Pesticide Mixtures Induce Immunotoxicity: Potentiation of Apoptosis and Oxidative Stress

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ABSTRACT

The three insecticides of interest were lindane (an organochlorine), malathion (an organophosphate) and piperonyl butoxide (PBO; a synergist). Based on minimum cytotoxicity ($\geq LC_{25}$), the following concentrations were chosen for the pesticide mixture studies: 70µM lindane (Lind), 50µM malathion (Mal) and 55µM PBO. In the AlamarBlue cytotoxicity assay, *individual* pesticide and *mixtures* of malathion/PBO (MP) and malathion/lindane (ML) prompted cytotoxicity with varying intensities (Mal 18.8%, Lind 20.4%, PBO 23.5%, ML 53.6% and MP 64.9%). Cytopathological analysis revealed apoptotic features in treated cells and the DNA Ladder Assay confirmed the presence of DNA fragments. The specific mode of cell death was examined via the 7-aminoactinomycin D (7-AAD) Staining Assay. Apoptosis was detected in each treatment (Mal 6.5%, Lind 12.0%, PBO 13.2%, ML 19.3% and MP 23.4%). Furthermore, 7-AAD staining in combination with fluorescent-labeled monoclonal antibodies, PE-CD45RB/220 and FITC-CD90, was performed. B-cells were more susceptible to Mal and PBO treatments than were T-cells. The pro-oxidant activity of the pesticides was monitored via the Dichlorofluorescin Diacetate assay. Exposure to pesticides for 15 minutes increased H_2O_2 production above the controls, Mal 21.1%; Lind 10.8%; PBO 25.9%; ML 26.8%; MP 37.8%. The activities of antioxidant enzymes, glutathione peroxidase (GSH-Px) and glutathione reductase (GR) were altered by these treatments. GR was significantly reduced for the pesticide mixtures only (control: 51.7; Mal: 48.2; Lind: 50; PBO: 52.3; ML: 40.5; MP: 42 Units/mg). GSH-Px activity was severely reduced for all the pesticide treatments (control: 44.9; Mal: 30.2; Lind:

30.6; PBO: 32.4; ML: 21.1; MP: 21.1 Units/mg). These results indicate that exposure to these pesticide and pesticide mixtures induces apoptosis and oxidative stress.

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$^{\bullet}OH$ – Hydroxyl Radical

$^{1}O_2$ – Singlet Oxygen

7AAD- 7-aminoactinomycin-D

ACh – Acetylcholine

AChE – Acetylcholinesterase

AhR – Aryl Hydrocarbon Receptor

AT – 3-Amino-2,3,4,-Triazole

BSA – Bovine Serum Albumin

cAMP – Adenosine 3',5'- Cyclic Monophosphate

CAT – Catalase

CMI – Cell Mediated Immunity

CO_2 – Carbon Dioxide

Con-A – Concavalin A

CTL – Cytotoxic T Lymphocytes

Cyt P450 – Cytochrome P450

DCF – 2',7'-Dichlorofluorescein

DCF-H – 2',7'-Dichlorofluorescene

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

DEX – Dexamethasone

DMPO – 5,5-Dimethyl-1-Pyrroline-N-Oxide

DNA –Deoxyribonucleic acid

EDTA – Ethylenediamminetetraacetic acid

EtOH – Ethanol

FBS – Fetal Bovine Serum

FITC – Fluorescein Isothiocyanate

G6PD – Glucose-6-Phosphate Dehydrogenase

GGTP – Gamma Glutamyl Transpeptidase

GPx – Glutathione Peroxidase

GR – Glutathione Reductase

GST – Glutathione-S-Transferase

GSH – Reduced Glutathione

GSSG – Oxidized Glutathione

H_2O_2- Hydrogen Peroxide

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

$HO_2^{\bullet}$ - Hydroperoxyl Radical

LC – Lethal Concentration

LGL – Large Granular Lymphocytes

Lind –Lindane

L-NAME – N-(Omega)-Nitro-L-Arginine Methyl Ester

LSM – Least Square Means

Mal – Malathion

MFO – Mixed Function Oxidase System

ML – Malathion/Lindane

MP – Malathion/Piperonyl Butoxide

NADPH- B-Nicotinamide Adenine Dinucleotide Phosphate Reduced Form

NaN_3 – Sodium Azide

NIEHS – National Institute of Environmental Health Sciences

$NO^{\bullet}$ - Nitric Oxide

$O_2^{-\bullet}$ - Superoxide Anion

$ONOO^-$ - Peroxynitrite

PBMNC – Peripheral Blood Mononuclear Cells

PBO – Piperonyl Butoxide

PBS – Phosphate Buffered Saline

PCB – Polychlorinated Biphenyl

PLA – Phospholipase A_2

PTIO – 2-Phenyl-4,4,5,5-Tetramethylimidazone-1-oxyl-3-oxide

ROS – Reactive Oxygen Species

R-PE – R-Phycoerythrin

RPMI 1640 – Roosevelt Park Memorial Institute Medium

SEM – Standard Error of Mean

SER – Smooth Endoplasmic Reticulum

SNP – Sodium Nitroprusside

SOD – Superoxide Dismutase

TBARS – Thiobarbituric Acid

TBE- Tris-Borate-EDTA

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

TEMPO – 2,2,6,6-Tetra-Methyl-1-Piperonyloxy

USEPA – United States Environmental Protection Agency

Chapter I INTRODUCTION

Pesticides are used in every realm of the environment to control undesired pests, such as insects, weeds, fungus and rodents. The pesticide industry is estimated at being a $30 billion- 2.6 million ton-a-year global industry (Repetto and Baliga 1996; Pretty et al. 1998). In 1997, it was estimated that the average American spent $44 and used 17 pounds of pesticide-related products in one year (Aspelin 1998). An estimated 85-90% of pesticides never even reach their target organisms (Repetto and Baliga 1996). It is very likely that many non-target organisms are exposed to multiple pesticides thouroughout their lifetimes, either sequentially or concurrently.

Following the onset of the Gulf War Syndrome and a report suggesting synergism between estrogenic environmental contaminants, the issue of multiple chemical exposures became an urgent topic for toxicology and public health officials (Arnold et al. 1996; USEPA 1998). The idea that the effect of a chemical mixture could be more *or* less potent than exposure to the individual compounds arose. Until recently, about 95% of all chemical studies were performed on individual chemicals (Groten et al. 1999). Today, however, more studies are examining the extent to which chemical mixtures impact organ systems. In particular, the effect of these mixtures on the immune system is of interest, especially due to rising occupational and public health concerns (Simmons 1995).

Certain compounds are capable of chemically interacting when combined in mixture. It is hypothesized that exposure to pesticide mixtures can result in additive, synergistic or antagonistic effects to immune cells, mainly because the metabolism of one pesticide can

affect the metabolism the other (Eaton and Klaassen 1996). This study was designed to examine the oxidative and immunotoxic effects of lindane, an organochlorine insecticide, piperonyl butoxide (PBO), a pesticide synergist, and malathion, an organophosphate insecticide individually and in simple mixtures on murine splenocytes *in vitro*. Lindane is a mixed function oxidase (MFO) inducer, PBO is a MFO inhibitor and malathion is an acetylcholinesterase inhibitor (Dikshith 1991).

Immunotoxicity is a challenging area of toxicology because the immune system is regulated by many external factors and feedback mechanisms (Descortes 2000; Voccia et al. 1999). Xenobiotics, such as certain pesticides, can be toxic to the immune system. Many have the potential to induce cytotoxicity via apoptosis or necrosis (Corcoran et al. 1994). These two modes of cell death have unique biochemical, morphological and physiologic properties (Wyllie et al. 1980; Darzykiewicz et al. 1997). Although it was once thought mature immune cells were resistant to apoptosis, this appears not to be the case (Perandones et al. 1993; Illera et al. 1993). Splenic immune cells are a mixture of mature immune cell types and are useful in assessing certain aspects of chemical toxicity (Li et al. 1999). The ability of many xenobiotics to induce apoptosis or necrosis can have severe implications on immune cell regulations, as well as regulation of other organ systems, such as the nervous or endocrine (Descortes 2000). The heightened sensitivity of the immune system, to even low levels of xenobiotics, make it an appealing system to study the effects of chemical toxicity (Sharma and Reddy 1987).

Xenobiotics also induce oxidative stress. Oxidative stress occurs when there is an imbalance in free radical generation or oxidants and antioxidant defense capability (Marks et al. 1996). Exposure to xenobiotics can often lead to excessive free radical generation and disrupt cellular processes, such as drug metabolism and ion homeostasis (Reed 1995; Younes 1999). Excessive free radical or reactive oxygen species (ROS) generation has been implicated as an initiator of apoptosis (McConkey et al. 1994; Corcoran et al. 1994). ROS and free radicals have been linked to a number of events associated with immune cell regulation or apoptosis, such as respiratory burst and intracellular calcium regulation. Also, apoptosis has been inhibited by the addition of antioxidants (Stoain et al. 1996; Knight 2000). The current literature supports the possibility that xenobiotic exposure results in increased ROS or free radical generation, and therefore mediates the induction of apoptosis in splenic immune cells.

1.1 Study Goals

The overall goal is to examine the relationship of pesticide mixtures and immunotoxic risk *in vitro*, as it pertains to oxidative stress.

1.2 Study Hypothesis

The hypothesis is that ROS and/or other free radical intermediates, generated during the metabolism of various pesticides, mediate the immunotoxic effects of environmental xenobiotics in mixture.

1.3 Study Rational

We predict that two or more pesticides may result in an additive, synergistic or antagonistic health effects, mainly because the metabolism of one will affect that of the other. Free

radicals have been suggested to enhance immune cell dysfunction and exposure to certain insecticides has resulted in immunotoxicity. It is reasonable to assume that these two events are interrelated.

1.4 Study Objective

The objective of this study is to broaden our understanding of the mechanisms by which simultaneous exposure to multiple chemicals causes splenocyte dysfunction in a mammalian model.

1.5 Specific Aims

There are two specific aims involved in the pursuit of this objective. The first specific aim is to identify if certain pesticide and pesticide mixtures induce apoptosis in murine splenocytes *in vitro* and if so is this induction additive, synergistic or antagonistic for the pesticide mixtures as compared to the individual pesticide.

The second specific aim is to determine the oxidative status of the treated splenocytes in terms of their pro-oxidant and antioxidant levels.

Chapter II LITERATURE REVIEW

Pesticides are agents used to kill or control undesired pests, such as insects, weeds, rodents, fungi, bacteria or other organisms. The term "pesticide" includes insecticides, herbicides, rodenticides, as well as disinfectants, fumigants and wood preservatives. These compounds have a vital role in controlling agricultural, industrial, home/garden and public health pests globally. Because they have the ability to reduce the level of vector born diseases and have offered lower cost, better quality goods and services to society, the public has been tolerant of their use (Aspelin 1998). However, these economic and health benefits are not achieved without potential risk and possible adverse health effects to humans, domesticated animals and the environment. It has been estimated that 85-90% of the pesticides applied in agriculture never reach their target organisms, but instead are dispersed in the air, water and soil (Repetto and Baliga 1996). Based upon such estimates, pesticide exposure is likely for non-target organisms.

2.1 Pesticide Usage

In 1995, the pesticide industry was estimated at being a $30 billion- 2.6 million ton-a-year global industry (Repetto and Baliga 1996; Pretty et al. 1998). It is estimated that two-thirds of this use occurs in the United States, Canada and Japan (Repetto and Baliga, 1996). In the US, as of 1997, there were about 890 registered active ingredients considered pesticides. A majority of these are conventional pesticides, which are chemicals produced exclusively or primarily for use as pesticides. There is also a class called "other" pesticide chemicals, such as petroleum or sulfur, which are made primarily for other purposes but are used as

pesticides, as well. Conventional pesticides and "other" pesticide chemicals account for 1.23 billion pounds of the pesticides used in the United States annually. Still yet, there are other compounds classified as pesticides and when these considered, this 1.23 billion pounds actually represents *only* one-fourth of the *total* US annual usage of all registered pesticide products. Based upon these figures, Americans per capita use is 4.6 pounds of conventional pesticide and "other" pesticide chemicals per year. If *all* compounds registered as pesticide and pesticide related products are taken into account, such as those described above as well as wood preservatives, biocides and chlorines/hypochlorines, then an estimated 4.63 billion pounds of pesticide and pesticide related products are applied per year in the United States alone. This is equivalent to 17 pounds of pesticide per year being used by the average American. On a dollar basis, we can estimate the US spent $11.9 billion, in 1997, on conventional pesticides and "other" pesticide compounds. In other words, in the US alone, an estimated $44 per capita per year is spent on conventional pesticide chemicals (Aspelin 1998). Furthermore in 1995, about 966 million pounds of just pesticide active ingredients were applied to agricultural crops in the US at a cost of about $7.7 billion. Despite the ten-fold increase in insecticide use from 1945-1989, total crop losses from insects have nearly doubled from 7% to 13% (USDA, 1996; Benbrook et al. 1996; Pimental et al. 1991; Pimental and Greiner 1997). This pesticide treadmill effect is partly due to destruction of beneficial organisms by pesticides that otherwise would have contributed to pest biocontrol (Croft 1990; Van Den Bosh and Messenger 1973).

The above estimates shed light on the dependence the world, and particularly the United States, has on pesticides and pesticide-related chemicals. From the health perspective, the

potential for occupational and public exposure to multiple chemicals, either concurrently or sequentially, is very likely over the course of an organism's lifetime. As awareness of chemical usage grows, so does interest in *what* type of effects these chemical exposures are having on an organism's health. The health effects of many pesticides have recently begun to be examined and more investigation is needed. In particular, the affect pesticides have on the immune system, both individually and in mixtures, must be examined more closely. The immune system is an excellent indicator of the overall health of an organism. The functioning of the immune system is delicately balanced to a complex array of cell-to-cell interactions, feedback mechanisms and amplification processes within other biological systems. If alterations in the immune system occur it is likely there will also be alterations in other systems as well (Sharma and Reddy 1987). In addition, very low levels of xenobiotic can induce immunotoxicity, often at concentrations much lower than those necessary to achieve target organ toxicity, making it a very sensitive indicator of toxicity (Burns et al. 1996). In light of these points, this study was designed to further investigate multiple pesticide exposure on immune cells *in vitro* and identify if any relationship between immunotoxicity and the oxidative status of these mature immune cells exist. The three chemicals chosen for the mixture study were malathion, lindane and piperonyl butoxide.

2.2 Malathion

Malathion ([(Dimethylphosphinothioyl)thio] butanedoic acid diethyl ester; O,O-dimethylphosphorodithioate of diethyl mercaptosuccinate; Carbophos; Maldison; Mercaptothion; and Sumitox) is a non-systemic, wide spectrum organophosphate insecticide (Figure 2.1; Immig 1998a; Dikshith 1991; USEPA 2000). Malathion is applied in million pound quantities worldwide because of its potent insecticide activity and relatively low mammalian toxicity. It is considered a general use pesticide and is often utilized in situations

where large urban populations or domesticated animals may be exposed (Rodgers and Ellefson 1992). Malathion's common uses are in commercial agriculture and in households to eradicate fruit flies and other household insects. In addition, malathion is often employed to protect domesticated animals from internal parasites (such as grubs and worms) or external pests (such as lice, ticks and mites), as well as for mosquito eradication in areas of high malaria outbreak (Barnett and Rodgers 1994; ExToxNet 1998; Immig 1998a).

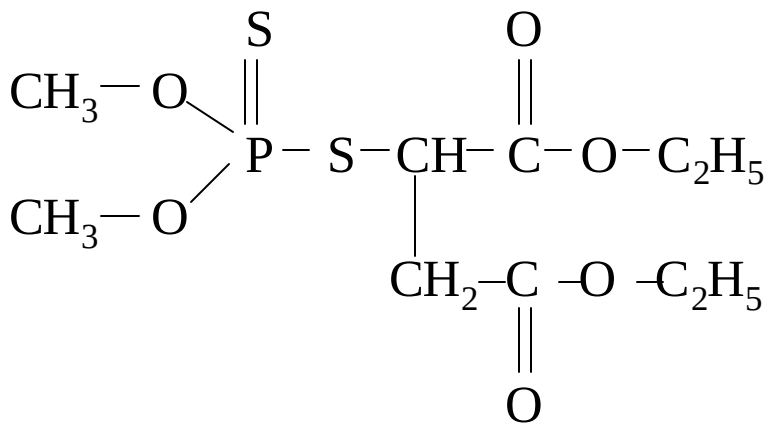

Figure 2.1. The chemical structure of malathion, an organophosphate insecticide (adapted from Dikshith 1991).

In the US alone, it an estimated 16.7 million pounds of malathion are applied annually. Approximately 12.5 million pounds are used in agriculture, mostly for cotton thourough the USDA Boll Weevil Eradication Program, and 3.4 million pounds are applied to non-agricultural uses, such as for the med fly quarantine, mosquito abatement, golf courses and home owner outdoor control (USEPA 2000). In 1999, malathion was also utilized in the West Nile Virus eradication effort (USEPA 2000b, 2001).

Malathion's low mammalian toxicity is attributed to its rapid detoxification by mammalian carboxylesterases present in the liver and other tissues (Barnett and Rodgers, 1994). Malathion is biotranformed to malaoxon and is degraded rapidly in the body. Malathion's acute effects depend mostly on the product's purity, vehicle of administration and dose (ExToxNet 1998).

Malathion's main biochemical action is on the nervous system. It is a known acetylcholinesterase (AChE) inhibitor, as are most organophosphorous compounds (Dikshith 1991; Chambers 1992; Barnett and Rodgers 1994; ExToxNet 1998). Under normal *in vivo* conditions the AChE enzyme binds acetylcholine (ACh), a neurotransmitter in the sympathetic and parasympathetic fibers, at neuromuscular junctions and some synapses of the central nervous system (Barnett and Rodgers 1994). Upon ACh binding to AChE, a signal is transmitted and ACh is immediately released to conclude the reaction (Chambers 1992). However, when an organophosphorate compound is present it mimics ACh and irreversibly phosphorylates the AChE active site. AChE loses catalytic activity and ACh

accumulates. When this occurs there is continued neurosignaling and hyperexcitability of neurons at these junctions.

This is followed by a number of neurotoxic effects proceed to cause deregulation in many other systems (Chambers 1992; Barnett and Rodgers 1994).

One system greatly influenced by neurotoxic effects of organophosphates, particularly in regard to the inhibition of esterase activity, is the immune system. Esterases, such as AChE, are vital membrane bound proteins that aid the immune system to interact with and destroy foreign organisms. For instance, neutrophils, a type of phagocytic leukocyte, require esterases to move about by chemotaxis. When organophosphates are present, there can be suppression of these esterases and, therefore, suppression of the chemotactic signals necessary for phagocytic cells (Pruett, 1992). This deregulation of the immune response results in a variety of events depending on the specific organophosphate, the dose exposed to, the species being studied, the nutritional and hormonal status of the organism, as well as the age and gender of the organism (Repetto and Baliga, 1996).

Although malathion provides a multitude of benefits to society, its high rate of usage also poses some concern for increased risk of exposure resulting in environmental or personal contamination. It is this increased risk of exposure and immune-esterase interactions, particularly to immuno-compromised individuals such as children and the elderly, which make the possible health risk due to malathion exposure of interest. In addition, very little has been studied about the effects of malathion when in chemical mixtures.

2.3 Lindane

Lindane (1,2,3,4,5,6-hexachlorocyclohexane; gamma HCH; gamma-BHC; in KWELL, Agrocide, Ambrocide, Benesan) is an organochlorine insecticide and fumigant (Figure 2.2; Dikshith, 1991). Lindane is commonly found in the technical grade hexachlorocyclohexane at 14-15% and it is the only isomer known to have insecticidal activity (Barros et al. 1991).

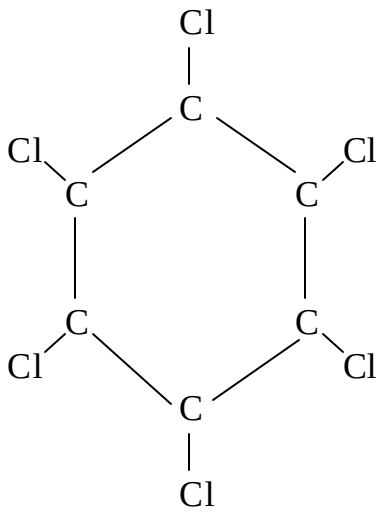

Figure 2.2. The chemical structure of lindane, the gamma isomer of hexachlorocyclohexane (HCH) (adapted from Dikshith 1991).

Since 1949, lindane has been marketed to treat against a wide range of soil-dwelling and plant-eating insects (Oesch et al. 1982; ExToxNet 1998; Safe2use 2000). From 1940 to the 1960's, lindane was used for all aspects of forestry and agriculture as well as in medical applications to eliminate mites and fleas. However, in 1975 the FDA issued an alert to the medical community regarding the potential harmful effects of lindane. In 1977, lindane was no longer commercially produced in the United States; however it is still being imported and formulated in the United States (ATSDR 1995). Today certain formulations of lindane are

classified as "Restricted Use Pesticides" and only certified applicators can handle them (ATSDR 1995, USEPA 2001). Most agricultural and the dairy industry uses have been cancelled by the USEPA because of its potential to bioaccumlate and promote carcinogenesis (Perocco, P et al. 1995; ExToxNet 1998; Safe2use 2000). The USEPA's Great Waters Program lists lindane as a pollutant of concern, due in part to these chemical properties and as well as its persistence in the environment (US EPA 2001c).

In 1983, most registered uses of lindane were banned in the US. Today, though, it is still legally registered as an insecticidal treatment for certain fruit and vegetable crops, in forestry and seed grains, as well as for use in dips, lotions, creams, and shampoos (1% solutions) to control fleas, ticks, lice, scabies and mites in humans, livestock and dogs (ExToxNet 1998; Safe2use 2000; USEPA 2001b). However, like many of the organochlorine insecticides, lindane's persistence in the environment and its biomagnification potential, as well as its ability to act as a potent central nervous system stimulant, particularly in children, continue to make it a health concern in many countries of the world (ExToxNet 1998). For this reason, lindane has already been banned in 18 countries and has restricted uses in 10 other nations. From 1987 to 1993, the USEPA's Toxic Release Inventory estimated that lindane releases to land and water were 1115 pounds (USEPA 2001c). The United States has a many advocates who would like to see its use banned completely, but the FDA has continued to allow it's use until "a safe, more effective alternative is available" to treat mites and lice (ExToxNet 1998; Safe2use 2000). In the United States, lindane is currently on the FIFRA's (Federal Insecticide, Fungicide, Rodenticide Act) Reregistration Eligibility Decisions list for

fiscal year 2001 (USEPA 2000c). Lindane is still used in ton quantities in India and is a major environmental contaminant in that country (Koner et al. 1998).

Lindane's main biochemical action, like other organochlorine insecticides, is as an inducer of the Mixed Function Oxidase system (MFO) (Konat and Clausen 1973; Srinvasan and Radhakrishnamurty 1983; Murdia et al. 1985; Junqueira et al. 1997; Barros 1991). The MFO system is a drug metabolizing system located in the smooth endoplasmic reticulum (SER) of most cells. It is also often called the monoxygenase system. The MFO system is essential to removing lipophilic xenobiotics from the body (Figure 2.3; Popov and Blaauboer 1991). The MFO utilizes cytochrome P450 (cyt P450) to interact with the toxicant. The general reaction catalyzed by cyt P450 is as follows:

$$NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + ROH$$

Where the substrate (RH) is a steroid, fatty acid, drug or other chemical that has an alkane, alkene, aromatic ring or heterocyclic ring. Thourough this process of events, the MFO eventually yields a more hydrophilic compound that can be readily excreted (ROH).

Lindane induces the MFO system by acting as a substrate molecule with a very low rate of biotransformation. Specifically, lindane will form an "enzyme-substrate complex" that last for relatively long periods of time and results in a decreased amount of enzyme available for other reactions. Such a decrease in enzyme availability is normally followed (within 1-12 hours of exposure) by an increased rate of enzyme synthesis. This event is called a "substrate-type" induction and results in increased levels of NADPH-cyt P450 reductase,

liver weight and levels of smooth endoplasmic reticulum (Popov and Blaauboer 1991; Junqueira et al. 1997). In addition, such induction potential has been shown to promote oxidative stress and membrane dysfunction in animals and cells exposed to lindane (Roux et al. 1979; Videla et al. 1990; Bagchi and Stohs 1993; Koner et al. 1998; Sahoo and Chainy 1998).

Immune dysfunction may be related to changes in the regulation of drug metabolizing enzymes, such as certain cyt P450's (Albright 1993). This dysfunction seems likely, since the immune system is responsible for interacting with xenobiotics and protecting against their noxious effects. It has been proposed that when certain receptors (such as aryl hydrocarbon receptors, Ahour) are activated, a cascade of events results and series of genes are activated, one of which is a family of cytochrome P450's (Albright 1993). Since immune cells have Ah receptors and cyt P450 enzymes, it is possible that gene activation could result. If such a scenario exisits, then it is also possible that induction of certain isoforms of cyt P450's, such as with lindane exposure, could lead to alterations in the immune response. There are, in fact, a number of studies that suggest lindane exhibits immunotoxic effects (Roux et al. 1978; Roux et al. 1979; Meade et al. 1984; Das et al. 1990; Barros et al. 1991; Meera et al. 1992; Meera et al, 1993; Dunier et al. 1994; Banerjee et al. 1996; Junqueira 1997; Sweet et al. 1998).

There is a significant amount of literature on lindane's target organ toxicity and the immunological effects it imposes *in vivo* and *in vitro*. However, there are areas regarding lindane's toxicity which must be explored, such as lindane's ability to induce apoptosis in

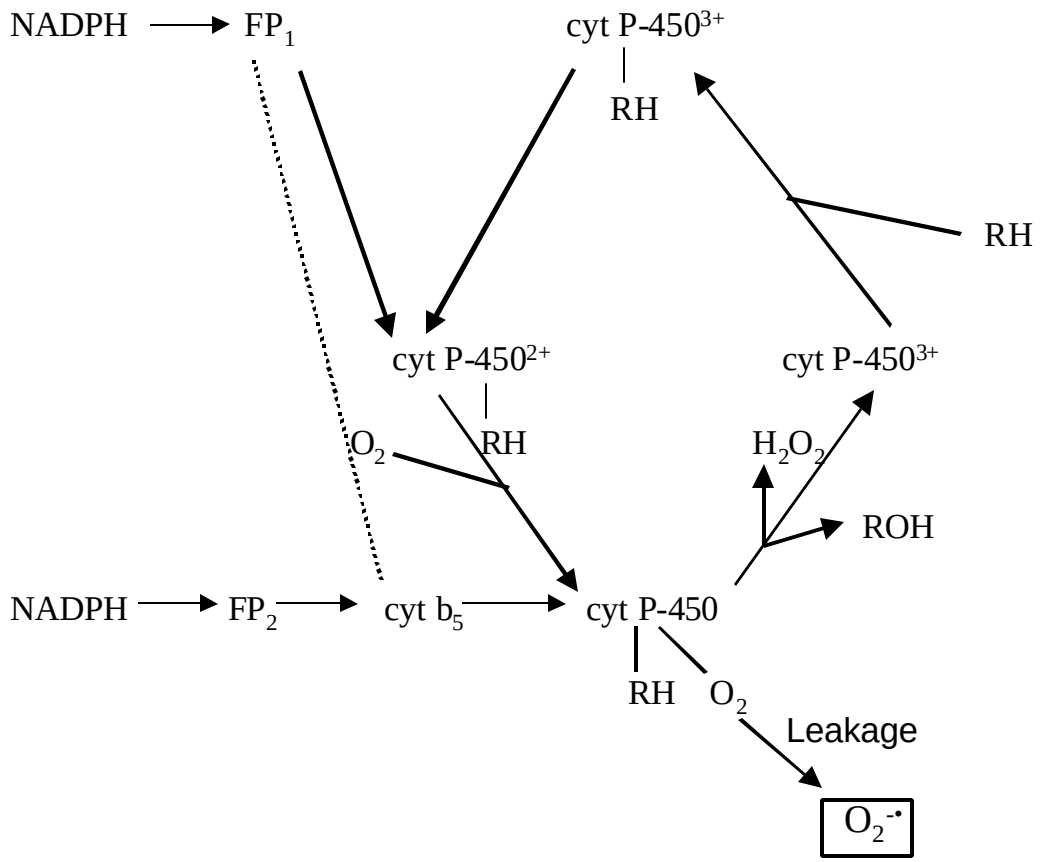

Figure 2.3. A schematic of the Mixed Function Oxidase system (MFO). The substrate (RH), such as a xenobiotic, combines with the oxidized form of cytochrome P450 (cyt P450) to form a complex that is reduced by an electron yielded by the NADPH-flavoprotein (FP_1) chain. This reduced RH-cyt P450 complex reacts with molecular oxygen and forms an oxygenated complex. This complex accepts another electron from the NADH-flavoprotein (FP_2) or cytochrome b_5 chain (or in some instances from the NADPH-FP_1 chain). The donation of this second electron activates the oxygen. Upon activation, one atom of "activated oxygen" is reduced to water, while the other is available to oxidize the substrate (RH) and form an intermediate complex (O_2-cyt P450-RH). This complex is unstable and quickly breaks up generating oxidized substrate (ROH) and releasing the cyt P450 for another cycle.

It is important to note that in some cases, if the MFO system is overwhelmed by substrate or other stressors, the activated oxygen can "leak" from the MFO system as superoxide anion ($O_2^{-\bullet}$). This especially occurs if there is a breakdown of the oxygenated cyt P450 substrate (O_2 -cyt P450-RH) complex. Thus $O_2^{-\bullet}$ can be dismutated to hydrogen peroxide (H_2O_2) and H_2O_2, upon reaction with metal ions, can generate hydroxyl radicals ($\bullet$OH). $\bullet$OH is highly destructive and can cause membrane disruption. If these events occur for prolonged periods of time, the MFO system cannot adequately biotransform xenobiotics and the metabolic process is disrupted (Adapted from Popov and Blaauboer 1991).

mature immune cells at low concentrations and it's toxicity to the immune system when in chemical mixtures.

2.4 Piperonyl Butoxide (PBO)

Piperonyl butoxide (PBO) (5-[[2-(2-Butoxyethoxy)ethoxy]methyl] 6-propyl-1,3-benzodioxole), a methylenedioxyphenyl compound, is a synergist used in combination with a wide variety of insecticides (Figure 2.4; Immig 1998b; Rose et al. 1999; USEPA 2000). Synergists are chemicals that lack pesticidal effects of their own but enhance the toxic properties of certain pesticide compounds (Franklin, 1976; USEPA 2000). PBO is most commonly used in combination with pyrethourins, pyrethouroids, rotenones and carbamates (USEPA 2000). PBO was first developed in 1947 using naturally occurring safrole as a key raw material. PBO is in Anvil, an insecticide mixture, which is used to control mosquitoes in outdoor residential or recreation areas. It is also in other pesticide products used indoors on plants and on pets to control insects such as fleas, tick and ants. PBO is stable to light and resist hydrolysis. Currently, PBO is under review by the US EPA for the FIFRA's (Federal Insecticide, Fungicide, Rodenticide Act) Reregistration Eligibility Decisions list for the fiscal year 2001 (USEPA 2000c; USEPA 2000d). Also, in July 2000, PBO was placed on the Restricted Use Products Report (USEPA 2001d).

