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Effects of pesticide exposure on the humoral immune response following *Streptococcus pneumoniae* vaccination

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Effects of pesticide exposure on the humoral immune response following *Streptococcus pneumoniae* vaccination

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Dissertation submitted to the School of Medicine at West Virginia University in partial
fulfillment of the requirements for the degree of:

Doctor of Philosophy
In
Microbiology, Immunology, and Cell Biology

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ABSTRACT

Effects of pesticide exposure on the humoral immune response following *Streptococcus pneumoniae* vaccination

As pesticide use rises in the US annually, the need for extensive characterization of the risks associated with these substances also increases. Herbicides represent the most frequently used type of pesticides. 3,4-dichloropropionanilide (propanil) and 2,4-dichlorophenoxyacetic acid (2,4-D) are two of the most commonly used herbicides. These experiments evaluated the effects of propanil and 2,4-D on the humoral immune response in C57BL/6 mice following heat-killed *Streptococcus pneumoniae* (HKSP) immunization. Intraperitoneal exposure of 150 mg/kg propanil on the same day as HKSP immunization enhances the splenic T-independent type 2 antibody response to phosphorylcholine (PC) antigen 4-6-fold. Maximal PC enhancement occurs 7 days post-exposure. Propanil exposure 9 days following HKSP immunization enhances the T-dependent antibody response to the pneumococcal surface protein A (PspA). 2,4-D exposure decreases the PC-specific antibody response in the bone marrow and the antibody serum titers 10 days post-exposure. Mixture exposure to the herbicides did not produce any synergistic effects. In addition, aspiration of the mixture of herbicides did not affect the antibody response. Ovariectomy and inhibition of ovarian steroid synthesis abrogates the PC-specific antibody enhancement in propanil-treated mice. Several studies examined the direct and indirect estrogenic activity of propanil. Interestingly, propanil enhances the PC-specific splenic antibody response independent of an estrogenic mechanism. Although male mice also had enhanced antibody responses following propanil exposure, orchiectomy did not affect the enhancement. Finally, propanil exposure did not enhance survival following a suboptimal immunization to a virulent strain of *S. pneumoniae*. Altogether, these results provide a number of findings that contribute to our understanding of the regulation of the humoral response to HKSP.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
2,4-DCP	2,4-dichlorophenol
ANOVA	one-way analysis of variance
AP	alkaline phosphatase
APC	antigen presenting cell
ASC	antibody secreting cell
asp	aspiration
BCR	B-cell receptor
BFU-E	erythroid burst-forming units
BM	bone marrow
BPA	bisphenol A
CFU	colony forming unit
CFU-E	erythroid colony forming units
CFU-G	neutrophils
CFU-M	macrophages
CFU-S	myeloid stem cells
CHR	contact hypersensitivity reaction
CNS	central nervous system
ConA	concanavalin A
CpG-ODN	unmethylated cytidyl guanosyl-oligodeoxynucleotide
CR1	complement receptor 1
CR2	complement receptor 2
DAG	diacylglycerol
DCA	3,4-dichloroaniline
DES	diethylstilbesterol
E2	17 β -estradiol
EDC	endocrine disrupting chemical
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunosorbent spot assay
EPA	Environmental Protection Agency
ER	estrogen receptor
FDC	follicular dendritic cell
FSH	follicle stimulating hormone
GM-CFU	granulocyte/macrophage progenitor cell
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptor
HEV	high endothelial venules
HKLM	heat-killed <i>Listeria monocytogenes</i>
HKSP	heat-killed <i>Streptococcus pneumoniae</i>
IC	immune complex
Ip	intraperitoneal
Iv	intravenous
LD ₅₀	lethal dose causing death in 50% of exposed animals
LH	luteinizing hormone

LOAEL	lowest observed affect effect level
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MZ	marginal zone
NK	natural killer cell
OVX	ovariectomized
PAMPS	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PC	phosphorylcholine
PFC	plaque-forming colony
PKC	protein kinase C
Po	peanut oil
PPS	pneumococcal polysaccharide
PspA	pneumococcal surface protein A
SC	subcutaneous
SCE	sister chromatid exchange
SLE	systemic lupus erythematosus
SRBC	sheep red blood cells
TCR	T cell receptor
TD	T-dependent
TH1	T-helper 1 antibody reponse
TH2	T-helper 2 antibody response
TI-2	T-independent type 2 antibody response
TLR	Toll-like receptor
WHO	World Health Organization

CHAPTER 1: GENERAL INTRODUCTION

PROPANIL

Background and General Toxicity

The pesticide 3,4-dichloropropionanilide (propanil) is an herbicide that is predominantly used for the control of weeds on commercial rice crops. Numerous studies have investigated the toxicological potential of propanil. It has been established in the literature that propanil has a number of immunotoxic effects. This report will provide a brief background of the general toxic effects of propanil exposure and primarily focus on the immunotoxicity studies that have been performed in both *in vivo* and *in vitro* models. In addition, epidemiological reports examining the immunotoxic effects of propanil on professional applicators and people living in areas of propanil use will be summarized.

Propanil is in the acetanilide chemical family (U.S. EPA OFFICE OF PESTICIDE PROGRAMS, 2003). Approximately 99% of the propanil used in the United States is for the control of weeds on rice crops (U.S. EPA OFFICE OF PESTICIDE PROGRAMS, 2003). Roughly 50-70% of all rice grown in the U.S. is treated with propanil (U.S. EPA OFFICE OF PESTICIDE PROGRAMS, 2003). A 2001 estimate by the Environmental Protection Agency (EPA) ranks propanil as the 17th most used pesticide in the U.S. The range of propanil application extends throughout the lower southern states along the Mississippi River and in some areas of California. Propanil is restricted exclusively to commercial use and it is readily degraded in the soil and water. The half-life in the soil is 1-3 days and 2-3 days in the water (Wauchope *et al.*, 1992). Given the lack of residential use of propanil and the short half-life, exposure of the

chemical is primarily restricted to applicators and residents who live near the treated farms. The preferred method of application is via groundboom sprayers and aerial methods (U.S. EPA OFFICE OF PESTICIDE PROGRAMS, 2003).

The most common routes of propanil exposure include inhalational, dermal, orbital, and to a lesser extent, oral. Upon entry into the organism, propanil is rapidly metabolized in the liver by the enzyme acylamidase into the major metabolite 3,4-dichloroaniline (DCA) (Williams *et al.*, 1966). Other metabolites of propanil include 2'-hydroxypropanil and 6'-hydroxypropanil (McMillan *et al.*, 1990a). DCA is subsequently metabolized to 6-hydroxy-3, 4-dichloroaniline and N-hydroxy-3, 4-dichloroaniline. Due to the rapid metabolism of the parent compound, propanil remains at detectable concentrations in the blood of rats for approximately 48 hours following exposure (Izmerov, 1984).

Following exposure to propanil, a number of toxic side effects are induced including hemolytic anemia, methemoglobinemia, and myelotoxicity. *In vivo* studies in Sprague-Dawley rats have shown that inhibition of the acylamidase activity with triorthotolyl phosphate decreased the amount of methemoglobinemia formed following a 400 mg/kg intraperitoneal (i.p.) exposure to propanil (Singleton *et al.*, 1973). In addition, i.p. injections of the metabolite N-hydroxy-3, 4-dichloroaniline into rats was found to induce the same amount of hemolytic anemia as the parent compound (McMillan *et al.*, 1991). *In vitro* studies analyzing the ability of propanil metabolites to oxidize hemoglobin in erythrocyte suspensions confirmed that N-hydroxy-3, 4-dichloroaniline was the most potent oxidizing metabolite (McMillan *et al.*, 1990a). Altogether, these studies indicate that metabolism of the parent compound is necessary for anemia.

Duration of the anemic effects differ according to the exposure concentration of propanil (McMillan *et al.*, 1990b). Analysis of blood samples from acutely exposed Sprague-Dawley rats treated with 300 mg/kg of propanil showed that methemoglobinemia effects peak at 4.5 hours post-exposure and these effects can be detected for as long as 24 hours (McMillan *et al.*, 1990b). However, 100 mg/kg of propanil induced a peak in methemoglobin levels at 1.5 hours and could be detected for 12 hours (McMillan *et al.*, 1990b). These data indicate that the anemic effects induced by propanil are brief and these effects do not persist following the clearance of propanil from the blood.

More recently, myelotoxic effects have also been established following propanil exposure (Malerba *et al.*, 2002; Blyler *et al.*, 1994). *Ex vivo* colony-forming assays from the bone marrow of acutely exposed C57Bl/6 mice demonstrated that the early stem cell progenitors, myeloid stem cells (CFU-S) and erythroid burst-forming units (BFU-E), are decreased in number following 200 mg/kg propanil exposure (Blyler *et al.*, 1994). However, the more differentiated cell types such as erythroid colony-forming units (CFU-E), granulocyte/macrophage progenitor cells (GM-CFU), macrophages (CFU-M), and neutrophils (CFU-G) are not reduced in number following propanil treatment (Blyler *et al.*, 1994). Similar effects on the early erythroid stem cells are seen with human cord progenitor cells following propanil treatment (Malerba *et al.*, 2002). *In vitro* exposure to cord blood demonstrated that the CFU-E and BFU-E progenitors had 50% of their growth inhibited (IC_{50}) by 234 and 441 μ M of propanil, respectively (Malerba *et al.*, 2002). The more differentiated CFU-GM progenitors had an IC_{50} of greater than 500 μ M, indicating similar susceptibility to the effects of propanil on the more immature progenitors in both

humans and mice (Malerba *et al.*, 2002). However, unlike mice, DCA is far less toxic to human CFU/BFU-E progenitors than its parent compound (Malerba *et al.*, 2002).

Initial chronic and acute exposure studies found that secondary immune organs are also affected after exposure to propanil (Barnett *et al.*, 1989; Ambrose *et al.*, 1972). Female and male rats exposed for 2 years to 400 ppm of propanil mixed in their feed have increased spleen weights, but the heart, kidney, liver and testes weights are unaffected (Ambrose *et al.*, 1972). In a subchronic study, female and male rats treated for 3 months with 1000 and 3300 ppm of propanil, respectively, did not have altered organ weights except increased spleen sizes (Ambrose *et al.*, 1972). Additionally, acutely exposed C57Bl/6 mice treated i.p. with 200 mg/kg of propanil had decreased thymus weights and increased spleen weights (Barnett and Gandy, 1989). These studies suggest that propanil may be more toxic to secondary immune organs than other nonimmune organs. The relevance of these immunotoxic effects will be discussed in more detail in later sections.

Other studies using common toxic endpoints such as mutagenicity, reproductive toxicity, and carcinogenicity have yielded negative results. Propanil has not been found to be mutagenic as determined in Salmonella reversion assays (McMillan *et al.*, 1988). The EPA has determined that propanil is not a carcinogen in humans (U.S. EPA OFFICE OF PESTICIDE PROGRAMS, 2003). Reproductive studies in rats have shown that chronic oral propanil exposure does not cause any abnormalities in the fertility of the parents nor does it affect the health of the offspring (Ambrose *et al.*, 1972). Propanil is classified in the “slightly toxic” toxicity class III for oral exposures and toxicity class IV

for inhalation and dermal exposures (U.S. EPA OFFICE OF PESTICIDE PROGRAMS, 2003).

Assessment of the effects of propanil on humans demonstrates that the greatest risk of toxic exposure is most hazardous with applicators and manufacturing workers. Examination of immune changes in farm-families exposed to propanil observed decreases in IL-2⁺ cells and natural killer (NK) cell counts (McClure *et al.*, 2001). However, these lower counts were associated with the preseason measurements and the cell counts rebounded during the spray season (McClure *et al.*, 2001). This could be due to the low particulate concentration of propanil in family homes lying adjacent to the spray fields (Richards *et al.*, 2001). Non-applicator personnel working in adjacent propanil-treated fields were also exposed to very low concentrations of propanil (Barnes *et al.*, 1987). These personnel were exposed to concentrations that were several orders of magnitude lower than the concentrations shown to be toxic (Barnes *et al.*, 1987). However, 61% of production workers exposed to propanil had chloracne indicating the higher risk of toxicity (Morse *et al.*, 1979).

Innate immune studies

The innate immune system is responsible for not only rapidly eliminating pathogens, but also initiating adaptive immune responses. Immunotoxic effects on cells of this system may also compromise the ability of an organism to control initial infections until an adaptive immune response can be generated. Two important effector cells of the innate immune system are macrophages and NK cells.

Propanil has been demonstrated to inhibit macrophage function. LPS-stimulated peritoneal macrophages harvested from mice 7 days following i.p. propanil exposure

(200 mg/kg) have reduced production of IL-6 (32% of control) and TNF- α (55 % of control) (Xie *et al.*, 1997b). In addition, oral exposure to propanil (400 mg/kg) also reduced the production of IL-6 (40% of control) (Xie *et al.*, 1997b). However, macrophage TNF- α production is more sensitive to the effects of an oral exposure to propanil. Forty mg/kg of propanil reduced the levels of TNF- α approximately the same amount as an i.p. exposure of 200 mg/kg of propanil (Xie *et al.*, 1997b). This data suggests that different routes of exposure can affect the extent of immunotoxicity on macrophages. However, the sensitivity of macrophages to the effects of propanil varies depending on the endpoint studied and the route of exposure.

Both Ca^{2+} release and NF- κ B regulate the production of IL-6 and TNF- α in macrophages. Ca^{2+} , in combination with diacylglycerol (DAG), stimulates protein kinase C (PKC). At the cytoplasmic membrane, PKC enhances the phosphorylation of some transcription factors for cytokine transcription, such as NF- κ B. Macrophages treated in vitro with propanil have a significant reduction in intracellular Ca^{2+} release (Xie *et al.*, 1997a). In addition, NF- κ B p65 subunit is decreased in the nucleus of propanil-treated macrophages (Frost *et al.*, 2001). The binding of NF- κ B to the TNF- α promoter is reduced in propanil-treated cells after 18 hours of in vitro exposure (Frost *et al.*, 2001). These data suggest the mechanism regulating the reduction in inflammatory cytokine production in propanil-treated macrophages is an effect on NF- κ B and Ca^{2+} .

NK cell function is also inhibited following propanil exposure. Mice treated i.p. with propanil and DCA have reduced NK cell cytotoxicity. NK cells harvested from the spleens of mice treated i.p. with 50 mg/kg of propanil have an approximate 20% reduction in their ability to lyse a target cell (Barnett *et al.*, 1992). The metabolite, DCA,

also inhibits the cytolytic ability of NK cells similar to the parent compound (Barnett *et al.*, 1992). The data suggests that propanil may reduce the capacity of cells of the innate immune system to fight new infections.

T cell toxicity

One of the first studied immunotoxic effects induced by propanil was observed on T cells (Barnett and Gandy, 1989). T cells are essential in facilitating a potent adaptive immune response. Briefly, CD4⁺ T cells are responsible for activating and coordinating the humoral immune response as well as cell-mediated immune responses. CD8⁺ T cells function primarily in executing cell-mediated immune responses. The thymus is the organ responsible for T cell development and maturation.

As mentioned previously, propanil induces thymic atrophy. Thymic atrophy is seen in acutely exposed mice approximately 2-4 days post-exposure (Cuff *et al.*, 1996). Thymic weight is reduced in these mice for up to 7 days following an i.p. dose of 200 mg/kg of propanil (Barnett and Gandy, 1989). By day 7, the thymus has begun to recover from the initial atrophy (Cuff *et al.*, 1996). After 14 days post-exposure, the thymus has fully recovered from the original insult (Cuff *et al.*, 1996). Analysis of the circulating T cell populations in the mesenteric lymph nodes and the spleen 7 days post-exposure shows that mature T cell numbers are unaffected as a result of the thymic atrophy (Zhao *et al.*, 1995). Failure to detect decreases in the mature T cell populations of immune organs may indicate that thymic atrophy is an endpoint to determine the extent of propanil exposure rather than a direct measure of damage to immune function.

