulfan. Time course studies demonstrated that caspase 8 activity in murine thymocytes, when exposed to endosulfan or permethrin, responded biphasically, with peak activation at 4 h. The observed decrease in caspase 8 activity after 4 h, coupled with the steady increase in caspase 9 activity, for these pesticides suggests a gradual switch in primary caspase pathways from extrinsic to intrinsic over time. Caspase 8 activity in permethrin-exposed murine thymocytes decreased in a dose-dependent manner, implying that only at low concentrations, permethrin-induced apoptosis is regulated via the extrinsic pathway.

Caspase pathways can act in concert with one another. Caspase 8 can activate Bid, a pro-apoptotic Bcl-2 family protein, that can stimulate release of cytochrome c from the mitochondria. Cytochrome c then activates caspase 9 in the intrinsic pathway. Permethrin and endosulfan combined increase apoptotic cell death in an additive manner (unpublished data), suggesting that exposure to this mixture should also increase caspase 9 or 8 activity in an additive manner. Exposure to mixtures of permethrin and endosulfan produced more than an additive effect on caspase 8 activity in thymocytes, but an antagonistic effect on caspase 9 activity. These data suggest that unlike exposure to permethrin or endosulfan alone, which utilizes both the intrinsic and extrinsic apoptotic pathways, the extrinsic pathway is the primary mechanism of apoptotic cell death in murine thymocytes exposed to permethrin/endosulfan mixtures.

Nuclear DNA is non-randomly cleaved into 180 base pair units during apoptosis. These DNA fragments can be observed as a "ladder" when electrophoresed on an agarose gel. Endosulfan and permethrin, alone or in combination, cause DNA laddering in murine thymocytes. Permethrin- and/or endosulfan-induced DNA ladder formation was inhibited, after 4 h, by caspase 8 and 9 inhibitors, alone and combined. This confirms that the extrinsic and intrinsic pathways of apoptosis were activated by these insecticides alone and as a mixture.

The extrinsic and intrinsic pathways have many points of cross-communication. For example, caspase 8 can activate Bid and, subsequently, caspase 9, as stated before. The only reported way by which caspases are activated is by other caspases. For example, caspase 8 can directly cleave and activate caspase 9 *in vitro* (Stennike et al. 1998). It is possible for caspase 8 to activate caspase 3. Caspase 3, subsequently, can activate caspase 9. The chemical reaction of these 2 insecticides could stimulate or block any number of pro-apoptotic or anti-apoptotic proteins.

Here we report evidence regarding potential mechanism(s) by which permethrin or endosulfan, as well as their mixture, cause immunotoxicity. Permethrin/endosulfan mixtures primarily cause activation of the extrinsic caspase pathway in murine thymocytes *in vitro*. Future studies should more thoroughly address the mechanism(s) of action introduced here, investigating specific areas of cross-talk between the apoptotic caspase pathways.