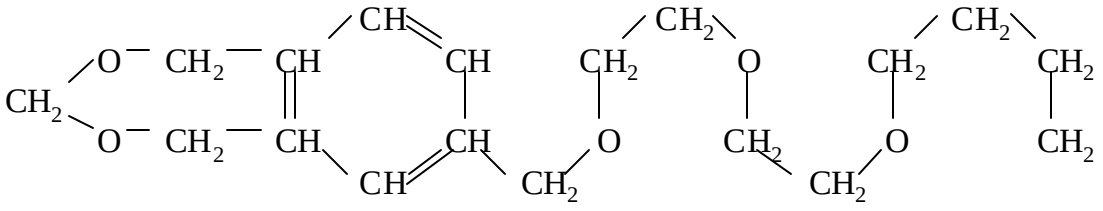

Figure 2.4. The chemical structure of piperonyl butoxide, a pesticide synergist and methylenedioxyphenyl compound (Adapted from Rose et al. 1999).

PBO's biochemical action is as an inhibitor of the MFO system and by doing so it can block the biotransformation of pesticides by insects (Anders 1968; Jaffe et al. 1968; Matthews et al. 1970; Conney et al. 1972; Goldstein et al. 1973; USEPA 2000). Specifically, PBO is a noncompetitive (irreversible) inhibitor because it covalently binds with the cyt P450 enzyme (Figure 2.3). The process, by which this occurs, is that PBO is biotransformed to a carbene compound and other reactive intermediates. The carbene interacts with the heme iron on cyt P450 and forms a stable metabolite-inhibitory complex with cyt P450. PBO is, like other pesticide synergists and methylenedioxyphenyl-derived compounds, a suicide substrate for the cyt P450 enzyme. The acute toxicity of PBO is relatively low, but by forming a stable complex, PBO is able to block the metabolism of other MFO substrates (Franklin 1976; Popov and Blaauboer 1991; Rose et al. 1999). This can be beneficial or detrimental depending on what the other substrates are and if bioactivation is necessary for a compound to induce toxicity (Franklin, 1976).

It has been indicated that the magnitude of PBO toxicity and MFO inhibition varies with mammalian species. This is an important concept when extrapolating results across species. Conney et al. (1972) showed that *in vivo* treatment of mice, rats and humans resulted in mice being 33 to 100 fold more sensitive to PBO's MFO inhibitory effects than rats. Also, humans were fed a dose of PBO much greater than the expected daily exposure levels and still showed no change in MFO potential suggesting it may be unlikely that humans are affected by PBO's inhibition on microsomal function.

It is also important to note that in mammals PBO is capable of both inhibition *and* induction of cyt P450. Following an acute dose of PBO, inhibition of cyt P450 occurs, then activity returns to normal and eventually an increase in activity observed (Wagstaff and Short 1971; Goldstein et al. 1973; USEPA 2000d). The inhibitory action occurs as described above, but the induction is more complicated. It has been shown, as mentioned with lindane, that when certain receptors on cells are chemically activated (such as AhR's) that specific cytochrome isoforms could also be activated (Rose et al. 1999). In light of the fact that immune cells have AhR's, their activation could possibly induce the delayed cyt P450 activation seen with PBO exposure, even though PBO is traditionally an inhibitor of cyt P450 (Albright 1993). It is also very possible, as mentioned above, that cyt P450 regulation, due to PBO exposure, could result in immune cell dysfunction since the two events may be related. Examination of PBO's ability to induce oxidative stress, immunotoxicity via apoptosis and its effect in multiple chemical exposures should be further investigated.

2.5 Pesticides, Reactive Oxygen Species and Oxidative Stress

2.5a *Types of Free Radicals and Reactive Oxygen Species*

Whenever discussing xenobiotic metabolism, the possible involvement of free radical species is of interest and should be considered. Free radicals are molecules which contain one or more unpaired electrons (Kehrer 1993; Younes 1999, Halliwell 1996). A free radical species may be highly reactive and can initiate chain reactions by extracting an electron from a neighboring molecule to complete its own orbital (Marks et al. 1996; Younes 1999). There are many forms of free radical species, such as oxygen-centered, carbon-centered ($R^{\cdot}$,

RCOO·) or nitrogen-centered ($NO^{\cdot}$, $ONOO^{-}$) (Kalyanaraman 1982; Kehrer 1993, Halliwell 1996). Many of these free radical species are formed when chemicals are metabolized to one or more reactive intermediates (Comporti and Pompella 1994). Typically, what often occurs is the mitochontrial electron transport system, which metabolizes molecular oxygen, can be disrupted. Free radicals, called reactive oxygen species (ROS), can form (Yu 1994). These include species such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), hydroperoxyl radicals (HO_2) and singlet oxygen ($^1O_2$).

Superoxide radical can be tended to the formation of many other reactive species, including hydroxyl radicals, hydrogen peroxide and hydroperoxyl radicals. The hydroxyl radical is the potentially most potent oxidant encountered in biological systems and can react readily with a variety of molecules, such as lipids, DNA and proteins (Yu 1994). Hydroxyl radicals are formed via the Fenton reaction:

(1) $$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

or by interaction with superoxide in the Haber-Weiss Reaction

(2) $$O_2^{-\bullet} + H_2O_2 \xrightarrow[H^+]{} O_2 + HO^{-} + {}^{\bullet}OH$$

Similar to the Fenton reaction, other reduced transition metals, such as copper, interact with hydrogen peroxide to generate $^{\cdot}OH$:

(3) $$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + {}^{\bullet}OH + OH^{-}$$

Hydrogen peroxide is not by definition an oxygen free radical but it is a nonradical ROS. It is a secondary product of one-electron reduction of $O_2^{-\cdot}$:

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

The reaction is called the dismutation of superoxide and is performed by superoxide dismutase (SOD) almost at diffusion-controlled rate ($k = 3x10^9\ M^{-1}s^{-1}$) (McCord and Fridovich, 1969; Yu 1994; Younes 1999). Singlet oxygen is, like hydrogen peroxide, not a free radical species but it is an ROS with oxidative properties. It is very short-lived, and although it's formation in biological systems has long been disputed, there is evidence that it does exist during phagocytosis (Younes 1999).

In addition to the oxygen-centered radicals, carbon-centered radicals can arise following hydrogen extraction from unsaturated bonds in fatty acids during lipid peroxidation or metabolism of certain xenobiotics (Kehrer 1993). Lastly, nitrogen-centered radicals occur naturally in mammalian organisms and are utilized in numerous physiological processes. For instance nitric oxide ($NO^\bullet$) is a signaling molecule for the immune system, it is important to many metabolic processes and is a paracrine messenger (vasoregulator). An excess of $NO^\bullet$, however, can result in cytotoxicity and tissue injury, particularly if it reacts with $O_2^{-\bullet}$ to form peroxynitrite ($ONOO^-$):

(5) $$NO^\bullet + O_2^{-\cdot} \rightarrow ONOO^-$$

Peroxynitrite, is a nonradical species that is very reactive and can induce a chain reaction of events, such as direct biological damage by excessively oxidizing –SH groups and also

leading to the production of nitric dioxide, which is a powerful initiator of lipid peroxidation (Halliwell 1996; Schmidt and Walter 1994; Muijsers et al. 1997).

2.5b *Sources of and Damage by Free Radicals and Reactive Oxygen Species*

Under conditions of normal metabolism, the continual formation of ROS and other free radicals is important for normal physiological functions (i.e. ATP generation, metabolic processes) and cellular redox reactions. However, excessive generation of free radicals can occur due to endogenous biological or exogenous environmental factors, such as chemical exposure, pollution or radiation (Figure 2.5). The main endogenous biological sources of reactive oxygen species are via: a) Soluble cell constituents (such as thiols, quinines, transition metals, epinephrine, metaloproteins, hemeproteins and flavoproteins), which can activate O_2 during autooxidation reactions and release ROS (Misra and Fridovich, 1972); b) Soluble cytosolic enzymes (such as xanthine oxidase and dihydroorotate dehydrogenase), which can generate ROS ($O_2^{-\bullet}$, $^{\bullet}OH$) via reduction of molecular oxygen in their catalytic cycles; c) Membrane-bound enzymes (such as NADPH oxidase) and electron transport systems (such as MFO), which can leak free radical species; d) Xenobiotic metabolizing enzymes in the endoplasmic reticulum or nuclear membrane (such as cyt P-450-dependent monooxygenase, cyt b_5 and NADPH-depend cytochrome reductases), which can activate molecular oxygen or allow superoxide to "leak" out; e) Phagocytic cells involved in inflammation, respiratory burst (such as neutrophils, macrophages and monocytes) and toxic agent removal, which can generate ROS (Yu 1994; Stoian et al.1996; Younes 1999);

f.) Ischemia/reperfusion following surgery or artery blockage, which can significantly increase levels of ROS (Reed 1995); and g.) Inhibition/reduction of antioxidant enzymes or molecules, which reduces the ability for an organism to handle increased levels of free radicals (Reed 1995).

In spite of numerous biological defense systems, increased free radical generation has the potential to result in oxidative stress. Oxidative stress occurs when the body cannot balance free radical generation with the body's defenses and often promotes cellular injury and tissue damage. This damage can range from DNA damage, membrane damage, mitochondrial swelling and lyses, calcium influx, protein damage and lipid peroxidation (Figure 2.6) (Reed 1995; Marks et al. 1996; Younes 1999).

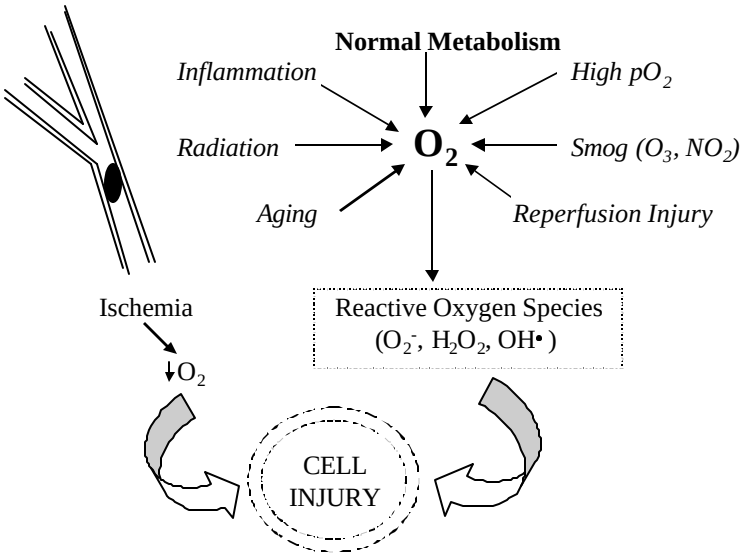

Figure 2.5. Sources of ROS and other free radicals. ROS and other free radical intermediates are formed during normal metabolic processes in oxygen respiring organisms. The levels are augmented, however, by exposure to other physical and environmental factors, such as xenobiotic exposure, pollution, radiation and aging processes (adapted from Marks et al. 1996).

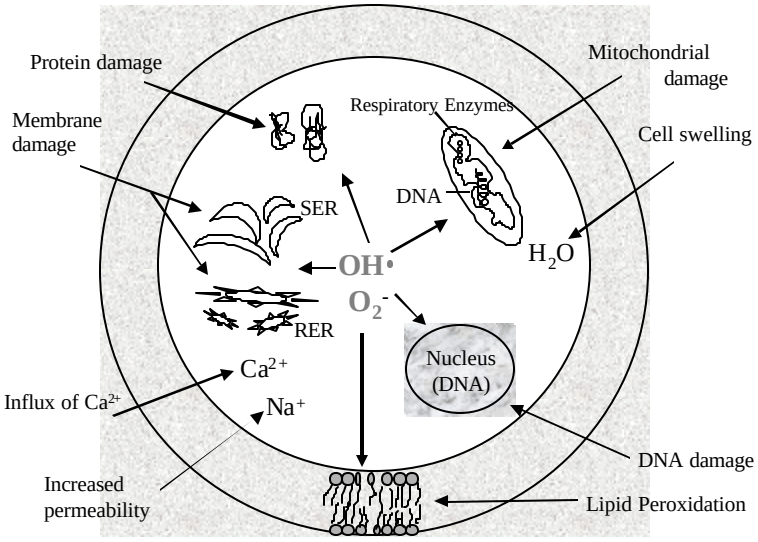

Figure 2.6. Cell injury by ROS or free radical-induced oxidative stress. Free radical-mediated cellular injury is often initiated by superoxide ($O_2^{-\bullet}$) or hydroxyl radicals ($^\bullet$OH). These ROS initiate lipid peroxidation of cellular, mitochondrial, nuclear and endoplasmic reticulum membranes. This increases cellular permeability to calcium and results in further mitochondrial swelling and damage. Amino acid residues on proteins can be oxidized and degraded by ROS. Nuclear and mitochondrial DNA can also be oxidized, leading to strand breaks and other damage. All of these events ultimately lead to cellular dysfunction and injury (adapted from Marks et al. 1996).

2.5c *Antioxidant Defenses: Primary and Secondary*

There are numerous cellular defenses, which under normal metabolic conditions, regulate the level of ROS and protect against the ill-effects of free radicals. These defenses can be classified into two categories: primary and secondary defenses.

The *primary* defenses consist of two groups, the antioxidant compounds and the antioxidant scavenging enzymes (Figure 2.7). Antioxidant compounds include vitamins C and E, as well as glutathione, β-carotene and uric acid. Vitamin E is an important intracellular oxidant measure of its highly lipophilic properties. It provides antioxidant action in and near lipid membranes by converting $O_2^{-\bullet}$, $^{\bullet}OH$, and $LOO^{\bullet-}$ to less reactive forms. Vitamin C is an effective extracellular antioxidant because of its hydrophilic nature and wide distribution in the body. It scavenges $O_2^{-\bullet}$ and $^{\bullet}OH$, neutralizes oxidants from neutrophils and aids in vitamin E regeneration from its oxidized state. Vitamin A and β-carotene are lipophilic and scavenge $O_2^{-\bullet}$ or react with peroxyl radicals. Uric acid is hydrophilic and scavenges $O_2^{-\bullet}$, $^{\bullet}OH$, and peroxyl radicals. It binds transition metals and prevents vitamin C oxidation. Glutathione (GSH) is a hydrophilic tripeptide and is critical to glutathione redox cycling and enzyme regulation. GSH reacts directly with $O_2^{-\bullet}$, $^{\bullet}OH$, or organic free radicals ($R^{\bullet}$, $RCOO^{\bullet}$) and is critical to the detoxification processes of xenobiotic metabolism (Yu, 1994; Marks et al. 1996).

Antioxidant scavenging enzymes include superoxide dismutase, catalase, glutathione peroxidase and reductase. Superoxide dismutase (SOD) dismutates superoxide radicals to

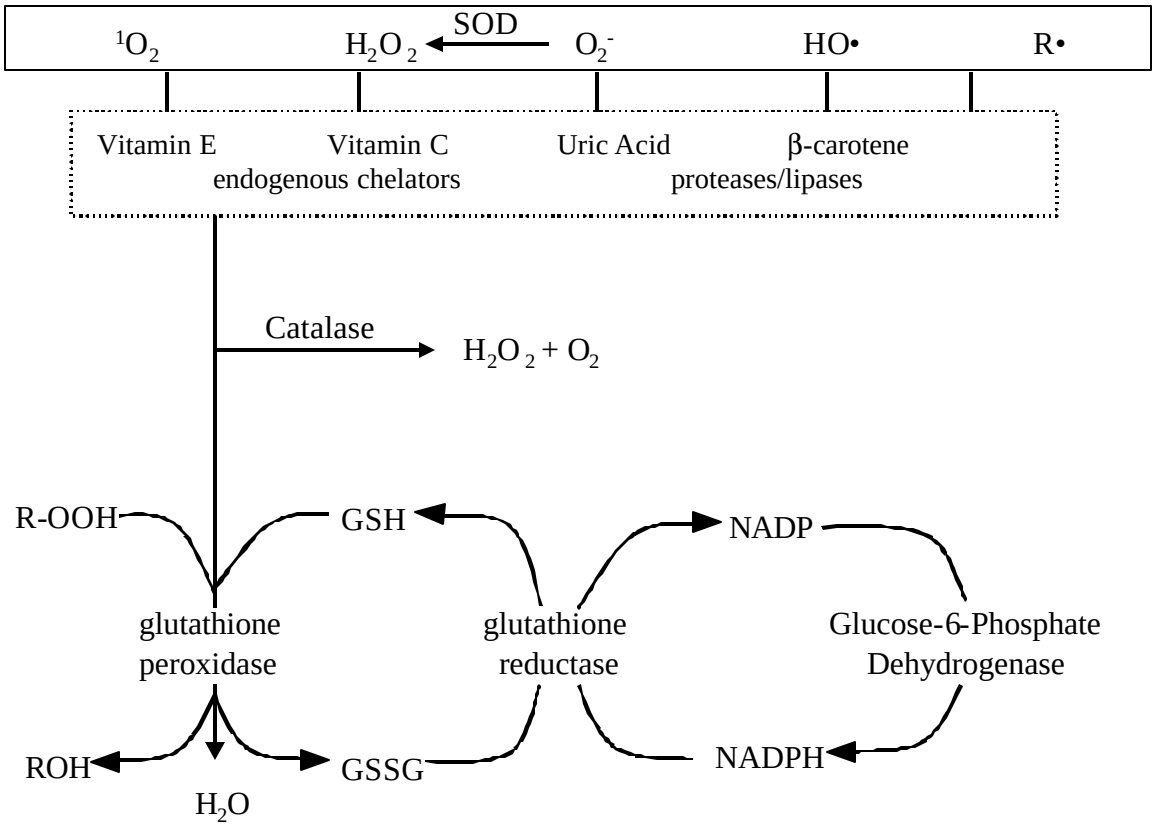

Figure 2.7. Biological antioxidant defense systems. All aerobic cells have a variety of chemical and enzymatic antioxidants that work to minimize the detrimental effects of free radicals and oxidative reactions within cells. The primary and secondary defenses are presented, as well as their locations of action in given oxidative processes. (Abbreviations SOD: superoxide dismutase; GSH:reduced glutathione; GSSG:glutathione disulfide or oxidized glutathione) (adapted from Kehrer 1993).

hydrogen peroxide (H_2O_2). SOD exists in all oxygen respiring organisms. In general, there are three forms of SOD-Cu/Zn (cytosolic), Mn (mitochondrial) and Fe. Exceptions have been noted in certain organisms and subcellular fractions. The activity of SOD varies among tissues but the highest amounts are in the liver, adrenal gland, kidney and spleen. Catalase decomposes H_2O_2 to water. Catalase is more effective at relatively high concentrations of H_2O_2 (Km for H_2O_2 is 1 mM), whereas at lower concentrations glutathione peroxidase (GPx;

selenium dependent) is more effective in catalyzing H_2O_2 breakdown. The Km for H_2O_2 for this enzyme is in the range of μM concentrations. GPx (selenium independent) decomposes organic hydroperoxides as well. This enzyme utilizes GSH, which is oxidized to GSSG, as a second substrate. Glutathione reductase (GR) catalyzes the reduction of low molecular weight disulfides (such as GSSG) using NADH as an electron source (Yu 1994; Marks et al. 1996).

The *secondary* defenses include lipolytic and proteolytic enzymes. Both groups of enzymes act as secondary lines of defense by "cleaning up" lipid or proteins that are damaged, altered or being turned over. This process removes reactive cellular components, which could otherwise result in further oxidative reactions (Yu 1994).

Overall, there are many defenses that help balance the oxidative status of cells within an organism. If these defenses are compromised, even if there is no external source of oxidative stress, the balance of ROS can be affected and cellular injury can result (Marks et al. 1996; Younes 1999). The defenses described here are critical to the survival of oxygen respiring organisms.

2.5d *Chemically-Induced Oxidative Stress*

As mentioned, the major source of organic free radicals and ROS is endogenous, particularly via the metabolism and bioactivation of xenobiotics or the reactivity of the actual parent compound. The consequence of increased free radical concentrations is either cellular injury or physiological dysfunction (Figure 6; Comporti and Pompella, 1994; Marks et al. 1996).

Many compounds have been found to result in free radical generation and have the potential to promote these types of injuries. For example, diquat and paraquat, bipyridyl herbicides, induce $O_2^{-\bullet}$ and H_2O_2 within cells (Reed 1995; Rose 1999). Also, carbon tetrachloride undergoes dehalogenation to form a reactive trichloromethyl radical. This metabolite can interact with macromolecules by abstracting H atoms and generating more radical species. A number of halogenated hydrocarbons are capable of undergoing such dehalogenation reactions. Analgetic and antipyretic drugs, such as acetaminophen, can be bioactivated and reduce molecular oxygen to superoxide molecules. Quinone related compounds, such as adriamycin and aflatoxin B, have the capability of transferring an e^- to molecular oxygen and resulting in superoxide formation. Lastly, other compounds such as bleomycin, a chemotherapeutic agent, and ethanol have the potential to induce toxicity via activation of molecular oxygen (Younes 1999).

These chemicals are only a few of many that have been implicated in inducing or promoting oxidative stress. The potential of lindane, malathion or PBO to promote oxidative stress has been examined to some degree in previous literature.

2.5e *Malathion-Induced Oxidative Stress*

Malathion is an organophosphate compound, and like other organophosphates, is detoxified via conjugation reactions with glutathione (Malik and Summer 1982). In a study on rat hepatocytes, increasing concentrations of malathion (0.25 mM to 30 mM) depleted GSH in a dose-dependent manner and carboxylesterase activity was inhibited. This type of inhibition, of the carboxylesterase-mediated malathion degradation, could direct malathion metabolism

toward GSH-dependent metabolism and result in further depletion of cellular GSH. The lack of GSH availability could have considerable influence on the metabolism and rate of detoxification of malathion, as well as other xenobiotics (Malik and Summer 1982).

Another study examined the effects of malathion (2 and 4 mM) on rat blood, liver and kidney homogenates *in vitro* (Lechner and Rahman 1986). Again, depletion of liver GSH content was noted and the concentration of GSH continued to decrease during the entire exposure period. The blood glutathione levels remained unchanged. The enzyme glutathione-S-transferase (GST) activity in liver homogenates increased two-fold, while the γ-glutamyl transpeptidase (GGTP) levels increased in the kidney and remained unchanged in the liver. Overall, the glutathione levels were reduced and it is predicted this is due to its role in malathion metabolism. It is thought that the reduction in glutathione is responsible for the increase in GST activity.

Furthermore, a study examined malathion exposure using human serum samples from individuals admitted for pesticide poisoning and found enhanced the levels of lipid peroxidation by-products, or thiobarbituric acid reacting substance (TBARS). Also, these patients had significantly increased levels of SOD, catalase and glutathoine peroxidase in their erythrocytes compared control subjects. GSH-S-transferase activity in serum samples was increased as well. Both, the level of blood and lymphocyte GSH and GR activity were significantly decreased. In addition, malathion significantly inhibited acetylcholinesterase activity in human lymphocytes (Banerjee et al. 1999).

In summary, glutathione and certain glutathione-linked enzymes are modulated by malathion exposure. This may attribute to the increased levels of TBARS in the blood, as well as the increased levels of SOD and catalase. Banerajee et al. (1999) point out that inhibition of AChE could result in the accumulation of ACh and increase lymphocyte mobility and cytotoxicity. High ACh concentrations have been associated with lymphocyte mortality due to depletion of lymphocyte glutathione and the compromised oxidative state of the cells.

It is clear that malathion has the potential to disrupt cellular antioxidant defenses in various cell systems and induce oxidative toxicity in hepatocytes, as well as human blood lymphocytes.

2.5f *Lindane-Induced Oxidative Stress*

In addition to inducing MFO activity, lindane enhances oxidative stress by interacting with the cell membrane, triggering the generation of ROS and other free radical intermediates and altering the levels of antioxidant molecules or enzymes (Barros et al. 1991; Bagchi and Stohs 1993; Samanta and Chainy 1997; Junqueira et al. 1997 and 1986; Koner et al. 1998; Sahoo and Chainy 1998; Banerjee et al. 1999).

Junqueira et al. (1986) treated rats with an acute dose (20 to 80 mg/kg; ip) and detected increased $O_2^{-\bullet}$, as well as enhanced cyt P450 expression in the liver homogenates 24 hours following exposure. TBARS increased in a dose dependent manner with increasing concentration of lindane. There was no fluctuation in glucose-6-phosphate dehydrogenase (G6PD), GR or GPx. SOD and catalase levels, however, were significantly reduced. In

1997, Junqueira et al. conducted another study to examine oxidative stress related parameters for up to one week following a 60 mg/kg dosing of rats. Again, the same trends as noted above were observed. However, there were also morphological changes noted in the mitochondria. Most of the fluctuations mentioned returned to control levels by day 3 following exposure. Barros et al. (1991) exposed rats to dietary lindane for 15 or 30 days (20 ppm). In this short-term exposure study, cyt P450 levels, TBARS in liver homogenates and superoxide levels all increased. However, SOD activity increased while no change in catalase, GR, GPx or G6PD was observed. A similar study was performed in 1983 (Srinivasan and Radhakrishnamurty) in which rats were fed 50 to 800 ppm lindane for 2 weeks. Increased cyt P450 and cyt b_2 levels were observed in the liver. Also, NADPH-cytC-reductase and aryl hydroxylase increased as well. A longer sub-chouronic study where rats were treated with 40 or 80ppm dietary lindane for 8 weeks showed increased TBARS in the serum and SOD in the red blood cells (Koner et al. 1998). Agrawal et al. (1991) treated rats with 300mg/kg lindane (ip) and reported reduced levels of glutathione in erythrocytes. Also, the GR, G6PD and glutathione-S-transferase were reduced 24 hours following treatment but no change in GPx levels was observed. A study by Bagchi and Stohs (1993) treated rat peritoneal macrophages and hepatic microsomal and mitochondrial fractions *in vitro* to lindane (0-200 ng/ml) for 30 minutes. An increase in chemiluminescence in all samples was observed indicating the generation of free radical species as a result of lindane exposure. This increase was particularly noted just 5-10 minutes following treatment and remained elevated for the entire 30 minutes.

The neurotoxicity of lindane was evaluated in an acute dosing study, where rats were treated with lindane (10 or 20 mg/kg; ip) and elevated TBARS were noted in the cerebral hemisphere of the rat brain homogenate. Cytosolic total, CN^- sensitive and CN^- resistant SOD, as well as Se-dependent and Se-independent GPx and catalase were decreased 24 hours after dosing. The GR was actually elevated at 24 hour following a reduction at 6 hour after treatment. The GSH and ascorbic acid levels also decreased due to exposure.

A study by Banerjee et al (1999) indicated increased TBARS in human serum and plasma after lindane poisoning. SOD and catalase activities in blood erythrocytes increased. Blood GSH levels were significantly reduced while serum glutathione-S-transferase activity, plasma GR and erythourocyte GPx were increased.

Videla et al. (1990) discussed lindane's ability to induce liver oxidative stress. They offer further explanation into lindane's ability to create reactive molecules. For instance, the generation of $O_2^{-\bullet}$ is demonstrated in many studies and it is very likely then this can lead to increased levels of H_2O_2, a potent pro-oxidant. Also, cyt P450 reacts with lindane, by abstracting a hydrogen from lindane and creating a free radical metabolite, as well as H_2O_2. Either of these molecules can induce oxidative stress by initiating cellular lipid peroxidation, which has clearly been measured in most studies involving lindane exposure. Lindane is very susceptible to GSH conjugation and lowers the concentration of this important antioxidant molecule, therefore reducing a cell's antioxidant capacity. It has been shown that just 4 hours following lindane treatment, GSH is significantly reduced and GSSG levels are dramatically enhanced.

In different species and cell types, as well as *in vivo* and *in vitro* treatments all conclusively indicate that ROS and organic radicals generation occurs from lindane exposure. Also, the modulation of antioxidant molecules clearly suggests that lindane is a potent inducer of oxidative stress and can cause severe physiological dysfunction in various organ systems.

2.5g *Piperonyl Butoxide-Induced Oxidative Stress*

PBO's ability to alter metabolic function via its biphasic action on the MFO system potentially enables it to disrupt the oxidative balance of cells (Franklin 1976; Popov and Blaauboer 1991). It is not PBO's intrinsic toxicity that is of concern in humans, but more it is the dual effect on oxidative detoxification and intoxication processes in the body. PBO is able to inhibit these reactions via two mechanisms. It can act as a substrate for mixed function oxidation and compete with other xenobiotics or it can bind to the cyt P450 to inactivate it. In addition, PBO also has the ability to activate xenobiotic metabolism by induction of the microsomal enzymes (Franklin 1976).

Numerous studies have been performed on mice, rats and humans to better understand PBO's toxicity. An *in vivo* study on rats treated with 2.4% dietary PBO caused increases in liver weight, gamma glutamyl transpeptidase activity and necrosis of hepatocytes (Fujitani et al. 1992). Also, Goldstein et al. (1973) fed rats up to 10000ppm PBO for two months and found increased liver size, smooth endoplasmic reticulum (SER) proliferation and increased cyt P450 levels at treatment above 5000ppm. Jaffe et al. (1968) treated mice in vivo to 160mg/kg PBO and saw significant reduction in microsomal hydroxylation reactions. All these finding are consistent with enhanced levels of oxidative stress. A study by James and

Harbison (1982) treated mice with 400mg/kg PBO (ip) and reported a significant decrease in total hepatic GSH. The effect was transient since levels returned to control values five hours following treatment.

Most studies have addressed PBO toxicity in terms of hepatotoxicity and it's effect on the MFO system. As mentioned earlier, PBO toxicity varies widely depending on the species and strain being examined, as well as dose and length of exposure. Such variations make it difficult to make general conclusions about PBO's ability to induce oxidative stress except that it stimulates biphasic activity and can modulate metabolizing enzymes of the liver. More studies on other cellular systems and antioxidant levels are necessary to clearly understand PBO's potential toxicity.

2.6 The Immune System, Apoptosis and Chemical Exposure

Immunity is a series of delicately balanced, complex, multicellular and physiological mechanisms that allow an organism to distinguish foreign material from "self" and neutralize or eliminate foreign matter from an organism (Burns et al. 1996). Many immune interactions are linked to other physiological functions and cellular processes, such as neural responses or endocrine hormone regulation (Descotes 2000). This intricate balance of organization and communication makes the immune system extremely difficult to study in some regards. Yet, its sensitivity and ability to respond to relatively low concentrations of xenobiotics has created a whole new approach to examining chemically-induced toxicity. In order to discuss how chemical exposures can influence the immune system, a description of the organs and cells, as well as the types of immunity is necessary.