The decrease in thymic weight is accompanied by a loss in total thymic cellularity, as well as an overall decline in the number of T cells in the various

developmental stages of maturation (Zhao *et al.*, 1995). Exposure doses as low as 100 mg/kg decrease the number of all the CD3⁺ and CD3⁻ subpopulations (Zhao *et al.*, 1995). Mice treated i.p. with 200 mg/kg propanil have the largest decrease in the percentage of CD3⁺CD4⁺CD8⁺ (DP) thymocytes 4 days post-exposure (Cuff *et al.*, 1996). This data indicates that the DP cells are the most sensitive of the thymocyte populations (Cuff *et al.*, 1996). The decrease observed in the immature population results in a downstream effect of decreasing the number of the more mature CD4⁺ single positive (SP) and CD8⁺ SP cells (Zhao *et al.*, 1995; Cuff *et al.*, 1996).

The primary mechanism by which propanil induces thymic atrophy is a result of increased levels of glucocorticoids (Cuff *et al.*, 1996; de la Rosa *et al.*, 2005). Acute i.p. propanil exposure with doses of 50 mg/kg in C57Bl/6 mice increases the levels of corticosterone in the serum to approximately 1000 ng/ml compared to approximately 60 ng/ml for control mice (de la Rosa *et al.*, 2005). The increase in corticosterone serum levels peaks within one hour following propanil exposure and returns to baseline levels within 24 hours (de la Rosa *et al.*, 2005). Inhibition of the glucocorticoid receptors (GR) with the GR antagonist RU486 or removal of the adrenal glands, the primary site for glucocorticoid production, abrogates the thymic atrophy induced by propanil (Cuff *et al.*, 1996; de la Rosa *et al.*, 2005). In response to the sudden loss in the thymocyte populations, the recovery of the thymocyte population is generated by an increase in the percentage of cycling DP and CD8⁺ SP cells 4 days post-exposure (Cuff *et al.*, 1996). However, the percentage of cycling thymocyte subpopulations returns to normal by day 7, indicating that recovery is initiated rapidly after exposure (Cuff *et al.*, 1996).

The extent of thymic atrophy induced by propanil can also be increased when combined with other herbicides (de la Rosa *et al.*, 2005). The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) does not decrease the number of DP or CD4⁺ SP cells following a 150 mg/kg i.p. exposure (de la Rosa *et al.*, 2005). However, the addition of 150 mg/kg of 2,4-D combined with a 150 mg/kg dose of propanil can induce a greater-than-additive decrease in the number of both the DP and CD4⁺ SP cell populations (de la Rosa *et al.*, 2005). This data demonstrates the potential for other chemicals to enhance the toxicity of propanil (de la Rosa *et al.*, 2005).

Analysis of T cell function after *in vivo* exposure to propanil has indicated that T cells are relatively resistant to the toxic effects of propanil. As mentioned previously, propanil exposure causes splenomegaly (Barnett and Gandy *et al.*, 1989; Ambrose *et al.*, 1972). The spleen is also an important secondary lymphoid organ. Therefore, the spleen was chosen for early immunotoxic characterization studies. Mixed lymphocyte reactions (MLR) are typically used to characterize CD4⁺ function. MLR of spleen cells from mice treated with an acute i.p. dose of propanil 1 week prior to harvest demonstrated that only at the highest doses tested of 400 mg/kg was there a significant decrease in the MLR (Barnett and Gandy, 1989). *In vivo* cell mediated immune responses were assessed using the contact hypersensitivity reaction (CHR) (Barnett and Gandy, 1989). The CHR was also decreased in animals treated with only the highest tested dose of 400 mg/kg (Barnett and Gandy, 1989). Finally, proliferation of splenocytes from mice treated *in vivo* with propanil was determined following *ex vivo* stimulation with the T cell mitogen, Concanavalin A (ConA). Again, only animals treated with the highest tested dose had significant reductions in proliferation (Barnett and Gandy, 1989). However, ConA

stimulated splenocytes from animals treated i.p. with 200 mg/kg of propanil had a significant reduction in IL-2 production (27% of control) and IL-6 production (45% of control) (Zhao *et al.*, 1998). Altogether, the studies characterizing the T cell toxicity of propanil indicate that several endpoints are required to establish an accurate inference of propanil's toxicity.

Mechanistic studies on the regulation of IL-2 in T cells demonstrated that *in vitro* propanil treatment decreases IL-2 transcription rate as well as IL-2 message stability (Zhao *et al.*, 1999). DNA binding of the IL-2 transcription factor, AP-1, is also reduced for up to 4 hours post-exposure in propanil-treated cells (Brundage *et al.*, 2004). In addition, propanil reduces the phosphorylation and the total protein levels of the subunit of AP-1, c-jun (Brundage *et al.*, 2004). These data suggest that propanil decreases the T-cell production of IL-2 by suppressing the activity of one of the subunits of a key transcription factor, however, the molecular mechanism by which propanil reduces phosphorylation is unknown.

B cell toxicity and the humoral response

As mentioned previously, propanil was found to be myelotoxic and induce splenomegaly. The bone marrow microenvironment is the site of B cell maturation prior to release into circulation. The spleen is an important site for early and late antibody production to bacterial pathogens (Martin *et al.*, 2001; Zandvoort *et al.*, 2002; Manz *et al.*, 2002). Approximately 40% of the lymphocytes in the spleen are B lymphocytes (Salazar *et al.*, 2005). Altogether, these organs represent two important sites for evaluating the immunotoxic effects of propanil on B cells.

Propanil exposure decreases the number of developing B cells in the bone marrow available for maturation. B cell maturation proceeds from the pro-B cell to pre-B cell and finally to the immature IgM⁺ B cell (Rosenberg *et al.*, 1994). *In vivo* i.p. propanil exposure at the lowest studied dose of 50 mg/kg decreased the number of murine pre-B cells in the bone marrow (~50% of control) 7 days post-exposure (de la Rosa *et al.*, 2003). Immature IgM⁺ B cells are also decreased in number (~50% of control) at doses of 150 mg/kg (de la Rosa *et al.*, 2003). However, this decrease in immature B cells does not result in a loss of circulating B cells as determined by an analysis of splenocyte populations (Salazar *et al.*, 2005). Total splenic B cell numbers, as well as the circulating follicular B cell population, remain unchanged following propanil exposure (Salazar *et al.*, 2005).

Although propanil exposure does not effect the number of B cells in the periphery, it can suppress B cell function. Exposure to 400 mg/kg of propanil suppresses splenic B cell proliferation after *ex vivo* stimulation with the B cell mitogen lipopolysaccharide (LPS) (Barnett and Gandy, 1989). Analysis of the antibody response to both T-independent type 2 (TI-2) and T-dependent (TD) model antigens found that these responses are more sensitive to propanil's toxic effects than B cell proliferation (Barnett and Gandy, 1989; Barnett *et al.*, 1992). Exposure to propanil on day 0 followed by immunization with sheep red blood cells (SRBC) on day 4 decreased the number of SRBC plaque forming cells (PFC) in a dose dependent fashion (Barnett and Gandy, 1989). Fifty mg/kg of propanil was the lowest dose to significantly reduce the number of SRBC PFC (67.5% of control) (Barnett and Gandy, 1989). Higher doses of propanil and DCA, 200 mg/kg and 150 mg/kg, respectively, were found to decrease the number of

PFC in animals immunized with DNP-Ficoll 4 days following propanil exposure (75% of control) (Barnett *et al.*, 1992). Altogether, these reports suggest that propanil exerts immunotoxic effects either directly on B cells or their supporting microenvironment critical for their normal function.

Adaptive immune response to bacterial challenges

In general, the collective *in vivo* studies on immune function indicate that propanil has suppressive effects on the immune system. Challenging the immune system with live bacteria provides important information toward understanding the relevance of altered immune function on individual lymphocyte populations. Live bacterial challenge studies provide information demonstrating the effect of decreased immune cell function on clearing infections caused by common pathogens. These studies are important for predicting risk of infection that is associated with a specific level of immunotoxicity.

The toxic effects of propanil on the cell-mediated immune response were studied using a *Listeria monocytogenes* infection model in C57BL/6 mice (Watson *et al.*, 2000). *L. monocytogenes* is an intracellular pathogen that requires an effective cell-mediated immune response to clear the infection. Animals were exposed to propanil and challenged with live *L. monocytogenes*. *Ex vivo* Con A and heat-killed *L. monocytogenes* (HKLM) stimulated splenocyte and liver cultures from the propanil-treated and *L. monocytogenes* challenged animals had an approximate 50% reduction in IFN- γ production (Watson *et al.*, 2000). However, analysis of spleen and liver endogenous cytokine levels from propanil-treated and *L. monocytogenes* infected animals showed no significant differences in IL-6, IFN- γ , TNF- α , and IL-1 β when compared to *L. monocytogenes*-infected control animals (Watson *et al.*, 2000). Additionally, animals

treated with 200 mg/kg of propanil and a LD₅₀ of *L. monocytogenes* did not demonstrate any greater susceptibility to infection than control animals (Watson *et al.*, 2000). This indicates that a 200 mg/kg exposure to propanil produces immunotoxic effects on cell-mediated immunocytes measured *in vitro*, but does not significantly affect the host's ability to mount an effective immune response. A limitation of this study is that *L. monocytogenes* infections can be initially controlled in mice lacking an adequate cytotoxic T cell response (Bancroft *et al.*, 1991). However, there have been no studies reported to date characterizing the effect of an acute propanil exposure on the immune response to a pathogen that predominantly requires a competent cytotoxic T cell response to clear the infection.

2,4-D

Background

2,4-D is a chlorinated phenoxy herbicide used for the control of broadleaf weeds throughout the year in both the public and private sector. 2,4-D is the most used pesticide ingredient in the home and garden sector in the United States and is the fifth most used active ingredient in the agricultural sector. 2,4-D is predominantly used in the Midwest, Great Plains, and the Northwest (U.S. EPA OFFICE OF PESTICIDE PROGRAMS, 2005).

2,4-D is readily metabolized in both the soil and the water. The half-life of 2,4-D in the water under aerobic and anaerobic conditions is 15 and 312 days, respectively. The half-life in the soil is approximately 59 days. Aerobic microbial metabolism is the major mechanism by which 2,4-D is degraded in both the water and soil. The major

metabolite of aerobic aquatic degradation is 2-chlorohydroquinone. Minor metabolites from aerobic microbial degradation include 2,4-dichlorophenol (2,4-DCP) and carbon dioxide. Conversely, anaerobic aquatic degradation results in the predominant formation of 2,4-DCP and carbon dioxide. The major metabolite of microbial degradation of 2,4-D in the soil yields 2,4-DCP (Veeh *et al.*, 1996).

The most common routes of exposure for 2,4-D include dermal and oral exposure, and to a lesser extent, inhalation (Garabrant *et al.*, 2002). Despite the rapid degradation of 2,4-D in the environment, exposure to humans is widespread. An analysis of 1000 randomly selected adults residing in the U.S. found that 12% had measurable levels of 2,4-D in the urine and 64% had detectable concentrations of the metabolite 2,4-DCP (Hill *et al.*, 1995). The median and maximum concentration of 2,4-D in the urine was 1.8 µg/liter and 37 µg/liter, respectively (Hill *et al.*, 1995). Following oral exposure in Fischer 344 rats, 2,4-D concentrations peak within the blood in 10-20 minutes (Pelletier *et al.*, 1989). Dermal exposures in rats demonstrates that 2,4-D is rapidly absorbed similarly to oral exposure and peaks 30 minutes post-exposure (Pelletier *et al.*, 1989). Upon entry into humans, 2,4-D is not metabolized (Knopp *et al.*, 1992). 2,4-D is widely distributed throughout the body due to its water solubility and does not accumulate to any specific organ (Pelletier *et al.*, 1989). The half-life of 2,4-D in the urine is approximately 17 hours and 11 hours in the plasma (Sauerhoff *et al.*, 1977). Due to the rapid clearance, inability to be metabolized, or locate to a particular tissue, 2,4-D is not believed to accumulate in repeatedly exposed humans (Garabrant and Philbert, 2002).

General toxicity

The toxicity of 2,4-D has been extensively studied. Numerous reports have evaluated the potential of 2,4-D to cause reproductive, carcinogenic, neurological, endocrinological and immunotoxic effects. The acceptable daily intake for 2,4-D as established by the World Health Organization (WHO) is .013 mg/kg/day (Zeljezic *et al.*, 2004). The conclusions from these studies have suggested that 2,4-D has very little overall toxicity (Munro *et al.*, 2002).

A number of genotoxic assays performed in a fibroblastic cell line found that 2,4-D does not increase the amount of DNA repair (Jacobi *et al.*, 1991; Clausen *et al.*, 1990; Galloway *et al.*, 1987). However, lymphocytes from human donors were exposed to a commercial formulation of 2,4-D in vitro and demonstrated that chromosomal aberrations were increased at concentrations that have been established by the WHO to lack any associated health risks (Zeljezic and Garaj-Vrhovac, 2004). The duration and concentration of lymphocyte exposure to 2,4-D has not been determined, thereby preventing an accurate assessment of genotoxic risks for lymphocytes (Zeljezic and Garaj-Vrhovac, 2004). Lymphocytes from humans exposed to 2,4-D in an occupational setting were analyzed for sister chromatid exchange (SCE) frequency and found no significant changes in SCE frequency in 2,4-D workers (Linnainmaa, 1983). Altogether, the literature supports the hypothesis that 2,4-D does not increase the risk for developing cancer (Munro *et al.*, 2002).

Adverse neurotoxic effects such as myotonia, characterized by difficulty relaxing skeletal muscles, can be observed in animals treated with high doses of 2,4-D (50-500 mg/kg) (Elo *et al.*, 1989). Locomotor effects have also been observed in rats at doses as low as 15 mg/kg/day, but these effects were transient (Mattsson *et al.*, 1997). Other

potentially lasting effects such as demyelination in the CNS have not been observed in animals (Garabrant and Philbert, 2002). Since the concentration of 2,4-D required to induce neurotoxic effects also causes systemic toxicity, 2,4-D is considered to be mildly toxic to the nervous system (Garabrant and Philbert, 2002).

Reproductive toxicity following 2,4-D exposure has been assessed in a number of animal studies. Maternal rats and rabbits exposed to doses in excess of 90 mg/kg/day had litters with reduced fetal weights and increased fetal variations (Charles *et al.*, 2001). Male mice and rats exposed chronically to 2,4-D did not produce any changes in the testes and did not affect reproductivity (Charles *et al.*, 2001; Charles *et al.*, 1996). There have been no published studies demonstrating that 2,4-D exposure induces reproductive or teratogenic effects in humans (Garabrant and Philbert, 2002; Munro *et al.*, 2002).

The ability of 2,4-D to disrupt the endocrine system is of recent interest and less studied compared with more common toxicological endpoints. The ability of 2,4-D to bind to the estrogen receptor was studied using an in vitro model in MCF-7 breast cancer cells (Lin and Garry, 2000). 2,4-D was found to induce estrogen receptor mediated growth at concentrations of 1 µg/ml (Lin and Garry, 2000). This concentration was roughly 500-fold less efficacious than E2 and is not considered to be estrogenic (Lin and Garry, 2000). Another approach analyzing the enzymes responsible for metabolizing E2 found that rats exposed to 375 mg/kg of 2,4-D had elevated amounts of CYP1A and CYP1B in the liver, kidney, and the mammary gland (Badawi *et al.*, 2000). These increased enzyme levels corresponded to a marked increase in estradiol and estrone (Badawi *et al.*, 2000). However, further dose response studies are needed to appropriately quantify the sensitivity for this effect. Finally, a preliminary study in

amphibians found that 2,4-D inhibited progesterone-dependent oocyte maturation, as well as, disrupt the actin cytoskeleton of oocytes (Lin and Garry, 2000). Additional studies are required to determine if 2,4-D is able to affect the progesterone pathway in mammals.