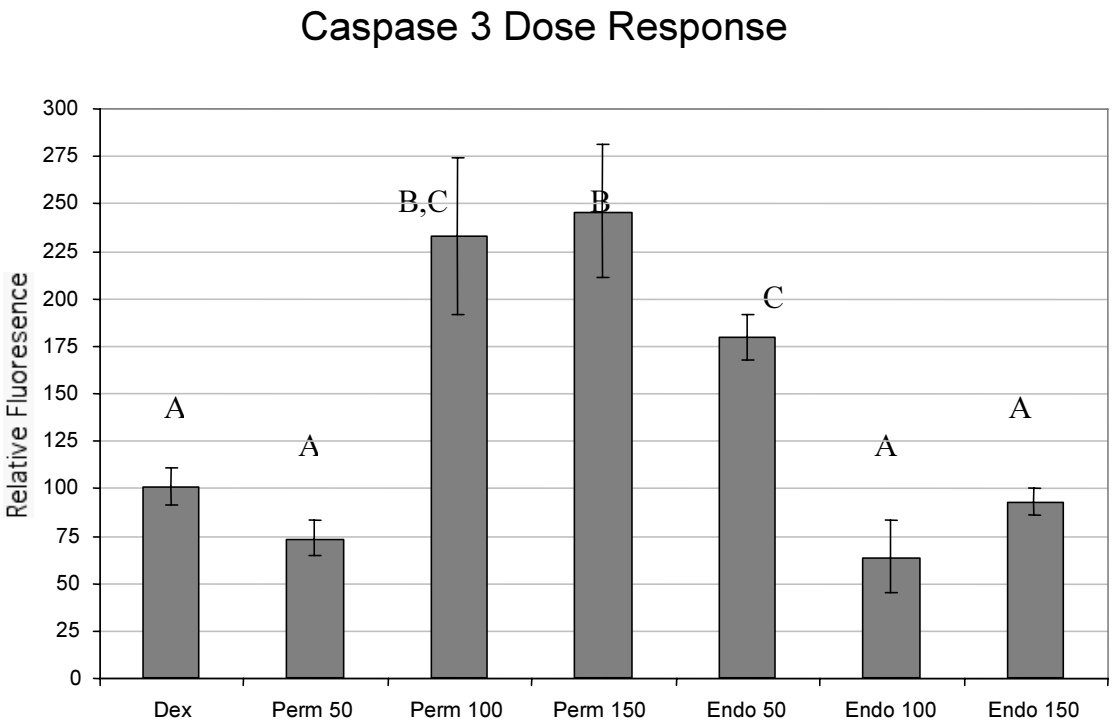

Figure 4.1. Caspase 3 activity in C57/BL6 murine thymocytes exposed to pesticides. Caspase 3 activity was measured using the Enzcheck Caspase 3 Assay Kit #2 (Molecular Probes) . Thymocytes were treated with Endo 50, 100, and 150 µM, Perm 50, 100, 150 µM for 12 hours. An Unt, EtOH, Dex 10 µg/ml were also performed. Relative fluorescence is shown.

Notes. **Treatments with similar letters were not significantly different ($p > 0.05$). All other treatment comparisons were significantly different. Values are the mean ± SEM of 4 experiments (Total of n = 8 per treatment).**