2.6a *Organs of the Immune System*

The organs of the immune system are divided into primary and secondary lymphoid organs. The *primary* lymphoid organs are the bone marrow and thymus. These organs support the production of mature T- and B-lymphocytes and myeloid cells, such as macrophages. The bone marrow is the site of origination of the pluripotent stem cells, a self-renewing cell from which all other hematopoietic cells are derived. The thymus is the site at which all T-cell precursors migrate and undergo selection for recognition of self or nonself antigens. The *secondary* lymphoid organs are the spleen, lymph nodes and mucosa-associated lymphoid tissues (MALT), which include the tonsils and Peyer's patches (Burns et al. 1996; Roitt et al. 1998). These organs have a highly organized microenvironment in which lymphocytes come into contact with exogenously derived antigens. They also serve as biological sieves. For instance, the spleen filters the blood by removing foreign antigens and any circulating dead cells (i.e. erythrocytes, platelets, granulocytes and lymphocytes). The lymph nodes, on the other hand, filter antigens from the fluid surrounding tissues. The key events that occur in the secondary lymphoid organs are: (1) specific antigen recognition; (2) clonal expansion of antigen-specific cells; and (3) differentiation of antigen stimulate lymphocytes (Sharma and Reddy 1987; Burns et al. 1996).

The spleen is divided into the white pulp region, consisting of lymphatic tissue, and the red pulp region, which contains erythourocyte rich blood (Sharma and Reddy 1987).

2.6b *Cells of the Immune System*

Immune responses are mediated by a variety of immune cells. Leukocytes are central to all immune responses, but other cells within tissues also participate by signaling to lymphocytes

and responding to cytokines released by T lymphocytes and macrophages. There are three major groups of leukocytes: lymphocytes, phagocytes and auxiliary cells. The lymphocytes consist of T cells, B cells and large granular lymphocytes (LGL). The phagocytes consist of mononuclear phagocytes, neutrophils and eosinophils. The auxiliary cells are basophils, mast cells and platelets.

Lymphocytes are responsible for specific immune recognition of pathogens. T lymphocytes develop in the thymus and B lymphocytes develop in the bone marrow. B cells have a specific surface receptor for a particular antigen. When recognizing that antigen, the B cell differentiates into a plasma cell and produces large amounts of the receptor molecule, or antibody. These antibodies bind to the specific antigen and induce further immune cell response. T lymphocytes consist of two different groups. Helper T cells interact with B cells to aid in division, differentiation and antibody production. The other, cytotoxic T cells, interact with the mononuclear phagocytes and aid in destroying antigens. T cells generate their effect by either releasing soluble proteins called cytokines, which act as secondary messengers and signal other cells, or by direct cell-cell interactions (Roitt et al. 1996).

The phagocytic cells internalize particles, such as infectious agents or xenobiotics, and destroy them. The mononuclear phagocytes are located in the blood (monocytes) and migrate to tissues where they become macrophages. These cells are antigen-presenting cells to T lymphocytes. Also, neutrophils are important phagocytic cells. They are derived just as macrophages and respond to certain stimuli, however they are much shorter lived.

The cytotoxic cells, which have the capacity to kill other cells, are either cytotoxic T cells, LGLs or eosinophils. LGL's, or natural killer cells, recognize surface antigens on tumor or infected cells. Eosinophils are a special group of phagocytic leukocytes that can damage large extracellular parasites. All cytotoxic cells damage other cells by dispensing the contents of their granules on or near an infected cell or antigen. This process is known as degranulation.

Lastly, two auxiliary cells that mediate inflammatory responses are basophils and mast cells. These cells contain mediators, which initiate inflammation in tissues as well as other immune reactions. Mast cells act on the blood vessel walls. Basophils are similar to mast cells but circulate in the blood.

The spleen has a very heterogeneous population of immune cells. B and T lymphocytes make up the majority of the splenic immune cell population, 65% and 25%, respectively. Macrophages make up approximately 4-5% of the splenic immune cell population and the remaining cells are mostly neutrophils and eosinophils (Li et al. 1999). The proper functioning of the spleen is an important determinant of organismal health and immune responsiveness. The cellular heterogeneity of the spleen, however, makes it difficult to predict how splenic immune cells will respond to particular chemical stimuli. Much attention has been focused on thymocyte and peripheral blood leukocyte responsiveness to xenobiotics, yet more research on mature splenic immune cells and chemical exposure is necessary.

2.6c *Immunity*

Immunity is classified as innate immunity, a non-specific immune response resulting during the genetic constitution of an organism, and acquired immunity, a specific response resulting from direct exposure to a foreign substance (Herbert and Wilkinson 1971; Sharma and Reddy 1987). Acquired immunity can be divided into two subclasses, humoral and cell mediated immunity (CMI). Humoral immunity is mediated by antibodies produced by B cells and CMI is mediated by macrophages, T cells and other phagocytic immune cells. CMI response is often related to delayed hypersensitivity or graft rejection (Herbert and Wilkinson 1971).

Previous research has reported that various chemical agents cause immune dysfunction in both humoral and CMI response (Casale et al. 1983; Johnson et al 1987; Barnett and Rodgers 1994; Banerjee et al. 1996b; Banerjee et al. 1998; Koner et al. 1998).

2.6d *Immunotoxicity*

Immunotoxicology is the discipline dealing with the interaction of test substances and the immune system (Banerjee et al. 1996). The objective of the discipline is to protect humans and animals against the harmful effects of chemical factors present in the environment and introduce/evaluate methods used for determination of interactions between these factors and immune system (Kacmar et al. 1999). Evaluation of immunotoxicity is challenging due to the impact external factors play on the immune system and the feedback mechanisms that regulate it (Descotes 2000; Voccia et al. 1999). There are four main areas that immunotoxicity can be evaluated: the *enhancement* of immune functions (immunomodulation); the *depression* of immune function (immunosuppression); the

overreaction of the immune system (allergic reactions); or the *failure* to recognize self (autoimmune reactions) (Descotes and Vial 1994; Kacmar et al. 1999; Neubert and Neubert 1999).

Xenobiotics have the ability induce any of the above events (Barnett and Rodgers 1994; Vial et al. 1996; Voccia et al. 1999). It is often difficult to predict how a given chemical will interact with the immune system since there are numerous factors that influence toxicity. For instance, external factors such as housing and handling of the animals, stress, exercise, and intrinsic factors such as hormonal status or genetic disposition make each case unique (Voccia et al. 1999; Descotes 2000). Also, feedback or compensatory mechanisms associated with immunoregulation could further complicate these predictions. In many instances, it is these compensatory mechanisms that trigger immune mediated diseases and cellular dysfunction (Voccia et al. 1999).

Although the obstacles are many, the adverse effects of xenobiotics on the immune system have become an increasingly diverse area of research. Pesticides and environmental contaminants are among the many compounds of interest that have the capability to induce various immune responses. For instance, studies have shown that chlordane, methylmercury, and carbofuran all induce immunotoxic effects on murine splenocytes *in vivo* (Johnson et al. 1987; Thompson et al. 1998; Jeon et al. 2001). As mentioned above, immunosuppression is a common result of chemical exposure and can be assessed by a variety of methods. In this study, the level of immune cell cytotoxicity and cell death mechanisms responsible for immunosuppression will be examined. Cytotoxicity refers to the cell-killing potential of a

chemical compound. In immune cells, cytotoxicity usually occurs via two specific mechanisms or modes of cell death, either apoptosis or necrosis (Wyllie et al. 1980; Darzynkiewicz et al. 1997; Boehringer-Mannheim 1998).

2.6e *Apoptosis and Necrosis*

Apoptosis and necrosis are two modes of cell death that have unique morphological, biochemical and physiological characteristics (Figure 2.8).

Necrosis is "accidental" cell death. It is a pathological process, which occurs when cells are exposed to a serious physical or chemical insult, such as in hypoxia, ischemia, temperature fluctuations, disruption of membrane structure and exposure to toxins. Necrosis begins with the impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Morphological characteristics include loss of membrane integrity and swelling of the cytoplasm, mitochondria and endoplasmic reticulum, which leads to cell rupture. Upon rupture, the contents of the cytosol, including lysosomal enzymes are released into the extracellular fluid. The major biochemical processes of necrosis are the loss of ion homeostasis, digestion of DNA and post-lytic random DNA fragmentation (late event). The physiological significance is that extensive tissue injury can result due to lysosomal enzyme action, inflammatory responses are likely to be triggered and phagocytosis by macrophages is increased leading to higher incidences of respiratory burst and ROS generation (Wyllie et al. 1980; Darzynkiewicz et al. 1997; Boehringer-Mannheim 1998). Apoptosis, in contrast, is a mode of cell death that occurs even under normal physiological conditions. It is considered "normal" cell death or a form of "programmed cell death." It is a process in which unwanted

or useless cells are eliminated during development and other normal biological processes. Most often it occurs during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system, endocrine dependent tissue atrophy, radiation and chemotherapeutic agents and via cell mediated immunity. Morphological features seen during apoptosis are membrane blebbing without a loss of membrane integrity, aggregation/condensation of nuclear chromatin, shrinkage of cytoplasm via cell dehydration, fragmentation of the cell into

smaller bodies, formation of membrane-bound vesicles (apoptotic bodies), loss of microtubules and mitochondrial leakage due to pore formation. The biochemical features are tightly regulated and ATP is necessary for apoptosis to occur. There is mobilization of intracellular ionized calcium, non-random mono- and oligonucleosomal fragmentation of DNA (ladder effect), release of factors (such as cytochrome-c) into cytoplasm from the mitochondria, caspase cascades are activated and alteration in membrane symmetry occurs (such as translocation of phosphatidylserine from cytoplasm to extracellular side of membrane).

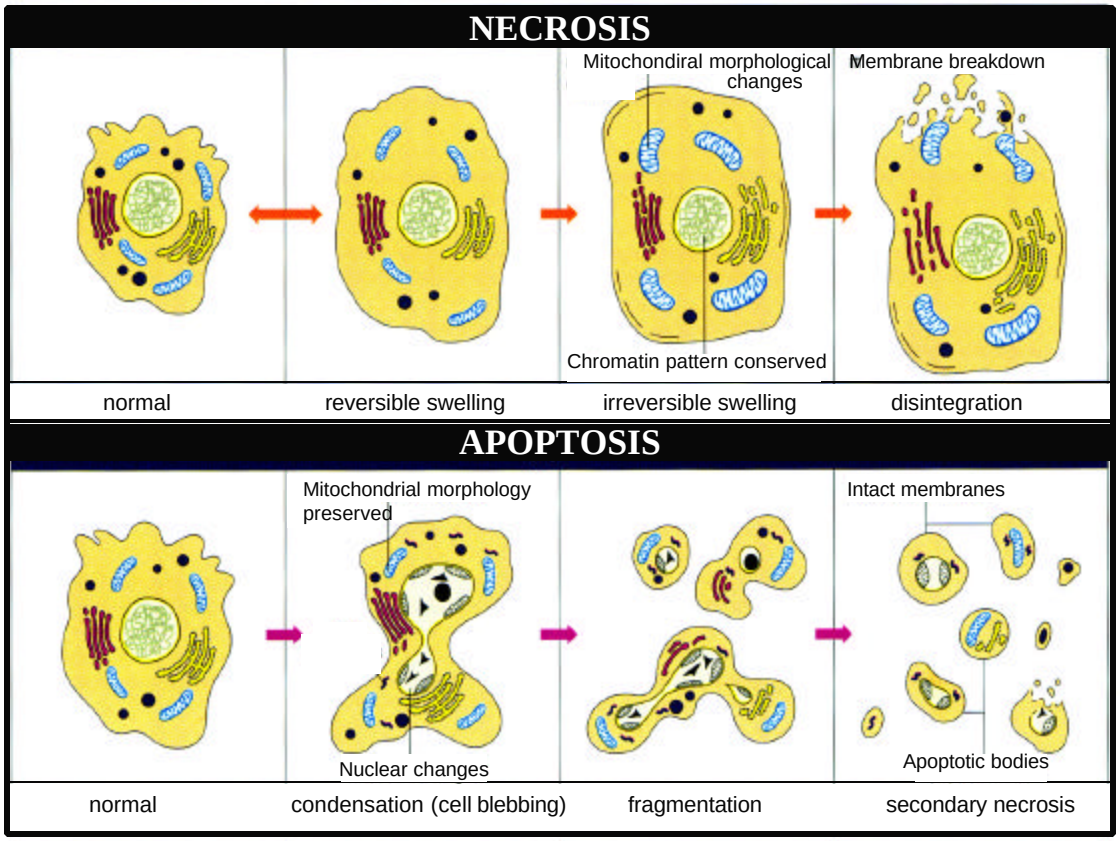

Figure 2.8. The morphological features of apoptosis and necrosis. Each mechanism of cell death has unique features. Necrotic cells have swelling of the mitochondria and total cell, the chromatin pattern is conserved and there is rupturing of the plasma membrane. Apoptosis on the other hand has dehydration of the cells resulting in cell shrinkage, mitochondrial morphology is conserved, nuclear changes are apparent and DNA fragmentation results. The membrane integrity remains intact even during fragmentation of the cell into smaller apoptotic bodies (Boehringer-Mannheim 1998, permission for use granted from Roche Biochemical Inc. by Mr. Neal Roach Jr., 2001).

The physiological processes characteristic of apoptosis are: occurrence to individual cells without much disruption to surrounding cells or tissue; induction by physiological stimuli (such as hormones or growth factors); phagocytosis of cell remains by macrophages is common; and lack of an inflammatory response (Wyllie et al. 1980; Cohen et al. 1992; Darzynkiewicz Z et al. 1997; Boehringer-Mannheim 1998). Another important feature, is

that not only is apoptosis dependent on ATP production, but it also must have ongoing protein and mRNA synthesis to occur (McConkey et al. 1994).

2.6f *Chemically-Induced Apoptosis*

Certain chemicals have been shown to induce immunotoxicity by triggering cell death via necrosis and/or apoptosis (Corcoran et al. 1994). Any significant loss of a cell population or particular cell type can be devastating to the proper functioning of an organ. There are numerous reasons why it is important to evaluate the level of apoptosis or necrosis in exposed cells. First, the two types of cell death differ markedly in context of how they develop and are two fundamentally different processes. Second, the occurrence and circumstances surrounding either event, permits inferences to be drawn regarding the intracellular mechanisms underlying them. Third, each event has different physiological implications by identifying predictable results of exposure (i.e. ROS generation, gene activation), compounds could be classified based upon toxic potential (Wyllie et al. 1980; Corcoran et al. 1994; Jacobson 1996).

The significance of identifying either event is a fundamental issue when addressing chemical exposures because under normal circumstances apoptosis is a natural event that occurs in living tissue by natural means, whereas necrosis is often a result of other mechanisms and is not induced by intrinsic cell factors. Although both events can occur in the same exposure system, induction of apoptosis could impair the steady-state kinetics of healthy tissues in an organism could be greatly impaired and result in unpredictable, deregulatory cellular responses (Wyllie et al. 1980; Corcoran et al. 1999). It should be mentioned, that a chemical

might have the potential to induce *both* apoptosis and necrosis in the same cell system (Corcoran et al. 1999).

It is suggested that immunotoxins stimulate apoptosis by exploiting the physiological mechanisms of the cell. Some of these suggested mechanisms include: the alteration of receptors on signal transduction proteins; the alteration of secondary messengers, such as calcium, cAMP, nitric oxide and other ROS; the alteration in gene expression; and the induction of DNA damage. Chemicals that have been found to induce apoptosis in immune cells are TCDD, glucocorticosteroids, ethanol, organotin compounds, gliotoxin and various chemotherapeutic agents (McConkey et al. 1994; Slukvin and Jerrells 1995;). In particular, ethanol, octylphenol, polychlorinated biphenyls and p-chloronitrobenzene have been implicated in inducing apoptosis in murine splenocytes (Slukvin and Jerrells 1995; Nair-Menon et al. 1996; Yoo et al. 1997; Li et al. 1999). The attempt of this study is to determine if lindane, PBO and malathion induce apoptosis and if so what type of response occurs when the chemicals are in mixture.

2.6g *Malathion Immunotoxicity*

Malathion exposure can be immunosuppressive or immunomodulating (enhancing). *In vivo* studies suggest cholinergic [1] doses, above 720 mg/kg, and noncholinergic doses, 0.1 mg/kg to 600 mg/kg, produce different effects. Cholinergic doses are proposed to stimulate physiological stress and enhance toxicity to malathion (Casale et al. 1983).

[1] Cholinergic doses stimulate acetylcholine's physiologic action in the cell and act as a neurotoxicant

Briefly, a summary of the potential immunological effects resulting from malathion exposure. *In vivo* exposure to malathion can suppress *or* enhance humoral immunity depending on dose or length of exposure. It has been reported that high (cholinergic) doses and prolonged exposure to low doses (subchouronic) suppressed humoral immune response (Casale et al. 1983; Barnett and Rodgers 1994; Banerjee et al. 1998). Another study reported that *in vivo* exposure to acute and subacute, noncholinergic doses of malathion elevated mouse humoral immunity and mitogenic proliferative responses (Rodgers et al. 1986a).

An *in vitro* study on murine splenocytes exposed to malathion (250 µg/ml) induced partial cytotoxicity. Also, purified malathion as low as 25 µg/ml suppressed cell mediated immunity by inhibiting cytotoxic T lymphocytes (CTL) response to alloantigens (Rodgers et al. 1985; Rodgers et al. 1986b). Addition of a NADPH fortified S-9 system to the in vitro system reversed the suppression of the CTL response as well as the increased levels of cytotoxicity (Rodgers et al. 1986b).

An established target of malathion's toxicity is adherent immune cell populations, such as macrophages, particularly to acute, noncholinergic doses. Malathion has been implicated in enhancing leukocyte functions and non-specific immune response by stimulation of mast cell degranulation. Mediators released during degranulation stimulate macrophage phagocytosis. Also, H_2O_2 generation is modulated, depending on *in vivo* or *in vitro* treatment with malathion. These finding have been cited for murine splenocytes, human PBMNC's and peritoneal murine macrophages (Rodgers and Ellefson 1990 and 1992; Pruett 1992; Barnett and Rodgers 1994; Rodgers and Xiong 1996; 1997a; 1997b; 1997c; 1997d). Another

important finding by Rodgers and Ellefson (1992) identified malathion's LOAEL and NOAEL for enhanced macrophage respiratory burst activity as 0.25 mg/kg and 0.1 mg/kg, respectively. Exposure to even very low concentrations of malathion may elevate nonspecific immune response.

Assessment of malathion toxicity has been difficult for many reasons. First, the effect of malathion *in vivo* and *in vitro* is inconsistant in many of the studies performed. Also, the dose and duration of exposure, as well as species used to study toxicity all have an impact on how immunity will be affected by malathion. Furthermore, the issue of metabolic activation of malathion to malaoxon as a necessary step for malathion toxicity is still being addressed (Rodgers and Ellefson 1990). A study in our laboratory has shown that malathion (99%) induced cytotoxicity of murine splenocytes at a more rapid rate than malaoxon (malathion's active metabolite) (unpublished data).

To date, there have been no published reports on apoptosis or necrosis in immune cells treated with malathion. A recent report revealed malathion was capable of inducing apoptosis in cultured human neuroblastoma cells (SH-SY5Y) (Carlson et al. 2000).

2.6h *Lindane Immunotoxicity*

Lindane exposure has been shown to repeatedly be immunosuppressive. For instance, when rats were fed 100 to 200 ppm, for 8 weeks, suppression of humoral immune response was observed (Koner et al. 1998). Also, when albino mice were fed 50 ppm lindane subchonically for 12 weeks, suppression of primary and secondary antibody response, as well as decreased

plaque formation was reported (Banerjee et al. 1996b). A study by Roux et al. (1978) reported decreased uridine uptake by RNA and reduced RNA synthesis after murine peritoneal macrophages were treated with 100 µM lindane, *in vitro,* for 12 hours. Also, a decreased rate of pinocytosis and ability of macrophages to phagocytize particles was observed. Concentrations of lindane above 63 µM (for 4 hour) caused a significant increase in arachadonic acid release from peritoneal macrophage membrane phospholipids of mice treated *in vitro,* indicating possible membrane disruption (Meade et al. 1984). Tithof et al. (2000) reported 100 µM lindane is capable of stimulating a phospholipase A_2 (PLA)-mediated release of arachadonic acid in rat neutrophils, *in vitro*. This event is also associated with increased in superoxide anion production. Production of ROS is a consequence of neutrophil PLA stimulation.

Exposure to low concentrations of lindane can result in a biphasic immune response, suggesting there is a period of stimulation followed by period of suppression (Meera et al. 1992). A 24-week treatment of mice with 0.012, 0.12, and 1.2 mg/kg of lindane in the diet, reported this type of biphasic response for delayed type hypersensitivity, IgM plaque formation and T cell mitogenic response. The study concluded there was a biphasic response for both cell mediated and humoral immunity. A follow-up to this study also reported that calcium uptake was significantly decreased during periods of immunosuppression and was increased during periods of immunostimulation. Calcium appears to have a role in the biphasic immune response reported previously (Meera et al. 1993).

Roux et al. (1979) treated stimulated human PBMNC with 100µM lindane *in vitro*. Mitogenic response was suppressed, as well as the ability of the PBMNCs to incorporate uridine. It appears lindane inhibits macromolecule biosynthesis in these human immune cells.

Since lindane is a contaminant of many water sources, a number of studies have examined the potential of lindane to disrupt fish immunity. For instance, immunosuppression was reported in rainbow trout injected (ip) with 50 or 100 mg/kg 41 and 46 days after dosing. A reduction in mitogenic lymphocyte proliferation, a decrease in phagocytic activity of blood neutrophils and a decrease in B-cell number, in the head kidney, was observed (Dunier et al. 1994). It should also be noted, however, no immunotoxic effect was observed when fish were fed lindane (1 mg/kg/day) for 30 days. Tilapian fish injected (ip) with lindane at 20 or 40 mg/kg for five days suffered a decrease in spleen total cellularity (white blood cells). No effect on phagocytosis was observed nor was any change in H_2O_2 production detected (Hart et al. 1997). Sweet et al. (1998) treated lake trout thymocytes with 10-100 µM *in vitro* for 6 to 24 hours. Over time thymocytes died via apoptosis, as well as via necrosis, from 60 to 100 µM lindane exposure as measured by the Annexin-PI staining assay. Thymocytes were also noted to undergo spontaneous apoptosis when placed in vitro conditions. It appears that in lake trout the time and temperature of treatment was a critical determinant in lindane toxicity.

There are currently no published studies on apoptosis or necrosis in mammalian splenic immune cells and lindane exposure.

2.6i *Piperonyl Butoxide Immunotoxicity*

Exposure to low doses of piperonyl butoxide has been implicated in stimulating hypersensitive immune response, particularly in atopic (history of allergic reactions) individuals. Prolonged or frequent exposure to PBO could even result in allergic disease (Diel et al. 1999a).

Diel et al. (1999b) obtained blood samples from atopic and nonatopic individuals. Mononuclear cells were isolated and dosed with 10 or 26 μM PBO. PBO (26 μM) inhibited lymphocyte proliferation, particularly in cells from the atopic individuals. Also, IL-4 and INF-gamma (immunologic cytokines) levels were modulated by PBO exposure. Basophil degranulation was also significantly increased (10μM PBO) for atopic individuals and only slightly in nonatopic individuals.

To date, no published studies on PBO's ability to induce of apoptosis or necrosis in immune cells has been published.

2.7 Reactive Oxygen Species, the Immune System and Apoptosis

Oxidative stress and apoptosis have both been associated with chemical exposures and toxicity. Reports have indicated that certain chemical exposures can result in the alteration of secondary messengers, such as free radicals or ROS, and these alterations have been linked to the induction of apoptosis in immune cells (McConkey et al. 1994; Corcoran et al. 1994). Free radicals influence gene expression, regulate cellular responses to cytokines, as well as proliferative events of a cell. All these events have also been implicated as possible

triggering mechanisms of apoptosis also. Although a direct link has been difficult to establish, the relationship between the two events cannot just be taken as coincidence as more evidence is gathered.

One link between ROS and immune system is a phenomenon known as respiratory burst. This is a process where phagocytic immune cells, such as neutrophils or macrophages, generate potent oxidant bactericidal agents, hypochlorous acid, $O_2^{-\bullet}$, H_2O_2 and $^{\bullet}OH$ to kill or destroy foreign molecules (Marks et al. 1996; Knight 2000). Macrophages also release ROS as signaling messengers to other immune cells (Knight 2000). It is possible that enhanced levels of ROS can lead to immune cell deregulation and result in apoptosis. Furthermore, Kobayashi et al. (1995) has proposed B-cells, and possibly NK cells and peripheral T cells, contain a superoxide generating system identical to that in phagocytes. Although the rate of $O_2^{-\bullet}$ generation is much lower than in phagocytic cells, the capability is present for these immune cells to generate ROS.

Other evidence supporting ROS/free radical involvement in apoptosis, is a study that indicated increased apoptosis in a murine cell line when H_2O_2 or menadione (Vit k-3) were added to cultures. Both are known stimulators of oxidative stress (Stoian et al. 1996). Also, when monocytes (phagocytic cells of the blood) were stimulated to release H_2O_2, apoptosis was induced in NK cells (Stoian et al. 1996).

Other findings have found the addition of antioxidant compounds or enzymes reduced the levels of apoptosis in cells. For instance, in one study mouse thymocytes were treated with

H_2O_2 (0.5-10 μM) and apoptosis was detected by the presence of a DNA Ladder. When Trolox, a Vitamin E analog and antioxidant, was added the amount of DNA fragmentation returned to control levels (Forrest et al. 1994). Apoptosis in human leukemic cells was significantly reduced when catalase was added to the cell culture prior to treatment to UV radiation exposure or low doses of chemotherapeutic agents (Gorman et al. 1997). Furthermore, murine thymocytes treated with dexamethasone (DEX), a known apoptotic agent, resulted in high levels (94%) apoptosis in culture. However, when catalase was added prior to DEX treatment, apoptosis was reduced by one-half (Torres-Roca et al. 1995). Yet in another study, rat thymocytes were treated with certain chemotherapeutic agents to trigger apoptosis. When the cells were pre-dosed with spin traps, such as 5,5-di-methyl-1-pyrroline-N-oxide (DMPO) or 2,2,6,6-tetra-methyl-1-piperidinyloxy (TEMPO), apoptosis was inhibited (Slater et al. 1995).

Disruption of cellular Ca^{2+} homeostasis has been linked to both the generation of ROS and increased apoptosis. Many enzymes, such as endonucleases, are activated by calcium levels. As noted in numerous studies, increased endonuclease activity results in DNA ladder formation, a hallmark of apoptotic cells (Corcoran et al. 1994; McConkey et al. 1994; Stoian et al. 1996). Also, rising intracellular Ca^{2+} concentration has been associated with increased ROS production and apoptosis in the case of TCDD (dioxin) exposure (McConkey et al. 1994).

A reduction in intracellular thiols, such as glutathione, has been reported with increased apoptosis. Glutathione is an antioxidant and scavenges ROS and free radical intermediates.

A decrease in the amount of thiols or reduction in the amount of reduced thiol can make cells vulnerable to increased levels of ROS or other free radical intermediate often resulting from chemical exposure (Buttke and Sandstrom 1994; Stoian et al. 1996).

There is a clearly established relationship between ROS/free radicals and apoptosis. Since ROS/free radical intermediates mediate many immune cell functions and apoptosis has been established in immune cell populations, it is likely these two events could arise simultaneously during certain chemical exposures. In the past decade a few studies have addressed ROS and/or apoptosis in immune cells. In particular, two different studies reported induced apoptosis in rat and murine thymocytes and concluded an association between the onset of apoptosis and the increase in ROS (Beaver and Waring 1995; Bustamante et al. 1997). Other studies treated thymocytes (murine and rat) with DEX and reported a relationship between increased ROS and apoptosis (Wolfe et al. 1994; Torres-Roca et al. 1996). A recent study, examined ROS and apoptosis in endothelial cells treated with menadione and reported a direct relationship between $O_2^{-\bullet}$ production and increased apoptosis (Warren et al. 2000).

In summary, these findings provide sufficient evidence for a relationship between ROS generation, immune cell regulation and apoptotic processes.

2.8 Chemical Mixtures

Exposure to multiple chemicals, either concurrently or sequentially, over the course of an organism's lifetime is likely. Chemicals are prevalent in every realm of the environment- in

food, water, soil, air and consumer products. Up until recently, about 95% of all chemical toxicity studies were performed on individual chemicals (Simmons 1995; Groten et al. 1999). Now, studies involving chemical mixtures are of interest due to concerns of occupational and public health (Simmons 1995).

In order to discuss interactions between chemicals in mixture, a common language or terminology must be established. Inconsistencies in the literature make it difficult to compare past studies because terms were loosely used. For this reason terminology is defined as described in *Casarett and Doull's Toxicology: The Basic Science of Poisons* (Eaton and Klaassen 1996).

When two chemicals are mixed and produce a stronger effect the term synergism, potentiation or additivity can be used. *Additivity* is when the combined effect of two chemicals is equal to the sum of the effect of each given agent alone (i.e. 2+2=4). *Synergism* is when the combined effect of two chemicals is greater than the sum of the effects of each agent given alone (i.e. 2+2=20). *Potentiation* occurs when one substance does not have a toxic effect on a certain organ or system but when added to another chemical makes that chemical much more toxic (i.e. 0+2=10). Also, potentiation, when not utilized to describe chemical interactions, refers to an enhancement or intensification of a particular event.

When two chemicals are combined and produce a weaker effect the term antagonism or inhibition are used to describe this interaction. *Antagonism* occurs when two chemicals are administered together and interfere with each other's actions or one interferes with the action

of the other (i.e. 4+6=8; 4+0=1). There are four classes of antagonism: functional, chemical (or inactivation); dispositional; and receptor (or blockers).

Each component of a mixture has a unique toxic potential and the ability to influence the toxicity of other components in the mixture by affecting toxicokinetic and toxicodynamic processes (Simmons 1995). Often when chemicals are in mixture, one compound will intensify the effect of other compounds by altering biotransformation processes, such as modulating certain metabolic enzymes or interrupting cellular receptors (Groten et al. 1999). In regard to the immune system, immunomodulation was reported when two compounds, with opposite effects on immune response, were mixed (Sharma and Reddy 1987). Recently, *in vitro* screening tests have gained popularity and are often the method chosen to screen a chemical mixture's toxicity due to their quickness, ease and reproducibility (Rodgers et al. 1986b; Groten et al. 1999). *In vitro* test are useful experimental tools but most must be supported by *in vivo* animal studies to be considered valid for public health conclusions.

In 1998, growing concern of chemical mixture exposures resulted in implementation of "Healthy People 2000" initiative by the USEPA. This initiative would move toxicologic studies away from the historical perspective of assessing individual environmental agents for solely mechanistic insight between human illness or toxicity and environmental exposure. Instead it challenged the scientific community to examine chemical mixtures and characterize their mechanisms of action. The initiative stressed the need for new experimental methods that could efficiently address chemical interactions and related health effects (USEPA 1998).