Immunotoxicity

Immunotoxic studies have suggested that 2,4-D has modest toxicity. Exposure to 2,4-D in CD-1 mice via a dermal administration and an ip immunization of sheep red blood cells (SRBC) on the following day decreased the number of SRBC-specific plaque forming cells (PFC) on day 5 post-immunization (Blakley *et al.*, 1986). The decrease in SRBC-specific PFC was dose dependent (Blakley and Schiefer, 1986). The lowest measured dose of 2,4-D to decrease SRBC-specific PFC was 100 mg/kg, which decreased the PFC 10% from controls (Blakley and Schiefer, 1986). In addition, acute dermal exposure did not affect the *ex vivo* proliferative responses of splenocytes to either the T cell mitogen, ConA, or the B cell mitogen, LPS (Blakley and Schiefer, 1986). Conversely, an acute oral exposure of 2,4-D in BDF₁ mice via gastric intubation and followed by an ip vaccination to SRBC on the next day, demonstrated that the number of SRBC-specific PFC was increased when measured on the fifth day of immunization (Blakley, 1986). The effect on PFC was not dose dependent and increased the number of SRBC PFC 1.5 to 2.4-fold following exposures of 50 to 200 mg/kg (Blakley, 1986). Animals treated with 2,4-D had normal proliferative responses in splenic T cells following ConA stimulation (Blakley, 1986). This suggests that the variation in SRBC PFC responses after oral or dermal exposure to 2,4-D is not likely due to changes in T cell function (Blakley, 1986). A proposed hypothesis for enhancement versus suppression of the antibody response in 2,4-D exposed animals is that 2,4-D acts as a

happen when given orally (Blakley, 1986). However, this hypothesis has not been experimentally tested.

Since 2,4-D is widely used for many different commercial and private applications, it is often applied in mixtures. Examining the effect of mixture interactions on the immune response is a more relevant method for evaluating immunotoxicity of 2,4-D. Initial studies demonstrate that mixture exposure with 2,4-D can enhance the immunotoxic effect of a single 2,4-D exposure.

A common commercial mixture of 2,4-D is Tordon 202C, which contains a 1:16 ratio of the herbicide picloram and 2,4-D (Blakley, 1997). The lowest tested dose of 2.5 mg picloram/kg/day and 50 mg 2,4-D/kg/day decreased the antibody response to SRBC 33% after 26 days of daily oral exposure (Blakley, 1997). An analysis of the effects of a single picloram or 2,4-D exposure was not performed, thereby preventing an interpretation of the mixture interaction. Another commonly occurring herbicide mixture includes propanil and 2,4-D. An acute ip exposure to 2,4-D did not affect pre-B or immature IgM⁺ B cells 2 days post-exposure (de la Rosa *et al.*, 2003). However, a 50 mg/kg mixture decreased both the pre-B and IgM⁺ B cells. When the propanil and 2,4-D mixture effect was analyzed on thymocytes, 200 mg/kg of 2,4-D decreased the number of CD4⁺CD8⁺ and CD4⁺CD8⁻. However, a 150/150 mixture decreased these thymocyte populations the same magnitude as a single 200 mg/kg exposure of 2,4-D. These findings suggest that the immunotoxic effects of 2,4-D may be enhanced when combined with other active herbicide ingredients.

There is little direct evidence that 2,4-D exposure affects human immune function. A published study on 10 farmers who used 2,4-D on their crops suggests that

there may be a risk for short-term immunosuppression (Faustini *et al.*, 1996). Blood samples were analyzed 7 days pre-exposure, 1-12 days post-exposure, and 50-70 days post exposure (Faustini *et al.*, 1996). CD4⁺, CD8⁺ T cells, and NK cells were all reduced 1-12 days post exposure (Faustini *et al.*, 1996). In addition, proliferative responses to ConA were decreased 50% from pre-exposure samples (Faustini *et al.*, 1996). By 50-70 days post exposure, the lymphocyte numbers and proliferation were normal. This data indicates that occupational exposure to 2,4-D exerts immunotoxic effects seen in animal studies. Given the widespread use of 2,4-D and its potential to be immunotoxic in greater-than-additive fashion when combined with propanil, further studies evaluating mixture effects on adaptive immune responses is warranted.

STREPTOCOCCUS PNEUMONIAE

Streptococcus pneumoniae is a gram-positive coccus that kills approximately 1 million children worldwide each year (Kruetzmann *et al.*, 2003). The antibody response to *S. pneumoniae* provides an excellent model for studying the humoral immune response to both TI-2 and TD antigens. The immunodominant antigen that elicits the TI-2 response is the cell wall polysaccharide phosphorylcholine (PC). One of the surface proteins that elicits a strong TD antibody response is pneumococcal surface protein A (PspA). Both of these antigens are important targets for vaccine development since robust antibody responses to these antigens are immunoprotective. In addition, these antigens serve as virulence factors for the bacteria. PC enables the bacterium to transport across endothelial and epithelial membranes (Cundell *et al.*, 1995; Tuomanen *et al.*, 1995). PspA inhibits the function of complement (Tu *et al.*, 1999).

This section is divided into five parts concentrating on literature relevant to regulation of the TI-2 antibody response. The first section will provide background information on the spleen, specific sites in the spleen involved in the response, and kinetics of the response. The second section will define the B cell subpopulations responsible for producing TI-2 antibodies. The third and fourth sections will describe mechanisms for enhancing or suppressing the TI-2 antibody response by the innate immune system and T cells. The final section will briefly compare and contrast the PspA and PC response for the purpose of identifying possible mechanisms for later discussion.

Site of humoral immune response

The central location for PC-specific antibody production is the spleen. The necessity of the spleen is made apparent in individuals with asplenia. Splenectomized patients are afflicted with chronic respiratory infections primarily caused by *S. pneumoniae* (Davidson and Wall, 2001). In addition, splenectomized individuals do not respond well to capsular polysaccharide vaccines (Davidson and Wall, 2001).

The marginal zone (MZ) in the spleen is the site primarily responsible for the TI-2 antibody responses (Guinamard *et al.*, 2000). The MZ of the spleen is defined as the compartment consisting of a broad band of cells surrounding the periarteriolar lymphoid sheath (PALS) containing T cells and the B cell follicles (Vos *et al.*, 2000). It has long been known that children under 2 years of age have poor responses to polysaccharide vaccines, which coincides with the maturation of MZ B cells (Timens *et al.*, 1989). In addition, TI-2 antigens rapidly localize to the MZ within 15 minutes after an ip injection (Harms *et al.*, 1996; van den Eertwegh *et al.*, 1992). Studies in Pyk-2 deficient mice, animals lacking a tyrosine kinase involved in growth factor receptor and chemokine

stimulation, showed that these mice do not develop MZ B cells (Guinamard *et al.*, 2000). However, the B cell populations in the bone marrow, the B1 cells in the peritoneum, and the serum Ig levels were all identical to wild type mice (Guinamard *et al.*, 2000). Subsequent immunization with TNP-Ficoll demonstrated that Pyk^{-/-} mice had dramatically reduced TNP-specific IgG2a (30% of control), IgG3 (2% of control), and IgM (30% of control) serum titers (Guinamard *et al.*, 2000). These observations strongly support the hypothesis that MZ B cells are responsible for producing the TI-2 specific antibodies.

As mentioned previously, TI-2 antigens are rapidly localized to the marginal zone. TI-2 antigen ligation of the B cell receptor (BCR) triggers B cell migration to the outer T cell borders in the spleen within the first 24 hours (Garcia *et al.*, 1999b). By 48 hours, the number of TI-2 antigen specific B cells increases in the T cell zones, in the red pulp, and along the red pulp: T zone junction (Vinuesa *et al.*, 2001). At 2-5 days, the antigen-specific B cells exponentially divide and differentiate into plasma cells primarily in extrafollicular foci in the red pulp of the spleen (Vinuesa *et al.*, 2001). B cell proliferation is the most rapid between days 3 and 5 (Vinuesa *et al.*, 2001). After day 7, the number of plasma cells decreases during the second week of the response. After day 14, the number of plasma cells remains static for at least 90 days (Garcia *et al.*, 1999b).

Responding B cell population

TI-2 responses are distinguished by early isotype class switching that occur more rapidly than T-dependent responses to protein antigens (Wu *et al.*, 1999). Isotype switching is observed as early as 3-4 days following immunization with polysaccharides (Leemans *et al.*, 1999). Six days following heat-killed *S. pneumoniae* (HKSP)

immunization, the IgG isotypes reach maximal levels (Wu *et al.*, 2002). The predominant isotypes produced to PC following HKSP vaccination are IgG3 and IgG2b and to a lesser extent IgG1 and IgG2a (Wu *et al.*, 1999).

The composition of responding B cells to a TI-2 response are predominantly high affinity B cells. Two days following antigen presentation, there are a 3-fold greater number of high affinity B cells entering cell cycle compared to low affinity B cells (Shih *et al.*, 2002). The three major idiotypes specific for PC that dominate the early humoral response are T15, M167 or M511, and M603 (Perlmutter *et al.*, 1984). T15 antibodies have the highest affinity of the three idiotypes. T15 is also the idotype expressed by the majority of PC-specific antibody secreting cells (ASC) (Feeney *et al.*, 1989; Martin *et al.*, 2001). These idiotypes have also been shown to undergo very little somatic hypermutation and do not enter germinal centers (Feeney and Thuerlauf, 1989; Wiens *et al.*, 2003). T15 plasmablasts found in both the spleen and peritoneal cavity largely express the B1 B cell phenotype 3 days post immunization (Martin *et al.*, 2001). The composition of MZ plasmablasts and B1 blasts is dependent on the route and dose of antigen administration (Martin *et al.*, 2001). A high dose of HKSP (Sharer *et al.*, 2003) given iv generates a larger T15 antibody response than a high dose given ip (Martin *et al.*, 2001). Low doses (Van Ravenzwaay *et al.*, 2003) given iv produce a reduced antibody response that is comparable to the high ip response. However, low ip doses initiate strong peritoneal B1 responses, which prevent sufficient amounts of antigen to reach the spleen thereby preventing a local splenic response (Martin *et al.*, 2001). It is currently unknown what the peritoneal B1 cells, splenic B1 cells, and MZ B cells contribute to the PC-specific serum titers.

Numerous studies reporting the importance of both MZ B cells and B1 cells in the TI-2 response have failed to reconcile the conflicting data demonstrating the contributions of these two B cell populations in TI-2 antibody responses. A recent report performed in transgenic mice (PV1TgL) containing B cells that are specific only for a synthetic TI-2 like Ag (PVP-360) suggests that B-1a cells are derived from MZ B cells (Whitmore *et al.*, 2004). Naïve PV1TgL mice do not express B-1 B cells but contain splenic MZ B cells. Three days and 5 days after immunization, antigen-specific B-1a cells begin to appear in the spleen and peritoneal cavity, respectively (Whitmore *et al.*, 2004). By days 5 and 8, the B-1a populations are the predominant B cell populations in the spleen and peritoneum, respectively. These data suggest that the MZ B cells may be the precursor population for B1a cells (Whitmore *et al.*, 2004). Altogether, these studies demonstrated that PC responses require both MZ B cells and B1 B cells for TI-2 antibody production.

Role of Innate Immunity

The MZ B cells are characterized by their high expression of the CD21/CD35 molecules on their surface. The CD21/CD35/CD19 coreceptor complex is critical for recognizing and binding complement components on the B cell. CD35, complement receptor 1 (CR1), binds the C3b and the C4b degradation components of the complement cascade (Vos *et al.*, 2000). CD21 (CR2) binds the complement component C3d. CD19 is a coactivator that is directly associated with CD21 and likely mediates the intracellular signaling of CD21 (Fearon *et al.*, 2000). The high expression of these complement binding receptors suggests that complement activation has an important role in TI-2 responses.

Components of the complement cascade are hypothesized to be one of the earliest signals necessary for TI-2 recognition. One of the roles of complement receptors in TI-2 responses is to lower the activation requirements necessary for antibody production. CD21/CD35 knockout mice vaccinated with 10^5 CFU HKSP and challenged with 5000 CFU of live *S. pneumoniae* had a survival rate of 33% compared to 100% survival rate for wild type animals (Haas *et al.*, 2002). However, increasing the immunization dose to 10^7 CFU HKSP for CD21/CD35^{-/-} mice allowed all the mice to survive the live challenge (Haas *et al.*, 2002). This study suggests that stimulation of the complement receptors lowers the threshold for B cell activation. Other reports have shown that both CD19 and CD21 lower the threshold for antigen stimulation in vitro. Mongini *et al.* demonstrated that CD21 and IgM receptor stimulation reduced the minimum concentration of antigen bound to mIgM required for entry into S phase by 100-fold (Mongini *et al.*, 1997). Deletion of CD19 in mice reduced the number of high-affinity plasma cells in response to an NP-Ficoll vaccination by approximately 40% (Shih *et al.*, 2002). However, activation of low-affinity plasma cells was completely abrogated in CD19^{-/-} mice (Shih *et al.*, 2002). These results indicate that the role of complement receptors in the TI-2 response is to lower the amount of antigen required for B cell activation.

Localization of antigen to the MZ is dependent on functional CR. Mice deficient in either the complement component C3 or the complement receptors CR1/2 are unable to localize TNP-Ficoll to the MZ (Guinamard *et al.*, 2000; Shih *et al.*, 2002). As expected, C3^{-/-} mice and mice depleted of complement via cobra venom factor subsequently have reduced IgG and IgM serum titers to TI-2 antigens (Guinamard *et al.*,

2000; Harms *et al.*, 1996). These data demonstrate that complement dependent localization of TI-2 antigens is crucial for normal antibody production.

The deposition of TI-2 antigens on the spleen occurs in specific sites and cell types. For example, complement bound antigens accumulate on follicular dendritic cells (FDC). FDC have high expression of both CD21 and CD35 (Peset Llopis *et al.*, 1996). Complement component C3d binds to Ab/Ag complexes, which form immune complexes (IC) that are recognized by CD21 (Fearon and Carroll, 2000). The IC are then transported to FDC and presented on its surface. Once presented, the IC are easily recognized by MZ B cells thereby aiding their activation (Fearon and Carroll, 2000). Because FDC present complement components efficiently, they are able to amplify an antibody response. A series of studies demonstrated that FDC enhance the Ab response 10-1000 fold in a CR2 dependent manner to ovalbumin (Qin *et al.*, 1998).

It is currently hypothesized that FDC have a role in the early isotype switching during the Ab response to TI-2 antigens. This hypothesis is supported by several findings. It has been observed that MZ B cells migrate to FDC within 24 hours following iv injection of Ag (Whipple *et al.*, 2004). Another study demonstrated that TI-2 antigens do not require the presence of specific Ig to produce C3d binding and this allows for a rapid uptake and presentation of TI-2 antigens by FDC thereby enabling rapid recruitment and activation of B cells (Peset Llopis *et al.*, 1996). When serum containing nonspecific antibody for pneumococcal polysaccharides (PPS) and complement were added to the spleens of vaccinated or naïve mice, no difference in PPS or C3d FDC localization was observed between the two groups (Peset Llopis *et al.*, 1996). However, inactivation of complement in the serum reduces PPS and C3d localization to FDC (Peset

et al., 1996). These studies suggest that a reduced requirement for specific Ig to initiate C3d binding enables a rapid uptake and presentation by FDC in the case of TI-2 antigens, thereby enabling rapid recruitment and activation of B cells (Peset Llopis *et al.*, 1996).

Other antigen presenting cells (APC) involved in early TI-2 antigens presentation and localization include MZ macrophages. MZ macrophages have been observed to rapidly take up TI-2 antigens shortly after injection (van den Eertwegh *et al.*, 1992). Phagocytosis of TI-2 antigens by MZ macrophages occurs independent of complement (van den Eertwegh *et al.*, 1992). Splenic deposition of TI-2 antigens is also dependent on the presence of MZ macrophages. Removal of MZ macrophages reduces the localization of TI-2 antigens to both the red pulp of the spleen and the MZ, while increasing deposition of antigens in the follicles (van den Eertwegh *et al.*, 1992). The increased localization of TI-2 antigens in the follicles corresponded with an increased humoral response to the antigen (van den Eertwegh *et al.*, 1992). This demonstrates that MZ macrophages have an important role in regulation of the TI-2 response, which is largely suppressive.