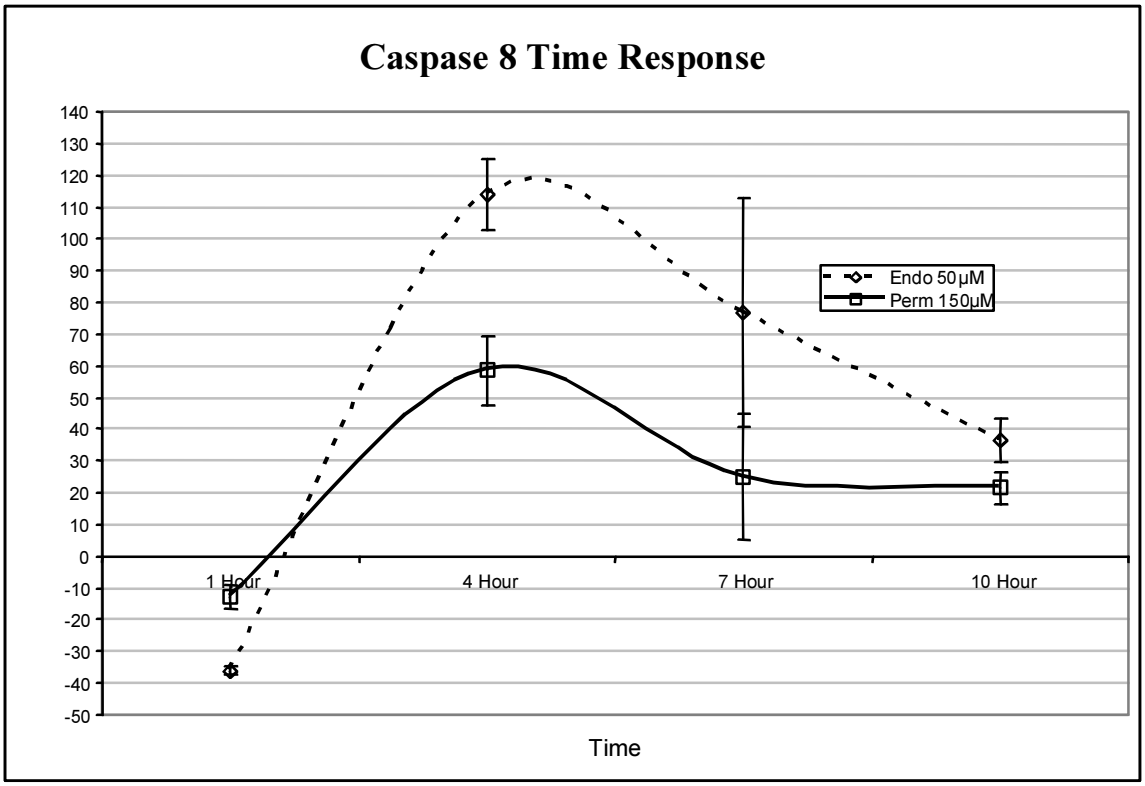

Figure 4.2. Effects of time on caspase 8 activity in C57/BL6 murine thymocytes exposed to pesticides. Caspase 8 activity was measured using the ApoAlert Caspase Fluorometric Assay Kit. Thymocytes were treated with Endosulfan and Permethrin at concentrations of 50 µM and 150 µM Respectively for 1, 4, 7, and 10 hours. Unt, EtOH, Dex 10µg/ml, and Pesticide + caspase 8 inhibitor controls were also performed. Relative fluorescence is shown.

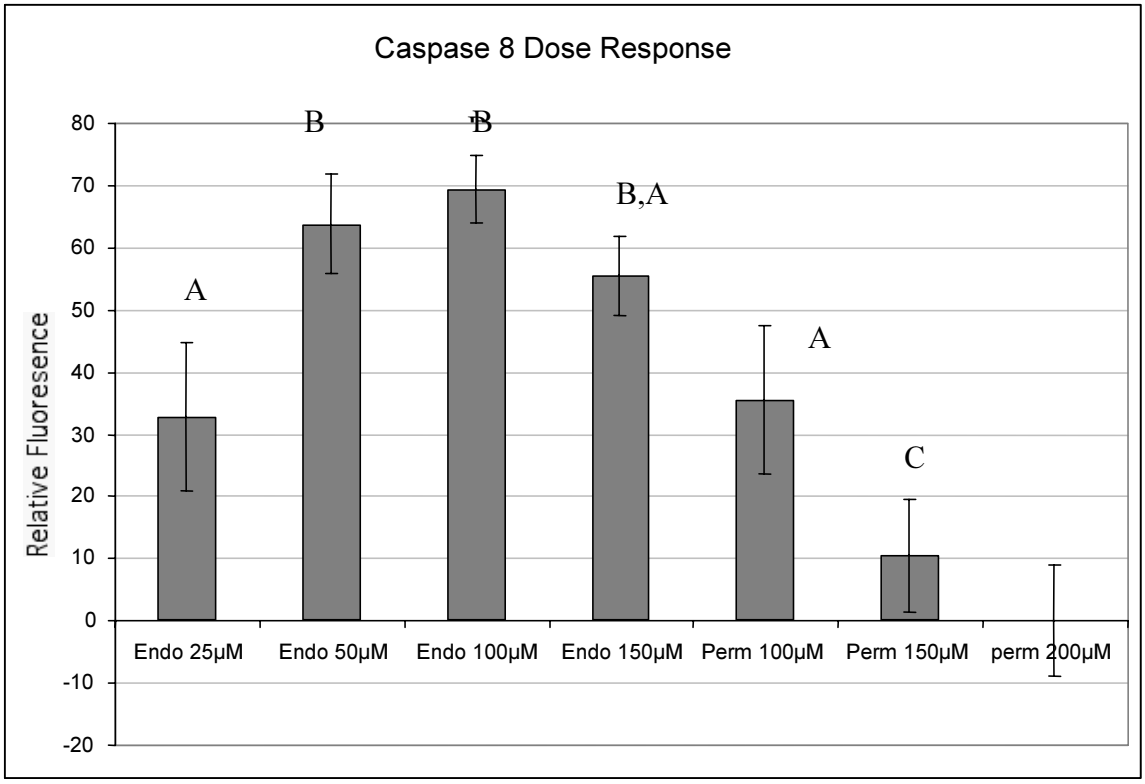

Figure 4.3. Caspase 8 activity in C57BL/6 murine thymocytes exposed to pesticides. Caspase 8 activity was measured using the ApoAlert Caspase Fluorometric Assay Kit. Thymocytes were treated with Endosulfan and Permethrin at concentrations of 25, 50, 100, and 150µM and 100, 150, and 200µM, respectively, for 12 hours. Unt, EtOH, Dex 10µg/ml controls were also performed. Relative fluorescence is shown. Data are presented ± SEM.