The urgency in the past decade to examine chemical mixtures may be due in impart to two events. The first was a study presented in 1996, which concluded mixtures of estrogenic-like compounds induced synergistic responses in yeast (Arnold et al. 1996). Although another study, in 1998, concluded inaccuracies in that report, the issue of chemical synergy became a topic of increased public concern (Arcaro et al. 1998). The second was the onset of the Gulf War Syndrome in the mid-1990's. Exposure to multiple chemicals was hypothesized to be the cause of this disorder. As a result, public concern about chemical mixtures and chemical interactions was a topic of increased discussion.

There have been studies published that addressed chemical mixture interactions but only one was performed on the immune system. This study dosed mice (ip) with a mixture of malathion/dieldrin (200 mg/kg:32 mg/kg) and examined the effects up to 21 days after treatment (Flipo et al. 1992). It was concluded that dieldrin alone suppressed humoral immunity, the rate of phagocytosis and antigen presentation of peritoneal cells. Malathion however enhanced humoral immunity and increased the rate of phagocytosis yet had no effect on antigen presentation of peritoneal cells. The malathion/dieldrin mixture reported a significant decrease in the level of antigen presentation by peritoneal cells. This indicated that malathion and dieldrin antagonized the effects of the other compound, at least in terms of the humoral immune response and rate of phagocytosis. It should also be noted the concentrations utilized in this study were high, being ½ the LD_{50} for each compound.

Briefly, in regard to the other mixture studies performed in other cell or organ systems. One study examined percutaneous exposure of a malathion/lindane mixture (200 mg/kg:50

mg/kg) for 30 days in guinea pigs. It was concluded that the mixture did not significantly change any parameters that were not already affected by the individual pesticide treatments and therefore the pesticide mixtures did not elicit any potentiation of effects in the guinea pig (Dikshith et al. 1987). Another study examined the effect of a malathion/carbaryl (4 mM: 4 mM) mixture on rat liver and kidney *in vitro*. In terms of liver glutathione and glutathione S-alkyltransferase levels, the mixture potentiated, or enhanced, the effect either pesticide had individually (Lechner and Abdel-Rahman 1986). A number of reports have examined PBO with other compounds, mostly because it always used in combination with other chemicals. One study examine the effect of PBO pre-treatment (1-1.25ppm) on malathion toxicity in fish and concluded that PBO altered the acute toxicity of malathion (LD_{50}) but the extent of alteration varied significantly among species (Shao-nan and De-fang 1996).

The studies on chemical mixtures are few and even fewer in regard to the immune system. More research on chemical mixtures is needed to gain a better understanding of multiple chemical toxicity.

2.9 Gaps in the Literature

There is a plenitude of published literature on individual pesticides and immunotoxicity, however, studies on lindane, PBO or malathion exposure, the induction of apoptosis or necrosis and murine splenocytes do not exist. One study, did examine lindane's effects on fish thymocytes and concluded apoptosis was induced in these immune cells (Sweet et al. 1998). Most of the literature specific to these chemicals and the immune system is focused on CMI or humoral immunity. Although these issues are very important in drawing

conclusions about a chemical's toxicity, it is also possible that apoptotic or necrotic events are involved as well. A few studies have actually examined the toxicity of other compounds, such as ethanol, octylphenol, PCB's and p-chloronitrobenzene, on splenocytes and indicated apoptosis was induced following exposure (Slukvin and Jerrells 1995; Nair-Menon et al. 1996; Yoo et al. 1997; Li et al. 1999). More studies on chemical exposure and splenocyte cytotoxicity are necessary to make general conclusions regarding chemically induced immunotoxicity.

Individual pesticide exposure and oxidative stress is another area that has been intensively studied for many years, yet no studies have specifically been performed to examine pesticide exposure and oxidative stress using murine splenocytes. A number of published reports have examined pesticide toxicity and oxidative stress using target organs, such as the liver, kidney or brain, or cell systems, such as hepatocytes and peritoneal macrophages, however not much research on the immune cells has been completed. At one time it was believed that mature immune cells, such as splenocytes, were resistant to apoptosis since they had escaped the clonal selection process. Perandones et al. (1993) indicated, however, that mature splenic T-cells are not resistant to apoptosis and Illera et al. (1993) concluded the same about mature B cells. In addition, the ability for apoptotic mechanisms to induce a series of signal transduction events, such as the generation of free radicals, upon toxic insult is very likely (Corcoran et al. 1994). A study that examined the effect of methylmercury (3 and 10ppm) *in vivo,* on mouse splenocytes following 4 weeks of treatment, indicated there was a reduction in the amount of reduced glutathione in the cells (Thompson et al. 1998). This study indicates that it is likely chemical exposure can induce oxidative stress in splenocytes. More

research in this area is needed to gain a better understanding of the consequences and mechanisms associated with mature splenic immune cell apoptosis, oxidative stress and chemical exposure.

As mentioned previously, there is a lack data on the effects chemical mixtures have on the immune system. Since, immune response to chemicals is a complex series of events it is difficult to make predictions about it's response to a chemical, and particularly to chemical mixtures. Although, immunotoxicity and individual chemical exposure is being studied more intensely more research must be done to explore the toxicity of chemical mixtures on the immune system. In particular, studies on mature splenic immune cells are limited and should be explored further.

This extensive literature review has offered a complete summary of issues important to environmental and immunotoxicology. It has also outlined areas that need more attention, in particular the relationship between oxidative stress, apoptosis and multiple chemical exposure. The objective and aims of this project will hopefully bridge this gap and answer questions regarding apoptotic or necrotic cell death pathways and oxidative stress in respect to xenobiotic exposure. Furthermore, this *in vitro* work could result in an accepted technique for screening pesticide mixture toxicity in cells.

2.10 Hypothesis, Rational and Objective

Our *hypothesis* is that ROS and/or other free radical intermediates, generated during the metabolism of various pesticides, mediate the immunotoxic effects of environmental xenobiotics in mixture.

Our *rational* is two-fold. *First*, it is predicted that two or more pesticides result in synergistic or antagonistic health effects, mainly because the metabolism of one will affect that of the other. *Secondly*, since free radicals have been suggested to enhance immune cell dysfunction and since exposure to certain insecticides has resulted in immunotoxicity, it is reasonable to assume these two events are related.

The overall *objective* of this study is to broaden our understanding of the mechanisms by which simultaneous exposure to multiple chemicals causes splenocyte dysfunction in a rodent model.

2.11 Specific Aims

1) To identify if certain pesticides and pesticide mixtures induce immunotoxicity via induction of apoptosis in splenocytes *in vitro*, and if so is this induction additive, synergistic or antagonistic for pesticide mixtures compared to individual pesticide treatments.
2) To investigate the role of reactive oxygen species in the above event by examining the pro-oxidant and antioxidant levels in splenocytes treated with pesticide and pesticide mixtures.

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Chapter III Immunotoxicity by Pesticide Mixtures: Potentiation of Apoptosis in Murine Splenocytes upon Concurrent Exposure to Pesticides

3.1 **Abstract**

Pesticides are used in every realm of the environment and exposure to multiple pesticides over the course of an organism's lifetime is very likely. Many reports have suggested that certain pesticides induce immunotoxicity, in some instances via apoptosis. Furthermore, studies have reported certain chemical exposures can induce oxidative stress. The *goal* of this study was to examine the relationship of pesticides on immunotoxic risk *in vitro*, as it pertains to oxidative stress. Specifically, the objective of this study was to identify if certain pesticide and pesticide mixtures induced cytotoxicity via apoptosis. The three insecticides of interest were lindane (an organochlorine), malathion (an organophosphate) and piperonyl butoxide (PBO; a synergist). All three pesticides were found to be cytotoxic to murine (C57BL/6) splenocytes *in vitro*. The cytotoxicity was both concentration- and time-dependent. Based on minimum cytotoxicity ($\leq LC_{25}$), the following concentrations were chosen for the pesticide mixture studies: 70 µM lindane (Lind), 50 µM malathion (Mal) and 55 µM PBO. In the AlamarBlue cytotoxicity assay an *individual* pesticide and *mixtures* of malathion/lindane (ML) and malathion/PBO (MP) prompted varying amounts of cytotoxicity (Mal 18.8%, Lind 20.4%, PBO 23.5%, ML 53.6% and MP 64.9%). Cytopathological analysis revealed apoptotic and necrotic features in cells treated with pesticides and pesticide mixtures and the DNA Ladder Assay confirmed the presence of DNA fragments, a hallmark of apoptosis. The specific mode of cell death was examined quantitatively via the 7-

aminoactinomycin D (7-AAD) Staining Assay, a flow cytometry assay that identifies apoptotic cells. Apoptosis was detected in each treatment (Mal 6.5%, Lind 12.0%, PBO 13.2%, ML 19.3% and MP 23.4%). Furthermore, to identify a subset of lymphocytes more susceptible to pesticides, 7-AAD staining in combination with fluorescent-labeled monoclonal antibodies, PE-CD45RB/220 (B-cell specific) and FITC-CD90 (T-cell specific), was performed. B-cells were more susceptible to Mal and PBO treatments than were T-cells. B or T lymphocytes responded similarly to the pesticide mixtures and comparable levels of apoptosis were observed. To conclude, splenocytes treated with these pesticides and pesticide mixtures were undergoing apoptosis, as well as a small degree of necrosis, and the effect elicited by the pesticide mixtures was additive compared to the individual pesticide treatments.

3.2 Introduction

Pesticides play a vital role in agricultural, industrial and residential pest control. They have offered the protection of crops in the field, thereby providing the society with abundant, inexpensive, wholesome, and attractive fruits and vegetables. Moreover, they have decreased public health concerns by reducing the level of vector-born diseases (Aspelin 1998). However, the widespread use and misuse of pesticides has created an awareness of the potential health hazards and the need to protect the consumer from residues in food. Humans can be exposed to a range of pesticides by accidental/suicidal poisoning, occupational exposure, bystander exposure to off-target drift from spraying and pesticide residues on agricultural produces. Additionally, humans and animals can be exposed to pesticides due to

environmental contamination of drinking water (both surface and ground water) and consumption fish from contaminated water reservoirs.

It is estimated that 85-90% of all pesticides applied never reaches their target organisms (Repetto and Baliga 1996). Based on such estimates, non-target organisms such as human and animals are likely to be exposed to a variety of pesticides creating potential health risks. In view of the widespread use and stability of some of pesticides in the environment, the potential of multiple chemical exposures, either simultaneously or sequentially, is extremely likely over the course of one's lifetime. Up until recently, most chemical studies were performed on individual chemicals (Groten et al. 1999). Research on chemical mixtures has increased due to an initiative set forth by the USEPA and NIEHS to promote a broader understanding of chemicals in mixtures and the mechanisms associated with multiple exposures (USEPA 1998). The pesticide mixture studies are of interest because exposure to multiple pesticides may alter the health effects, since the presence of one pesticide could influence the metabolism of the other.

The three pesticides: Lind, an organochlorine and mixed function oxidase (MFO) inducer (Konat and Clausen 1973; Barros et al. 1991; Junqueira et al. 1997), Mal, an organophosphate and acetylcholinesterase inhibitor (Chambers 1992; Barnett and Rodgers 1994; Immig 1998) and PBO, a synergist and MFO inhibitor (Jaffe et al. 1968; Goldstein et al. 1973). These pesticides are widely used in the public and industrial sector. They are commonly found in over the counter products, as well as in chemical mixtures used in agriculture, horticulture and in controlling pathogen carrying vectors (ExToxnet 1998).

Although, the toxicity of these pesticides is well documented in the literature, there is no evidence of what health effects result when they are in mixtures.

Many pesticides cause impairment or suppression of the immune system and are a risk to public health. The toxicity of these xenobiotics, singly and in combination, on the immune system, is the focus of this study. The immune reponse in governed by a series of delicately balanced, complex, multicellular physiological mechanisms that at times is vulnerable to very low levels of chemical toxicity (Burns et al. 1996; Voccia et al. 1999). One possible outcome of immunotoxicity is death of immune cells. There are two major mechanisms of cellular death: necrosis and apoptosis. Necrotic cell death is an unregulated, passive process resulting from severe damage to the cell that includes loss of membrane integrity, swelling of the cytoplasm and mitochondria leading to cell rupture. Upon rupture, lysosomal enzymes are released into the extracellular fluid creating extensive tissue injury and inflammatory response (Darzynkiewicz et al. 1997). In contrast, apoptosis is a highly regulated, energy-dependent process leading morphological changes such as plasma membrane blebbing, aggregation of nuclear chromatin and shrinkage of cytoplasm forming membrane bound vesicles (McConkey et al. 1994; Boehringer-Mannheim 1998). Biochemically, apoptosis is associated with non-random mono- and oligo-nucleosomal fragmentation of DNA, release of cytochrome c from mitochondria into the cytoplasm and alteration of membrane symmetry (such as phosphatidylserine flip-flop). Physiologically, individual cells undergo apoptosis without disrupting surrounding tissue, phagocytosis of cell-remains is common and there is usually no inflammatory response (Wyllie et al. 1980; Darzynkiewicz et al. 1997; Boehringer-Mannheim 1998).

Because the immune system plays a central role in the maintenance of an organism's health, the interaction of xenobiotics with various component of immune system has become an area of profound interest. Suppression of immune responses by certain pesticides has been suggested to be the basis of increased allergy, hypersensitivity and malignancy (Gleichmann et al. 1989). Therefore, there is an urgent need to obtain more information regarding the manner in which pesticide mixtures cause immunotoxicity. The present study examined the characteristics of pesticide-induced cell death, assessing a variety of criteria for apoptosis versus necrosis in cells. We present evidence that splenic immune cells in culture are susceptible to low doses of pesticides (Mal, Lind and PBO) and that pesticide mixtures produce an additive increase in the amount of both apoptosis and necrosis compared to the individual pesticide treatments.

3.3 **Materials and Methods**

3.3a *Animals*

Eight- to twelve-week old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were used for all experiments. The animals were maintained in accordance with U.S. Department of Health and Human Services Guide *for the Care and Use of Laboratory Animal*s (National Research Council, 1996). The animals were housed in polycarbonate cages with hard wood chip bedding in rooms in which the temperature (21 ± 2° C), humidity (50 ± 10%), and light cycle (12 h/12 h lights) were controlled.

3.3b *Chemicals*

Mal (purity 99%), Lind (purity 99.5%) and PBO (purity 98%) were purchased from Chem Services (West Chester, PA) and stock solutions (37.5 mM) were prepared using 100% denatured ethanol. A working solution was made from each stock solution using complete phenol red-depleted, RPMI-1640 media (Gibco BRL, Rockville, MD; 10% FBS [Atlanta Biologics, Norcross, GA], 2 mM L-glutamine, 0.1 mM non-essential MEM amino acids, 50 units/ml penicillin + 50 µg/ml streptomycin and 10 mM HEPES Buffer [Gibco BRL]) so that the final concentration of ethanol in reaction mixture would not exceed 0.3%. Pesticide solutions were made in 4X stock solutions and diluted with media *or* other pesticide solutions and cells. Appropriate controls were included in all experiments. These included: concavalin A (ConA; 10 µg/ml), a proliferative agent, (Sigma Chemical, St. Louis, MO) or dexamethasone (10 µg/ml) (DEX), an apoptotic agent (Sigma), or 10 mM hydrogen peroxide (H_2O_2), a necrotic agent at high doses, or ethanol 0.3% (EtOH), the pesticide solvent.

3.3c *Isolation of Splenocytes*

Isolation of the cells was performed as described in Ahmed et al. (1994a; 1994b). Immediately after the mice were sacrificed via cervical dislocation, the body weight (24.75 g $\pm$ 2.28) was measured and the spleen was removed aseptically. Quickly, the spleen weight (0.0754 g $\pm$ 0.025) was obtained and 10 ml of cold, incomplete phenol-red depleted RPMI-1640 media was added. The spleen was dissociated by *gently* teasing against a metallic 60-mesh wire sieve (Sigma). The cells were pelleted at 250 x g for 8 minutes at 4^{o}C (IEC Centra GP8R, International Equipment, Needham Heights, MA). The cells were resuspended in erythourocyte lysis buffer containing 1ml of cold complete phenol red-

depleted RPMI-1640 media and 3 ml cold ACK lysis buffer (0.15 M ammonium chloride, 1 mM potassium bicarbonate and 0.1 mM EDTA, pH 7.4; Sigma) (Ahmed et al. 1985; Gogal et al., 2000b). The cell suspension was incubated for 3 minutes on ice and diluted to 15 ml total volume with cold complete RPMI-1640 media. Cells were pelleted as previously described and washed once each with complete and incomplete media, respectively. The washed cells were resuspended in incomplete media and counted on a CASY I Cell Counter and Analyzer System (Scharfe Systems Gmblt, Reutlinger Germany). Cells were diluted to $5x10^6$ cell/ml and kept on ice until use. Final preparations routinely contained ~90% lymphocytes on examination of Wright's stained smears under microscope.

3.3d *AlamarBlue Cytotoxicity Assay*

The splenocyte cytotoxicity was monitored utilizing the fluorimetric indicator, AlamarBlue (Accumed International, Westlake, OH), which detects cellular metabolic activity. The greater the level of metabolic activity, or corresponding cell viability, the more reduction of the AlamarBlue dye. AlamarBlue data are presented as the percent decrease in fluorescence from the untreated cells, which has the greatest level of cell survival (McGahon et al. 1995; Zhi-Jun et al. 1997). The isolated cells were seeded at $5x10^5$ cells/well in a 96-well, U-bottomed tissue culture plate (Corning, Corning, NY). Treatments (100 µl/well) were added to the wells prior to cell seeding. Dose response, time response and mixture studies were performed using this assay.

After incubation, the cells were rinsed with PBS (Gibco BRL) at 37^{o}C and the 96-well plate was centrifuged at 250 x g for 8 minutes, at 25^{o}C. The media were removed and complete

RPMI-1640 media and AlamarBlue (1/10 of total well volume) pre-warmed to 37°C were added to each well. The plate was incubated for 24 hours (37°C, 5% CO_2, 95% humidity) and the fluorescence was measured on the Cytofluor™ II Multiwell Plate Reader (530 nm excitation, 590 nm emission and gain 35; Perspective Biosystems, Farmington, MA). Results were obtained as arbitrary fluorescence units and expressed as the percent decrease compared to the untreated cell mean percent (Ahmed et al. 1994a; deFries and Mitsuhashi 1995; Zhi-Jun et al. 1997). This was performed by obtaining the average values of the control wells (untreated) for each compound (C) and the raw fluorescence value of the treatment (T) and calculating the percent decrease compared to the untreated [(C-T)/C x 100%]. This value was a measure of the level cytotoxicity each treatment induced.

3.3e Cytologic Identification of Apoptotic Cells

Splenocytes ($5x10^5$/well) were treated with pesticides as described in A*lamarBlue Mixture Studies* using a 96-well U-bottomed plate (Lind 70 µM, Mal 50 µM, PBO 55 µM and mixtures of ML or MP). Additional treatments included untreated 0 hour (UNT), UNT 16 hours, EtOH 0.3% and 10 µg/ml DEX. After 16 hours, the plate was centrifuged (250 x g for 10 minutes at 4°C), the media were removed and the cells were rinsed with PBS. Cells were resuspended in 4 parts incomplete, RPMI-1640 media and 2 parts 0.5% BSA/PBS solution (Donner et al. 1999; Gogal et al. 2000a). Each cell suspension (two wells for each treatment or 1 x 10^6 cells) was added to a cytocentrifugation chamber (Sakura, Tokyo Japan). The chambers were centrifuged (17 x g for 5 minutes at 23°C; Cyto-TEK, Mile Scientific, Elkhart, IN). Slides were allowed to dry and then stained with Modified Wright Stain (Sigma). Coverslips placed on the slides after the addition of Permount (Fisher Scientific,

Pittsburgh, PA), were dried for 24 hours and examined under oil immersion light microscopy (250X) on the Olympus AH-2 Vanox-T Light Microscope. Ektachourome 64 slides (Kodak) were taken of representative cells (Donner et al. 1999; Gogal et al. 2000a). Apoptotic cells were identified based upon chromatin condensation, nuclear and membrane blebbing, cell shrinkage and apoptotic body formation. Necrosis was identified by excessive cell debris, cell ghost and cell swelling (McGahon et al. 1995).

3.3f *DNA Ladder Assay*

Splenic immune cells were treated as previously described in *Cytologic Identification of Apoptosis*. At 4 and 8 hours of treatment, 4 wells were combined per treatment into one sterile microcentrifuge tube. The wells were rinsed with 37^{o}C PBS and the rinses were added to the microcentrifuge tube. After centrifugation, the pellet was suspended in 200 µl of cold PBS. The procedures of the Apoptotic Ladder Kit were then followed (Boehringer Mannheim, Mannheim, Germany). Briefly, the cells were lysed, isopropanol was added, the samples were filtered thourough a glass fibered fleece, upon which the nucleic acids bind to the surface. Impurities were removed via two rinses and then the purified DNA was eluted. The samples were stored for up to two weeks in the –20^{o}C. A 1% agarose gel (with ethidium bromide) was prepared with Tris-Borate-EDTA Buffer (TBE), pH 8.0 and run at 75V for 45 minutes. The gel was viewed under UV light and the ethidium bromide/DNA complexes were visible. The presentation of a ladder pattern indicates an apoptotic cell population, a smear pattern suggests a necrotic population and a band at the top of the lane represents intact genomic DNA.

3.3g *7-amino-actinomycin D (7-AAD) Staining Assay*

7-AAD is a fluorescent, DNA-binding agent that intercalates between cytosine and guanine bases (Molecular Probes, Eugene, OR). The more disrupted and permeabilized the cellular membrane, the more 7-AAD that enters the cell and binds to the DNA. The principle is that 7-AAD staining intensity reflects the loss of membrane integrity. Since early apoptotic cells have more membrane integrity than late apoptotic/necrotic cells, the staining intensities should vary for these two populations. Typically, three degrees of staining intensity can be measured via flow cytometry and discriminate between live, early apoptotic and late apoptotic/necrotic cell populations (Schmid et al. 1994a; 1994b; Philpott et al. 1996; Donner et al. 1999).

Splenocytes ($5x10^5$/well) were treated with pesticides as described in *Cytologic Identification of Apoptosis*. After 16 hours of treatment, cells were rinsed with 37°C PBS and centrifuged at (250 x g, for 10 minutes at 4°C). The media were 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) was added to all wells except the unstained treatment, to which only supplemented buffer was added. The plate was placed on ice, in the dark and incubated no more than 30 minutes (Donner et al. 1999; Gogal et al. 2000a). Cells were processed on a Beckman-Coulter Epics XL/MCL flow cytometer (Hialeah, FL). Apoptotic cells were identified and quantified based upon the method described in Schmid et al. (1994a; 1994b). Briefly, the cells were first gated based upon forward scatter (or size) and side scatter (or granularity). These cells were then analyzed for their ability to take up the 7-AAD. The $7AAD^{dull}$ are the live cells, $7AAD^{moderate}$ are the early apoptotic and $7AAD^{bright}$

are the late apoptotic or necrotic populations. The values were reported as percentages of the total cells counted (n=5000 events).

3.3h *7-AAD Staining with Monoclonal Antibodies*

7-AAD's spectral properties allow it to used in combination with fluorescein-isothiocyanate (FITC) and R-phycoerythourin (R-PE) cell surface labels (Schmid et al. 1994a; 1994b). In this experiment, 7-AAD stain is used in combination with two monoclonal antibodies, one labeled with FITC and one with R-PE. These antibodies bind and label the cell type they are specific for and permit the quantitation of B and T lymphocyte populations in treated versus untreated samples. The antibody specific for T-cells, a FITC-conjugated rat anti-mouse CD90.2 (Thy 1.2) (BD Pharmingen, San Diego, CA), and for B-cells, a R-PE conjugated rat anti-mouse CD45R/B220 (BD Pharmingen), were incubated with splenocytes.

Splenic immune cells (~90% lymphocytes) were treated as described in the *7AAD Staining Assay*. Working solutions of: FITC-rat anti-mouse CD 90.2 (Thy-1.2); R-PE rat anti-mouse CD45R/B220; isotype FITC rat $IgG_{2a,kappa}$; and isotype R-PE rat $IgG_{2a,kappa}$ monoclonal antibodies were prepared, just prior to addition to the cells, in sterile PBS to a concentration equivalent to 1 µg/ 1 x 10^6 cells. Isotype matched negative controls for each fluorescent probe was employed for each experiment to assess non-specific binding. The cells were incubated with the proper isotype or antibody solution for 20 minutes at 4°C, in the dark, gently on an orbital shaker (Gogal et al. 2000b). The cells were rinsed with PBS, centrifuged (250 x g for 10 minutes at 4°C), and 7-AAD was added as described in the *7-AAD Staining Assay*. The cells were incubated for no more than 30 minutes at 4°C, in the dark, and

processed on a Coulter Epics XL/MCL flow cytometer (Hialeah, FL). The data were processed as described for the 7-AAD assay except this time the 7-AAD results were presented for the total cells, FITC positive (T-cells) and R-PE positive (B-cells) cells (Schmid et al.1994a; 1994b; Ahmed et al. 1994).

3.3i *Statistical Analysis*

SAS v. 8.0 software (SAS Institute Inc., Cary, NC) was used for data analysis. The data from each cytotoxicity assay was analyzed by utilizing pre-specified contrast determined during the experimental design. The Mixed Procedure allowed for comparisons between pre-specified treatments to determine whether statistical differences were evident. Experimental means were used to calculate the least square means (LSM) $\pm$ standard error of the LSM (SELSM). Data are presented as the mean $\pm$ SEM. If $p \leq 0.05$, the treatments *were* statistically different (or if $p > 0.05$ the treatments *were not* statistically different). The exception was the AlamarBlue time study, which was analyzed using the General Linear Model procedure.

3.4 **Results**

3.4a *AlamarBlue Cytotoxicity Assays*

3.4a.1 Dose Response for Cytotoxicity

A percent decrease in fluorescence corresponds to the percent cytotoxicity by each treatment. The following concentrations of each compound, extrapolated from the dose response curve,

induced 15 to 25% cytotoxicity ($\leq LC_{15\text{-}25}$): 50 µM Mal, 70 µM Lind and 55 µM PBO. These concentrations were also used in the mixture studies (Figure 3.1).

3.4a.2 AlamarBlue Time Response Mixture Assay

The AlamarBlue fluorescence was measured after 4, 8 and 16 hours of treatment. There was an increase in cytotoxicity thouroughout the 16 hours treatment period, particularly for the mixture treatments (Figure 3.2). There was increased cytotoxicity in the Lind (70 µM) and PBO (55 µM) treatments, however Mal (50 µM) induced no increase after 8 hour of treatment. The individual treatments *were* significantly different from the corresponding mixture treatments ($p \leq 0.05$).

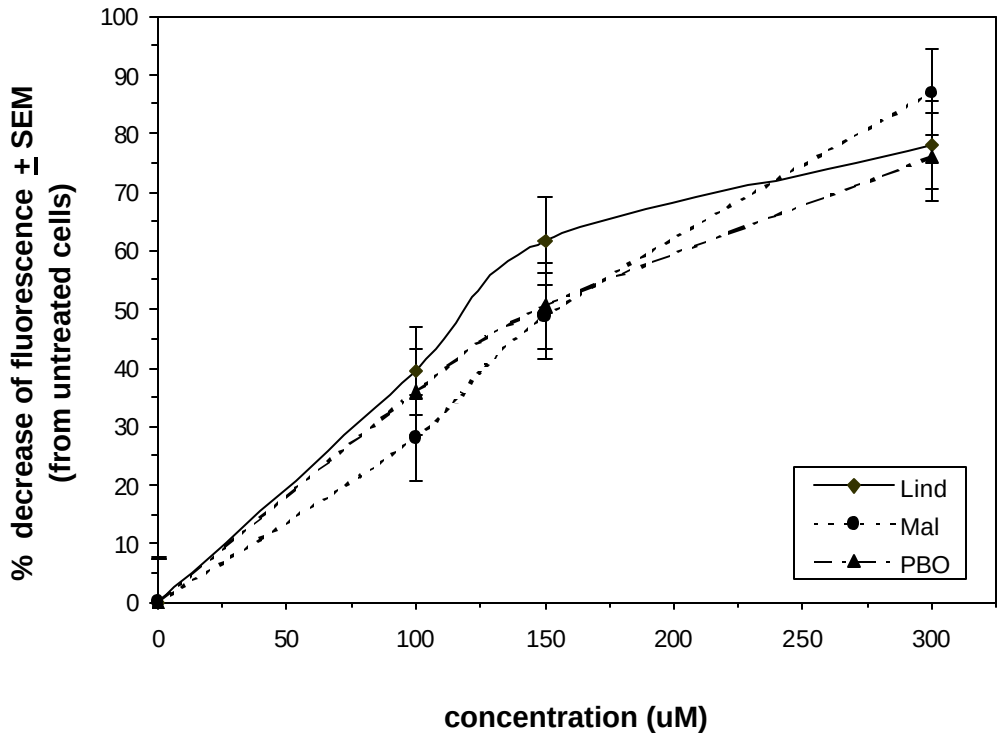

Figure 3.1. Dose response assays measuring the cytotoxicity of Mal, Lind and PBO (0 to 300 μM) were performed using the AlamarBlue Cytotoxicity Assay. C57BL/6 murine splenocytes were treated with 0, 100, 150 and 300 μM of each compound for 16 hours in a 96-well tissue culture plate. AlamarBlue was added to each well and 24 hours following, a fluorescence measurement was taken (Ex:530 nm, Em:590 nm). The raw arbitrary fluorescence units were converted to the percent decrease in fluorescence, as compared to the untreated cells. This value represents the percent cytotoxicity of each treatment.

Notes:
Data are presented as the mean ± SEM of 4 experiments (total of n =16 per treatment).
PBO 100 μM and PBO 150 μM *were not* statistically different ($p > 0.05$).
All other treatments for each specific compound *were* statistically different ($p \leq 0.05$).