In addition to complement, other innate immune factors that regulate early isotype switching to TI-2 antigens include pro-inflammatory and anti-inflammatory cytokines as well as toll-like receptors (TLR). Neutralizing the pro-inflammatory cytokine TNF- α via an injection with monoclonal antibody 1 day post-HKSP vaccination suppressed the IgG anti-PC response 2-3 fold (Khan *et al.*, 2002). Other pro-inflammatory cytokines such as IL-6 and IFN- γ have been shown to moderately influence early PC isotype switching. Mice deficient in IL-6 and IFN- γ have significantly reduced IgG2a and IgG2b serum titers, respectively (Khan *et al.*, 2002). The anti-inflammatory cytokines have the

opposite effect on control of isotype switching. Mice deficient in IL-4 and IL-10 production have elevated IgG2a serum titers (2-fold) and increased IgG1 and IgG3 titers, respectively (Khan *et al.*, 2002). The peak splenic mRNA production for both the pro-inflammatory and anti-inflammatory cytokines occurs between 2-6 hours post-vaccination and returns to baseline within 24 hours. This indicates that these cytokines influence the beginning of the TI-2 response.

Other innate signaling molecules such as, toll-like receptors (TLR), also play a role in antibody responses to polysaccharides. TLR2^{-/-} mice have significantly reduced PC titers 7 days following vaccination with either purified polysaccharides or HKSP (Khan *et al.*, 2005; Sen *et al.*, 2005). Survival to a lethal challenge of *S. pneumoniae* is also mildly compromised in naive TLR2^{-/-} mice (Khan *et al.*, 2005). However, vaccinated TLR2^{-/-} mice survive a lethal challenge similarly to normal mice (Khan *et al.*, 2005). In addition, TLR stimulation enhances a polysaccharide response. Mice treated once with the TLR9 ligand unmethylated CpG-containing oligodeoxynucleotide (CpG-ODN) and alum and vaccinated twice with purified polysaccharide have enhanced primary and secondary antibody titers (Sen *et al.*, 2006). CpG and alum treatment produced the greatest enhancement on day 21 (Sen *et al.*, 2006). This data suggests that TLR are important for stimulating the TI-2 antibody response.

Role of T cells

The precise role of T cells in regulating the TI-2 humoral response is poorly understood. T cells are required for normal TI-2 responses. However, T cell deficient mice are able to produce antibody responses to polysaccharides, albeit, very poorly (Briles *et al.*, 1982b). Mice deficient in $\alpha\beta$ T cells have a 6-10 fold reduction in IgG3,

IgG2b, IgG1, but not IgM PC specific serum antibody, indicating their importance for isotype switching (Wu *et al.*, 1999). Removal of $\gamma\delta$ T cells, however, does not significantly affect PC serum titers (Wu *et al.*, 1999). The relative importance of CD4⁺ and CD8⁺ T cells has produced conflicting results. CD4⁺ T cells are more effective at stimulating the anti-PC serum response than CD8⁺ T cells following HKSP vaccination (Wu *et al.*, 2002). Injection of 2×10^6 CD4⁺ T cells into athymic mice increased the IgG PC-specific serum response approximately 100-fold compared to a 5-fold increase in animals given CD8⁺ T cells (Wu *et al.*, 2002). However, more recent findings suggest that CD8⁺ T cells have a greater impact on TI-2 antibody responses than CD4⁺ T cells (Kobrynski *et al.*, 2005). CD8^{-/-} mice vaccinated with 23-valent pneumococcal polysaccharide vaccine Pneumovax failed to produce detectable polysaccharide antibody in the serum. In contrast, CD4^{-/-} mice did not have significantly different titers compared to wt mice in response to the Pneumovax vaccination (Kobrynski *et al.*, 2005). A possible explanation for the discrepancy between these studies is that CD8⁺ cells are involved in responses to soluble polysaccharides and CD4⁺ cells are critical for whole bacterial responses (Kobrynski *et al.*, 2005).

It has long been known that polysaccharide antigens cannot be presented on a MHC molecule. However, it is apparent that T cells contribute to PC antibody responses. Recent data suggests that T cells are activated in a noncognate fashion, which does not involve T cell receptor (TCR) antigen recognition in the context of MHC. Transgenic mice with T cells expressing only a TCR that is incapable of responding to a protein Ag did not affect PC-specific serum titers following HKSP vaccination (Wu *et al.*, 2002). In addition, transgenic mice deficient in MHCII peptide loading have normal serum PC

titers (Wu *et al.*, 2002). These results indicate that specific recognition of antigen by the TCR is not required for normal TI-2 antibody responses.

Several studies have demonstrated that other T cell coactivating molecules are important for noncognate T cell polysaccharide responses. These coactivators have also been shown to be important for isotype switching. For example, studies have shown that B7:CD28 interactions are required for normal TI-2 responses. Blockade of B7 interactions with CD28 via *in vivo* administration of CTLA-4 Ig prior to HKSP vaccination suppresses PC-specific IgG sera titers but does not affect PC-specific IgM titers (Wu *et al.*, 2002; Wu *et al.*, 1999).

CD28 stimulation on T cells also upregulates the expression of CD137 (4-1BB) on T cells (Wu *et al.*, 2003). Ligands for CD137 are found on APC such as dendritic cells and B cells. Several experiments have demonstrated the importance of CD137 in the TI-2 response. Mice deficient in CD137 expression have significantly reduced IgM and IgG anti-PC responses following HKSP vaccination (Wu *et al.*, 2003). In addition, *in vivo* administration of anti-CD137 mAb to HKSP vaccinated mice increases proliferation in splenic CD4⁺ T cells in a B7 dependent manner 14 days post-immunization (Wu *et al.*, 2003). Anti-CD137 administration also was found to increase B cell and CD8⁺ T cell proliferation (Wu *et al.*, 2003). These data demonstrate that CD28 and CD137 cooperatively regulate splenic proliferation and isotype switching following HKSP vaccination.

A third coactivating molecule on the T cell that is also necessary for PC Ig production is CD40L. CD40L-KO mice have a marked reduction in anti-PC IgM and a complete abrogation of anti-PC IgG responses following HKSP vaccination (Wu *et al.*,

1999). Antibodies specific for CD40 also act as an adjuvant for T-independent antibody responses. Anti-CD40 treatment increases the number of NP-specific ASC 25-fold following NP-Ficoll immunization (Garcia *et al.*, 1999a). The increase in NP-specific ASC does not occur until 6 days post-immunization (Garcia *et al.*, 1999a). The growth of plasmablasts is also sustained in the red pulp as demonstrated by immunohistochemistry (Garcia *et al.*, 1999a). These data suggests that CD40 ligation increases the TI-2 response by extending proliferation rather than increasing the number of B cells recruited to the response.

Finally, CD1, the MHC class I-like molecule, presents several types of glycolipids and nonpeptide Ags (Kobrynski *et al.*, 2005). Mice that do not express CD1 have undetectable polysaccharide serum titers following Pneumovax vaccination (Kobrynski *et al.*, 2005). CD1 expressing APC are hypothesized to present polysaccharide antigens to CD8⁺ T cells, thereby contributing to their activation. $\beta_2m^{-/-}$ mice, which lack functional CD1, MHC class I, and FcRn, have suppressed polysaccharide antibody responses following Pneumovax vaccination (Kobrynski *et al.*, 2005). However, reconstitution of $\beta_2m^{-/-}$ mice with normal CD8⁺, B cells, and macrophage/monocytes restored the polysaccharide antibody response to those of normal mice (Kobrynski *et al.*, 2005). Altogether, these data demonstrate the panoply of coactivators that affect PC responses.

PspA response

This section will briefly compare and contrast the major differences between the development of the antibody response to the TD protein antigen, PspA, with the PC antibody response. The differences between the PspA response and PC response are due primarily to the TD and TI-2 nature of the responses. T-dependent responses involve

cognate T cell responses, affinity maturation of B cells in germinal centers, and high antibody output during the second week of the antibody response. Both TD and TI-2 responses require CD21 receptors, early cytokine production, and T cell-associated coreceptors.

TD antigens, such as PspA, are processed and presented by several types of APC such as dendritic cells, macrophages, and B cells. The antigen bound to APC then migrates to the PALS region of the spleen and present the antigen to T cells. B cells migrate through the high endothelial venules (HEV) into the T cell zone. B cells expressing a specific mIg for the immunizing antigen are upheld. Next, a primary focus of activated B cells and T cells localize at the T:B cell border as early as 2 days post-immunization (Jacob *et al.*, 1991). B cells rapidly proliferate in the primary foci, which peaks by day 8 (Jacob *et al.*, 1991). Between 4 and 12 days, some of these B cells in the primary foci will continue to differentiate into plasma cells and migrate to the red pulp where they will produce antibody of low affinity during the primary phase of the TD response (Liu *et al.*, 1991).

The production of high affinity antibodies during TD responses depends on the formation of germinal centers. Germinal centers are characterized by three cellular components: FDC, B cells, and T cells (Han *et al.*, 1995). Formation of germinal centers is observed by day 8 and persists until day 16 in the spleen (Jacob *et al.*, 1991). Germinal center formation requires several costimulating molecules including those that are also required for PC responses.

CD21/CD35, CD40L, and B7 costimulation are all required for establishment and maintenance of germinal centers. B cells that do not express CD21 and CD35 fail to

survive selection in germinal centers (Fischer *et al.*, 1998). Deletion of both CD21 and CD35 on B cells also markedly reduces serum antibody titers to TD antigens (Croix *et al.*, 1996). Administration of anti-CD40L antibody at the time of vaccination with a TD antigen severely reduces both germinal center formation and the sera antibody titers (Han *et al.*, 1995). CD40L is also necessary for maintaining germinal centers as demonstrated when anti-CD40L is administered late in the response after germinal centers have already formed (Han *et al.*, 1995). In addition to CD40L, B7-2 is necessary for normal germinal center formation. Administration of anti-B7-2 antibody at the time of vaccination decreases the number of germinal centers formed and serum titers by 50% (Han *et al.*, 1995).

PspA-specific antibodies are measurable in the serum by day 6 and maximal by day 10 (Wu *et al.*, 2000). The predominant isotypes produced are IgG2b, IgG1, and IgG2a (Wu *et al.*, 1999). CD4⁺ T cell help is required for at least the first 6 days during the PspA response (Wu *et al.*, 2000). However, PC responses only require CD4⁺ T cell help for the first 2 days (Khan *et al.*, 2004).

As mentioned previously, costimulatory molecules present on T cells are required for normal antibody responses and isotype switching for TD and TI-2 responses. B7-2 but not B7-1 stimulation is necessary for normal PspA responses (Wu *et al.*, 2000). In addition, mice deficient in CD40L have nearly undetectable anti-PspA IgG responses (Wortham *et al.*, 1998). As opposed to the PC response, deletion of CD137 does not affect the PspA response (Wu *et al.*, 2003).

Studies suggest that early cytokine production has a greater impact on the PspA response compared to the PC response. TNF- α is required during the first 48 hours

following vaccination for the PspA response (Khan *et al.*, 2002). Other pro-inflammatory cytokines such as IFN- γ and IL-6 are also required for IgG PspA switching (Khan *et al.*, 2002). Lack of anti-inflammatory cytokines IL-4 and IL-10 were both found to increase IgG anti-PspA titers (Khan *et al.*, 2002). The effect of IL-10 deletion produced a similar effect of general IgG increase to both PC and PspA antigens, however the increase was not consistently significant (Khan *et al.*, 2002).

ENDOCRINE REGULATION OF THE IMMUNE SYTEM

Endocrine regulation of the immune system is a well-established phenomenon. The predominant classes of sex steroids produced by the gonads that regulate the immune response are estrogens, androgens, and progestins. The humoral immune response, in particular, is strongly influenced by sex hormones. This is evidenced by a number of observations. Autoantibody-mediated diseases afflict females at a much higher rate than males. For example, systemic lupus erythematosus (SLE) and Sjögrens' syndrome afflict women 9 times more frequently than men (Ansar *et al.*, 1985). In addition, female mice have higher IgM and IgG serum titers than males (Eidinger *et al.*, 1972).

This section will provide background information on previous hormone studies that identified immunomodulating effects leading to altered antibody responses. The three major classes of sex hormones are discussed. The effect of estrogen on the humoral response is given the greatest emphasis. The estrogen section will also present data demonstrating similar immunomodulating effects on the antibody response, thymus, and BM that is observed in both 17 β -estradiol (E2) and propanil-treated animals.

ESTROGEN

Autoimmune vs. nonautoimmune animals

The sex hormone E2 is one of the first steroid hormones to be identified as an immunomodulator. Early studies were performed in the autoimmune mouse strain, NZB/W. This strain will spontaneously develop lupus-like symptoms that eventually are lethal.

Initial studies found that when male NZB/W mice were castrated and treated with E2, the mortality rate was greatly increased and resembled the mortality rate of female NZB/W mice (Roubinian *et al.*, 1978). Subsequent studies found that E2 increased the antibody responses in autoimmune mice (Brick *et al.*, 1985). Castrated male NZB/W mice treated with E2 for 10 weeks increased the number of IgM PFC to the TI-2 antigen TNP-Ficoll 3-4-fold (Brick *et al.*, 1985). E2 also increased the number of IgG and IgM PFC 3-fold to the TI-1 antigen, TNP-LPS. The IgG and IgM PFC responses to the TD antigen SRBC was increased 2-fold following E2 treatment (Brick *et al.*, 1985). In contrast, nonautoimmune DBA/2 mice did not have significantly changed PFC to TNP-Ficoll, TNP-LPS, or SRBC following identical E2 treatment (Brick *et al.*, 1985). These early studies suggested that E2 stimulates the antibody response only in autoimmune mice.

Subsequent studies established that E2 exposure increases the antibody responses in nonautoimmune rats and mice, as well. Male Sprague Dawley rats treated with E2 3 days prior to SRBC immunization had a 4-fold increase in antibody titers 6 days post-immunization (Myers and Petersen, 1985). E2 treatment 3 days prior to the secondary immunization also yielded a similar increase in antibody titers (Myers and Petersen, 1985). However, in contrast to earlier findings demonstrating that E2 increases in the

number of PFC, the number of PFC was not affected in the nonautoimmune mice suggesting that E2 treatment increased antibody synthesis in the plasma cells (Myers and Petersen, 1985).

A more prolonged exposure to E2 induced a greater enhancement of the humoral response. Castrated C57BL/6 mice were chronically exposed to E2 via pellet implantation for 4-6 months. The total number of IgG and IgM ASC in the spleen were increased 6-fold and 3-fold, respectively (Verthelyi and Ahmed, 1998). The chronic E2 exposure is characterized by a decrease in both the percent and number of B cells in the spleen, which suggests that more splenic B cells become activated (Verthelyi and Ahmed, 1998). The total number of IgG bone marrow ASC was also increased 12-fold in E2 exposed mice (Verthelyi and Ahmed, 1998). In addition to increasing the number of ASC in the spleen and BM, the amount of immunoglobulin produced by each plasma cell is also increased (Verthelyi and Ahmed, 1998). Morphometric analysis of the spots obtained from ELISPOT assays demonstrated that the density and area of the splenic and BM ASC were significantly increased in chronically E2 treated mice (Verthelyi and Ahmed, 1998). Finally, vaccination of the E2 exposed mice with a variety of autoantigens such as dsDNA and actin or killed whole bacteria such as *Actinobacillus pleurpneumoniae* or *Brucella abortus* yielded increased antibody serum titers to all tested antigens (Verthelyi, and Ahmed, 1998).

Altogether, these data established that E2 increases the antibody response in both nonautoimmune and autoimmune animals. The antibody response is increased both by increasing the number of plasma cells and increasing the immunoglobulin synthesis by each plasma cell. The data also suggest that the increased response is the result of

polyclonal activation. However, the initial evidence presented conflicting data on the duration of an E2 exposure required for an increased antibody response.