Notes. **Treatments with similar letters were not significantly different ($p > 0.05$). All other treatment comparisons were significantly different. Values are the mean ± SEM of 4 experiments (Total of n = 8 per treatment)**

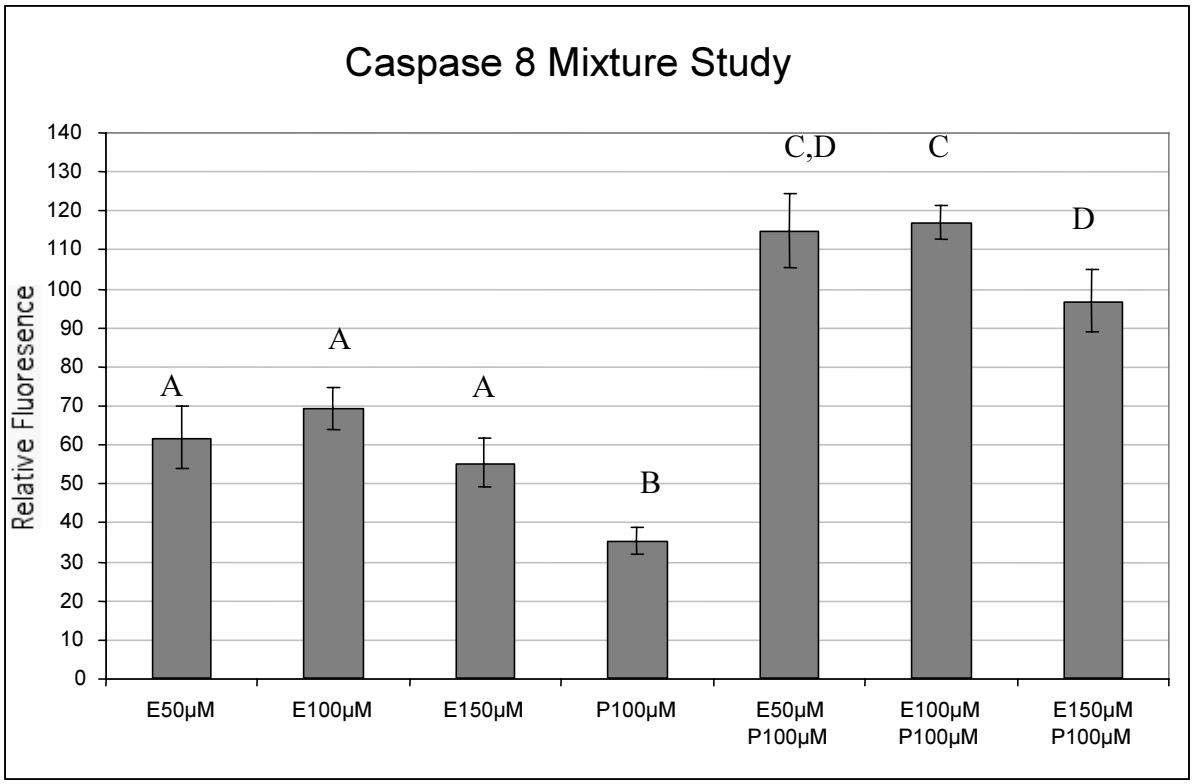

Figure 4.4. Caspase 8 activity in C57BL/6 murine thymocytes exposed to pesticides or pesticide mixtures. Caspase 8 activity was measured using the ApoAlert Caspase Fluorometric Assay Kit. Thymocytes were treated with Endo 50, 100, and 150 µM, Perm 100 µM, Endo 50/Perm 100 µM, Endo 100/Perm100 µM, and Endo 150/Perm 100 µM for 12 hours. Unt, EtOH, Dex 10µg/ml, and Pesticide + Caspase 8 inhibitor controls were also performed. Relative fluorescence is shown.