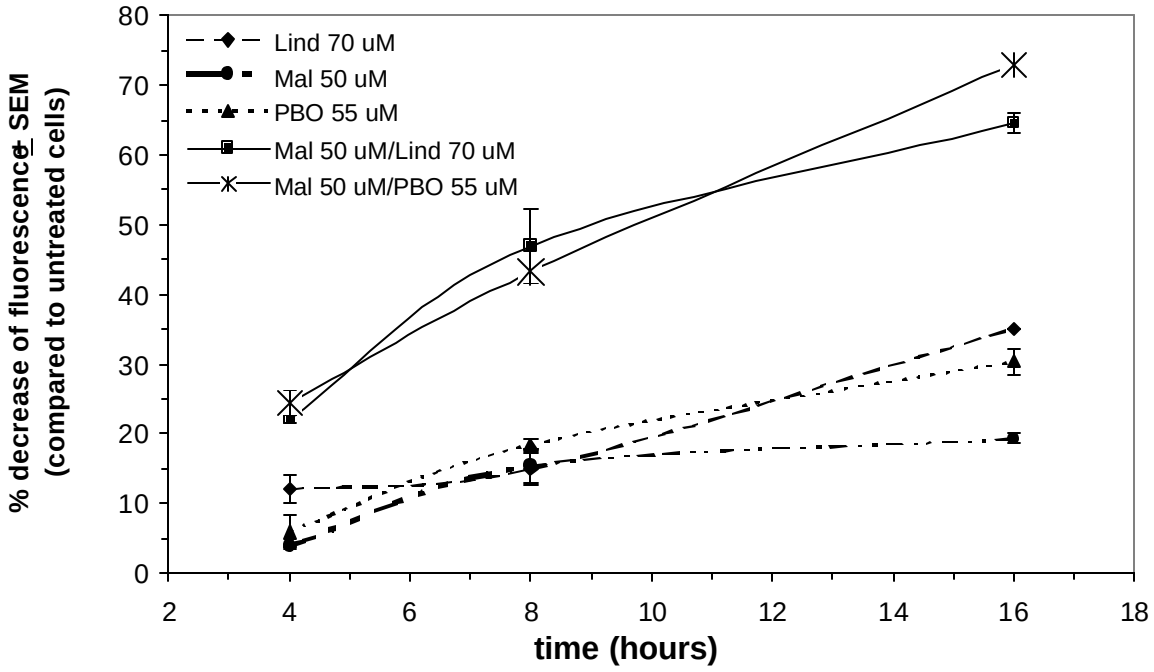

Figure 3.2. Time response of the cytotoxicity for pesticides and pesticide mixtures were performed using the AlamarBlue Cytotoxicity Assay. C57BL/6 murine splenocytes were treated with Mal 50 μM, Lind 70 μM, PBO 55 μM, ML or MP for 4, 8 and 16 hours. The % decrease in fluorescence was calculated as described for Figure 3.1.

Notes.
Data are presented as the mean ± SEM of one experiment (total n = 4 per treatment per time point with the exception of the untreated n = 8).

3.4a.3 AlamarBlue Cytotoxicity Assay: Mixture Study

Cytotoxicity was significantly greater in the mixture treatments (ML 53.6% and MP 64.5%) than in the individual pesticide treatments (Mal 18.8%, Lind 20.4% and PBO 23.5%; $p \leq 0.0001$; Figure 3.3).

3.4b *Cytologic Identification of Apoptosis*

Apoptotic and necrotic features were present in many of the treatments. The untreated (Unt) 0 hour cells were rounded, uniform in shape and intensely stained, characteristic of healthy cell population (A; Figure 4a). The Unt 16 hours (B) cells had some loss of staining uniformity and intensity compared to 0 hour. Also, cell debris and lysed cells were present, characteristic of necrotic cells. The DEX 10 µg/ml (C), a positive control for apoptosis, had significant apoptotic features, such as cell blebbing, cell shrinkage and apoptotic body formation. As expected, necrotic cells were also present. Mal 50 µM (D; Figure 4a), Lind 70 µM and PBO 55 µM had some cellular irregularities with only a few observations of apoptotic cells (E; F; Figure 4b). These observations included membrane perturbations and cell shrinkage. PBO treated cells had indications of cell lysis and membrane blebbing, as well as phagocytosis of a likely apoptotic cell. Comparatively, the pesticide mixtures contained increased numbers of apoptotic cells and cell disruption than the individual treatments. ML treated cells had more lysis and membrane perturbations, as well as cell shrinkage and apoptotic body formation than the individual pesticide treatments. The MP slides showed significant signs of cell swelling and debris, characteristic of necrosis. However, there were also significant indications of apoptosis, such as cell shrinkage, blebbing, chromatin condensation and fragmentation (G; H; Figure 4b).

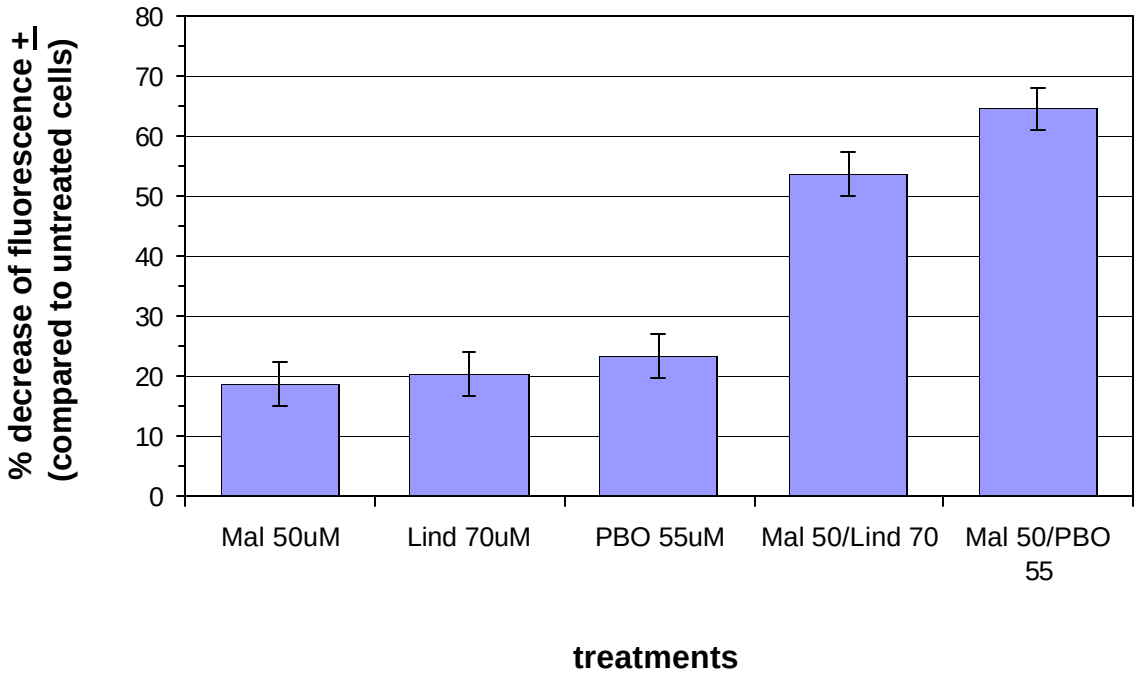

Figure 3.3. Cytotoxicity induced by pesticides or pesticide mixtures was assessed using the AlamarBlue Assay. C57BL/6 murine splenocytes were treated with Mal 50 µM, Lind 70 µM, PBO 55 µM, ML or MP for 16 hours. The percent decrease in fluorescence was calculated as described in Figure 3.1.

Notes. Data are presented as the mean ± SEM of 5 experiments (n = 4/treatment/experiment with the exception of the untreated n =12/experiment). The individual pesticide treatments *were* statistically different than their corresponding mixture treatments ($p \leq 0.0001$).

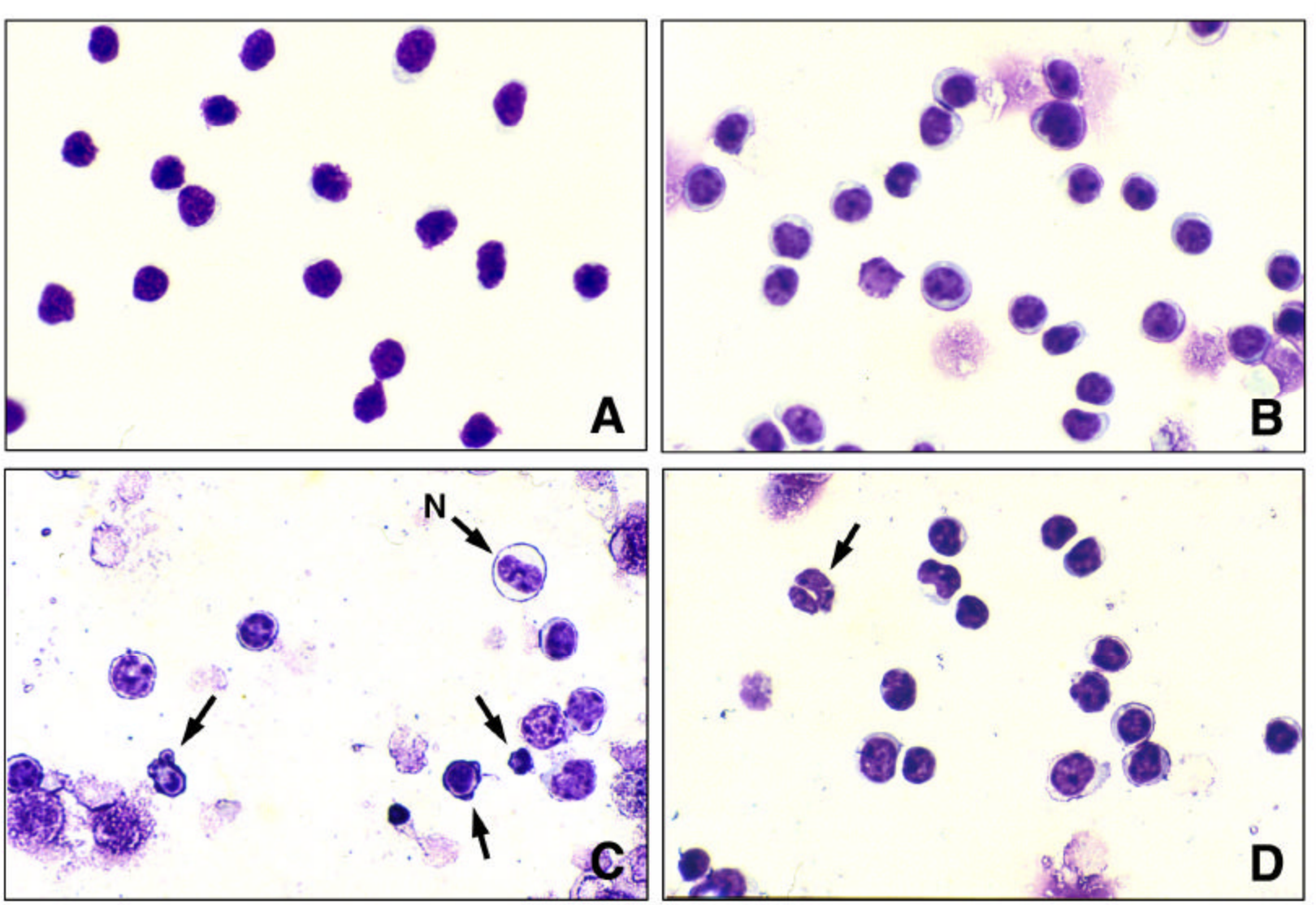

Figure 3.4a. Cytologic identification of apoptotic or necrotic features in C57BL/6 murine splenocytes treated with pesticides and pesticide mixtures for 16 hours. (A) Unt 0 hour (B) Unt 16 hours (C) DEX 10 µg/ml (D) Mal 50 µM. magnification = 250x; ➔ = apoptotic cells; **N** = necrotic features.

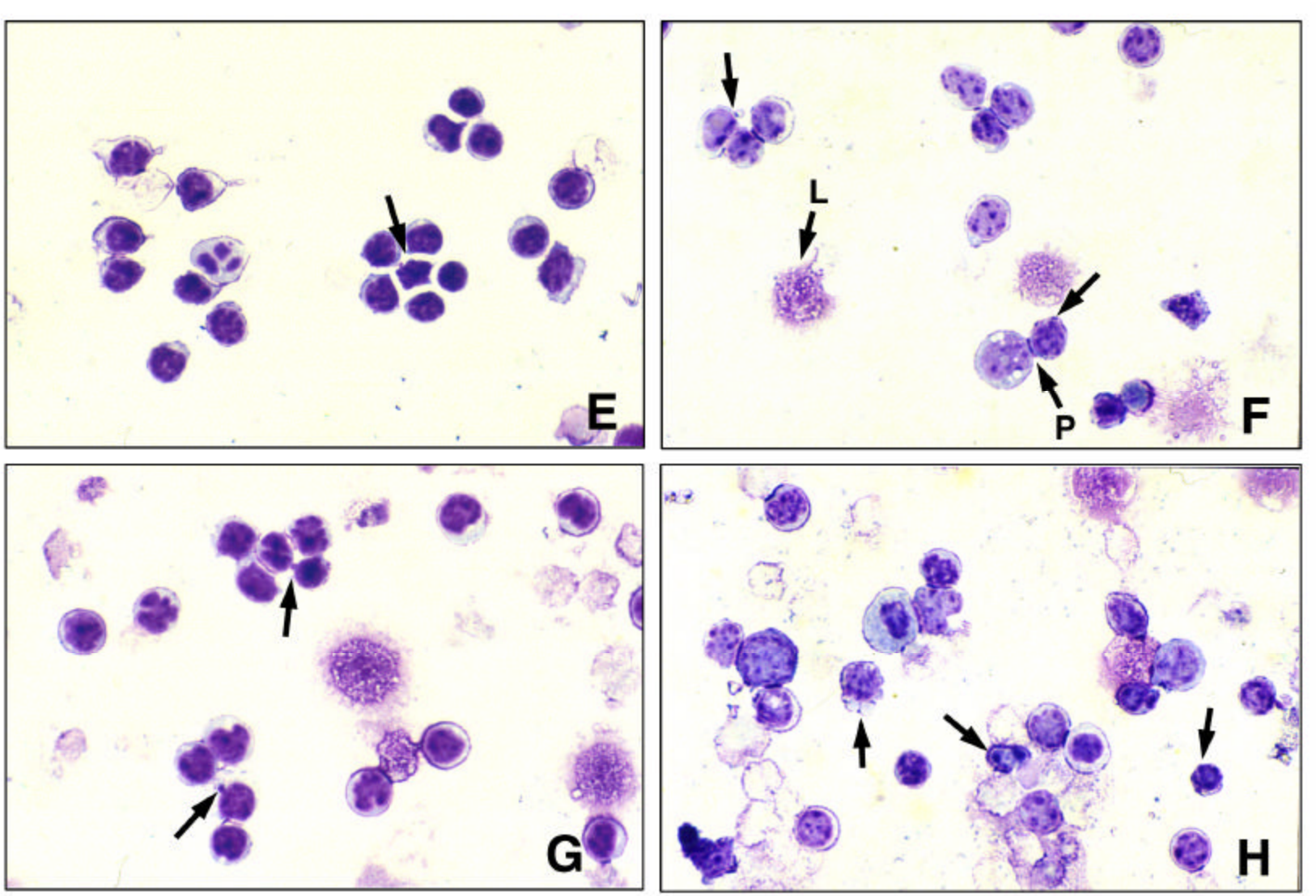

Figure 3.4b. Cytologic identification of apoptotic or necrotic features in C57BL/6 murine splenocytes treated with pesticides and pesticide mixtures for 16 hours. (E) Lind 70 µM (F) PBO 55 µM (G) ML and (H) MP. magnification = 250x; ➔ = apoptotic cells; **P** = phagocytosis; **L** = lysis.

3.4c DNA Ladder Assay

Splenocytes were treated with pesticides for 4 and 8 hours. By 4 hours of treatment, a DNA ladder was evident in all lanes except the Unt 0 hour (Figure 3.5). The DEX 10 μg/ml, ML and MP had vivid ladder formation. By 8 hours the ladder bands increased in intensity for all treatments, suggesting an increase in apoptosis with time. At each time point, the mixtures were comparable in intensity to the DEX treated. A smearing effect was present in many of the sample lanes, suggestive of the presence of randomly cleaved DNA.

3.4d *Flow Cytometric Analysis with 7-AAD*

Experimentally, the 7-AAD staining assay was performed to examine the percent of early apoptotic and dead (or necrotic/late apoptotic) cells in each treatment. A representative of the flow output is in Appendix A Figure A.1. At 0 hour the cell population was gated on and this gate was monitored after 16 hours of treatment for each sample. Any shift in the forward scatter/side scatter positioning of the cells was identified and the percent of cells remaining in the gate was measured (V-gate; Figure 3.6). The untreated had the greatest population in the gate (36.8%) after 16 hours, whereas the pesticide mixtures (ML 18.2%; MP 13.2%) and DEX (11%) had the least. The MP was not statistically different from the DEX treatment, nor was the EtOH and Unt 16 hours samples ($p > 0.05$).

The percent of live, early apoptotic and dead cell populations was also obtained from the output. From the early apoptotic data, the mixtures induced a greater increase in apoptotic cells than did the individual treatment, however, all were significantly greater than the untreated ($p \leq 0.05$; Figure 3.7). By subtracting the control (Unt 16 hours) percent from the pesticide or pesticide mixture treatments, the increase in apoptosis above the Unt 16 hours

sample untreated was as follows: Mal 6.5%, Lind 12.0%, PBO 13.2%, ML 19.3% and MP 23.4%.

In Table 3.A, the percent of cells for each staining intensity, or that were live, early apoptotic or dead for each treatment, are listed. The live cell populations were greatest for the Unt 16 hours (52.4%) and least for the DEX (17.5%), ML (30.6%) and MP (25%). The percent of dead cells was similar across most treatments.

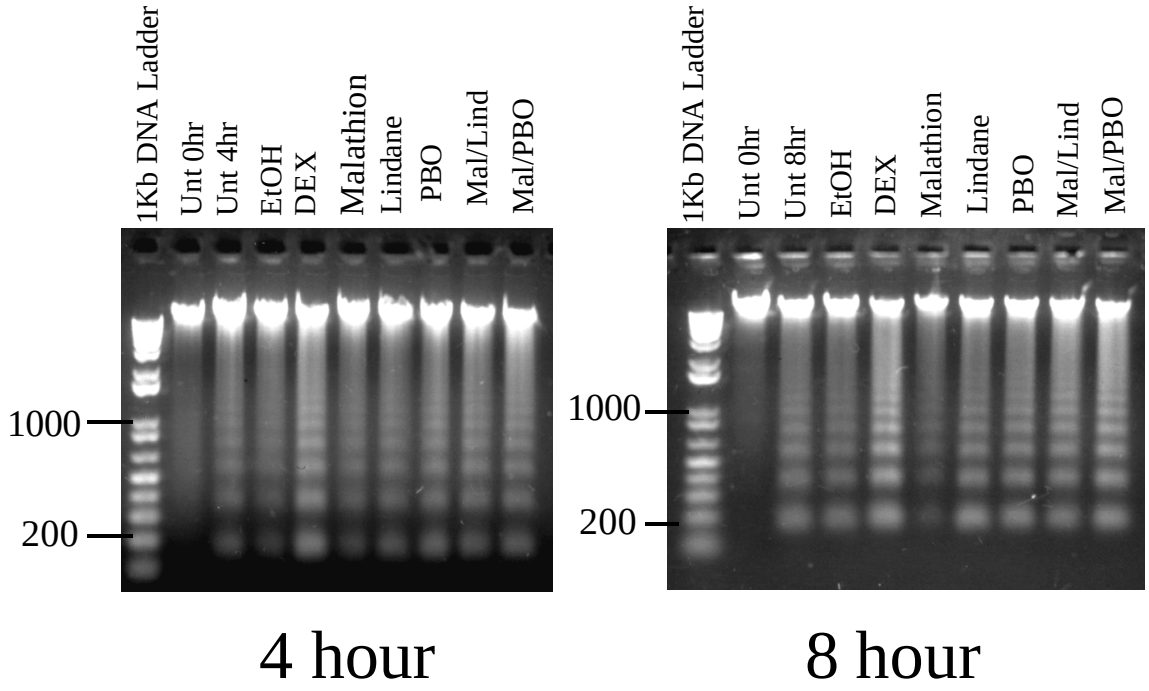

Figure 3.5 Detection of a DNA Ladder in C57BL/6 murine splenocytes treated for 4 or 8 hours with pesticide or pesticide mixtures. The treatments included: Unt 0 hour, Unt 4 hours or 8hours, EtOH 0.3%, DEX 10µg/ml, Mal 50 µM, Lind 70 µM, PBO 55 µM, ML or MP. Samples were prepared using the Apoptotic Ladder Kit and run on a 1% agarose gel with TBE buffer, pH 8.0.

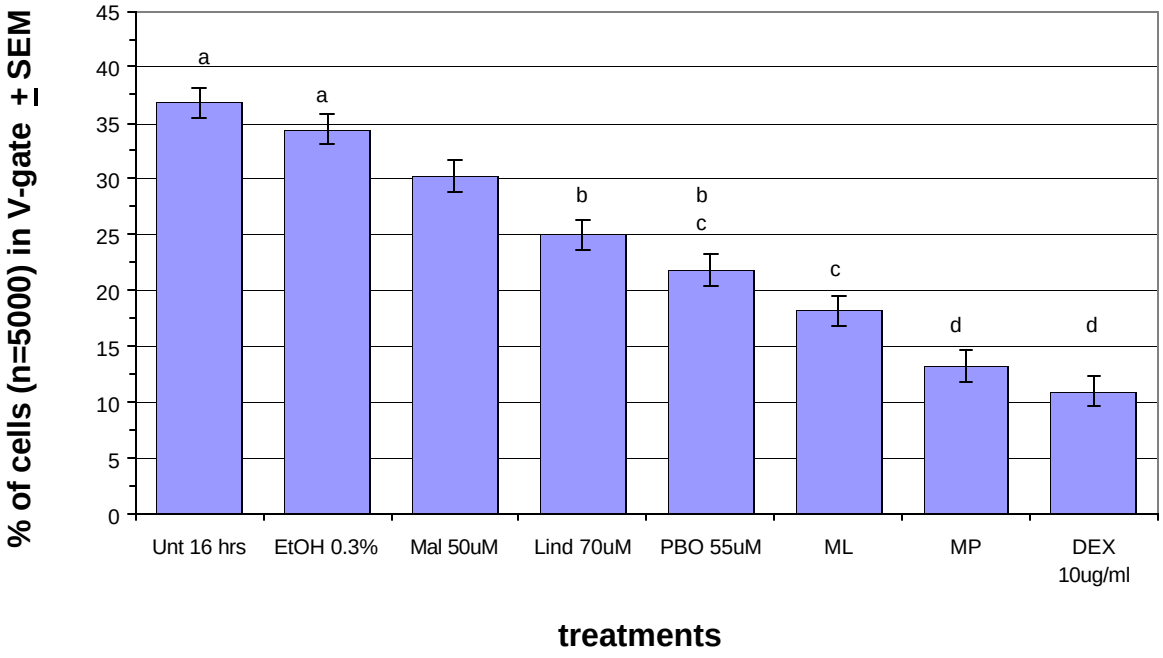

Figure 3.6. The percent of cells in the designated V-gate following treatment with pesticides or pesticide mixtures was assessed using flow cytometry. C57BL/6 murine splenocytes were treated with Mal 50 µM, Lind 70 µM, PBO 55 µM, ML or MP for 16 hours. An Unt 16 hours, EtOH 0.3% and DEX 10 µg/ml were also performed. At 0 hour, a gate (V-gate) was drawn around the majority of the cell population. The percent of cells in this V-gate following treatment was measured by analyzing cell size (forward scatter) and granularity (side scatter) for each sample. The mean percent ± SEM is shown.

Notes. Treatments with *similar* letters *were not* significantly different ($p > 0.05$). All other treatments *were* significantly different ($p \leq 0.0005$). Values are the mean $\pm$ SEM of 9 experiments (total of n = 20 per treatment).

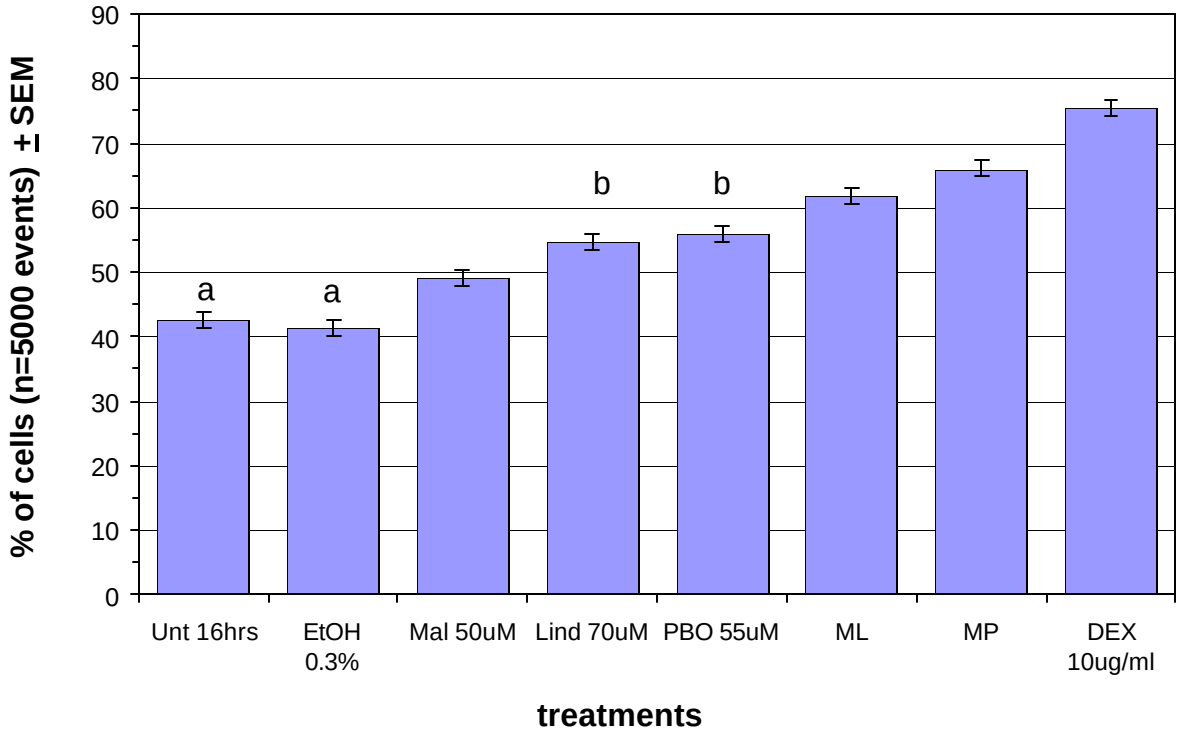

Figure 3.7. The percent of early apoptotic cells following treatment with pesticides or pesticide mixtures was measured on the flow cytometry using the 7-AAD Staining assay. C57BL/6 murine splenocytes were treated with Mal 50 μM, Lind 70 μM, PBO 55 μM, ML or MP for 16 hours. An Unt 16 hours, EtOH 0.3% and DEX 10 μg/ml were also performed. Following treatment 10 μg/ml 7AAD-DNA binding dye was added. Samples were examined via flow cytometry for three degrees, or intensities, of staining, $7AAD^{dull}$ (live cells), $7AAD^{moderate}$ (early apoptotic cells) and $7AAD^{bright}$ (dead/late apoptotic). The percent of cells (of n = 5000 events) stained $7AAD^{moderate}$,the early apoptotic cell population is shown. Data are presented as the mean $\pm$ SEM. The percent of live and dead cell populations is summarized in Table 3.A.

Notes. Treatments with similar letters were *not* significantly different ($p > 0.05$). All other treatment comparisons *were* statistically different ($p \leq 0.0001$). Values are the mean $\pm$ SEM of 9 experiments (total of n = 20 per treatment).

TABLE 3.A. The percent of live, early apoptotic or dead cells following treatment with pesticides or pesticide mixtures as assessed by the 7-AAD Staining assay. C57BL/6 murine splenocytes were treated with Mal 50 μM, Lindane 70 μM, PBO 55 μM, ML or MP for 16 hours. An Unt 16 hours, EtOH 0.3% and DEX 10μg/ml were also performed. The percent of cells (of n = 5000 events) stained as $7AAD^{dull}$ (live), $7AAD^{moderate}$ (early apoptotic) and $7AAD^{bright}$ (dead) is presented. Data is the mean ± SEM.

	Cell Status		
Treatments	live	early apoptotic	dead
Untreated	52.4 (± 1.53)[a]	42.6 (± 1.25)[A]	4.94 (± 0.73)
EtOH 0.3%	52.3 (± 1.53)[a]	41.3 (± 1.25)[A]	6.29 (± 0.73)
Mal 50uM	45.0 (± 1.53)	49.1 (± 1.25)[B]	5.91 (± 0.73)
Lind 70uM	36.5 (± 1.53)[b]	54.6 (± 1.25)[B]	7.06 (± 0.73)
PBO 55uM	37.7 (± 1.53)[b]	55.8 (± 1.25)	6.45 (± 0.73)
Mal 50uM/Lind 70uM	30.6 (± 1.53)	61.9 (± 1.25)	7.50 (± 0.73)
Mal 50uM/PBO 55uM	25.0 (± 1.53)	66.0 (± 1.25)	8.96 (± 0.73)
DEX 10 ug/ml	17.5 (± 1.53)	75.4 (± 1.25)	7.17 (± 0.73)

Notes. Statistical comparisons were made for all treatments within each cell status. Treatments with similar letters *were not* significantly different ($p > 0.05$). All other treatment comparisons were significantly different ($p \leq 0.0001$). Statistical analysis of dead cell populations was not performed. Values are the mean ± SEM of 9 experiments (total of n = 20 per treatment).

3.4e *Flow Cytometric Analysis with 7-AAD and Monoclonal Antibodies*

The 7-AAD Staining Assay was performed in combination with fluorescently labeled monoclonal antibodies, R-PE:CD45R/B220 and FITC:CD90.2, specific for B cells and T cells, respectively. A representative of the flow output is in Appendix B Figure B.1. Cells were sorted for being FITC or R-PE positive, then the live, early apoptotic and dead cell populations were identified for both B (R-PE +) and T (FITC +) cells. The B and T cell populations were statistically compared for each treatment. The early apoptotic cell results indicated B cells had higher levels of apoptosis in the Unt 16hours, EtOH, Mal and PBO treatments than did T cells (Figure 3.8; $p \leq 0.05$). However, there were no significant differences between B and T cell populations for Lind, ML, MP or DEX treatments ($p > 0.05$).

The 7AAD Staining results, the percent of live, early apoptotic and dead cell populations, for B cells (R-PE:CD45R/B220 positive) and T cells (FITC:CD90.2 positive) are listed in Table 3.B. In the Unt 16 hours sample, 46.4% of B cells were live, or viable. All other treatments had reduced B cell viability compared to the Unt, particularly the pesticide mixtures. The Unt 16 hours had the lowest percent of early apoptotic B cells (44%) and this percent increased for all other treatments (with the exception of EtOH). DEX had the greatest level of apoptotic B cells (70%). The EtOH 0.3% and Unt 16 hours *were not* statistically different ($p > 0.05$) in terms of the live or early apoptotic cell populations. Furthermore, PBO *was not* statistically different from the MP mixture for either the live or early apoptotic cell population ($p > 0.05$). Mal also was not statistically different from the corresponding mixture treatments, ML and MP, for the early apoptotic cell population ($p > 0.05$). ML and MP

elicited the same percent of apoptosis in B cells. Lind and MP had the greatest amount of dead cells (19%), while the Unt had the lowest level (9.5%).