A conclusive time course of E2 exposure necessary to enhance the humoral response is currently unknown. For example, peripheral blood mononuclear cells (PBMC) from rhesus macaque females treated for 18 hours with E2 increases the percent of IgG and IgA ASC (Lu *et al.*, 2002). In contrast to the macaque studies, the lupus prone BWF1 mice require 2 months of chronic E2 pellet exposure until IgG and IgM anti-DNA serum titers are increased (Yurino *et al.*, 2004). Variation in animal models and lack of antibody kinetic studies precludes a conclusion of the duration of E2 exposure required for an enhanced response.

E2 sensitive B cell subpopulations

It is hypothesized that the amplification of the ASC by E2 disproportionately affects certain B cell subpopulations. The B cell subpopulations, B1 B cells and MZ B cells, have both been shown to be targets for E2 induced proliferation and activation. B1 B cells from BWF1 mice cultured with E2 for 4 days had an approximate 2-fold increase in IgM production (Yurino *et al.*, 2004). However, the classically defined B cell population, B2 B cells, cultured under identical conditions did not produce significantly different concentrations of IgM (Yurino *et al.*, 2004). Another study found that B1 B cells from C57BL/6 mice exposed to E2 for 2-3 months and pretreated with bromelain had increased numbers of splenic bromelain-specific PFC. However, the number of B2 B cell PFC in the spleen was not affected (Ansar *et al.*, 1989).

The MZ B cell population is also sensitive to the effects of E2. R4A-IgG2b BALB/c mice are a nonautoimmune strain of mouse that produce an easily detectable

autoimmune B cell that is normally removed during the course of maturation (Grimaldi *et al.*, 2006). When these mice are treated with E2 chronically, they develop a lupus-like phenotype (Grimaldi *et al.*, 2001). Phenotypic analysis of the splenic anti-DNA ASC demonstrates that MZ B cells are increased 4-fold compared to control animals (Grimaldi *et al.*, 2001). In contrast, follicular B cells are not significantly increased (Grimaldi and Diamond *et al.*, 2001). Altogether, these studies suggest that the E2-induced increase in ASC is not the result of polyclonal activation, since particular subsets are affected. These data also suggests that the subpopulations of B cells affected by E2 exposure are also involved in the production of TI-2 antibodies.

Mechanisms for E2-induced antibody enhancement

The expression of ER α has been suggested to be the receptor regulating the increased antibody response. The ER α and ER β are expressed on lymphocytes including B cells, suggesting a mechanism for a direct effect of E2 on B cells (Grimaldi *et al.*, 2002). Analysis of ER mRNA expression in peritoneal B1 and splenic B2 B cells from 8-12 month old BWF1 mice treated chronically with E2 demonstrated that ER α is significantly increased in B1 B cells compared to control animals (Yurino *et al.*, 2004). However, ER α expression in B2 B cells is unaffected (Yurino *et al.*, 2004). ER α has been shown to regulate the increased humoral immune response following chronic E2 exposure. Nonautoimmune C57BL/6J/129 castrated male mice treated with E2 5 days/week for 2.5 weeks have significantly increased numbers of IgA, IgG, and IgM ASC in both the spleen and BM (Erlandsson *et al.*, 2003). Deletion of ER α in mice abrogates the increased ASC in the spleen or BM (Erlandsson *et al.*, 2003). However, ER β ^{-/-} mice had similar ASC responses as control mice (Erlandsson *et al.*, 2003).

Altogether, these data suggest that the E2-induced B cell activation is mediated through ER α .

E2 exposure elicits a number of changes in the genetic expression profile of B cells that decreases tolerance. BALB/c mice treated with E2 for 3-6 weeks have increased expression of the proteins VCAM-1 (40%), CD22 (40%), SHP-1 (20%), and Bcl-2 (20%) (Grimaldi *et al.*, 2002). VCAM-1 functions as a ligand for VLA-4 and is hypothesized to protect B cells from apoptosis during early B cell development. CD22 and SHP-1 regulate the antigen threshold required for B cell activation. Bcl-2 increases survival of autoreactive B cells. The percent increase in these proteins induced by E2 is sufficient to alter B cell survival (Grimaldi *et al.*, 2002).

It is hypothesized that increased expression of these proteins decreases the amount of stimulation required for activation and protects against apoptosis during B cell development. For example, Bcl-2 expression is also increased in the bone marrow of pro/pre B cells and immature B cells (Grimaldi *et al.*, 2002). Supporting experiments show that E2 exposure decreases the percentages of BM B220⁺ cells, but transitional splenic type 2 B cells, which differentiate into mature splenic B cells, are increased (Grimaldi *et al.*, 2001). In addition, transitional splenocytes from E2-treated mice demonstrated that these cells were resistant to apoptosis (Grimaldi *et al.*, 2002). These data suggest that certain B cells during development in both the BM and spleen have anti-apoptotic expression patterns that results in increased survival.

Other studies evaluating the effect of E2 on B cell maturation revealed that autoreactive B cells are eliminated from circulation, albeit less effectively than in normal mice (Grimaldi *et al.*, 2006). R4A-IgG2b BALB/c mice treated with E2 for 5-6 weeks

have a 4-fold increase in mature autoreactive B cells compared with control animals (Grimaldi *et al.*, 2006). Autoreactive B cells survive in greater numbers throughout maturation in the BM and the spleen (Grimaldi *et al.*, 2006). High affinity autoreactive B cells survived in slightly greater numbers than low affinity autoreactive B cells in E2 treated mice to enter into the splenic transitional pool from the BM, suggesting that some autoreactive B cells are eliminated in the BM similarly to normal animals (Grimaldi *et al.*, 2006). However, E2 dramatically increased the number of high affinity autoreactive B cells that survived the splenic transitional stages to enter the mature B cell population (Grimaldi *et al.*, 2006). Normal mice had a 3-fold reduction in high affinity autoreactive B cells during the transitional to mature stages, but the number of high affinity autoreactive B cells in E2 treated mice was not reduced (Grimaldi *et al.*, 2006). Interestingly, E2 treatment did not affect the deletion of low affinity autoreactive B cells during the differentiation of transitional B cells into mature B cells (Grimaldi *et al.*, 2006). These data indicate that E2 increases the number of autoreactive B cells by impairing the removal of high affinity autoreactive B cells during the immature and transitional phases of maturation.

Effects of E2 on B cell maturation in the BM

The effects of E2 on lymphopoiesis further upstream in the B cell maturation stages in the BM indicate a suppressive effect. The number of B220⁺ cells in the BM is decreased 5-fold following 2.5 weeks of treatment in C57BL/6J/129 castrated mice (Erlandsson *et al.*, 2003). Furthermore, the E2 decreases the number of pro-B cells, pre-B cells, and immature IgM⁺ B cells 70-80% compared to control animals (Erlandsson *et al.*, 2003). Selected deletion of the ER α and ER β in mice demonstrated that both

receptors are required for the E2-mediated decrease in the pro-B and IgM⁺ B cell populations (Erlandsson *et al.*, 2003). ER α alone regulates the E2-mediated deletion of pre-B cells (Erlandsson *et al.*, 2003).

Effects of E2 on the thymus

E2 also affects T cell development by inducing thymic atrophy and T cell apoptosis. E2 decreases the thymocyte population approximately 80% of controls (Screpanti *et al.*, 1989). The decrease is primarily in the CD4⁺CD8⁺ population (Screpanti *et al.*, 1989). The mechanism regulating E2-mediated thymic atrophy is Fas/FasL dependent (Do *et al.*, 2002). In contrast with antibody responses and lymphopoeisis, E2-mediated thymic atrophy requires both ERs for the maximal effect (Erlandsson *et al.*, 2001). The increase in thymic atrophy is correlated with changes in the populations of T cells in the spleen of developing mice, however, no studies have been performed to determine if these changes affect adaptive immune responses (Erlandsson *et al.*, 2001). Altogether, these data emphasize the diverse immunomodulating affects of estrogen.

PROGESTERONE

The other major immunomodulatory female sex hormone is progesterone. Analysis of the effects of progesterone on immune function and antibody production suggest an inhibitory or an opposing effect to estradiol. *In vitro* progesterone treatment on PBMC from rhesus macaques inhibits the number of IgG and IgA ASC approximately 90% from controls (Lu *et al.*, 2002). Progesterone has also been found to induce TH2 cytokine production. *In vitro* culture with progesterone of human cloned T cells from multiple sclerosis patients induced all of the clones to produce IL-4 and suppressed inflammatory

cytokine production (Correale *et al.*, 1998). The progesterone regulated protein, progesterone induced blocking factor (PIBF), regulates some of the immunomodulatory effects of progesterone. *In vitro* treatment with PIBF on ConA stimulated murine splenic CD4⁺ and CD8⁺ T cells induced both cell populations to produce the TH2 cytokines IL-4, IL-10, and IL-3 (Szekeres-Bartho and Wegmann, 1996). However, the expression of the TH1 cytokine, IFN- γ , was not affected (Szekeres-Bartho and Wegmann, 1996).

Progesterone may downregulate the antibody response by also affecting apoptosis and bcl-2 expression. Progesterone decreases the expression of bcl-2 in estrogen treated Jurkat T cells and increases apoptosis (McMurray *et al.*, 2001). It is currently unknown if lymphocytes express progesterone receptors (Bouman *et al.*, 2005). These data suggest that progesterone antagonizes the effects of estrogen.

Progesterone is hypothesized to have the greatest affect on mucosal immune responses. Subcutaneous progesterone exposure increased the number of antigen specific ASC in the vagina and the uterus following intranasal or vaginal immunization with cholera toxin B (Johansson *et al.*, 1998). An opposing study demonstrated that progesterone exposure decreases resistance to a viral challenge (Gallichan and Rosenthal, 1996). Mice treated with progesterone prior to i.n. immunization with glycoprotein B from herpes simplex virus increased the ratio of IgG to IgA vaginal titers (Gallichan and Rosenthal, 1996). In addition, progesterone administration or elevated levels of progesterone associated with the diestrus stage of the estrus cycle increase viral load following vaginal HSV challenge (Gallichan and Rosenthal, 1996). Altogether, these data present a contrasting set of data concerning the effect of progesterone on mucosal challenges that differs according to antigen exposure.

ANDROGENS

Androgens represent the third major sex hormone produced by the gonads. Numerous studies have demonstrated that testosterone suppresses the humoral response. The androgen receptor is expressed on B cells (Benten *et al.*, 2002). NZB/W female mice chronically treated with testosterone had a 3-5 fold increase in survival time compared to controls (Roubinian *et al.*, 1979). In addition, female OVX NZB/W mice treated with testosterone had a 2-fold reduction in the number of IgM PFC following TNP-Ficoll immunization (Brick *et al.*, 1985). The numbers of IgG and IgM PFC were also reduced in the testosterone treated females 2-3 fold following TNP-LPS immunization (Brick *et al.*, 1985). Androgen replacement therapy in patients with Klinefelter's syndrome demonstrated a significant reduction in serum IgA, IgG, and IgM serum titers (Kocar *et al.*, 2000). Experiments suggest that compounds that are antiandrogenic do not have contrasting effects on the immune system. The antiandrogen, flutamide, does not significantly affect the anti-IgM response following SRBC immunization and chronic flutamide treatment (O'Connor *et al.*, 2002; Ladics *et al.*, 1998). These studies indicate that androgens are immunosuppressive with regards to humoral responses and antiandrogens are neither immunostimulatory nor immunosuppressive.

SUMMARY

These experiments employed the HKSP immunization model to study the effects of herbicide exposure on the humoral immune response. Previous reports demonstrated a number of immunotoxic effects induced by propanil and 2,4-D. These studies were

conducted to determine if these previously observed immunotoxic effects affect an adaptive immune response to a common bacteria.

The experiments in Chapter 2 establish an environmentally relevant route of herbicide exposure to study these immunotoxic effects. Chapter 3 characterizes the effects of propanil, 2,4-D, and 1:1 mixture of the two herbicides on the PC and PspA-specific responses following HKSP immunization. Chapter 4 determines if propanil selectively enhances TI-2 antibody responses as well as determine the time of exposure and antigen concentration requirements for the enhanced antibody response. Chapter 5 examines the role of the endocrine system in the enhanced antibody response following propanil exposure. Finally, Chapter 6 explores the effect of the propanil-induced antibody enhancement on the survival to a virulent *S. pneumoniae* challenge.

**CHAPTER 2: COMPARISON BETWEEN ASPIRATION
AND IP ROUTES OF HERBICIDE EXPOSURE ON THE
IMMUNE SYSTEM**

INTRODUCTION

Previous published data on the immunotoxic effects of propanil exposure have been performed using an ip exposure (de la Rosa *et al.*, 2005). As mentioned in the General Introduction, human exposure to propanil occurs primarily via inhalational, dermal and oral routes. The goal of these experiments is to establish a model to study an environmentally relevant route of exposure for propanil. This new exposure model will be applied to contrast and compare the effects of propanil exposure on immune function that has previously been established to be susceptible following ip exposure. In addition, mixture interactions will be studied in the new exposure model.

An aspiration route of exposure was chosen based on the result of preliminary experiments performed in the laboratory. Dermal studies found that propanil was not readily transported across the skin (data not shown). As mentioned in the General Introduction, previous experiments have demonstrated that oral exposures to propanil require approximately twice the concentration to induce the same toxicity as ip exposures (Xie *et al.*, 1997b). For these reasons, an inhalational model was studied.

Propanil has previously been shown to induce thymic atrophy that is easily measured by decreases in thymic weight and in the number of thymocyte subpopulations (de la Rosa *et al.*, 2005). In addition, 2,4-D induces a greater-than-additive effect on the thymus when administered with propanil (de la Rosa *et al.*, 2005). One of the common routes of occupational exposure to 2,4-D includes inhalation (Garabrant and Philbert, 2002). For these reasons, thymic atrophy was chosen as the endpoint for evaluating the efficacy of herbicide exposure by aspiration. Long-term goals for this research include

evaluating the effects on a humoral response following vaccination. Therefore, the effects of an aspiration exposure were also studied in HKSP immunized animals.

METHODS

Animals

Six to eight week old C57Bl/6 female mice were purchased from Hill Top Lab Animals (Scottdale, PA). Mice were housed in microisolator cages in pathogen free conditions at West Virginia University's animal facility. Mice were kept on a 12 hr light-dark cycle and allowed to acclimate to the facility for 1 week. These studies were conducted in accordance with all federal and institutional guidelines for animal use and were approved by the West Virginia University Institutional Animal Care and Use Committee.

Pesticides

Propanil, 3,4-dichloropropionanilide, 99% pure was purchased from Chem Service. 2,4-D, Dimethylamine salt of 2,4-dichlorophenoxyacetic acid was purchased from Universal Cooperatives. 2,4-dichlorophenoxyacetic acid, 99.5% pure was purchased from Chem Service.

Herbicide exposures

For aspiration, pesticides were diluted in 12 μ l of olive oil and 8 μ l DMSO. Three or 4 mg of purified pesticide was dissolved in DMSO/olive oil for a 150-mg/kg or 200 mg/kg treatment, respectively. Mixture exposures were prepared by dissolving both herbicides in the 20 μ l olive oil and DMSO solution. Animals were lightly anesthetized with 150 μ l of a 5mg/ml solution of Nembutal prior to pesticide exposure. Mice were

suspended vertically and the tongue was extended with forceps to prevent swallowing of the herbicides. Herbicides were pipetted at the base of the tongue and subsequently inhaled.

For ip exposures, propanil was dissolved in peanut oil at a concentration of 15 mg/ml or 20 mg/ml for 150 mg/kg or 200 mg/kg exposures. Commercial formulation of 2,4-D was diluted in sterile PBS at the appropriate concentration for a 150 mg/kg or 200 mg/kg injection. Herbicides were injected ip in 200 μ l volumes.