Notes. **Treatments with similar letters were not significantly different ($p > 0.05$). All other treatment comparisons were significantly different. Values are the mean ± SEM of 4 experiments (Total of n = 8 per treatment).**

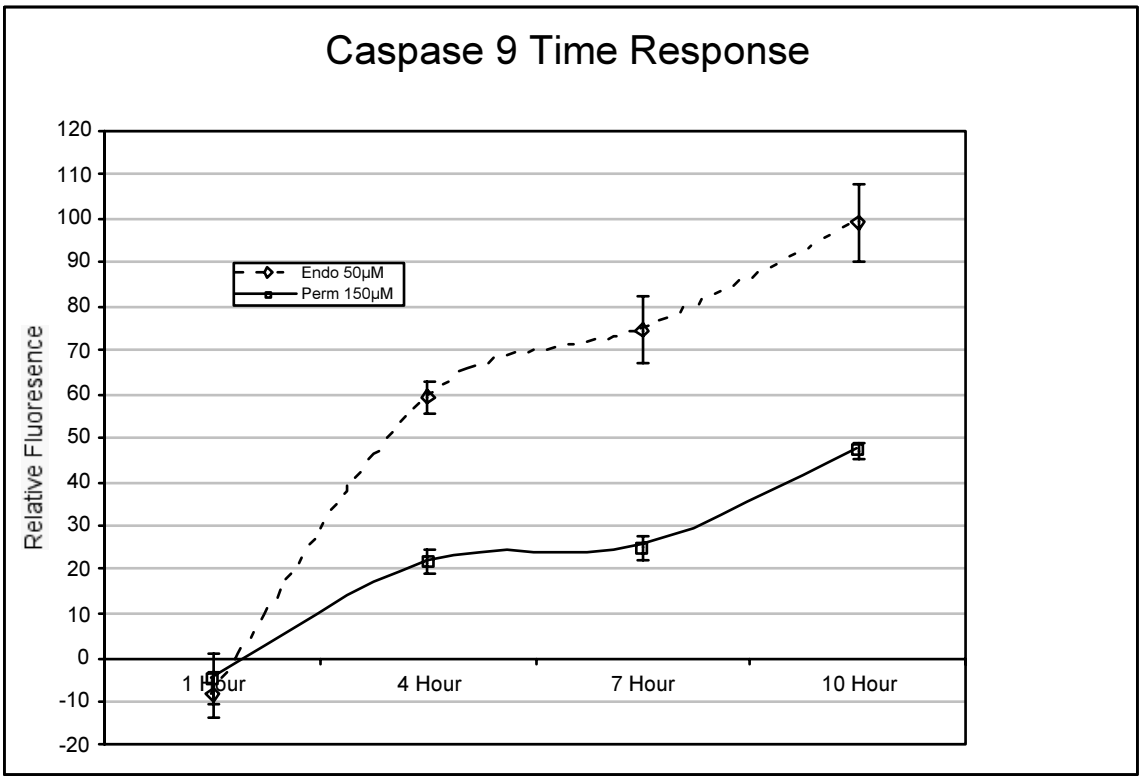

Figure 4.5. Effects of time on caspase 9 activity in C57/BL6 murine thymocytes exposed to pesticides. Caspase 9 activity was measured using the Caspase 9/Mch6 Fluorometric Protease Assay Kit (MBL). Thymocytes were treated with Endosulfan and Permethrin at concentrations of 50 µM and 150 µM Respectively for 1, 4, 7, and 10 hours. Unt, EtOH, Dex 10 µg/ml, and Pesticide + caspase 8 inhibitor were also performed. Relative fluorescence is shown. Data are presented ± SEM.