In the Unt 16 hours sample 57.8% of T cells were live, or viable (Table 3.B). All other treatments had reduced cell viability compared to the Unt (except Mal and EtOH). As with the B cells, the loss of live cells was most severe for the pesticide mixtures and DEX. The percent of early apoptotic T cells followed the same trend as B cells. The individual pesticide treatments were statistically different than the pesticide mixtures in the live and early apoptotic cell populations ($p \leq 0.05$). EtOH was not statistically different from the untreated for either the live or early apoptotic cells ($p > 0.05$). The MP treatment had the greatest amount to dead cells (20.6%) while the untreated had the lowest (7.35%).

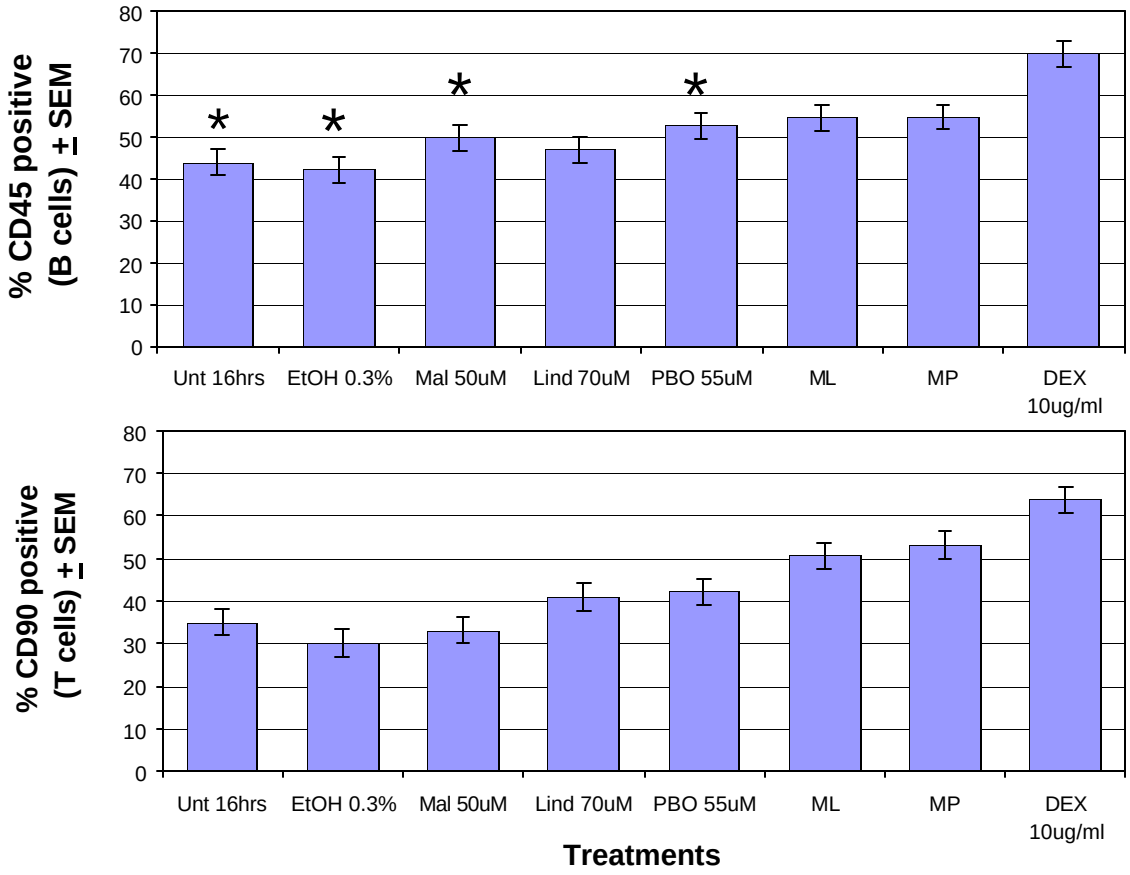

Figure 3.8. The percent of early apoptotic T (CD90.2 positive) and B (CD45R/B220 positive) lymphocytes following treatment with pesticides or pesticide mixtures as assessed by the 7-AAD Staining assay. C57BL/6 murine splenocytes were treated with Mal 50 µM, Lind 70 µM, PBO 55 µM, ML or MP for 16 hours. An Unt 16 hours, EtOH 0.3% and DEX 10µg/ml were also performed. Following treatment, fluorescently labeled monoclonal antibodies (1 µg/1x10^6 cells; R-PE:CD45R/B220 and FITC:CD90.2) were incubated with the cells for 20 minutes on ice, in the dark. Antibodies solutions were rinsed away and 10 µg/ml 7AAD-DNA binding dye was added. The cells were analyzed by flow cytometry for FITC or R-PE fluorescence then the 7AAD staining intensity for each subset of lymphocytes was measured.

Notes. B and T cell populations for each treatment were statistically compared. The B cell treatments labeled with * had significantly higher levels of apoptosis than did the corresponding T cell population for that same treatment ($p \leq 0.05$). No statistically significant differences between B and T cell populations any of the other treatments was observed ($p > 0.05$). All values are the mean percent of apoptotic cells ± SEM for 6 experiments (total of n = 14 per treatment).

TABLE 3.B. The percent of live, early apoptotic and dead B cell (R-PE:CD45R/B220 positive) and T cells (FITC:CD90.2 positive) as assessed by the 7AAD Staining assay. C57BL/6 murine splenocytes were treated and analyzed as described in Figure 3.8.

	B Cell Status		
Treatments	CD45+:live	CD45+:early	CD45+:dead
untreated	46.4 ± (1.93)^{a}	44.0 ± (1.87)^{A}	9.50 ± (1.33)
EtOH 0.3%	44.2 ± (1.93)^{a}	42.3 ± (1.87)^{A}	13.5 ± (1.33)
Mal 50uM	36.8 ± (1.93)^{b}	50 ± (1.87)^{ABC}	13.0 ± (1.33)
Lind 70uM	33.9 ± (1.93)^{bc}	47 ± (1.87)^{AB}	19.0 ± (1.33)
PBO 55uM	30.2 ± (1.93)^{cd}	52.7 ± (1.87)^{C}	17.0 ± (1.33)
Mal 50uM/Lind 70uM	28.0 ± (1.93)^{de}	54.7 ± (1.87)^{CD}	17.3 ± (1.33)
Mal 50uM/PBO 55uM	26.2 ± (1.93)^{de}	54.9 ± (1.87)^{CD}	18.9 ± (1.33)
DEX 10 ug/ml	15.9 ± (1.93)^{f}	70.0 ± (1.87)^{E}	14.6 ± (1.33)
	T Cell Status		
Treatments	CD90+:live	CD90+:early	CD90+:dead
untreated	57.8 ± (3.29)^{a}	34.9 ± (3.08)^{A}	7.35 ± (2.43)
EtOH 0.3%	60.3 ± (3.29)^{a}	30.2 ± (3.08)^{A}	9.58 ± (2.43)
Mal 50uM	58.8 ± (3.29)^{a}	33.0 ± (3.08)^{A}	8.15 ± (2.43)
Lind 70uM	44.5 ± (3.29)^{b}	41.0± (3.08)^{A}	14.5 ± (2.43)
PBO 55uM	46.6 ± (3.29)^{b}	42.2 ± (3.08)^{A}	11.2 ± (2.43)
Mal 50uM/Lind 70uM	35.1 ± (3.29)^{c}	50.8 ± (3.08)^{B}	14 ± (2.43)
Mal 50uM/PBO 55uM	26.2 ± (3.29)^{cd}	53.1 ± (3.08)^{B}	20.6 ± (2.43)
DEX 10 ug/ml	20.8 ± (3.29)^{d}	63.8 ± (3.08)^{C}	15.5 ± (2.43)

Notes. B and T cell populations were statistically analyzed separately. Treatments with *similar* letters *were not* significantly different ($p > 0.05$). All other treatment comparisons were significantly different ($p \leq 0.05$). Statistical analysis on the dead cell population is not presented.

3.5 **Discussion**

Low concentrations of Mal, Lind, PBO and mixtures of ML and MP had the potential to induce splenocyte cytotoxicity *in vitro* as noted in the AlamarBlue Assay. The pesticide mixtures, ML and MP, induced an additive increase in cytotoxicity compared to the corresponding individual treatments. To further identify the specific type of cell death as apoptotic or necrotic, three different assays were performed: cytologic identification of apoptotic or necrotic features, DNA ladder formation and 7AAD staining. Each experiment indicated a small degree of necrosis in all treatments, particularly the ML and MP. However, there was also a significant amount of apoptotic cells detected in each treatment. Furthermore, the ML and MP mixtures additively enhanced apoptosis compared to the corresponding individual pesticide treatments. B cells were slightly more susceptible than T cells, in terms of increased apoptosis, for certain pesticide treatments however the effect was not pronounced.

The lack of data on chemical mixtures and the immune system makes this study unique. Since, exposure to low concentrations of chemicals is more realistic for most organisms and many past studies have focused on LD_{50} concentrations of chemicals, low doses of each chemical had to be identified for this study. These doses were chosen with the idea that when two pesticides were placed in mixture, the cytotoxicity would be less than 100%. The AlamarBlue (AB) assay was used to measure the amount of cytotoxicity induced by each treatment and identify the doses of each pesticide that would be utilized thouroughout the

study. The AB assay has been utilized as a cytotoxicity assay in previous studies (Mossman 1983; Larson et al. 1997; Nociari et al. 1998).

The concentration of each pesticide that induced 15-25% cytotoxicity ($\leq LC_{25}$) was extrapolated from the dose response curves (Figure 3.1). EtOH (0.3%) had no statistically significant effect on cell viability when compared to the untreated ($p > 0.05$), however concentrations of above 0.3% should be used with caution, particular when mature immune cells are involved (Slukin and Jerrells 1995). To rule out any concerns regarding this solvent, a preliminary study indicated ethanol, up to 0.8%, had no significant effect on the AB fluorescence compared to the untreated (data not shown). The 300 μM treatment for each compound was not statistically different from the H_2O_2 (10 mM) treatment ($p > 0.05$) suggesting high concentrations of each compound had the potential to induce toxicity similar to the positive control. Overall, an increasing linear trend between the increase in cytotoxicity and increasing pesticide concentrations was observed.

A time response, using the concentrations chosen from the dose response curve, indicated 16 hours of treatment caused the maximum response from the cells (Figure 3.2). Incubation time is an important factor when using primary cells, such as splenic immune cells, to assess toxicity since they cannot remain viable in culture for long periods of time. Previous studies have examined immune cells in vitro and utilized this same incubation time (Perandones et al. 1993; Illera et al. 1993). Furthermore, the alamarBlue assay indicated an additive increase in cytotoxicity for each pesticide mixture as compared to the corresponding individual pesticide treatments (Figure 3.3). This finding is invaluable since previous work in this area

has been limited and there have been no published studies on immunotoxicty and treatment with mixtures of ML or MP.

As mentioned, there are two modes of cell death, necrosis and apoptosis. Since cytotoxicity was clearly demonstrated in the alamarBlue assay, the next objective was to determine if this cytotoxicity was a result of apoptotic or necrotic mechanisms. To clarify this issue three different assays were utilized to identify morphological, biochemical or physiological aspects of either necrosis or apoptosis. Each assay showed features characteristic to apoptotic cells, with also some indications of low level necrosis as well.

The cytologic identification of morphological features associated with necrotic or apoptotic cells revealed individual pesticide treatments had low to moderate levels of apoptotic cells (Figure 3.4a and 3.4b). PBO had a few cells that were enlarged or had vacuole formation indicating it also induced necrosis. More importantly, however was the induction of apoptosis and necrosis in the pesticide mixtures. ML had moderate to high levels of apoptotic cells while MP had a high level of both apoptotic and necrotic cells. The presence of both necrosis and apoptosis in immune cell cultures, particularly those exposed to low levels of toxins is not unexplainable (Corcoran et al. 1994). It is likely that both events can occur simultaneously, as we have noted here. The cellular morphology of either pesticide mixture was similar to the DEX treatment, a positive control for apoptosis (Torres-Roca et al. 1995; Donner et al. 1999). The Unt 0 hour had no indication of cell death or debris. Unt 16 hours had very low levels of cell debris, yet the presence of some dead cells is not surprising. It is known that when placed in culture mature immune cells can undergo spontaneous

apoptosis (Perandones et al. 1993; Illera et al. 1993). Since the other cells appear unaffected, cell death is not suggested to be primarily necrotic since lyses would have induced more cell injury.

The DNA Ladder assay is based upon the principle that during apoptosis cellular nuclear DNA is non-randomly cleaved into 180-200 base pair units. When run on an agarose gel, this DNA ladder can be detected and is an indicator of apoptotic cells (Wyllie et al. 1984; Darzynkiewicz et al. 1997; Boehringer-Mannheim 1998). Initially, the splenocytes were incubated with pesticides for 16hours but DNA ladders were present in all samples that were incubated, including the Unt (data not shown). Interestingly, when samples were incubated with pesticides for only 4 hours laddering was detected in the pesticide treated samples, particularly in the PBO, ML and MP treatments (Figure 3.5). DEX also had an intense ladder verifying its efficacy as a positive control for apoptosis. The intensity of the ladders increased between the 4 and 8 hour treatments. DNA fragmentation has been suggested to be one of the first irreversible events to trigger mature immune cell apoptosis (Duke et al. 1983; Goldstein et al. 1991). With this in mind it does not seem unreasonable that this laddering effect was noted so early in the incubation, while cytotoxicity was very low. It is even possible to infer that DNA fragmentation may trigger the onset of other apoptotic mechanisms in the cells, however this has yet to be examined. There was some degree of DNA smearing at both time points, suggestive of necrotic cellular events, such as randomly cleaved DNA. The intensity of the smearing increased over time. The presence of necrotic cells in culture is consistent with the cytologic results also.

The 7AAD staining of splenocytes monitored any shifts in forward scatter/side scatter positioning. An Unt 0 hour sample was analyzed as the healthy population of cells and was gated upon, V-gate. This gate was monitored and a decrease in the less cells remaining in the gate was an indicator of the potential toxicity each pesticide and pesticide mixture had on splenocytes. Compared to the Unt 16 hours sample all others had less cells in the V-gate. The pesticide mixtures, ML and MP, and DEX treatments were most toxic to the cells as they had the least amount of cells remaining in the V-gate after 16 hours in culture (Figure 3.6). 7AAD staining has also been shown to be a reliable, quantitative method for detecting apoptosis in immune cells (Philpott et al. 1996; Donner et al. 1999). It allows for the measurement of remaining membrane integrity, which can be correlated to the cell status. Three intensities were measured and the percent of live, early apoptotic and dead cells was given (TABLE 3.A). Trends similar to the previous experiments were noted, including an additive increase in apoptotic cells for the pesticide mixtures compared to the individual pesticide treatments. Also, there was a significant level of early apoptosis in the Unt 16hours samples, again suggesting cells undergo a certain level of spontaneous apoptosis in culture (Perandones et al. 1993, Illera et al. 1993). The level of dead cells never exceeded 9% indicating that a small amount of the cell death was attributed to possible necrotic processes.

Since splenocytes are a mixture of different immune cells, mostly B and T lymphocytes, it was of interest to examine if either subset had increased sensitivity to the pesticides and pesticide mixtures. B cells were slightly more sensitive than T cells to both Mal and PBO treatment as noted by the higher levels of early apoptosis. However, Lind, ML, MP and DEX treatments induced the same level of apoptosis in both B and T cells. In addition, B cells

were more susceptible to culture conditions and had a greater level of apoptosis in the Unt 16hours. Furthermore, EtOH induced a higher level of apoptosis in the B cells as well. This is consistent with a previous finding, which linked higher levels of apoptosis in B cells treated with 0.4% or greater EtOH as compared to T cells (Slukin and Jerrells). The results from this study reported a higher level of dead cells in both subsets than the 7AAD only staining results. This could be explained by the increased handling involved in the staining procedure and the sensitivity of cell following treatment.

The consistency in results from all three experiments strengthens the claim that these pesticides and pesticide mixtures are inducing apoptosis in splenocytes. There is no one assay that can conclusively detect and measure apoptosis or necrosis in cells. Therefore, it is important to examine multiple parameters, as done in this study, before drawing conclusions. Particularly, since apoptosis is a very rapid event and it is also likely that both modes of cell death will occur simultaneously (Corcoran et al. 1994). Previous studies have indicated Mal induced apoptosis in a neuroblastoma cell line and Lind induced apoptosis in fish splenic immune cells so the idea these pesticides are capable of inducing apoptosis is not new (Carlson et al. 2000; Sweet et al. 1998). However, the toxic additivity of ML and MP is an important finding in regard to chemical interactions and immunotoxicity, particularly since there has been limited literature in this area. In addition, there is much to be understood about the mechanisms associated with the induction of apoptosis during chemical exposures (Descotes 2000). In this regard, it is of interest to examine the pro-oxidant and antioxidant status of splenocytes treated with these pesticides and pesticide mixtures prior to and during the induction of apoptosis. Since oxidative stress has been implicated in both chemical

metabolism and apoptosis, measuring indices of it may offer insight to the oxidative status of cells undergoing apoptosis (Comporti and Pompella 1994; Buttke and Sandstrom 1994; Slater et al. 1995).

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Chapter IV Pro-Oxidant and Antioxidant Status of Murine Splenocytes upon Exposure to Multiple Pesticides *In Vitro*

4.1 **Abstract**

The widespread use of pesticides in the environment increases the likelihood of multiple pesticide exposure, either concurrently or sequentially, over an organism's lifetime. Earlier we reported that exposure to certain pesticides at low concentrations causes cytotoxicity (both apoptotic and necrotic) in murine (C57BL/6) splenocytes *in vitro* in both concentration- and time- dependent manner. Because reactive oxygen species (ROS) are known to be involved in chemical-induced cytotoxicity, we examined the pesticide-induced oxidative stress for lindane (Lind; an organochlorine), malathion (Mal; an organophosphate) and piperonyl butoxide (PBO; a synergist) individually and in combination (mal/lind (ML) and mal/PBO (MP)), in murine (C57BL/6) splenocytes *in vitro*. The pro-oxidant and antioxidant statuses of the cells were monitored as indices of oxidative stress. The pro-oxidant activity of the pesticides was monitored by the generation of hydrogen peroxide (H_2O_2) via the 2',7'-dichlorofluorescin diacetate assay (DCFH-DA). Exposure to pesticides for 15 minutes significantly increased H_2O_2 production above the controls, Mal 21.1%; Lind 10.8%; PBO 25.9%; ML 26.8%; MP 37.8%. The activities of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) were not altered by these treatments. However, the levels of glutathione reductase (GR) and glutathione peroxidase (GPx) activities were significantly altered when cells were exposed to pesticides and/or pesticide mixtures for 12 hours. Thus, GR levels were significantly reduced for the pesticide mixtures (control 51.7; Mal 48.2; Lind 50; PBO 52.3; ML 40.5; MP 42 Units/mg) and GPx activity was severely

reduced for all pesticide treatments (control 44.9; Mal 30.2; Lind 30.6; PBO 32.4; ML 21.1; MP 21.1 Units/mg). These results indicate that exposure to Mal, Lind and PBO induces immunotoxicity, at least in part, via oxidative stress by both increased pro-oxidants and decreased antioxidants levels in cells. Exposure of splenocytes to pesticide mixtures increased ROS, such as H_2O_2, and decreased GPx activity at least in an additive manner compared to the individual pesticide treatments.

4.2 Introduction

Pesticides are used in all aspect of the environment-in public health programs, agriculture and for non-agricultural purposes (Banerjee et al. 2001). Although pesticides have reduced vector born diseases and offered lower cost and better quality food stuffs, the economic and health benefits do not come without potential risk and undesirable health effects. The public has been tolerant of the use of pesticides because of the benefits they provide; yet human, animal and environmental health suffer due to repeated or excessive exposure. The widespread use of pesticides has created a global health concern, particularly because an estimated 85-90% of pesticides applied never even reach their target organisms (Repetto and Baliga 1996).

Often exposure to pesticides is accidental and mild, yet in some cases severe pesticide poisoning occurs, an estimated 3 million cases annually, with 99% of those being in third world countries (Banerjee et al. 2001). From the health perspective, the potential for occupational or public exposure to multiple pesticides, either concurrently or sequentially, is

very likely over the course of an organism's lifetime (Simmons 1995). Despite existing knowledge, many health related side effects of pesticide exposure, particularly of pesticide mixtures, is still not well understood.

ROS, such as superoxide anions ($O_2^{-\bullet}$) and H_2O_2 are produced thouroughout the cells during normal aerobic metabolism. The intracellular concentration of ROS is a consequence of both their production and their removal by various antioxidants. A major component of antioxidant system in mammalian cells consists of three enzymes, i.e., SOD, CAT and GPX. These enzymes work in concert to detoxify $O_2^{-\bullet}$ and H_2O_2 in cells. It has been established that many pesticides are capable of inducing oxidative stress by overwhelming or modulating cellular drug metabolizing systems (Dikshith 1991). Oxidative stress occurs when there is an imbalance between free radical generation and antioxidant defenses. It often results in severe pathological consequences, such as membrane disruption, DNA damage and protein damage and cytotoxicity (Marks et al. 1996; Younes 1999).

The pesticides utilized in this study are malathion (Mal), lindane (Lind) and piperonyl butoxide (PBO). Mal is a general use organophosphate pesticide and acetylcholine esterase (AchE) inhibitor. It is this property that makes it such a potent insecticide with relatively low mammalian toxicity (Rodgers and Ellefson 1992). Mal is commonly used in commercial agriculture, households, and protection of domesticated animals and mosquito eradication (Barnett and Rodgers 1994; ExToxNet 1998; Immig 1998). In the US alone, it is estimated 16.7 million pounds of Mal are applied annually. Mal is rapidly biotransformed and excreted; however it has been reported to be a neuro- and immunotoxicant (Dikshith 1991;

Pruett 1992; Barnett and Rodgers 1994; Repetto and Baliga 1996). Lind, an organochlorine and mixed function oxidase (MFO) inducer, is no longer produced in the United States but is imported for use in forestry, seed grains treatment and flea/tick/scabies shampoos (ExToxNet 1998; USEPA 2001b). It is extremely persistent in the environment and has the potential to biomagnify. Lind has been reported to induce oxidative stress and membrane dysfunction in in rat hepatocytes, erythrocytes and cerebral hemisphere (Barros et al. 1991; Dikshith 1991; Junqueira et al. 1997; Koner et al.1998; Sahoo and Chainy 1998). PBO, a pesticide synergist and MFO inhibitor, is used in combination with pyrethourins, pyrethouroid, rotenone and carbamates to enhance their insecticide toxicity (USEPA 2000). PBO is often used on indoor plants and on pets to control fleas, ticks and ants. It is thought to have relatively low toxicity but as an MFO inhibitor, it is implicated in disrupting drug or chemical metabolism (Anders 1968; Jaffe et al. 1968; Conney et al. 1972; USEPA 2000).

The immune system is extremely sensitive to pesticide exposure and very low levels of chemical may induce a response (Sharma and Reddy 1987). We have reported earlier (Ref. Chapter III) that the three pesticides (Mal, Lind and PBO) induce immunotoxicity by causing cytotoxicity in spleenocytes in culture via both apoptosis and necrosis mechanisms. These cells are mature immune cells of the spleen, consisting ~90% of B and T lymphocytes, as well as macrophages, neutrophils and eosinophils, and are useful in examining the toxic potential of chemicals (Li et al. 1999). Oxidative stress has been implicated in immune cell cytotoxicity but very little is understood about the mechanisms behind this relationship (McConkey et al. 1994; Corcoran et al. 1994; Banerjee et al. 2001).

In the present study, we have examined the effects of each pesticide and pesticide mixture, ML and MP, for their potential to stimulate oxidative stress in murine splenocytes *in vitro*. Our results suggest that exposure to the above pesticides causes accumulation of ROS and depletion of certain key antioxidant enzyme levels that may lead to oxidative stress in the murine splenocytes in culture.

4.3 Materials and Methods

4.3a *Animals*

Eight- to twelve-week old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) was used for all experiments. The animals were maintained in accordance with U.S. Department of Health and Human Services Guide *for the Care and Use of Laboratory Animal*s (National Research Council, 1996). The animals were housed in polycarbonate cages with hard wood chip bedding in rooms in which the temperature (21 ± 2° C), humidity (50 ± 10%), and light cycle (12 h/12 h lights) were controlled.

4.3b *Pesticide Solutions*

Mal (purity 99%), Lind (purity 99.5%) and PBO (purity 98%) were purchased from Chem Services (West Chester, PA) and stock solutions (37.5 mM) were prepared using 100% denatured ethanol. A working solution was made form the stock solution using complete phenol red-depleted, RPMI-1640 media (Gibco BRL, Rockville, MD; 10% FBS [Atlanta Biologics, Norcross, GA], 2 mM L-glutamine, 0.1 mM non-essential MEM amino acids, 50 units/ml Penicillin/50 µg/ml streptomycin and 10 mM HEPES Buffer [Gibco BRL]) so that

the final concentration of ethanol in reaction mixture did not exceed 0.3%. Pesticide working solutions were made in 4X stocks and exposed to cells in media to provide a 1X in culture conditions. To ensure cells were responsive to treatment, either Concavalin A (ConA; 10 µg/ml), a proliferative agent, (Sigma Chemical, St.Louis, MO), or Dexamethasone (1µg/ml) (DEX), an apoptotic agent, (Sigma) were used. In addition, to exclude any effects due to the minute quantities of ethanol as a solvent, control cells were cultured with 0.3% ethanol.

4.3c *Isolation of Splenocytes*

Immediately after the mice were sacrificed via cervical dislocation, the body weight was measured and the spleen was removed aseptically. Quickly, the spleen weight was obtained and 10ml of cold, incomplete phenol-red depleted RPMI-1640 media was added. The spleen was dissociated by gently grinding against a 60-mesh wire screen (Sigma). The cells were pelleted at 250 x g, for 8 minutes at 4^{o}C (IEC Centra GP8R, International Equipment, Needham Heights, MA). Erythourocyte lyses were performed by suspending the cell pellet in 1 ml of cold, complete, phenol red-depleted RPMI-1640 media, and adding 3 ml cold ACK lyses buffer (0.15 M ammonium chloride, 1 mM potassium bicarbonate and 0.1 mM EDTA, pH 7.4; Sigma) (Perandones et al. 1993; Ahmed and Sriranganathan 1994; Donner et al. 1999). The suspension was incubated for 3 minutes on ice and diluted to 15 ml total volume with cold, complete RPMI-1640 media. Cells were pelleted as previously described and rinsed two times with complete and incomplete media, respectively. The rinsed cells were counted on the CASY I Cell Counter and Analyzer System (Scharfe Systems Gmblt, Reutlinger Germany) and diluted to 5x10^{6} cell/ml.

4.3d *Assay for Reactive Oxygen Species: DCFH-DA Oxidation Assay*

The DCFH-DA assay was adapted to monitor pro-oxidant status of cells. The theory in using 2',7'-Dichlorofluorescin Diacetate is that upon entering the cell, it becomes hydrolyzed to a nonfluorescent analog 2'-7'-dichlorofluorescene (DCF-H) and is trapped in the cell. In the presence of certain ROS intermediates, such as H_2O_2 or low-molecular weight peroxyl radicals or nitric oxide (NO), DCF-H is oxidized to a highly fluorescent 2'-7'-dichlorofluorescein (DCF) molecule that can be measured in the presence of hydrogen peroxide. ROS are mostly generated during oxidative respiratory burst or by phagosomal peroxidase activation (Das and Misra 1994; Rothe and Valet 1990; Wang et al. 1996; Wang and Joseph 1999).

DCFH-DA → DCF-H → DCF (fluorescent)

The generation of intracellular ROS was performed using the DCFH-DA method described in Bass et al. (1983; Das and Misra 1994) with slight modifications. Briefly, 25 mM 3-amino-2,3,4-triazole (AT; Sigma), in PBS (Cellgro), was added to a suspension of splenocytes (5 x 10^6/ml) in complete RPMI media (Murray et al. 1980). AT, a catalase inhibitor, prevents catalase from scavenging H_2O_2. Preliminary studies verified AT at 25 mM concentration was not cytotoxic to the cells. Cells were incubated, on ice, for 15-17 minutes, in the dark. DCFH-DA (5 mM or 2.44 mg/ml in 100% sterile EtOH; Acros, NJ) was added to suspension for a final concentration of 5 μM or 1 μl/ml. The mixture was incubated for 15 minutes at 37°C, in the dark. An unstained sample was also incubated as a negative control.

A flat bottom 96-well tissue culture plate (Costar) was used for the following treatments: hydrogen peroxide 150 μM (H_2O_2), unstained (Unst), ethanol 0.3% (EtOH), DCF only, Mal (50 μM), Lind (70 μM), PBO (55 μM), ML and MP. An aliquot of 5 x 10^5 cells was transferred to each well. An Unst 0 hour and DCF only sample were observed for DCF fluorescence on a Beckman-Coulter Epics XL/MXL flow cytometer. A separate culture plate was set up for each time point (15, 30, 45, 60 minutes) and all were incubated at 37°C, 95% humidity and 5% CO_2, in the dark. A plate was removed at each specified time point and the samples were observed via flow cytometry for DCF fluorescence. The DCF fluorescence peak of the DCF only was subtracted from all the other samples using the Coulter Epics Software v.1.5 Overton subtraction. The subtracted value represents the percent increase in DCF fluorescence due to treatment effect. Each time point had a unique DCF only value, used specifically for that time point only.

4.3e *Antioxidant Enzyme Assays*

Washed splenic immune cells were aliquoted into a round bottom 96-well tissue culture plate (Costar) with the following treatments: Unt, EtOH 0.3%; Mal 50 μM; Lind 70 μM, PBO 55 μM, ML and MP. For the Adrenochrome and CAT assays, cells from eight wells (5 x 10^5cells/well) were pooled to obtain a total of 4 x 10^6 cells per sample. For the GR and GPx assays, cells from twelve wells (5 x 10^5cells/well) were pooled to obtain a total of 6 x 10^6 cells per sample. A time-response curve was performed and 12 hour was found to be sufficient to observe treatment differences. Plates were incubated for 12 hours (37°C, 5% CO_2, 95% humidity) and all the wells for a treatment were combined into one 1.5ml tube. PBS (100ul) was added to each well, the samples were centrifuged (1000 x g for 10 minutes

at 4°C) and the plate was placed on ice. Two rinses were performed in this manner using cold sterile PBS to rinse the wells, as well as wash the cell pellet. Cells were resuspended in 80 µl of cold PBS for Adrenochrome and CAT assays and 100 µl for GR and GPx assays.

For the adrenochrome assay, this suspension was freeze-thawed twice at –20°C. Samples were centrifuged (20,000 x g, 10min, 4°C) and the clarified supernatant was transferred to a 0.5ml sterile tube. Samples were stored at –20° C (short-term) or –70°C (long-term) for enzyme assays. For the CAT, GR and GPx assays, the 100 µl cell suspension was sonicated, on ice, for two-5 second intervals at setting 1.0 (Fisher Scientific Sonic Dismembrator F550; USA). Samples were centrifuged (20,000 x g for 10min at 4°C) and the clarified supernatant was transferred to a 0.5 ml sterile tube. The CAT, GR and GPx samples were not frozen. Protein concentrations in all samples was estimated with the Bradford Protein Assay using bovine serum albumin as standard (Bradford 1976; Sigma).