Bacterial preparation and immunization with R36A

S. pneumoniae strain R36A, an avirulent, nonencapsulated strain, was grown to mid-log phase in Todd-Hewitt broth (Becton Dickinson, Sparks, MD) + .05% yeast extract (Becton Dickinson) and stored at -80°C . For immunization, stock was cultured in a candle jar for 18 hrs at 37°C on blood agar plates (Becton Dickinson). A few characteristic colonies were selected and suspended in 200 ml Todd-Hewitt broth + .05% yeast extract. Bacteria were grown at 37°C to an absorbance reading at 650nm of 0.4 and heat killed for 4 hours in a 60°C water bath. A final concentration of 10^9 CFU/ml was established in PBS based on colony counts. Sterility was confirmed by culture. Heat-killed stock was stored at -20°C in 1 ml aliquots. Mice were immunized ip with 2×10^8 CFU on the same day as herbicide exposure.

Organ preparation

Mice were euthanized with 100 μ l Nembutal Sodium Solution (50 mg/ml, Abbott Laboratories, North Chicago, IL). Spleen and thymus wet weights were recorded. Spleens were mechanically dissociated through Spectra nylon mesh (Spectrum Labs, Rancho Dominguez, CA) in complete cell media containing RPMI-1640 (BioWhitaker,

Walkersville, MD), 10% heat inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc, Logan, UT), 10 mM HEPES (Sigma), 1 mM L- glutamine (Gibco, Rockville, MD), 5×10^{-5} M 2-mercaptoethanol (Sigma), 100 U/ml penicillin (Gibco), and 100 μ g/ml streptomycin (Gibco). Thymuses were mechanically dissociated between microscope slides in complete cell media. Red blood cells in the spleen and thymus were lysed with Tris-buffered ammonium chloride. Cell suspensions were washed twice and counted using a hemacytometer. Viability was determined using Trypan blue dye exclusion.

Measurement of antibody secreting B cells (ASC) in the spleen

Acrowell™ 96 well filter plates (Pall Life Sciences, Ann Arbor, MI) were coated with 50 μ l PC-BSA (Biosearch Technologies, Novato, CA) (10 μ g/ml) overnight at 4°C. In all subsequent steps, plates were washed with PBS + .01% Tween-20. Plates were blocked with 200 μ l/well complete medium + 25% FBS for 2 hours at 37°C. Plates were washed and cells (100 μ l/well) were then added at a concentration of 5×10^6 cells/ml or 1×10^6 cells/ml. All samples were plated in triplicate. Plates were incubated for 4-6 hours at 37°C in a 5% CO₂ incubator. After washing, goat anti-mouse alkaline phosphatase (AP) conjugated IgG or IgM antibodies (Southern Biotechnology Associates, Birmingham, AL), diluted 1/250 in PBS + 1% BSA + .05% Tween-20, were added to the appropriate wells (100 μ l/well). Plates were incubated overnight at 4°C and washed. SIGMAFAST 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma-Aldrich, St. Louis, MO) were dissolved in distilled water and added at 100 μ l/well. Color development was stopped by washing with distilled water. The number of spots/well was counted using a dissection microscope (Olympus Optical Co., Melville,

NY). The number of ASC was calculated by using the mean number of spots from triplicate wells. The number of ASC was normalized to 1×10^6 B cells.

Flow cytometric analysis

Splenic cells were stained with the appropriate combinations of rat anti-mouse B220-APC (RA3-6B2), rat anti-mouse CD4-FITC (GK1.5), or rat anti-mouse CD8 α -PE (53-6.7) (BD PharMingen, San Diego, CA). All steps were performed in PBS supplemented with 1% FBS and 0.04% sodium azide (Sigma). Briefly, 1×10^6 cells were stained in a total volume of 25 μ l of antibodies at the appropriate concentrations for 25 minutes on ice in the dark. After incubation, cells were washed twice and fixed in 0.04% paraformaldehyde overnight at 4°C (Fisher Scientific, Pittsburgh, PA). The following day cells were washed twice to remove the paraformaldehyde and resuspended in 1 ml of staining media. For each sample, 10,000 cells were collected for analysis on a Becton-Dickinson FACScan (Becton Dickinson Immunocytometry Systems, Mansfield, MA). Analysis was performed using WinMDI software (Joseph Trotter, Scripps Institute, San Diego, CA). Population percentages, obtained from flow cytometric analysis, were used to calculate the absolute cell number by multiplying the percentage of cells in a population by the total number of cells harvested per organ.

Statistics

One-way analysis of variance (ANOVA) was performed for all statistical analyses using a Tukey-Kramer t-test to perform multiple comparisons between all treatment groups. A significance level of $p \leq 0.05$ was used for all tests. Statistical analysis was performed using JMP software (SAS Institute Inc., Cary, NC). All experiments were

performed 3 or more times with similar results. The figures are representative data from one experiment.

RESULTS

Aspirated herbicides decrease thymic weight similarly to an ip injection.

Mice were treated with either a 150 mg/kg or 200 mg/kg concentration of herbicides. Animals were exposed to either individual propanil or 2,4-D treatments or a 1:1 mixture of the two herbicides. Mice were exposed to the herbicides via either an ip or aspiration route of exposure. Animals that received the herbicides via the aspiration route did not remain unconscious as long as animals receiving herbicides via the ip route.

Propanil-treated mice exposed via the aspiration route were unconscious for approximately 45 min compared to 30 min in animals receiving Nembutal alone.

However, ip injected mice receiving propanil were unconscious for 6-8 hours. A 150/150 mg/kg ip mixture herbicide exposure to the mixture of herbicides significantly decreased the thymic weights in a greater-than-additive fashion compared to the single herbicide exposures (Figure 1). Aspiration exposure to the mixture of herbicides was not significantly different from the ip mixture exposure (Figure 1). In addition, 150 mg/kg single herbicide exposures were not significantly different between both aspiration and ip routes of exposure (Figure 1). Aspirated or ip injected 200 mg/kg single propanil and aspirated or ip injected mixture herbicide exposures produced statistically similar thymic atrophy (Figure 1). However, single 2,4-D exposure was an approximate LD₅₀ when the herbicide was aspirated (data not shown). Surviving mice exposed to 2,4-D by aspiration induced similar decreases in thymus size compared to ip injected animals (Figure 1).

Aspirated herbicides decrease the number of thymocytes similar to ip injected herbicides.

Thymocyte populations were analyzed from the thymuses of herbicide-treated animals. The total number of thymocytes from 150 mg/kg and 200 mg/kg in mixture treated animals were decreased similarly in both aspirated or ip injected groups (Figure 2). Aspirated 200 mg/kg propanil and 2,4-D significantly decreased the number of thymocytes compared to aspirated controls (Figure 2). In contrast, ip injected 200 mg/kg propanil and 2,4-D were not significantly decreased from ip vehicle controls (Figure 2).

Analysis of the $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ subpopulations demonstrate a similar magnitude of reduction in mixture treated animals via either the aspiration or ip route of exposure (Figures 3A, 3B, 3C). Single exposures produced similar decreases in thymocyte subpopulations regardless of aspiration or ip injection (Figures 3A, 3B, 3C). The statistical significance of single exposures varied with concentration and route of exposure, however, the general effect of the herbicides on the thymocyte subpopulations is similar regardless of the route of exposure (Figures 3A, 3B, 3C).

Aspirated herbicides do not affect the number of ASC.

Since herbicide exposure induced thymic atrophy similarly in aspirated and ip injected animals, the effect of aspirated herbicides on the antibody response to an ip HKSP immunization was examined. Previous experiments demonstrated that propanil and 2,4-D mixture exposure increases the number of PC-specific ASC following HKSP immunization (de la Rosa dissertation). Mice were immunized with HKSP and treated with herbicides via either aspiration or ip injection. Mixture herbicide exposure

increased the number of IgG and IgM PC-specific ASC when animals were ip injected with a 150/150 mg/kg herbicide mixture (Figure 4). However, aspirated herbicides did not significantly increase the number of PC-specific ASC compared to aspirated vehicle mice (Figure 4).

DISCUSSION

These experiments begin to explore how the toxic effects of the herbicides 2,4-D and propanil differ when animals are exposed via different routes. These experiments use established immunotoxic effects induced by ip exposure to evaluate the efficacy of an environmental route of exposure on immune function. Propanil and mixture treatments produced similar immunotoxic effects on the thymus when animals were aspirated or ip injected. 2,4-D treatments were more lethal when animals were aspirated instead of ip injected. The cause of increased mortality for aspirated 2,4-D compared to ip or dermal exposures is not currently known. However, the thymic atrophy induced in the surviving animals was similar in both exposure models. Animals exposed to herbicides via aspiration did not have significantly different antibody responses. Altogether, these data demonstrate the importance of considering exposure routes when evaluating the toxicity of chemical exposures.

Propanil induces thymic atrophy by increasing glucocorticoids in ip injected mice. Although aspirated propanil treatment induces similar thymic atrophy as ip injection, the mechanism has not been studied. However, given the similarity of the response in ip vs. aspiration, it is plausible that the mechanisms are the same. Aspirated herbicides did not exhibit the overt neurotoxicity that is observed in ip injected animals. This result

suggests that the hind leg paralysis and the period of unconsciousness induced by the toxicity from ip injected herbicides is not involved in thymic atrophy.

The route of immunization and the site of the antibody response might be responsible for the inability of aspirated herbicides to enhance the antibody response. IP immunizations typically pass through the subdiaphragmatic lymphatics (Smith *et al.*, 1996). Since the mechanism that is responsible for enhancing the antibody response following herbicide exposure is probably different from the one regulating thymic atrophy, it is unknown what is required for splenic effects. Respiratory exposure to herbicides may induce a more localized effect. Since ip immunization does not produce strong antibody responses in the lung, a different route of immunization may be necessary. Follow-up studies may utilize an antigen that elicits a strong local antibody response via respiratory challenge. Preliminary experiments with live aspirated *S. pneumoniae* were unsuccessful due to a high mortality associated with herbicide and bacterial challenge. Death was observed within hours of exposure, suggesting acute respiratory distress. Future experiments may attempt an immunization several days prior to herbicide exposure to allow for complete clearance of the antigen and a reduction in inflammation.

Altogether, these data demonstrate the importance of route of administration when analyzing toxicity of chemical exposure. Thymic atrophy is readily induced by herbicide exposure and the sensitivity of the assay may serve as an endpoint for determining pesticide exposure. However, effects on humoral responses are dependent on the primary site of antibody production.

Figure 1. Aspirated herbicides decrease thymic weight similarly to ip injection.

C57BL/6 female mice (5/group) were treated with vehicle control, 150 or 200 mg/kg propanil, 150 or 200 mg/kg 2,4-D, or a 150/150 or 200/200 mixture. Mice were exposed via aspiration or ip injection and thymic weights were recorded on day 2. Values represent the mean $\pm$ SD. * Significantly different from vehicle, $p \leq .05$. # indicates a mixture interaction.

Figure 2. Aspirated herbicides decrease the number of thymocytes similarly to ip injection.

C57BL/6 female mice (5/group) were treated with vehicle control, 150 or 200 mg/kg propanil, 150 or 200 mg/kg 2,4-D, or a 150/150 or 200/200 mixture. Mice were exposed via aspiration or ip injection and thymocyte numbers were counted on day 2. Values represent the mean number of thymocytes $\pm$ SD. * Significantly different from vehicle, $p \leq .05$. # indicates a mixture interaction.

Figure 3. Aspirated herbicides decrease thymocyte subpopulations similar to ip injection.

C57BL/6 female mice (5/group) were treated with vehicle control, 150 or 200 mg/kg propanil, 150 or 200 mg/kg 2,4-D, or a 150/150 or 200/200 mixture. Mice were exposed via aspiration or ip injection. The thymocyte subpopulations (A) CD4⁺CD8⁻, (B) CD4⁻CD8⁺, and (C) CD4⁺CD8⁺ were enumerated by flow cytometry on day 2. Values represent the mean number of thymocytes $\pm$ SD. * Significantly different from vehicle, $p \leq .05$. # indicates a mixture interaction.

Figure 4. Aspirated herbicides do not affect the number of PC-specific ASC.

C57BL/6 female mice (5/group) were treated via aspiration or ip injection with vehicle control or a 150/150 mixture and vaccinated ip with 2×10^8 CFU HKSP. Numbers of PC-specific IgG and IgM ASC were determined by ELISPOT assay on day 7. Values represent the mean $\pm$ SD of ASC/ 1×10^6 B cells. * Significantly different from vehicle, $p \leq .05$.

**CHAPTER 3: THE POLYSACCHARIDE ANTIBODY
RESPONSE AFTER *STREPTOCOCCUS PNEUMONIAE*
VACCINATION IS DIFFERENTIALLY ENHANCED OR
SUPPRESSED BY 3,4-DICHLOROPROPIONANILIDE AND
2,4-DICHLOROPHENOXYACETIC ACID**

The polysaccharide antibody response after *Streptococcus pneumoniae* vaccination is differentially enhanced or suppressed by 3,4-dichloropropionanilide and 2,4-dichlorophenoxyacetic acid

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Running title: Propanil and 2,4-D alter TI-2 antibody levels

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ABSTRACT

Propanil (3,4-dichloropropionanilide) and 2,4-D (2,4-dichlorophenoxyacetic acid) are commonly used herbicides that have toxic effects on the immune system. The present study determined the effect of exposure to these chemicals on the immune response to a bacterial vaccine. The antibody responses to the T-independent type 2 antigen, phosphorylcholine (PC) and the T-dependent antigen, pneumococcal surface protein A (PspA) were characterized in C57BL/6 mice following heat-killed *Streptococcus pneumoniae* (HKSP) immunization and single or mixture herbicide exposure. Propanil exposure significantly increased the number of PC-specific IgM, IgG2b, and IgG3 antibody secreting B cells (ASC) in the spleen 4-6 fold over control animals in a dose dependent manner. However, the number of ASC in the bone marrow and serum titers were comparable in control and propanil-treated mice. In contrast, 2,4-D exposure decreased the number of PC-specific IgM and IgG bone marrow ASC 2-3 fold from control animals. The decrease in bone marrow ASC in 2,4-D-treated mice corresponded to a 3-4 fold decrease in PC-specific IgM, IgG2b, and IgG3 serum titers compared to control mice. The number of ASC in the spleens of 2,4-D-treated mice was, however, comparable to control mice. The antibody response to PspA was not affected by any of the treatments. There were no mixture interactions between the two herbicides in any of the responses measured. These results characterize the primary PC-specific antibody response in the bone marrow, spleen, and serum following HKSP vaccination and herbicide exposure. The differential effects of propanil and 2,4-D on the antibody response to a bacterial vaccine demonstrate the potential of chemical exposure to augment or suppress immune responses to vaccines and infectious diseases.

INTRODUCTION

The class of pesticides, commonly referred to as herbicides, is extensively used both commercially and by individuals making them ubiquitous in the environment. The immune system, in particular, is reported to be sensitive to the toxic effects of herbicides (Faustini *et al.*, 1996; Short and Colborn, 1999). Since infectious diseases are a major cause of morbidity and mortality worldwide, it is important to determine if exposure to these compounds compromises the immune response to an infection. 2,4-dichlorophenoxyacetic acid (2,4-D) and 3,4-dichloropropionanilide (propanil) are commonly used herbicides marketed as a chemical mixture under the product names of NOX-D and Herbanil 368 (Crop Protection Handbook, 2003). Previous research has demonstrated that 2,4-D and propanil are immunotoxic to the primary immune organs and to specific immune cell functions, however, the effects of an exposure to a mixture of these herbicides on an *in vivo* immune response has not been studied (de la Rosa *et al.*, 2003; de la Rosa *et al.*, 2005; Barnett and Gandy, 1989; Blakley, 1997). This study was performed to determine if exposure to these herbicides, either alone or as a mixture, altered the primary humoral immune response to the model bacterial vaccine, heat-killed *Streptococcus pneumoniae* (HKSP).