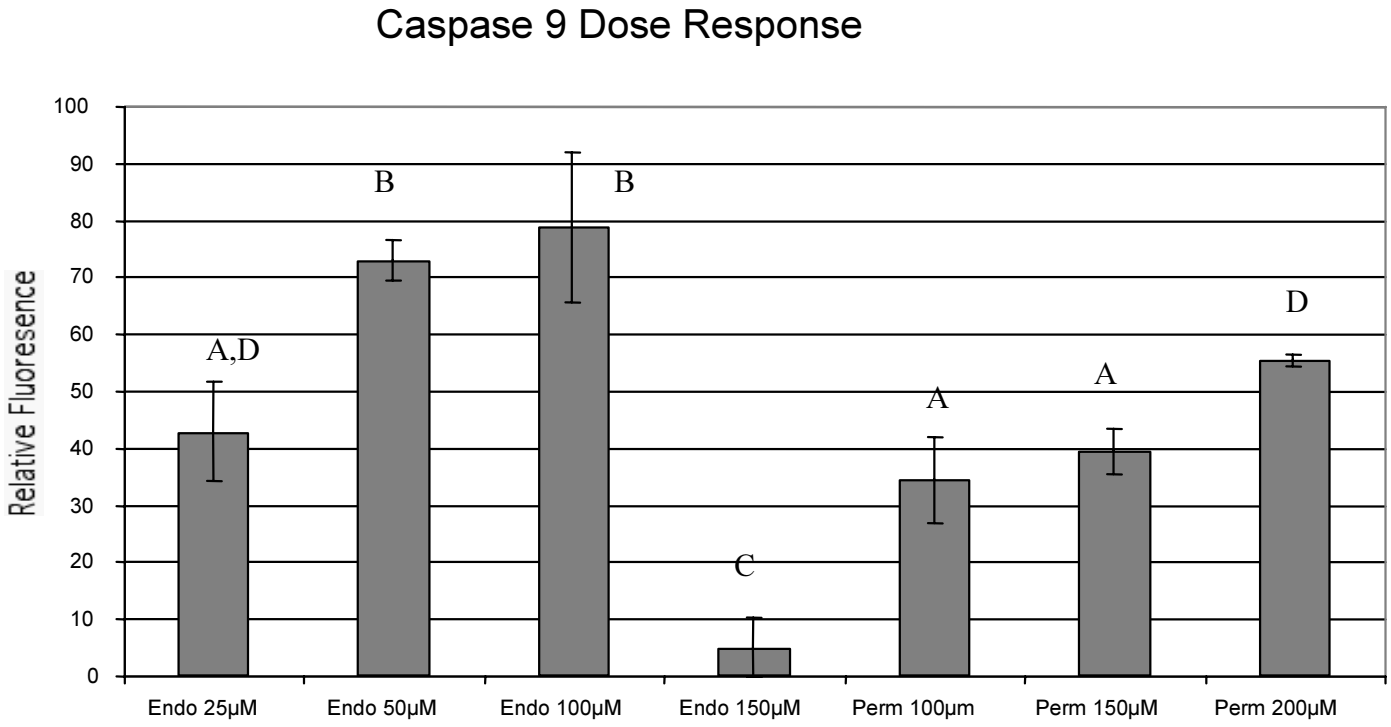

Figure 4.6 Caspase 9 activity in C57BL/6 murine thymocytes exposed to pesticides. Caspase 9 activity was measured using the Caspase 9/Mch6 Fluorometric Protease Assay Kit (MBL). Thymocytes were treated with Endosulfan and Permethrin at concentrations of 25, 50, 100, and 150, and 100µM, 150µM, and 200µM, respectively for 12 hours. Unt, Etoh, Dex 10µg/ml, and Pesticide + Caspase 9 inhibitor controls were also performed. Relative fluorescence is shown. Data are presented ± SEM.

Notes. **Treatments with similar letters were not significantly different ($p > 0.05$). All other treatment comparisons were significantly different. Values are the mean ± SEM of 4 experiments (Total of n = 8 per treatment).**