4.3e.1 Superoxide Dismutase Assay

Superoxide dismutase catalyzes the dismutation of superoxide radicals. $O_2^{-\bullet}$, according to the reaction:

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

Epinephrine is stable in acidic solution but auto-oxidizes with increasing ease as the pH is elevated. Thus, at pH 10.2 and at 30°C, the auto-oxidation of epinephrine is rapid, with a linear rate (after an initial short lag) of conversion to the oxidation product, adrenochrome (Misra 1985). By measuring the rate at which SOD decreases this $O_2^{-\bullet}$ -dependent

autoxidation reaction, the specific activity of SOD in each sample can be calculated using this Adrenochrome assay (Misra and Fridovich 1972). *A unit of SOD is defined as the quantity of SOD required to produce a 50% inhibition in the rate of the conversion of epinephrine to adrenochrome, under specified conditions*. The specific activity of the enzyme was measured similar to the method described in Misra and Fridovich (1972) with slight modification. Briefly, in a 0.5ml quartz cuvette, 485ul of 30°C, 0.05M $NaHCO_3$-Na_2CO_3 buffer (pH 10.2, 10^{-4} M EDTA; Sigma) and 15ul of epinephrine 10^{-2} M (DL-epinephrine; store $\leq$ 5 days; Sigma) were mixed and a kinetic rate (ΔA/min; 480 nm; 30°C) was obtained on the Shimadzu UV Spectrophotometer with CPS Temperature controller (Model UV160; Kyoto, Japan). The amount of epinephrine was adjusted until a rate of 0.025 $\pm$ 0.002 was obtained. Then $\leq$ 25 µl of sample was added and the reaction rate was recorded. The change in rate, or ΔA/min, for each sample was compared to the epinephrine control. The percent the sample inhibited epinephrine auto-oxidation was calculated as percent inhibition (see below). Epinephrine only samples were taken every 6 samples and adjusted as necessary to keep 0.025 per minute rate. Each sample was measured in triplicate and a total of four experiments were performed. Specific activity (Units/mg protein) was calculated using the ΔA/min as follows:

A = Epinephrine ΔA/min at 480 nm
B = Sample ΔA/min
C = Protein content, mg/ml
Percent Inhibition (PI)= (1- [B/A]) x 100
VS_{50} = Volume (µl) of sample needed for 50% inhibition; contains 1Unit of SOD

Units of SOD/ ml = $1000/VS_{50}$

$$\text{Specific Activity} = \frac{\text{Units of SOD/ml}}{\text{mg protein/ml}} = \text{Units of SOD/mg protein}$$

4.3e.2 Catalase Specific Activity

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

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

By monitoring the rate of breakdown of H_2O_2 spectrophotometrically the specific activity of catalase, in each sample, can be calculated. *One unit of catalase is equivalent to the amount of protein necessary to decompose 1 mM of H_2O_2 per minute under specified conditions at 25^oC* (Beers and Sizer 1952).

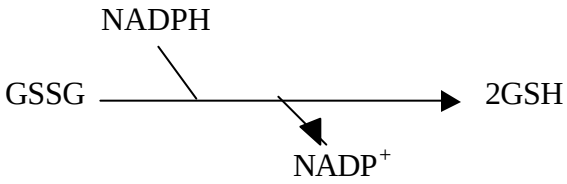
The following procedure was adapted from Beers and Sizer (1952) and the *Worthington Enzyme Manual* (1972) to determine the specific activity of catalase. Briefly, the following reagents were added to a 0.5 ml quartz cuvette,: 167 µl of substrate, (0.053 M H_2O_2; Sigma) in 0.05 M potassium phosphate buffer, pH 7.0, 25^{o}C), ~25µl of sample and bring to a final volume of 0.5 ml with 25^{o}C, 0.05 M potassium phosphate buffer, pH 7.0 and the rate of decrease in absorbance at 240 nm was monitored on a Shimadzu UV Spectrophotometer at 25^{o}C. Each sample was measured in triplicate and a total of four experiments were performed. The specific activity was calculated using the extinction coefficient of H_2O_2 43.6 M^{-1} cm^{-1}as follows (Beers and Sizer 1952; Kukucka and Misra 1993):

$$\text{Units/ml} = \frac{\Delta A/\text{min at 240 nm} \times \text{cuvette volume (ml)} \times \text{dilution factor} \times \text{1cm lightpath}}{43.6\ M^{-1}\ cm^{-1} \times \text{sample volume used (ml)}}$$

$$\text{Specific Activity} = \frac{\text{Units/ml}}{\text{mg/ml protein}}$$

4.3e.3 Glutathione Reductase Specific Activity

Glutathione reductase reduces oxidized glutathione (GSSG) to restore intracellular concentrations of reduced glutathione (GSH). It uses the cofactor NADPH for this reduction. By monitoring the oxidation of NADPH to $NADP^+$ on a UV spectrophotometer at 340nm, the specific activity (units/mg protein) of GR can be calculated. *One unit of GR is equivalent to the amount of enzyme necessary to catalyze the oxidation of 1 mM NADPH per minute.*



The measurement of GR was adapted from the Carlberg and Mannervik method with slight modifications (1985; Sies and Akerboom1984; Anderson et al. 1983). Briefly, in a 0.5 ml quartz cuvette, add 175µl of assay reagent (freshly prepared with 1 ml 1.0 M Potassium Phosphate buffer, pH 7.6; 2 ml 0.05% BSA solution 0.1 M KPO_4 buffer; 2 ml 1 mM NADPH in sterile dH2O; and 2 ml oxidized glutathione (GSSG) in sterile dH_2O; Sigma), $\leq$ 25 µl of sample and volume was brought up to 0.5 ml with 25°C dH_2O. The ΔA/min of the reaction was monitored on the Shimadzu UV spectrophotometer (340 nm, 25°C). Each sample was measured in triplicate and a total of four experiments were performed. The specific activity was calculated using the extinction coefficient of NADPH 6.22 x 10^{-3} μM^{-1} cm^{-1} (Merck 1996):

(Note: A reference blank containing all components except the sample was used for obtaining the background rate. This background rate was subtracted from all other rates prior to calculating the specific activity).

$$\text{Units/ml} = \frac{\Delta A/\text{min } 340 \text{ nm} \times \text{cuvette volume (ml)} \times \text{dilution factor} \times 1 \text{ cm lightpath}}{6.22 \times 10^{-3}\ \mu M^{-1}\ cm^{-1} \times \text{volume of sample (ml)}}$$

$$\text{Units/mg protein} = \frac{\text{Units/ml}}{\text{mg/ml protein}}$$

4.3e.4 Glutathoine Peroxidase Specific Activity

Glutathione peroxidase catalyzes the reduction of hydroperoxides by utilizing reduced glutathione (GSH) as a reductant. By coupling this reaction with GR, the specific activity (units/mg protein) of GPx can be calculated by monitoring the reduction of NADPH spectrophotometrically at 340 nm. One unit of GPx is equivalent to the amount of enzyme necessary to reduce 1 µM of NADPH per minute at 37°C under specified conditions (Tappel 1978).

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

$$GSSG + NADPH \rightarrow 2GSH + NADP^+$$

The measurement of GPx was adapted from Tappel with slight modifications (1978; Mannervik 1985). Briefly, in a 0.5 ml quartz cuvette, add 462.5 µl of fresh, 37°C coupling mixture (0.25 mM reduced glutathione, 0.12 mM NADPH, 1 unit/ml of GR (from dried bakers yeast) in Tris-HCl buffer, pH 7.6; Sigma), 12.5 µl of fresh cumene hyrdoperoxide (1.0 mg/ml by weight; Sigma) and $\leq$ 25 µl of sample. The total volume in the cuvette was adjusted to 0.5 ml with Tris-HCl buffer (Fisher Scientific), pH 7.6, 37°C. The ΔA/min was recorded on the Shimadzu UV spectrophotometer (340 nm, 37°C). Each sample was measured in triplicate and a total of four experiments were performed. The specific activity

was calculated using the extinction coefficient of NADPH 6.22 x 10^{-3} μM^{-1} cm^{-1} (Merck 1996).

(Note: A reference blank containing all components except the sample was used for obtaining the background rate. This background rate was subtracted from all other rates prior to calculating the specific activity).

$$\text{Units/ml} = \frac{\Delta A/\text{min at 340 nm} \times \text{volume of cuvette (ml)} \times \text{dilution factor x 1cm light path}}{6.22 \times 10^{-3}\ \mu M^{-1}\ cm^{-1} \times \text{volume of sample (ml)}}$$

$$\text{Units /mg protein} = \frac{\text{Units/ml}}{\text{mg/ml protein}}$$

4.3f *Statistical Analysis*

SAS 8.0 software (SAS Institute Inc., Cary, NC) was used for data analysis. The DCFH-DA results were analyzed using a randomized complete block design with no sub-sampling. The enzyme assays were analyzed using a randomized complete block design with sub-sampling. Pre-specified contrasts were outlined in the experimental design. The Mixed Procedure allowed for the comparison of each treatment to other specified treatments to determine whether a statistical difference was evident. Data are presented as least square means (LSM) $\pm$ standard error of the LSM (SEM). If $p > 0.05$ then the treatments were not statistically different.

4.4 **Results**

4.4a *Assay for Reactive Oxygen Species: DCFH-DA Oxidation Assay*

Figure 4.1 shows the measurement of intracellular reactive oxygen intermediates using the flow cytometric DCFH-DA assay, with the addition of 25 mM 3-amino-2,3,4-triazole (AT),

15 minutes following pesticide treatment. AT was added to inhibit catalase, a potent scavenger of H_2O_2, which could compete with DCF-H for H_2O_2 and possibly reduce DCF fluorescence (Murray et al 1980; Darr and Fridovich 1986). Such competition was noted, for when treatments were incubated with AT the DCF fluorescence was greater than those same treatments without AT (Appendix C Table C.1 and. C.2). The percent increase in DCF fluorescence for each sample compared to the DCF only treatment is presented and an increase in intracellular ROS for all treatments was observed (Figure 4.1). The H_2O_2 treatment (positive control) had the greatest DCF fluorescence (86%) followed by MP (38%). The Lind treatment had the least DCF fluorescence (11%). All other treatments were similar in the extent of their DCF fluorescence (Mal 21%, PBO 26%, ML 27%). Mal and ML were not statistically different ($p > 0.05$), while Lind and ML were statistically different ($p \leq 0.05$). Also, Mal to MP and PBO to MP were statistically different ($p \leq 0.05$) in their levels of DCF fluorescence. Splenocytes were also measured for DCF fluorescence at 30, 45 and 60 minutes following treatment (Appendix C Table C.2). The peak fluorescence ranged between 15 and 30 minute following exposure, depending on the treatment, and decreased from 30 to 60 minutes.

The addition of NO modifying chemicals was performed, with no AT present, and cells were analyzed for DCF fluorescence via flow cytometry (Table 4.A). L-NAME (1 mM; N-(omega)-nitro-L-arginine methyl ester; Oxis Intl. Eugene, OR), a NO synthase inhibitor, did not increase DCF fluorescence. When it was added in the presence of pesticide mixtures, the calculated percent increase in DCF fluorescence was the same as with no L-NAME present. PTIO (200 μM; 2-phenyl-4,4,5,5-tetramethylimidazone-1-oxyl-3-oxide; Oxis Intl.), a NO

scavenger, actually increased the amount of DCF fluorescence. In addition, when PTIO was added in the presence of pesticide mixtures, the calculated percent increase in DCF fluorescence was the same as with no PTIO present. Also, the addition of SNP (1 mM, Sodium Nitroprusside, Sigma), a NO donator, increased DCF fluorescence (data not shown).

4.4b *Adrenochrome Assay*

Prior to beginning the enzyme assays, it was necessary to determine the length of incubation that was appropriate to observe fluctuations in enzyme activity. The SOD Adrenochrome Assay was used to identify the length of incubation (Appendix D Figure D.1). It was determined, based on this and other preliminary data (such as cell cytotoxicity studies, refer to Chaper III), that 12 hours of incubation was sufficient to examine potential changes in enzyme activity.

Figure 4.2 shows the specific activity (units/mg protein) of SOD in each sample after 12 hours of incubation with pesticide and pesticide mixtures (Unt, Mal 50 µM, Lind 70 µM, PBO 55 µM, ML, MP). Statistically, there was no treatment effect observed in splenocyte SOD levels for any of the pesticide or pesticide mixture treatments ($p \leq 0.05$).

4.4c *Catalase*

Figure 4.3 shows the specific activity (units/mg protein) for catalase in each sample. There were no statistically significant differences ($p \leq 0.05$) between any of the treatments. It should be noted however that there was a significant decrease in catalase activity between all treatments (except EtOH) and the Unt 0 hour ($p \leq 0.05$; data not shown).

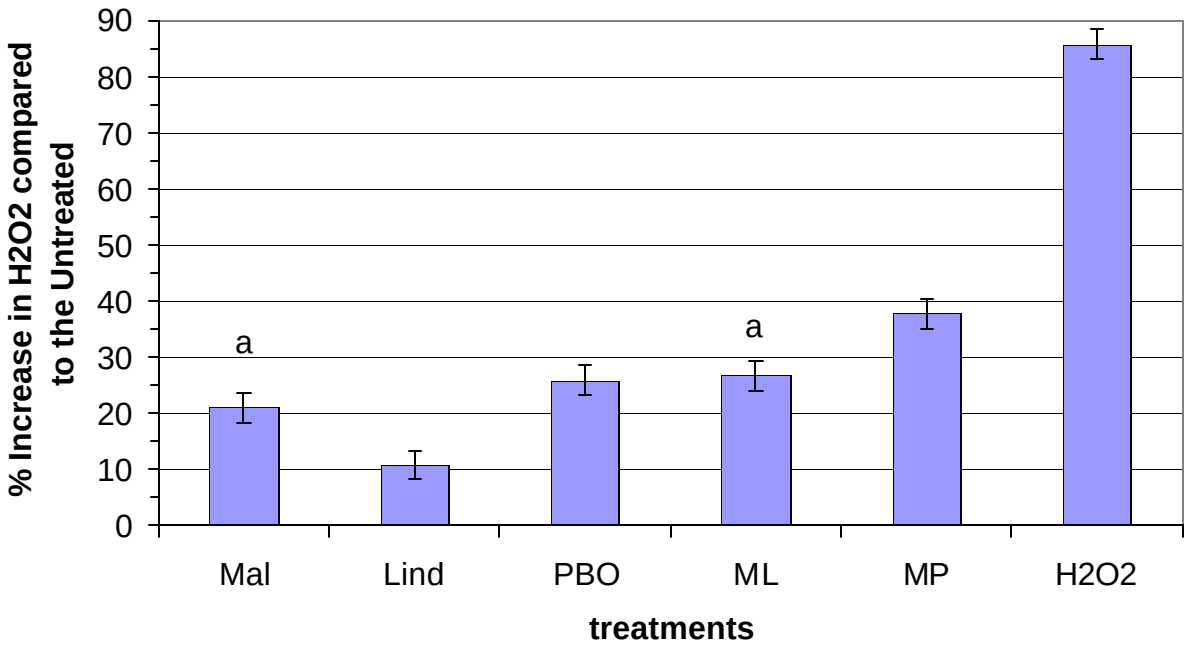


Figure 4.1. Measurement intracellular reactive oxygen intermediates by the DCFH-DA assay, with the addition of 25 mM 3-amino-2,3,4-triazole (AT), 15 minutes following treatment. Splenocytes remained either untreated or were dosed with pesticides and pesticide mixtures (Mal 50 µM, Lind 70 µM, PBO 55 µM, ML and MP) or H_2O_2 (150 µM) for 15, 30, 45 and 60 minutes (only 15 minutes shown). AT was incubated with the cells for 15 minutes, then DCFH-DA (5 µM) was added and cells were incubated for an additional 15 minutes. DCFH-DA stained splenocytes were added to the chemical treatments, in a 96-well tissue culture plate, and incubated for 15 minutes (37^oC, 5% CO_2, 95% humidity, in the dark). Flow cytometry analysis of samples was performed and DCF fluorescent peaks were obtained for each treatment.

Notes. The DCF only fluorescence peak area was subtracted from the DCF fluorescence peak area for each treatment. This value, the percent increase in DCF fluorescence, is presented above for each treatment as the mean of 3 experiments $\pm$ SEM. Treatments with *similar* letters were NOT statistically different ($p > 0.05$) from each another. All other contrast examined were statistically different ($p \leq 0.05$).

TABLE 4.A

DCFH-DA Assay for splenocytes treated with pesticide mixtures in the presence of L-NAME, PTIO or SNP to investigate the role nitric oxide has in DCF fluorescence. Solutions were mixed and incubated as described in Figure 4.1, except 1 mM L-NAME or 200 μM PTIO was to the cells immediately following isolation and incubated for 20 minutes before DCFH-DA (5 μM) was added, also no AT was added. 1 mM SNP was added to the samples when the DCFH-DA was added. Cells were incubated for 15 minutes at 37°C in the dark and were aliquoted into treatments. Treatments were set up for 15, 30, 45 and 60 minutes (only 15 minute shown) and 0 hour samples were taken for all treatments except those with pesticides. Samples were analyzed via flow cytometry and the percent increase was calculated as described in Figure 4.1.

Treatments (DCF +)	% Increase in DCF Fluorescence[1]	Corrected % Fluorescence
ML	19	*na*
MP	15	*na*
L-NAME	3	0 [2]
L-NAME + ML	23	20 [2]
L-NAME + MP	19	16 [2]
PTIO	30	0 [3]
PTIO + ML	50	20 [3]
PTIO + MP	46	16 [3]
SNP	30	*na*

Notes:
The % fluorescence describes the increase in fluorescence for each sample above these designated samples:

[1] DCF only -fluorescence
[2] DCF + L-NAME fluorescence
[3] DCF + PTIO fluorescence
na indicates no subtraction was performed

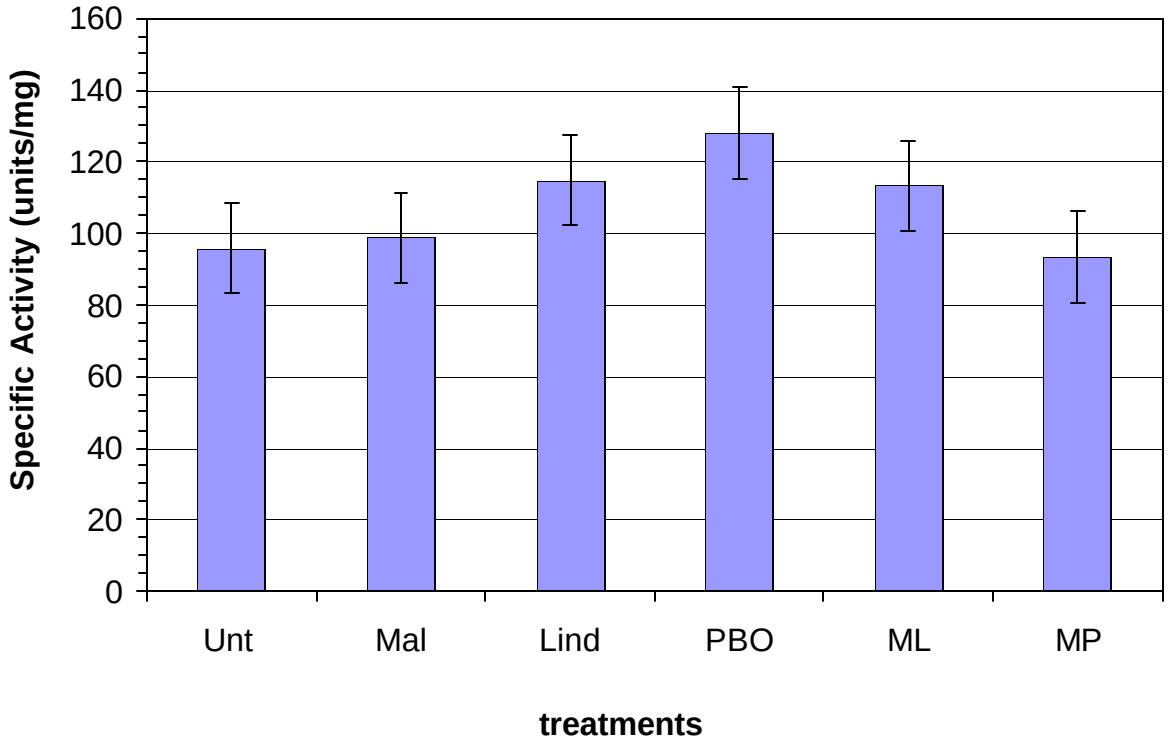


Figure 4.2. Measurement of superoxide dismutase (SOD) activity in splenocytes treated with pesticide and pesticide mixtures using the Adrenochrome Assay. Cells were incubated 12 hours with pesticide and pesticide mixtures (Unt, Mal 50 μM, Lind 70 μM, PBO 55 μM, ML, MP). A rate of 0.025 A/min was achieved by mixing 0.05 M $NaHCO_3$-Na_2CO_3 buffer (pH 10.2, 10^{-4} M EDTA) and a volume of epinephrine (10^{-2} M stock) on UV spectrophotometer (30^oC; 480 nm). Then the rate, or ΔA/min, each sample inhibited this Epi autooxidation was obtained and used to calculate the specific activity (Units/mg protein).

Notes. The specific activity of each sample is presented above as the mean of 5 experiments $\pm$ SEM. Statistically, no treatment effect by any of the pesticide or pesticide mixtures was observed ($p \leq 0.05$).

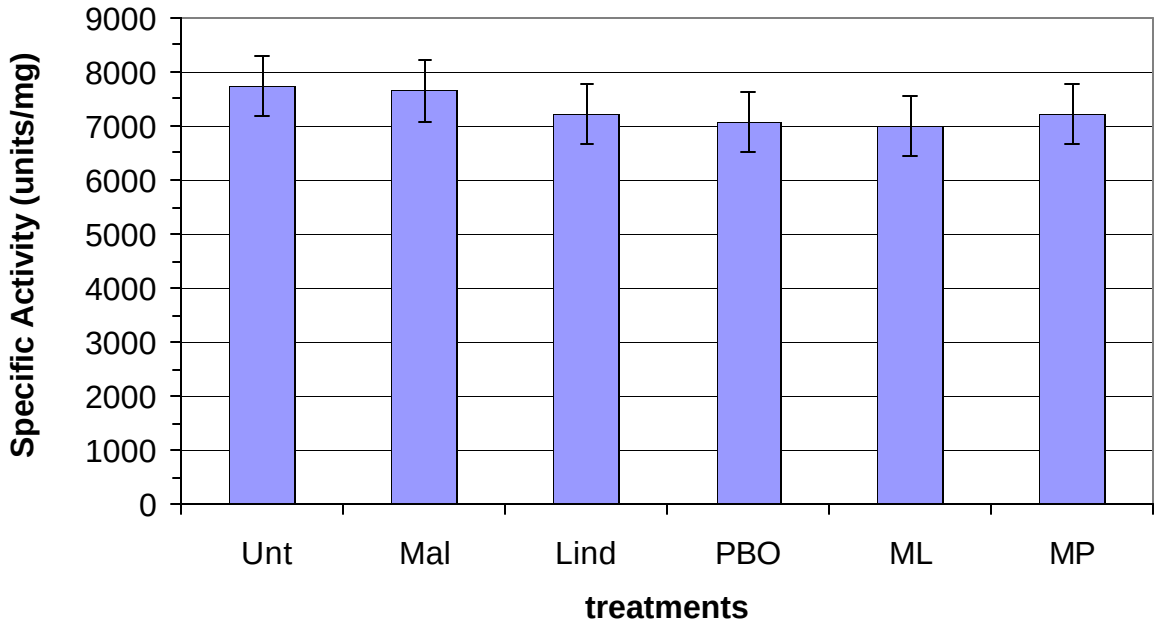


Figure 4.3. Measurement of catalase specific activity in splenocytes treated with pesticide and pesticide mixtures by monitoring the breakdown of H_2O_2 spectrophotometrically at 240 nm. Cells were incubated 12 hours with pesticide and pesticide mixtures (Unt, Mal 50 µM, Lind 70 µM, PBO 55 µM, ML, MP). The rate, or ΔA/min, each sample degraded H_2O_2 was measured by mixing 167 µl substrate (0.053 M H_2O_2) with 0.05 M potassium phosphate buffer (pH 7.0) and a certain sample volume (final volume is 0.5 ml) and obtaining a rate on a UV spectrophotometer (25°C, 240 nm). This rate was then used to calculate the specific activity (Units/mg protein).

Notes. The specific activity is presented as the mean of 5 experiments $\pm$ SEM. Statistically, no treatment effect by any of the pesticide or pesticide mixtures was observed ($p \leq 0.05$).

4.4d *Glutathione Reductase*

Figure 4.4 shows the specific activity of GR in splenocytes treated with pesticides and pesticide mixtures. There *were* statistically significant differences ($p \leq 0.05$) between the untreated and both pesticide mixtures (ML and MP). In addition, the mixtures and each of their corresponding individual pesticide treatments *were* statistically different in terms of GR specific activity. It should be noted that there was significant changes in the GR activity for all treatments (except PBO) and the untreated 0hour sample ($p \leq 0.05$) (data not shown).

4.4e *Glutathione Peroxidase*

Figure 4.5. shows the specific activity of GPx in splenocytes treated with pesticides and pesticide mixtures. Treatments with *similar* numbers were NOT statistically different from each other ($p > 0.05$). However, treatment effects were observed between all pesticide treatments and the untreated ($p \leq 0.05$). Also, for both the ML and MP treatments GPx activity was statistically different from the corresponding individual pesticide treatments. It should also be noted the untreated 0 hour sample had a significantly greater level of GPx than all the other treatments ($p \leq 0.05$).

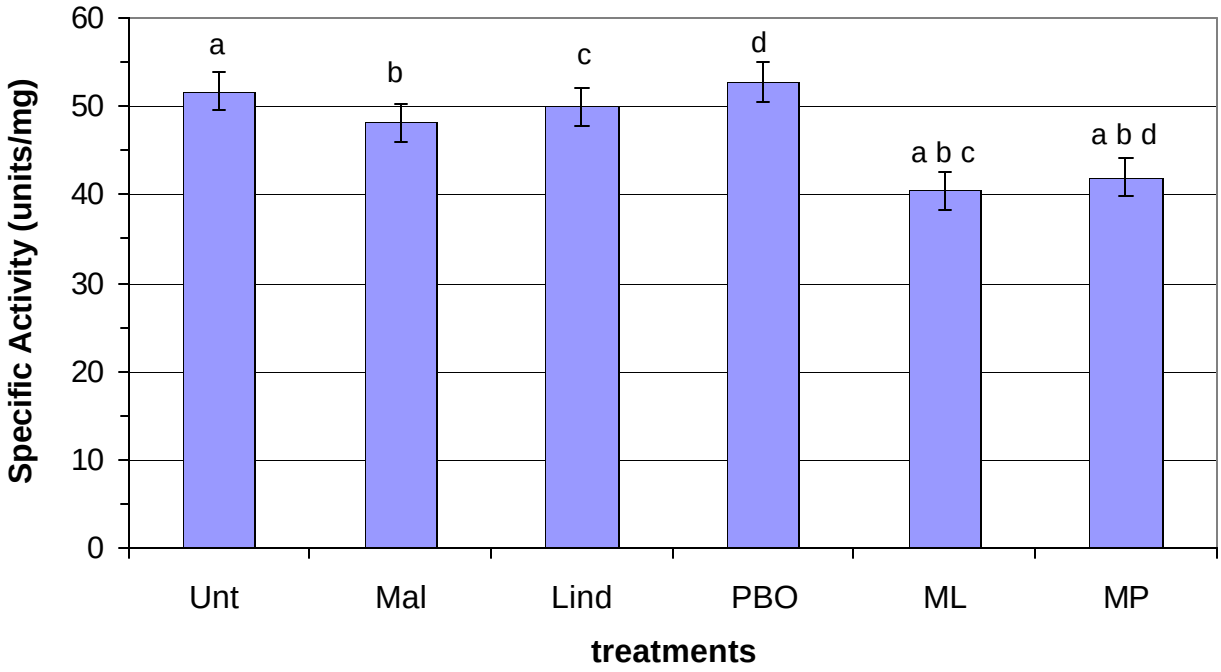


Figure 4.4. Measurement of GR activity in splenocytes treated with pesticide and pesticide mixtures by monitoring the oxidation of NADPH spectrophotometrically at 340 nm. Cells were incubated 12 hours with pesticide and pesticide mixtures (Unt, Mal 50 µM, Lind 70 µM, PBO 55 µM, ML, MP). The rate, or ΔA/min, each sample oxidized NADPH was obtained by mixing 175 µl of cocktail (containing 1 ml 1.0 M Potassium Phosphate buffer, pH 7.6; 2 ml 0.05% BSA solution 0.1 M KPO_4 buffer; 2 ml 1 mM NADPH in sterile dH2O; and 2 ml oxidized glutathione (GSSG) in sterile dH_2O), $\leq$ 25 µl of sample and 25°C distilled water (final vol. 0.5 ml) and measuring on a UV spectrophotometer (25°C; 340 nm). This rate was then used to calculate the specific activity (Units/mg protein) for each sample.

Notes. The specific activity is presented as the mean of 5 experiments $\pm$ SEM. Treatments with *similar* letters *are* statistically different from each other ($p \leq 0.05$).