Propanil is an amide class herbicide that induces thymic atrophy and splenomegaly. Numerous studies have established that exposure to propanil can inhibit the function of a variety of immune cell populations including macrophages, T cells, and natural killer cells (Barnett and Gandy, 1989; Barnett *et al.*, 1992; Xie *et al.*, 1997; Zhao *et al.*, 1998). Murine studies have demonstrated that propanil inhibits the antibody response in the spleen to the model T cell-independent-type 2 (TI-2) antigen DNP-Ficoll

and the T-dependent (TD) antigen sheep red blood cells (SRBC) (Barnett and Gandy, 1989; Barnett *et al.*, 1992). 2,4-D is a chlorinated phenoxy compound designed as a synthetic form of the plant hormone auxin (Munro *et al.*, 2002). Reports on the immunotoxic effects of 2,4-D are inconclusive. Studies using a mouse model demonstrated that oral exposure to 2,4-D at the time of vaccination with SRBC increased the number of antibody producing cells (ASC) in the spleen (Blakley, 1986). Conversely, another report found that exposure to a mixture of 2,4-D and the herbicide picloram decreased the number of plaque-forming cells in the spleen following SRBC vaccination (Blakley, 1997). Thus, additional studies are needed to determine the effects of 2,4-D on the humoral immune response.

S. pneumoniae kills approximately 1 million children aged 5 years or younger annually and remains one of the most common causes of pneumococcal death worldwide. Due to its prevalence, it is a well-characterized model for studying the humoral immune response. Vaccination with HKSP elicits a TD antibody response and a TI-2 antibody response (Wu *et al.*, 1999). Pneumococcal surface protein A (PspA) is a TD antigen on *S. pneumoniae* that acts as a virulence factor by inhibiting the functions of complement (Tu *et al.*, 1999). Phosphorylcholine (PC) is a cell wall polysaccharide and the immunodominant antigen that elicits the TI-2 antibody response. PC is a virulence factor that functions by transporting the bacterium across the epithelial and endothelial membranes (Cundell *et al.*, 1995; Tuomanen *et al.*, 1995). Current vaccine strategies use a 23-valent polysaccharide vaccine or a 7-valent conjugate vaccine, both of which produce a robust anti-polysaccharide response (Bogaert *et al.*, 2004). The kinetics of the

serum antibody response and the predominant isotypes produced to PspA and PC are well characterized (Wu *et al.*, 2000; Wu *et al.*, 1999).

Our laboratory focuses on studying potential synergistic effects on the immune system following exposure to a mixture of propanil and 2,4-D. Previous studies show that exposure to either propanil or 2,4-D alone induced thymic atrophy and reduced the number of double positive ($CD4^+CD8^+$) thymocytes (de la Rosa *et al.*, 2005). In the bone marrow, exposure to either herbicide alone decreased the number of pre-B ($B220^+CD43^+IgM^{lo}$) cells and IgM^+ B ($B220^+CD43^+IgM^{hi}$) cells (de la Rosa *et al.*, 2003). However, exposure to the mixture of propanil and 2,4-D caused greater-than-additive decreases in the same cell populations in the thymus and bone marrow suggesting that interactions of the mixture increased the toxicity of the individual chemicals (de la Rosa *et al.*, 2003; de la Rosa *et al.*, 2005).

Based on the previous reports on the immunotoxic effects of propanil and 2,4-D, it was originally hypothesized that exposure to the herbicides would inhibit the humoral immune response after vaccination with HKSP and that exposure to the mixture would be more immunotoxic than the individual compounds. However, in contrast to the hypothesis, the results demonstrated that propanil and 2,4-D differentially affected the immune response to HKSP and there were no apparent interactions between the two herbicides. Exposure to propanil significantly increased the number of PC-specific ASC in the spleen. 2,4-D had no effect on the PC response in the spleen but significantly decreased the number of PC-specific ASC in the bone marrow. The decrease in ASC in the bone marrow after exposure to 2,4-D correlated with a significant decrease in the PC-specific serum antibody titers. There was no effect on the response to PspA by any of the

treatments. In addition, these results extend our knowledge of the humoral immune response to vaccination with HKSP by characterizing the primary antibody response to PC in the bone marrow and the spleen in conjunction with the serum antibody response.

MATERIALS AND METHODS

Mice.

Six to eight week old C57Bl/6 female mice were purchased from Charles River Farms (Wilmington, DE). Mice were housed in microisolator cages in pathogen free conditions at West Virginia University's animal facility. Mice were kept on a 12 hr light-dark cycle and allowed to acclimate to the facility for 1 week. Food and water was provided *ad libidum*. These studies were conducted in accordance with all federal and institutional guidelines for animal use and were approved by the West Virginia University Institutional Animal Care and Use Committee.

Chemicals.

Propanil (3,4-dichloropropionanilide, 99% pure) was purchased from Chem Service (West Chester, PA). Commercial-grade 2,4-D amine (47.2% dimethylamine salt of 2,4-dichlorophenoxyacetic acid, 52.8% inert ingredients, Universal Cooperatives, Inc., Minneapolis, MN) was purchased from Southern States Cooperative (Morgantown, WV).

Bacterial preparation and immunization.

S. pneumoniae strain R36A (a gift from Meenal Elliott, West Virginia University) an avirulent, nonencapsulated strain, was used for all experiments. Strain R36A was chosen because it is a commonly used strain of *S. pneumoniae* and the kinetics of the serum antibody response and the predominant isotypes to PC and PspA have been well established (Wu *et al.*, 1999; Wu *et al.*, 2000). Strain R36A was grown to mid-log phase in Todd-Hewitt broth (Becton Dickinson, Sparks, MD) + .05% yeast extract (Becton Dickinson) and stored at -70°C. For immunization, stock was cultured in a candle jar for 18 hrs at 37°C on blood agar plates (Becton Dickinson). A few characteristic colonies

were selected and suspended in 200 ml Todd-Hewitt broth + .05% yeast extract. Bacteria were grown at 37°C to an absorbance reading at 650nm of 0.4. Bacteria were heat killed for 4 hours at 60°C. A final concentration of 10^9 CFU/ml was established in PBS based on colony counts. Sterility was confirmed by culture. Heat-killed stock was stored at -20°C in 1 ml aliquots. Mice were immunized intraperitoneally (i.p.) with 2×10^8 CFU.

Exposure of mice to herbicides.

Mice (5-6/ group) were treated with either single doses of herbicides (propanil or 2,4-D) or a 1:1 mixture of both herbicides by i.p. injection within one hour of HKSP vaccination. Mice were treated with a range of concentrations of herbicide based on milligrams of herbicide/ kilogram of body weight (mg/kg). Propanil was dissolved in peanut oil and animals were treated with 25, 50, 100, or 150 mg/kg. 2,4-D was diluted in sterile PBS and mice were treated with 150 mg/kg. 2,4-D concentration was based on the amount of active 2,4-D in the commercial preparation. The route of exposure and the doses used were based on previous studies that demonstrated a mixture interaction at 150 mg/kg propanil and 150 mg/kg 2,4-D on thymocyte populations (de la Rosa *et al.*, 2005). Control animals were treated with the vehicle peanut oil only as previous studies determined that there was no difference between animals treated with the peanut oil vehicle compared to the PBS vehicle.

Preparation of spleen and bone marrow cell suspensions.

Mice were euthanized with 100 µl Nembutal Sodium Solution (50 mg/ml, Abbott Laboratories, North Chicago, IL) on days 3, 5, 7, 10, and 14 following herbicide exposure and vaccination. Spleen wet weights were recorded. Spleens were mechanically dissociated through Spectra nylon mesh (Spectrum® Labs, Rancho Dominguez, CA) in

complete cell media containing RPMI-1640 (BioWhitaker, Walkersville, MD), 10% heat inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc, Logan, UT), 10 mM HEPES (Sigma, St. Louis, MO), 1 mM L- glutamine (Gibco, Rockville, MD), 5×10^{-5} M 2-mercaptoethanol (Sigma), 100 U/ml penicillin (Gibco), and 100 μ g/ml streptomycin (Gibco). To collect bone marrow cells, one hind leg was removed from each animal. Femur and tibia were flushed with complete media for single cell suspensions. Red blood cells in the spleen and bone marrow populations were lysed with Tris-buffered ammonium chloride. Cell suspensions were washed twice and counted by hemacytometer in Trypan blue.

Flow cytometric analysis.

Cells were stained with the appropriate combinations of rat anti-mouse B220-APC (RA3-6B2), rat anti-mouse CD23-PE (B3B4), rat anti-mouse CD21-FITC (7G6), rat anti-mouse CD4-FITC (GK1.5), or rat anti-mouse CD8 α -PE (53-6.7) (all from BD PharMingen, San Diego, CA). All steps were performed in PBS supplemented with 1% FBS and 0.04% sodium azide (Sigma). Briefly, 1×10^6 cells were stained in a total volume of 25 μ l of antibodies at the appropriate concentrations for 25 minutes on ice in the dark. After incubation, cells were washed twice and fixed in 0.04% paraformaldehyde overnight at 4⁰C (Fisher Scientific, Pittsburgh, PA). The following day cells were washed twice to remove the paraformaldehyde and resuspended in 1 ml of staining media. For each sample, 10,000 cells were collected for analysis on a Becton-Dickinson FACScan (Becton Dickinson Immunocytometry Systems, Mansfield, MA). Analysis was performed using WinMDI software (Joseph Trotter, Scripps Institute, San Diego, CA). Population percentages, obtained from flow cytometric analysis, were used

to calculate the absolute cell number by multiplying the percentage of cells in a population by the total number of cells harvested per organ. Marginal zone B cells are defined as B220⁺CD21/35^{hi} CD23^{neg/low}, follicular B cells are defined as B220⁺CD21/35^{int} CD23^{hi} (Oliver *et al.*, 1997). B cells are defined as all cells that are B220⁺ and include marginal zone B cells and follicular B cells.

Measurement of antibody secreting B cells (ASC) in the bone marrow and spleen.

Acrowell™ 96 well filter plates (Pall Life Sciences, Ann Arbor, MI) were coated with 50 µl PC-BSA (Biosearch Technologies, Novato, CA) (10 µg/ml) or 50 µl PspA (10 µg/ml) (PspA was a generous gift from Clifford Snapper, USUHS) overnight at 4°C. In all subsequent steps, plates were washed with PBS + .01% Tween-20. Plates were blocked with 200 µl/well complete media + 25% FBS for 2 hours at 37°C. Plates were washed and cells (100 µl/well) were then added at a concentration of 5x10⁶ cells/ml or 1x10⁶ cells/ml. All samples were plated in triplicate. Plates were incubated for 4-6 hours at 37°C in a 5% CO₂ incubator. Plates were washed and goat anti-mouse alkaline phosphatase (AP) conjugated IgG, IgG1, IgG2a, IgG2b, IgG3, or IgM antibodies (Southern Biotechnology Associates, Birmingham, AL), diluted 1/250 in PBS + 1% BSA + .05% Tween-20, were added to the appropriate wells (100 µl/well). Plates were incubated overnight at 4°C and washed. SIGMAFAST 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma-Aldrich, St. Louis, MO) were dissolved in distilled water and added at 100 µl/ well. Color development was stopped by washing with distilled water. The number of spots/well was counted using a dissection microscope (Olympus Optical Co., Melville, NY). The number of ASC was calculated

by using the mean number of spots from triplicate wells. Data are expressed as the number of ASC per 1×10^6 B cells or as the number of ASC per spleen or bone marrow.

Measurement of PC- and PspA- specific titers.

Serum samples were prepared via blood collected from the heart. Immulon 2 plates (for PC) or Immulon 4 plates (for PspA) (ThermoLabsystems, Franklin, MA) were coated overnight at 4°C with 5 µg/ml PC-BSA or 10 µg/ml PspA (50 µl/well). Plates were washed, blocked with 3% BSA + PBS at 37°C for 2 hours. Plates were washed and 100 µl/well of twofold dilutions of sera in PBS +1% BSA were added starting at 1/400 for the IgG and IgM ELISAs and 1/250 for the IgG subclasses. Plates were then incubated for 1 hour at 37°C and washed. AP conjugated antibodies (100 µl/well) were added for 1 hour at 37°C. Plates were washed and phosphatase substrate tablets (Sigma-Aldrich) were dissolved in PNPP (p-Nitrophenyl Phosphate, Disodium Salt) substrate buffer. Plates were developed and absorbance read at 405nm on a µQuant spectrophotometer (Bio-Tek instruments, Winooski, VT) using KCJunior software (Bio-Tek instruments). To determine the titer a standard pooled sera was diluted and plated on each ELISA plate. The titer for each sample was determined by comparison to the standard sera when the OD 405nm for the standard was 0.200 at a 1:3200 dilution for IgM and IgG or at 1:2000 for IgG2b and IgG3. These dilutions were chosen because they are in the linear part of the curve for the respective isotypes.

Measurement of ex vivo PC-specific antibody production.

Spleen cells were cultured *in vitro* with no additional stimulation for 5 days at 37°C and 5% CO₂ at a concentration of 5×10^5 cells/ml in 500 µl complete media in 48 well tissue culture plates (Costar, Corning Inc., Acton, MA). All cultures were

performed in duplicate. The protein synthesis inhibitor cycloheximide was added to replicate samples at a concentration of 75 µg/ml (50 µl/well) to distinguish the amount of antibody produced *de novo* in culture from preformed antibody secreted during the culture period (Dhanjal *et al.*, 1992). Supernatants were collected and antibody ELISAs were performed as described above. *De novo* antibody synthesis was determined by subtracting the absorbance readings from cycloheximide treated cells from absorbance readings from non-cycloheximide treated cells.

Statistics.

One-way analysis of variance (ANOVA) was performed for all statistical analyses using a Dunnett's t-test to compare herbicide treated animals with control animals. A significance level of $p \leq 0.05$ was used for all tests. Identification of possible mixture interactions was determined using a partial factorial design. A mixture interaction was defined as the sum of the responses of the individual components of the mixture is significantly different from the response of the mixture treatment. Statistical analysis was performed using JMP software (SAS Institute Inc., Cary, NC). All experiments were performed 3 or more times with similar results. The figures are representative data from one experiment.

RESULTS

Propanil exposure increased the number of PC-specific antibody secreting B cells in the spleen.

To determine if propanil and 2,4-D had either individual effects or cooperative effects on the humoral response in the spleen, mice were vaccinated with HKSP and treated with either a single dose of propanil (150 mg/kg), 2,4-D (150 mg/kg), or a mixture (150/150 mg/kg). Serum titers to the PC antigen are detectable by day 4 and peak at day 6-7 following HKSP vaccination (Wu *et al.*, 2000; Wu *et al.*, 1999). Splenic ASC have been reported to peak 2-3 days prior to serum titers (Verheul *et al.*, 1990). Based on these reports, days 3, 5, 7, and 10 post-exposure were chosen to analyze the *in vivo* time course of the PC-specific antibody response.

Initial experiments in vehicle control animals immunized with HKSP demonstrated that IgM ASC were detectable at day 3 (30 ± 10 ASC/ 1×10^6 B cells, Figure 1A) and peak 5 to 7 days post-exposure (144 ± 73 ASC and 252 ± 42 ASC, respectively, Figure 1A). There was no statistical difference between day 5 and day 7. By day 10, the number of splenic ASC in control animals had decreased 2-fold (83 ± 35 ASC). The predominant isotypes produced were IgM, IgG2b, and IgG3 and all had similar kinetics (Figure 1A, 1B, and 1C, respectively). Comparable results were obtained when the number of ASC per spleen were determined (Figure 1D, 1E, and 1F). IgG1 and IgG2a were below the limit of detection by ELISPOT at all time points.

Following exposure to propanil or the mixture of propanil and 2,4-D, the number of PC-specific IgM (Figure 1A, 1D), IgG2b (Figure 1B, 1E), and IgG3 (Figure 1C, 1F) ASC were significantly increased 5 days after immunization (3-4 fold over vehicle

control). Similar fold increases were determined for the number of ASC per whole organ (Figure 1D, 1E, 1F) and for the number of ASC normalized to one million B cells (Figure 1A, 1B, 1C) in all experiments. There was a statistically significant 4-6-fold increase in the number of IgM, IgG2b, and IgG3 ASC in the animals treated with propanil alone or the mixture at day 7 post-exposure (Figure 1A - 1F). By day 10, the number of ASC was declining in all groups, but was still significantly increased 3.5 fold in propanil and mixture-treated animals over control animals (Figure 1A - 1F). 2,4-D exposure alone did not alter the splenic ASC response compared to the control at any of the days measured (Figure 1A - 1F). Propanil and mixture-treated animals produced statistically similar ASC responses in the spleen, which suggests that the propanil component of the mixture is responsible for the increase in ASC in the spleen.