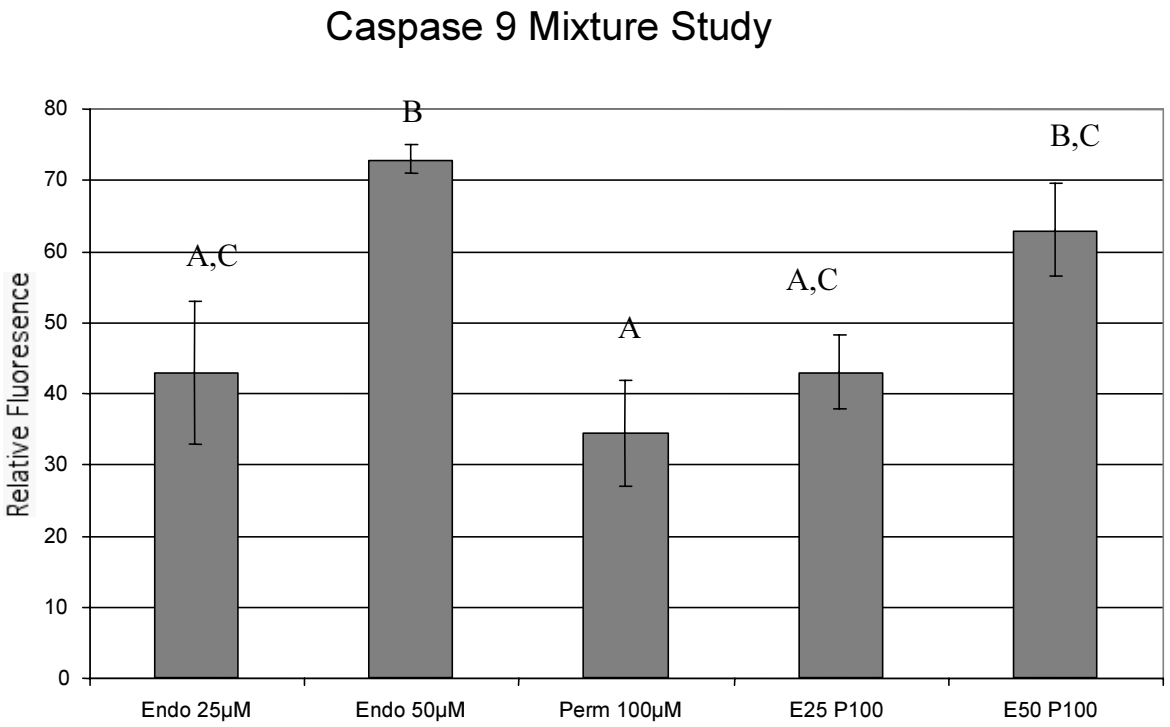

Figure 4.7. Caspase 9 activity in C57BL/6 murine thymocytes exposed to pesticides or pesticide mixtures. Caspase 9 activity was measured using the Caspase 9/Mch6 Fluorometric Protease Assay Kit (MBL). Thymocytes were treated with Endo 25 and 50 µM, Perm 100 µM, Endo 25/Perm 100 µM, and Endo 50/Perm 100 µM for 12 hours. Unt, EtOH, Dex 10µg/ml, and Pesticide + Caspase 9 inhibitor were also performed. Relative fluorescence is shown. Data are presented ± SEM.

Notes. **Treatments with similar letters were not significantly different ($p > 0.05$). All other treatment comparisons were significantly different. Values are the mean ± SEM of 4 experiments (Total of n = 8 per treatment).**