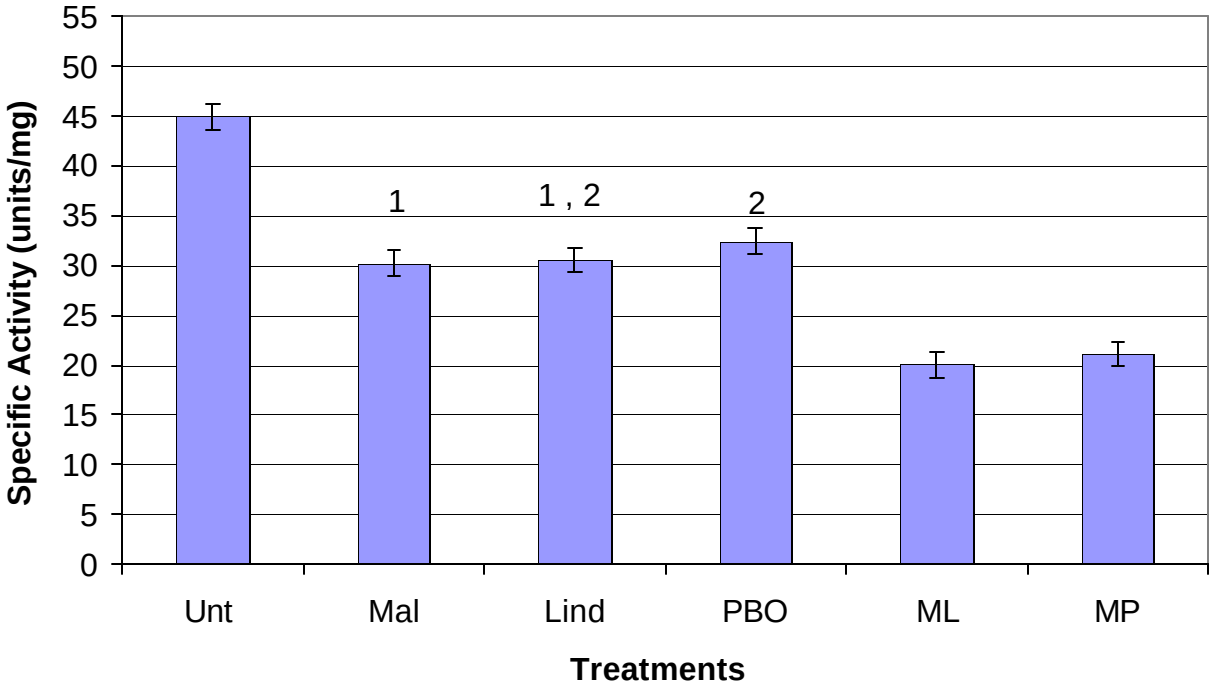


Figure 4.5. Measurement of GPx activity in splenocytes treated with pesticide and pesticide mixtures by monitoring the oxidation of NADPH spectrophotometrically at 340 nm. Cells were incubated 12 hours with pesticide and pesticide mixtures (Unt, Mal 50 µM, Lind 70 µM, PBO 55 µM, ML, MP). The rate, or ΔA/min, each sample oxidized NADPH was obtained by mixing 462.5 µl of coupling mixture (0.25 mM reduced glutathione, 0.12 mM NADPH, 1 Unit/ml of GR (from dried bakers yeast) in Tris-HCl buffer, pH 7.6), 12.5 µl of fresh cumene hyrdoperoxide (1.0 mg/ml by weight), $\leq$ 25µl of sample and 0.05 M Tris-HCl buffer, pH 7.6, 37^{o}C (final volume 0.5 ml). This rate was then used to calculate the specific activity (Units/mg protein).

Notes. The specific activity is presented as the mean of 5 experiments $\pm$ SEM. Treatments with *similar* numbers were NOT statistically different from each other ($p > 0.05$). However, a treatment effect was observed between all the treatments as compared to the untreated sample ($p \leq 0.05$). In addition, ML and MP GPx levels were statistically different from the corresponding individual pesticide treatments.

4.5 Discussion

This study examined the potential malathion, (50), lindane (70), PBO (55) and mixtures of those pesticides (ML and MP) had in increasing pro-oxidant levels and modulating antioxidant enzymes. The pro-oxidant levels were examined and each chemical, in particular each mixture, increased the amount of intracellular ROS. Also, the antioxidant enzyme activity was monitored and no effect on either SOD or CAT in any of the treatments was observed. However, GR was moderately decreased for the pesticide mixtures and GPx was severely reduced in all treatments. It appears these pesticides, particularly the mixtures, induced oxidative stress and interfere with the glutathione enzyme system.

The role ROS or free radicals play in the induction of apoptosis, particularly in immune cells, has been an area of discussion for some time (McConkey et al. 1994; Corcoran et al. 1994). Previous evidence supports this relationship between the two events (Forrest et al. 1994; Beaver and Waring 1995; Torres-Roca et al. 1995; Stoian et al. 1996; Gorman et al. 1997; Bustamante et al. 1997). However, none of these reports examined the level of free radical generation in splenocytes as a result of chemical exposure. In the previous chapter, apoptosis was detected in splenocytes treated with these chemicals. In this study, an increase in intracellular ROI's, such as H_2O_2 or nitric oxide (NO), was measured after 12 hours of incubation with pesticide and pesticide mixtures. The mixtures had a greater level of ROI production than any of the individual treatments, however the effect was not additive. In addition, an increase in DCF fluorescence occurred when AT was added to the cultures. Since AT is an inhibitor of catalase, a scavenger of H_2O_2, and inhibition of it increased fluorescence, it is clear that H_2O_2 is being generated upon chemical exposure. Other studies

have indicated that addition of H_2O_2 in murine cells increased the level of apoptosis in that culture, implicating the increase in intracellular ROI as a possible inducer of apoptosis (Stoian et al. 1996). Also, two studies on thymocyte apoptosis detected increases in ROI production with increases in apoptosis (Torres-Roca 1995; Bustamante et al. 1997).

Another common ROI is NO. NO has been implicated in oxidizing DCF-H to DCF, as well as apoptotic processes, nitric oxide pathways were observed (Gunasekar et al. 1995; Brune et al. 1997; Dimmeler and Zeiher 1997; Gabriel et al. 1997; Wang and Joseph 1999). The addition of SNP increased DCF fluorescence confirming the presence of NO would result in the oxidation of DCF-H in this experimental system. L-NAME, is an inhibitor of nitric oxide synthase (NOS) and therefore inhibits NO production (Brune et al. 1997; Dimmeler and Zeiher 1997). L-NAME had no effect of fluorescence when added with DCF only and when added in the presence of the pesticide mixtures (+DCF) it did not decrease the fluorescence from the levels observed with pesticide mixtures. Therefore, it is reasonable to conclude that NOS is not involved in the increase in ROI noted in the DCFH-DA assay. PTIO, an NO scavenger, was expected to compete with DCF-H for NO if NO was being generated due to pesticide exposure. PTIO actually increased DCF fluorescence when added with DCF only. However, when it was added to the pesticide mixtures, it had no effect on the level of fluorescence generated by pesticide exposure. This indicates that NO is not the intracellular ROI generated by the cells following exposure to pesticide mixtures. The increase of DCF fluorescence due to PTIO is explainable if PTIO was scavenging even the small levels of NO and freeing superoxide anions to form more H_2O_2.

The increase in pro-oxidant levels in treated splenocytes implies the possible onset of oxidative stress. This led to the examination of certain key antioxidant enzymes. Often antioxidant enzyme levels are used as indices oxidative stress. Relatively few reports have been published that examine the specific activity of antioxidant enzymes in murine splenocytes, neither being *in vitro* (Azenabor and Hoffman-Goetz 1999; Avula and Fernandes 1999).

This study examined the levels of four antioxidant enzymes in cells exposed to the pesticide and pesticide mixtures. There was no treatment effect observed on either SOD or CAT specific activity. A previous study of human serum samples from individuals poisoned with Lind or Mal indicated increased levels of both enzymes following exposure (Banerjee et al. 1999). However, in rats the dose and length of exposure seemed to play a role. In rats acutely dosed with lindane, a decrease in SOD and catalase was observed, while in short-term, low dosing experiment SOD levels were enhanced but catalase was not affected (Junqueira et al. 1986; Barros et al. 1991). A subchouronic study on rats treated with moderate doses of lindane had increased SOD (Koner et al. 1998). In summary, it appears the species, dose and length of exposure determines the extent to which each of these compounds influences SOD and catalase activity. This makes it difficult to relate previous findings to this study on murine immune cells. However, the findings in this study do provide insight into SOD and catalase enzyme response to pesticide exposure in murine splenocytes.

Although there was no effect on these enzymes following 12 hours of treatment, it is useful to know in freshly harvested splenocytes, from C57BL/6 mice, that SOD levels were 125 Units/mg protein and the catalase levels were 9333 Units/mg protein. Also, interesting is after 12 hours in culture with no treatment, SOD levels were not significantly different than at 0 hours (96 Units/mg), whereas catalase levels had dropped to 7730 Units/mg and were significantly different than the 0 hour values. It has been important to consider what occurs to immune cells when put in culture. Perandones et al. (1993) and Illera et al. (1993) point out immune cells in culture undergo a certain level of spontaneous apoptosis. When examining enzyme regulation and apoptosis, it is critical to consider the state of the cells upon harvesting. In the previous chapter, we reported there was negligible levels of apoptosis in splenocytes at 0 hour (upon harvesting) but by 12 hours there was ~25% apoptosis in the untreated cultures. It is evident, spontaneous apoptosis was occurring and could attribute to the drop in catalase enzyme activity or possibly a drop in catalase acitivity activates spontaneous apoptosis. Ellerby and Bredesen (2001) examined the levels of antioxidant enzymes in cultured cells and stress the importance in examining cells at 0 hours, prior to treatment or culture conditions, particularly when looking at biochemical enzymes in cells prone to spontaneous apoptosis.

A significant decrease in GR activity was observed in the pesticide mixture treatments only. Since GR restores the level of reduced glutathione (GSH), an important antioxidant molecule, depletion of this enzyme could affect the ability of the cells to metabolize these compounds. GSH is important role in Mal and Lind metabolism (Banerjee et al. 2001). With this in mind, it is likely reduction of GR has the potential to induce oxidative stress in

splenocytes treated with these pesticide mixtures. The onset of oxidative stress can result in cell injury and possibly cell death and, as mentioned earlier, could even be implicated in the triggering apoptosis (Ref. Chapter III). Previous studies on these chemicals and GR provide varied results. Most experiments were conducted in liver tissue or blood samples and not immune cells. For instance, rat GR was not affected by a single injected dose or short-term feeding of lindane, however, a study that injected rats with a high dose of lindane reported suppression of GR, 24 hour following treatment (Junqueira et al. 1986; Barros et al. 1991; Agrawal et al. 1991). Furthermore, humans poisoned with lindane had an increase in serum GR however Mal significantly decreased blood and lymphocyte GR (Banerjee et al. 1999). It is difficult to compare GR fluctuations in these chemical treatments since the effect is dependent upon the species, the dose and length or frequency of exposure. Also, in some instances the GR samples were frozen at $-20^{o}C$, which has not been recommended when quantitating total enzyme levels (Carlberg and Mannervik 1985).

At 0 hour, GR levels were at 58.3 Units/mg protein. By 12 hours in culture the levels had significantly dropped to 51.7 Units/mg. Again, as with catalase, this enzyme is slightly down regulated when the cells are placed in culture. One study indicated the levels of GR in the rat spleen were third highest of any tissue examined, with just the liver and kidney being greater (Rall and Lehninger 1952). Few studies on immune cells have examined GR levels, therefore there is little literature to support the Units/mg found in these splenocyte cultures.

GPx was also measured and a significant decrease in activity was observed. Since GPx is a vital defense mechanism against peroxidative damage of biological membranes and the

generation of free radicals, inhibition could lead to cellular oxidative stress (Mannervik 1985; Agrawal et al. 1991). This is likely, especially since the levels of hydrogen peroxide were elevated, as noted in the DCFH-DA assay. Previous studies indicated human erythrocytes had an increased level of GPx activity upon Mal or Lind poisoning (Banerjee et al. 1999). However, acute exposure (80 ppm) or short term feeding of low doses (20 mg/kg/day) of lindane did not have any effect on rat liver GPx levels (Junqueira et al. 1986; Barros et al. 1991). Yet, Agrawal et al. (1991) did indicate a significant reduction of GPx following acute exposure (300 mg/kg) of lindane in rat erythrocytes. Rat cerebral brain homogenates also had a significant decrease in GPx following acute low dose exposure (Sahoo and Chainy 1998). As with GR, it is difficult to compare GPx fluctuations following chemical exposure since the effect appears to be dependent upon the species, the dose and length or frequency of exposure.

At 0hour, GPx control level was 76.9 Units/mg protein. By 12 hours in culture the levels had significantly dropped in the untreated samples to 44.9 Units/mg. Again, as with catalase and GR, this enzyme was down regulated when cells were placed in culture.

Overall, this study provides a foundation for *in vitro* studies on immune cells, chemical exposure and enzyme analysis. Increased ROI generation was detected and was more enhanced for the pesticide mixtures than the individual pesticides. Also, after 12 hours of exposure, prior to peaks in apoptosis (Ref. Chapter III), GR levels were reduced by the pesticide mixtures and GPx was down regulated by all treatments. This clearly indicates the modulation of the GSH enzyme system and the potential onset of oxidative stress following

exposure to these pesticides, particularly the pesticide mixtures. Furthermore, the specific activity calculated for each enzyme in these splenocyte cultures, is one of the few published studies to examine these enzymes in the splenocytes of C57BL/6 mice and will be useful to future studies. Future work should be focused on the glutathione associated enzymes and the GSH:GSSG ratio of cells. This may provide a better understanding of the mechanisms associated with oxidative stress and the induction of apoptosis noted in the previous chapter.

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Conclusions

1. Malathion 50 μM, Lindane 70 μM and PBO 55 μM induced cytotoxicity in C57BL/6 murine splenocytes. An additive increase in cytotoxicity was induced by the pesticide mixtures, ML and MP, compared to the corresponding individual pesticide treatments.

2. Apoptosis was detected in splenocytes treated with pesticide and pesticide mixtures in three assays: Cytologic Identification of Apoptosis, DNA Ladder Formation and 7AAD Staining. Each assay also detected a small degree of necrosis in the cells. The pesticide mixtures induced greater toxicity than the corresponding individual pesticide treatments.

3. The 7AAD Staining Assay reported additive increase in early apoptotic cells compared to the individual pesticides.

4. B lymphocytes had a greater level of apoptosis than T lymphocytes for the Mal and PBO treatments. The mixtures and Lind treatments induced a similar amount of B and T cells to be early apoptotic.

5. An increase in intracellular reactive oxygen intermediates was detected following pesticide and pesticide mixture exposure, as measured in the DCFH-DA Oxidation Assay.

6. Superoxide dismutase and catalase specific activity were not significantly altered by the pesticides and pesticide mixtures.

7. Glutathione reductase specific activity was not changed by individual pesticides but was significantly reduced by the pesticide mixtures.

8. Glutathione peroxidase specific activity was significantly reduced by individual pesticides and pesticide mixtures.

Thus, the pesticides examined in this study, at certain concentrations, induced immunotoxicity by causing both apoptotic and necrotic death of splenic immune cells *in vitro*. Further, the pesticides induced oxidative stress by increasing pro-oxidants and decreasing some of the major antioxidants in these cells. Overall, pesticide mixtures induced a greater level of toxicity and oxidative stress compared to the individual pesticide treatments.

Future Work

Future research will focus on the oxidative status of splenocytes treated with these pesticides and pesticide mixtures. For instance the glutathione pathway will be more closely examined by measuring the ratio of reduced to oxidized glutathione, as well as by determining if a selenium deficiency is attributing to the reduction in glutathione peroxidase activity. In addition, antioxidant compounds, such as vitamin E or vitamin C, will be added to the treatments to observe any amelioration in pesticide toxicity. Furthermore, electron paramagnetic resonance studies will be performed to characterize which reactive oxygen species are involved.

Eventually, these *in vitro* results will be compared to results obtained from a future *in vivo* study using the same pesticides and pesticide mixtures. A long-term goal is to also perform these pesticide exposure studies on elderly and juvenile mice and characterize any age-related differences.

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Appendix A. *Flow Cytometry Output: Histograms for 7AAD Staining Assay*

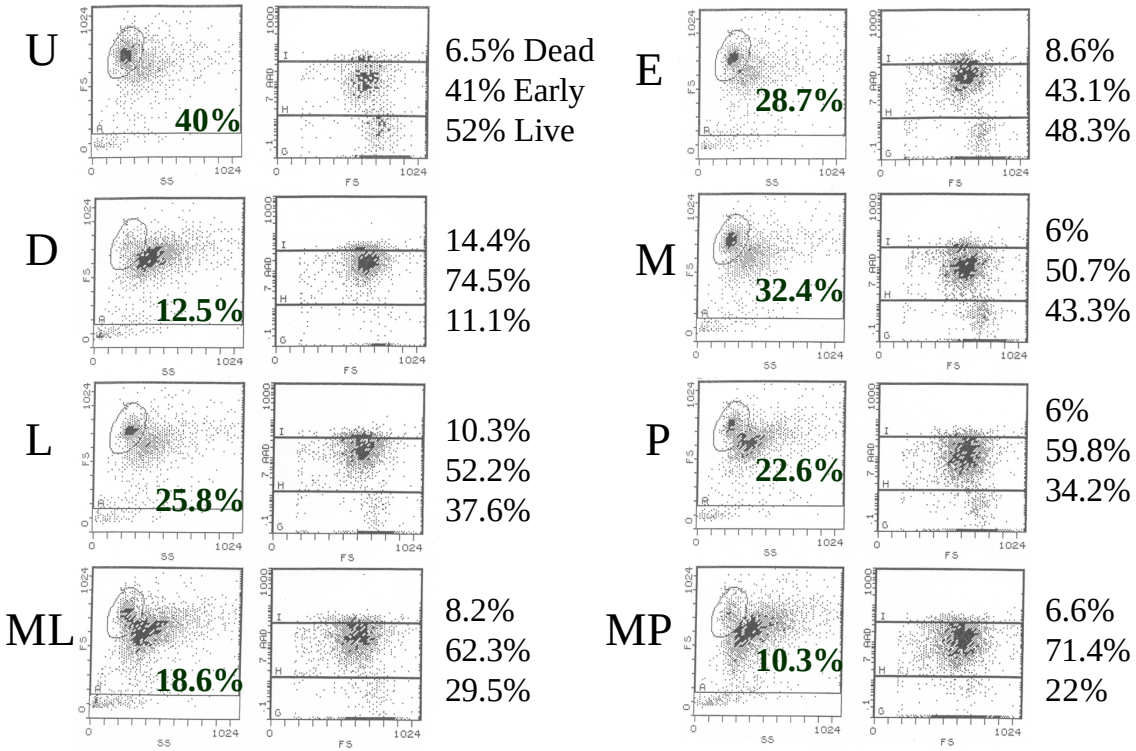

Figure A.1. Histograms representative of flow cytometry output for the 7-AAD Staining Assay. The first column represents ouput for the V-gate. At 0 hour cells were measured by size (forward scatter; FS; y axis) and granularity (side scatter; SS; x axis). A gate was drawn at 0 hour around the densest cell population (included ~80% of the total cells). Dispersal of cells from this gate was monitored following treatment and the percent of cells remaining in the gate was measured. C57BL/6 murine splenocytes were treated with Mal 50 μM (M), Lind 70 μM (L), PBO 55 μM (P), ML or MP for 16 hours. An Unt 16 hours (U), EtOH 0.3% (E) and DEX 10 μg/ml (D) were also performed. Refer to the *7AAD Staining Assay* protocol for the details of experimental set up. The percent of cells (of n = 5000 events) remaining in the gate after treatment is presented as the V-gate value. The second column has histograms with 3 distincet divisions. Each division is specific to a 7AAD staining intensity, $7AAD^{dull}$ (live), $7AAD^{moderate}$ (early apoptotic) and 7AADbright(dead). The percent of cells (of n = 5000 events) stained, in each intensity, was given for each sample. Data is a representative of one replicate in one experiment.

Conclusion: The histograms of flow output offer a visual representation of assessing treatment effect. The shift of cells from within the V-gate to outside is an indicator of the potential toxicity induced by each treatment. Particularly it can indicate when a cell populations is undergoing apoptosis. For instance, cells often shourink and become more granulated when apoptotic. When measured on the flow cytometer, these changes would be reflected by a shift in the population toward the lower right corner of the histogram. As noted from these representative histograms, the cell populations did shift from the gate toward the lower right quadrant.

Also, the ability to gate on three different staining intensities indicates the versatility of the 7AAD staining assay. However, it is critical to use caution when designating the gates that separate the intensities. It is essential to analyze various controls and monitor the samples over time to observe the progression of staining intensity.

Appendix B. *Flow Cytometry Output: Histograms for 7AAD Staining Assay with Monoclonal Antibodies.*

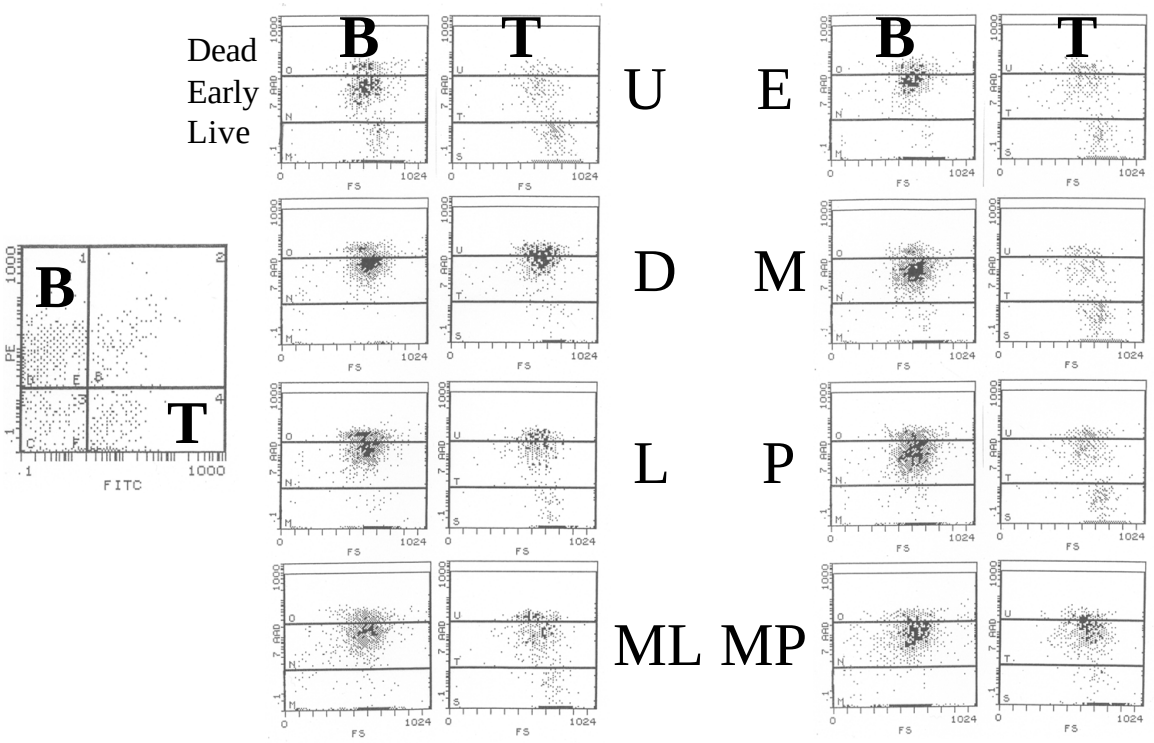

Figure B.1. Histograms representative of flow cytometry output for the 7-AAD Staining Assay in combination with two fluorescently labeled monoclonal antibodies-- R-PE:CD45R/B220 and FITC:CD90.2 (Thy-1.2). C57BL/6 murine splenocytes were treated with Mal 50 µM (M), Lind 70 µM (L), PBO 55 µM (P), ML or MP for 16 hours. An Unt, EtOH 0.3% and DEX 10 µg/ml were also performed. Refer to the *7AAD Staining Assay with Dual Monoclonal Antibodies* protocol for experimental details. Cells were sorted by flow cytometry for R-PE or FITC fluorescence (left histogram). Both B and T cell populations were gated on and measured for 7AAD staining intensity, $7AAD^{dull}$ (live), $7AAD^{moderate}$ (early apoptotic) and $7AAD^{bright}$ (dead) (refer to histograms in columns 2-5). The percent of B and T cells for each intensity was given. Data is representative of one replicate in one experiment.

Conclusion: 7AAD's unique spectral properties make it versatile to use in combination with other fluorescent probes, such as R-PE and FITC. By doing this, specific subsets of cells, in this case B and T lymphocytes, were observed for live, early apoptotic or dead cell populations following treatment with pesticides and pesticide mixtures. This data was used to assess the toxicity induced by each pesticide and pesticide mixture.

Appendix C: *The DCFH-DA Oxidation Assay*

TABLE C.1

Measurement of intracellular reactive oxygen intermediates in splenoyctes, treated with pesticides and pesticide mixtures for 15, 30, 45 and 60 minutes, using the DCFH-DA Oxidation Assay without the addition of 3-amino-2,3,4-triazole, a catalase inhibitor.

treatments	15 minute	30 minute	45 minute	60 minute
EtOH	2 (+ 1.44)	5.4 (+ 2.02)	6.2 (+ 2.73)	5.5 (+ 2.5)
Malathion	14.1 (± 1.44)	18.2 (± 2.02)	7.9 (± 2.73)	0.9 (± 2.5)
Lindane	6.6 (+ 1.44)	10.4 (+ 2.02)	4.1 (+ 2.73)	0.24 (+ 2.5)
PBO	7.1 (± 1.44)	9.3 (± 2.02)	2.9 (± 2.73)	0.98 (± 2.5)
Mal-Lind	14.2 (± 1.44)	18.3 (± 2.02)	13.3 (± 2.73)	5.3 (± 2.5)
Mal-PBO	11.1 (+ 1.44)	12.9 (+ 2.02)	8.5 (+ 2.73)	2.9 (+ 2.5)
H2O2	85.1 (± 1.44)	82.3 (± 2.02)	67.3 (± 2.73)	61.6 (± 2.5)

Note: The mean percent of five blocks (experimental units) ± standard error or mean (n = 5) is shown. The percent represents the increase in DCF fluorescence for each sample compared to the DCF only sample. The DCF fluorescence peak is at 30 minutes. Refer *Assay for Reactive Oxygen Species* protocol for experimental details.

TABLE C.2

Measurement of intracellular reactive oxygen intermediates in splenoyctes, treated with pesticides and pesticide mixtures for 15, 30, 45 and 60 minutes, using the DCFH-DA Oxidation Assay with the addition of 3-amino-2,3,4-triazole, a catalase inhibitor.

treatments	15 minute	30 minute	45 minute	60 minute
EtOH	14 (+ 2.6)	17.9 (+ 3.21)	16.9 (+ 3.72)	9 (+ 3.54)
Malathion	21.1 (± 2.6)	18.1 (± 3.21)	11.8 (± 3.72)	3 (± 3.54)
Lindane	10.8 (+ 2.6)	15.1 (+ 3.21)	13.6 (+ 3.72)	8.2 (+ 3.54)
PBO	25.9 (± 2.6)	22.7 (± 3.21)	15.5 (± 3.72)	7.4 (± 3.54)
Mal-Lind	26.8 (± 2.6)	28.7 (± 3.21)	16.4 (± 3.72)	13 (± 3.54)
Mal-PBO	37.8 (+ 2.6)	30.3 (+ 3.21)	17.6 (+ 3.72)	12.8 (+ 3.54)
H2O2	85.8 (± 2.6)	81.4 (± 3.21)	67.1 (± 3.72)	58.3 (± 3.54)

Note: The mean percent of 3 blocks (experimental units) ± standard error or mean (n = 3) is shown. The percent represents the increase in DCF fluorescence for each sample compared to the DCF only sample. The DCF fluorescence peak is between 15 and 30 minutes, depending on the treatment. Refer *Assay for Reactive Oxygen Species* protocol for experimental details.

Conclusion: The addition of 3-amino-2,3,4-triazole (AT), a catalase inhibitor, enhanced the level of DCF fluorescence, as can be noted by comparing the datasets. The peak time of DCF fluorescence ranged between 15 and 30 minutes depending upon the treatment.

Appendix D: *Adrenochrome Assay: Time Response*

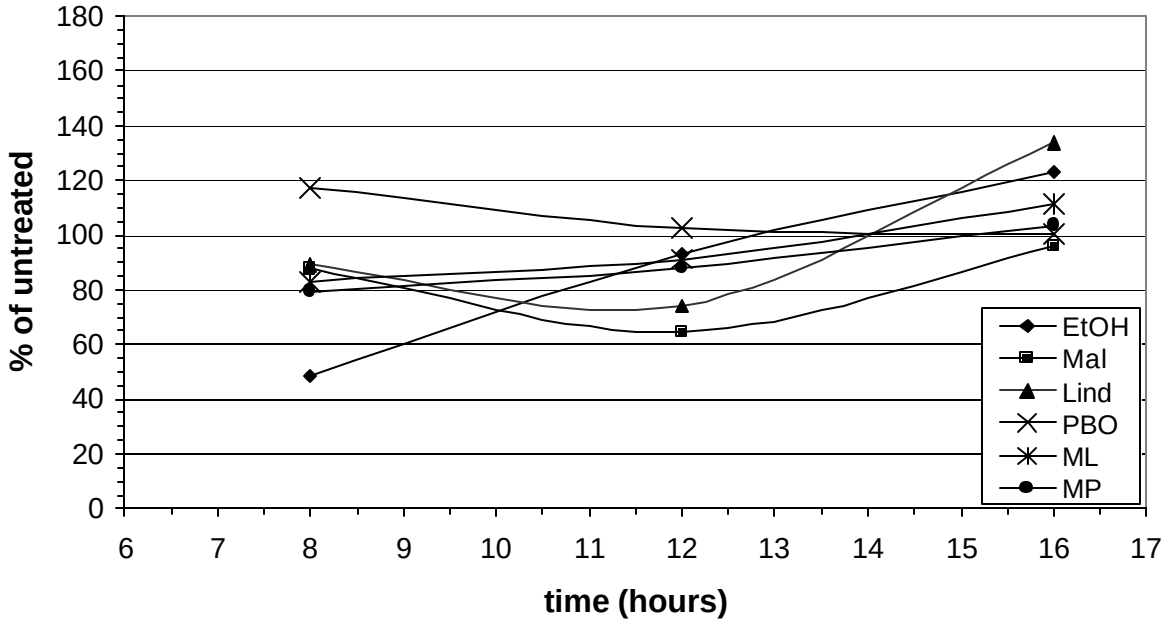

Figure D.1. Measurement of SOD at 8, 12 and 16 hours following pesticide treatment. Splenocytes treated with pesticide and pesticide mixtures were measured for superoxide dismutase (SOD) activity using the Adrenochrome Assay. Cells were incubated for 8, 12, and 16 hours with pesticide and pesticide mixtures. The rate each sample inhibited epi autooxidation was used to calculate the specific activity (Units/mg protein) for each sample. Data above is presented as the percent each sample varied from the untreated for the same time point.

Notes. The means of two experiments are shown (n = 2). Refer to the *Adrenochrome Assay* for protocol on experimental details.

Conclusion: The 12 hour time point was chosen to examine the level of antioxidant enzymes since some degree of effect was observed.

VITA

Christine Lee Rabideau was born on July 4, 1973 in Plattsburgh, New York. In 1986, her family moved to Underhill Center, Vermont. She graduated from Mt. Mansfield Union High School in 1991. Christine attended St. Michael's College in Colchester, Vermont and graduated with a B.S. in Environmental Science in 1995. In 1995 and 1996, Christine participated in two Department of Energy ORISE Student Internships. At Savannah River Ecology Lab (Aiken, SC) she studied aquatic toxicology and at Oak Ridge National Lab (Oak Ridge, TN) she studied the health effects associated with electromagnetic field exposure. In December 1996, Christine moved to Blacksburg to work as a VMRCVM Laboratory Specialist in Virology and Molecular Toxicology. In August 1999, after having been a part-time graduate student, she entered into the graduate research program full-time. Upon graduating, Christine will relocate to Fort Collins, Colorado where she will join her fiancé, Mr. Michael Battaglia. She will work as a Faculty Research Associate at Colorado State University's, Department of Environmental Health, Center for Environmental Toxicology and Technology. Christine and Michael will marry on September 1, 2001 in Vermont.