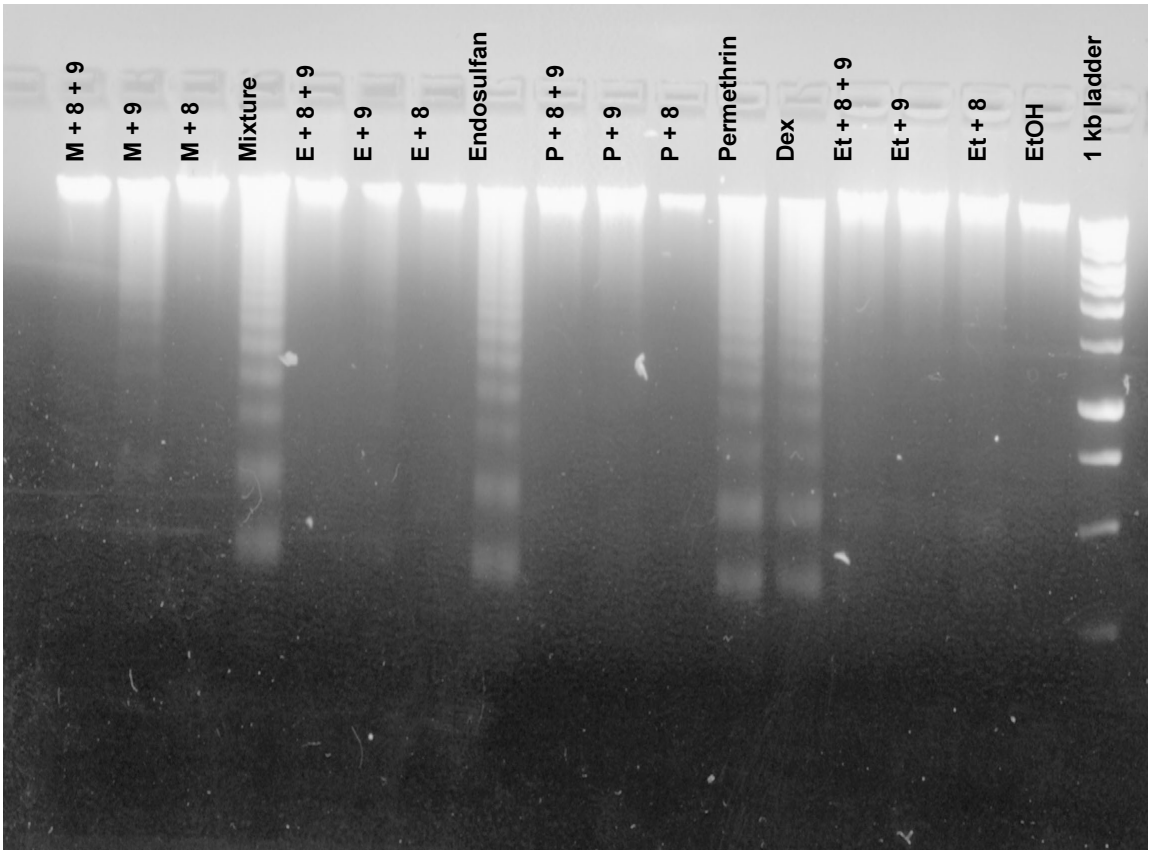

Figure 4.8. Detection of DNA Ladder in C57/BL6 mouse thymocytes treated for 4 hours with pesticides or pesticide mixtures, with and without caspase inhibitors. The treatments included EtOH, EtOH + caspase 8 inhibitor, EtOH + caspase 9 inhibitor, EtOH + caspase 8+9 inhibitors, Dex 10 µG/ml, permethrin 100 µM, permethrin + caspase 8 inhibitor, permethrin + caspase 9 inhibitor, permethrin + caspase 8+9 inhibitors, endosulfan 50 µM, endosulfan + caspase 8 inhibitor, endosulfan + caspase 9 inhibitor, endosulfan + caspase 8+9 inhibitor, mixture, mixture + caspase 8 inhibitor, mixture + caspase 9 inhibitor, and mixture + caspase 8+9 inhibitor.

Conclusion

The first specific aim of this study was to investigate whether endosulfan and permethrin induce apoptosis in murine thymocytes *in vitro* and if so whether mixtures of these pesticides enhance or inhibit thymocyte cytotoxicity. Both endosulfan and permethrin induced apoptosis. When the pesticides were combined there was an additive effect on thymocyte apoptosis. In the second study we examined if caspases are involved in the apoptotic pathways of thymocytes exposed to individual pesticides or pesticide mixtures. The specific caspases we studied were 3, 8 and 9. Significant caspase 3 activity was observed in thymocytes exposed to both pesticides. Caspase 8 and 9 were studied to ascertain by which caspase pathway thymocytes underwent apoptosis when exposed to pesticides alone or in combination. Caspase 8 and 9 were both activated in thymocytes exposed to pesticides alone or in combination. Thus, the extrinsic and intrinsic pathways were activated. A mixture study was performed and caspase 8 activation had a slight synergistic effect while caspase 9 activation had a slight inhibitory effect. Therefore, the caspase 8 pathway is used predominantly in thymocytes exposed to permethrin and endosulfan mixtures. The immunotoxic effect of these pesticides via multiple caspase pathways is clear, but needs to be investigated further.

Future Studies

Future studies on this subject should involve an in depth study of the caspase pathways involved, looking specifically at cytochrome c, Bcl-2, and Bid. It is obvious in our studies that both pathways are activated, but a thorough look at these proteins will give a better understanding of which is the dominant pathway. Antioxidents have also been implicated in caspase activation and should be studied as a possible trigger for pesticide induced thymocyte apoptosis. Another avenue of studies that should be performed is to look at immunosuppression, specifically looking at CD4 and CD8 Tcells in the spleen, including an *in vivo* challenge study.

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Vita

James John Keenan was born on July 31st, 1977 in Massapequa NY. James, with his parents John and Dorothy and sisters Kim and Kristen, settled down in Fairfax, VA at the age of 11. James attended Robinson High School in Fairfax where he was a member of both the band and the baseball team. James, at the age of 16 visited his older sister Kim at Virginia Tech and VT was subsequently the only school he applied to. Jimmy was accepted into the college of Agriculture and 4 years later was awarded a degree in Animal and Poultry Sciences. Since the fall of 2000, Jimmy has been working on his master's thesis project in the department of Biomedical Sciences and Pathobiology at the Virginia-Maryland Regional College of Veterinary Medicine.