Previous studies had demonstrated that propanil treatment induces splenomegaly (Barnett and Gandy, 1989). The spleen weights were determined after herbicide exposure and HKSP vaccination. Propanil and the mixture of propanil and 2,4-D, but not 2,4-D alone, caused an increase in spleen weight at 7 and 10 days post-exposure (Figure 2). However, flow cytometric analysis of the major cell populations in the spleen at all time points determined that there were no significant changes in any of the treatment groups in the number of total B220⁺ B cells, CD21/35^{hi} CD23^{neg/low} marginal zone B cells, CD21/35^{int} CD23^{hi} follicular B cells, CD4⁺ T cells, and CD8⁺ T cells (Table 1, representative data from day 7 post-exposure and vaccination). The total number of bone marrow cells was also comparable for the vehicle ($28.5 \pm 1.8 \times 10^6$), propanil ($27.6 \pm 3.5 \times 10^6$), 2,4-D ($25.4 \pm 3.8 \times 10^6$), and mixture treated groups ($27.4 \pm 3.2 \times 10^6$).

Additional experiments were performed to determine the dose response to propanil that enhanced the ASC response in the spleen. The lowest observed adverse effect level (LOAEL) dose of propanil that induced an increase in PC-specific ASC over control animals was 50 mg/kg (Figure 3A – 3F). A 25 mg/kg dose of propanil failed to increase the number of splenic ASC over control animals (Figure 3A – 3F). A higher dose of propanil of 200 mg/kg increased the ASC response 4-6 fold, similar to the 150 mg/kg dose (data not shown).

Ex vivo splenic antibody production is increased in propanil-treated mice.

To determine if there was a concomitant increase in antibody production from the spleens of animals that had an increase in ASC, spleens were harvested 7 days post-exposure and immunization and cultured *in vitro* for 5 days. The supernatants were harvested and IgM and total IgG determined by ELISA. Spleen cells from propanil and mixture-treated animals produced antibody at concentrations 3-4 fold higher than spleen cells from the vehicle control or 2,4-D- treated animals (Figures 4A and 4B). Analyses performed from day 5 and day 10 post-exposure and immunization had similar results (data not shown).

2,4-D decreased the number of PC-specific ASC in the bone marrow.

The number of PC-specific ASC in the bone marrow was also measured on days 3, 5, 7, and 10 post-exposure and vaccination. Individual isotypes were not detectable in the bone marrow at days 3 and 5 after immunization. However, IgM and total IgG were detectable at the early time points (Figure 5A – 5D). 2,4-D and mixture herbicide exposure decreased the number of PC-specific IgM and IgG ASC in the bone marrow approximately 2-fold by day 5 compared to the vehicle controls (Figure 5A – 5D). There

was a significant decrease at days 7 and 10 (2-3 fold) in ASC in the bone marrow (Figures 5A – 5D). Propanil-treated animals had responses comparable to the vehicle controls at all time points (Figure 5A – 5D). These results suggest that the 2,4-D component of the mixture is responsible for the reduction in ASC in the bone marrow.

Propanil exposure did not increase the PC-specific titers, however 2,4-D exposure decreased the PC-specific titers.

PC-specific titers were measured to determine if the increase in ASC in the spleens of propanil-treated mice, or the decrease in bone marrow ASC in 2,4-D-treated mice, altered serum titers. Individual isotypes were not detectable at day 3. Propanil and vehicle control animals had comparable titers of IgM, IgG2b, and IgG3 at 5, 7, and 10 days post-exposure and immunization (Figure 6A, 6B, and 6C). These results demonstrate that the increased splenic ASC in propanil-treated mice do not affect the serum antibody titers. However, 2,4-D and mixture treatments significantly reduced the PC-specific IgM, IgG2b, and IgG3 isotypes 3-4 fold from vehicle control and propanil-treated animals by day 10 (Figure 6A, 6B, and 6C). The serum titers of mixture and 2,4-D-treated groups were not significantly different, indicating that 2,4-D is the chemical component of the mixture responsible for the decrease in titers. The results also indicate a correlation between the reduced bone marrow ASC response and the decrease in serum antibody levels.

Herbicide exposure does not affect the antibody response to PspA.

To determine if exposure to propanil, 2,4-D, or the mixture would affect the response to a TD antigen, mice were treated with the herbicides and vaccinated with HKSP. The peak serum antibody response to PspA has been demonstrated to be at 14

days post-vaccination (Wu *et al.*, 1999). The results demonstrate that the number of splenic IgM and total IgG PspA-specific ASC was comparable to the vehicle control in the propanil, 2,4-D and mixture-treated animals 14 days after herbicide exposure and vaccination with HKSP (Figure 7A – 7D). The PspA-specific serum antibody titers were also not affected by herbicide exposure and were comparable for all of the treatment groups (data not shown). The PspA-specific ASC in the bone marrow were below the limit of detection at day 14.

DISCUSSION

Propanil and 2,4-D are immunotoxic herbicides commonly applied together as a mixture (Crop Protection Handbook, 2003; Farenhorst and Prokopowich, 2003). Previous findings have demonstrated that the two herbicides exert greater-than-additive immunotoxic effects on the primary immune organs, the thymus and bone marrow (de la Rosa *et al.*, 2003; de la Rosa *et al.*, 2005). However, the present study demonstrated that there was no subsequent effect on the mature lymphocyte populations in the spleen. The increase in total spleen cell number in the mixture-treated group is probably due to the presence of immature precursor cell populations as determined by differential analysis (not shown). Furthermore, increased immunotoxicity due to mixture exposure did not occur in the humoral response to HKSP examined in the present study. At the 150 mg/kg 1:1 mixture tested, the mixture combination failed to produce a greater-than-additive effect on ASC or the serum antibody levels. Splenic PC-specific ASC were increased to the same extent in both propanil and mixture-treated mice, suggesting that propanil was responsible for the increase in splenic ASC. Reduction in the number of bone marrow PC-specific ASC and serum antibody titers was dependent on 2,4-D treatment. These experiments indicate that propanil and 2,4-D modulate the immune response to vaccination with HKSP independently.

Propanil magnified the splenic PC-specific ASC response without altering the kinetics of the response or shifting the isotype composition. The earliest observed increase due to propanil exposure was 5 days post-exposure. Maximal effects were observed at day 7 and ASC decreased by day 10. Both T helper 1 (Th1) and Th2 cytokines alter the antibody response. IFN- γ , a Th1 cytokine, promotes IgG3 production

and TGF- β , a Th2 cytokine, promotes a shift to IgG2b production (Stavnezer, 1996). The retention of the predominant PC-specific isotypes IgG3 and IgG2b suggests that propanil does not skew the cytokine profile of the immune response. The kinetics of the splenic antibody response in propanil and vehicle-treated animals were similar, suggesting that proliferation and activation is not unregulated as would be indicated by a continued increase in ASC at day 10.

Despite a several fold increase in the number of PC-specific ASC in the spleen, and an increased *ex vivo* production of PC-specific antibody by splenocytes from propanil-treated animals in comparison to the control, the serum antibody titers were comparable for the two groups. There are several possible explanations for this result. First, it is possible that the increased amount of antibody produced in propanil-treated animals remains localized to the spleen. Second, the antibodies produced in the spleen in propanil-treated mice may be rapidly catabolized if serum antibodies levels are at a saturated concentration, therefore no increase in serum titers would be detected. However, the second possibility is unlikely since mixture-treated animals also had increased splenic ASC but reduced serum titers. Several earlier reports established the bone marrow as the primary source of serum antibodies (reviewed in Benner *et al.*, 1981). Exposure to propanil had no effect on the number of bone marrow PC-specific ASC. In contrast to propanil, 2,4-D decreased PC-specific ASC in the bone marrow and the decrease correlated with a decrease in PC-specific titers. Splenic ASC were comparable to control animals after 2,4-D exposure. Taken together, the results suggest that 2,4-D decreased serum PC-specific titers by decreasing bone marrow ASC and demonstrates the importance of bone marrow ASC for PC-specific titers during the

primary response to HKSP. The ability of a chemical to decrease the number of plasma cells in the bone marrow could have long-term implications for maintaining circulating levels of protective antibody after immunizations.

In contrast to the results presented here, propanil exposure has previously been reported to suppress the number of plaque-forming cells after immunization with the TI-2 antigen DNP-Ficoll, and the TD antigen, SRBC (Barnett and Gandy, 1989; Barnett *et al.*, 1992). The nature of the antigen may be important for the immunomodulation of the immune response by propanil. DNP-Ficoll and PC are both model TI-2 antigens. However, in the present study, PC is presented in the context of whole HKSP, a complex particulate immunogen. It was previously demonstrated that the requirements for the humoral response to PC, after immunization with HKSP, is substantially different than after immunization with purified polysaccharide preparations (Wu *et al.*, 1999). Although the PC antibody response is classically considered to be T-independent, Wu *et al.*, (1999) demonstrated that the IgG isotype responses to PC after immunization with HKSP was decreased in T cell receptor β knock-out mice. In addition, they demonstrated that CD4⁺ T cells and CD8⁺ T cells contributed to an optimal antibody response, and the PC response was significantly decreased in CD40L knockout mice (Wu *et al.*, 1999). Further studies demonstrated that noncognate T cell help was required for an optimal PC response after HKSP immunization (Wu *et al.*, 2002). Therefore, propanil could affect one of the components necessary for the response to PC after immunization with HKSP that is not required after immunization with soluble DNP-Ficoll.

The time of exposure to the herbicide may also be important to the subsequent effects on the immune response. In the previous studies that demonstrated propanil

suppressed the plaque-forming cell response to SRBC and DNP-Ficoll, the antigens were administered 3 days after propanil exposure (Barnett and Gandy, 1989; Barnett *et al.*, 1992). In the present study, propanil was administered at the time of HKSP vaccination. This may suggest that propanil is acting as an adjuvant to affect innate immune mechanisms and enhance the immune response to PC.

The antibody response to vaccination with HKSP is influenced by the early innate immune response. *S. pneumoniae* has pathogen associated molecular patterns on its surface that can stimulate signaling pathways through pattern recognition receptors such as toll-like receptor-2 (TLR2) (Yoshimura *et al.*, 1999). It was recently demonstrated that the IgG2b and IgG3 antibody response to PC after immunization with heat-killed *S. pneumoniae* type 14 is decreased in TLR-2 knockout mice (Khan *et al.*, 2005). The complement pathways are also important for the innate immune response to *S. pneumoniae* that can influence the subsequent adaptive immune response (Brown *et al.*, 2002). Conjugation of the complement component, C3d, to pneumococcal capsular polysaccharide, has been demonstrated to enhance the antibody response to the polysaccharide dependent on the dose of antigen (Test *et al.*, 2001). Similarly, preliminary studies in our laboratory suggest that the dose of HKSP is important, as mice vaccinated with suboptimal doses of HKSP did not have an increase in ASC. The effect of propanil on specific components of the innate immune response has not been investigated.

Propanil may alter the immune response through interactions with the endocrine system. Propanil induces thymic atrophy primarily through the induction of glucocorticoids, however, inhibition of glucocorticoid production does not completely

abrogate thymic atrophy (Cuff *et al.*, 1996; de la Rosa *et al.*, 2005). In addition, glucocorticoids are reported to inhibit Th1 responses and enhance Th2 responses (Ashwell *et al.*, 2000; Miyaura and Iwata, 2002). Propanil did not alter the major isotype response to PC, which suggests that the T cell response and subsequent production of cytokines driving B cell switching was not affected. This suggests that additional mediators may play a role in the immunotoxic effects of propanil. Propanil also decreases the pre-B cell and IgM⁺ B cell populations in the bone marrow via an unknown mechanism (de la Rosa *et al.*, 2003). Similar to propanil, 17 β -estradiol exposure has been shown to induce thymic atrophy and decrease immature B cell populations in the bone marrow (Erlandsson *et al.*, 2003). Chronic exposure to 17 β -estradiol has been reported to increase ASC to bacterial and autoantigens in C57BL/6 mice (Verthelyi and Ahmed, 1998). In addition, exposure to 17 β -estradiol was demonstrated to increase activation of the marginal zone B cell population and lead to the production of autoantibodies by the marginal zone B cells (Grimaldi *et al.*, 2001). Marginal zone B cells are crucial in the generation of the immune response to TI-2 antigens (reviewed in Zandvoort and Timens, 2002). Mice deficient in marginal zone B cells have a deficient antibody response to TI-2 antigens (Guainamard *et al.*, 2000). If propanil induced the production of 17- β estradiol it could enhance the antibody response to PC through the effects of 17- β estradiol on the marginal zone B cell population. Preliminary studies in our laboratory have demonstrated that propanil does not increase the number of PC-specific splenic ASC in ovariectomized mice suggesting an important potential role for 17 β -estradiol.

The mechanism by which 2,4-D decreased the bone marrow PC-specific ASC is unknown. However, there are several possibilities. Homing of plasma cells from the spleen to the bone marrow is dependent on the expression of the chemokine receptor, CXCR4, on splenic plasma cells and its ligand, CXCL12, in the bone marrow (Erickson *et al.*, 2003). The splenic ASC in 2,4-D-treated mice could be defective in the expression of CXCR4. It is also possible that production of CXCL12 in the bone marrow could be defective. Finally, support of plasma cells in the bone marrow could be affected due to damage to the bone marrow microenvironment.

In contrast to the effect on the TI-2 antigen, PC, exposure to propanil, 2,4-D or the mixture had no effect on the response to the TD antigen, PspA. The number of PspA-specific ASC in the spleen and the serum titers to PspA were comparable to the controls for all of the treatment groups. This could suggest that the effects of both propanil and 2,4-D occur early after vaccination when the TI-2 antigen response is being generated. If propanil has effects on the innate immune system or on marginal zone B cells which are critical to the response to TI-2 antigens, as discussed above, the effects may not impact the subsequent response to the TD antigen, PspA. A second possibility is that the time of exposure to the herbicides during the immune response to each antigen is critical. In the experiments presented here, herbicide exposure was on the day of HKSP vaccination. The peak response to PC was determined 7 days post-exposure while the peak response to PspA is at day 14. If the herbicides mediate their effect during the time antigen-specific B cells are undergoing activation or expansion, and the effect is short-term, then the later response to PspA may not be affected. Similar to PspA, propanil exposure does

not increase the number of splenic IgM ASC to SRBC, another TD antigen, when administered at the time of SRBC immunization (de la Rosa, unpublished results).

As the use of herbicides escalates, it is necessary to have an accurate understanding of the risks associated with their use. This report illustrates the importance of studying immunotoxic effects using naturally occurring microbial pathogens and the diverse effects that different compounds can have. The enhanced antibody response after exposure to propanil has implications for the potential of this class of compounds to be environmental factors in autoimmune disease. In contrast, other compounds, such as 2,4-D, may impair the ability of the host to mount an appropriate protective immune response after vaccination.

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Figure 1. Propanil and mixture exposure increases the number of PC-specific ASC in the spleen.

C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture. Spleens were removed at days 3, 5, 7, and 10. Numbers of PC-specific IgM (A, D), IgG2b (B, E), and IgG3 (C, F) ASC were determined by ELISPOT assay. All values represent the mean $\pm$ SD of ASC per 1×10^6 B cells (A, B, and C) or per spleen (D, E, F). Propanil and mixture treatments are significantly different from vehicle control on days 5, 7, 10 for IgM, IgG2b, and IgG3, $p \leq .05$.

Figure 2. Propanil and mixture exposure increase spleen weight.

C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture. Spleens were removed at days 3, 5, 7, and 10 and the wet weights determined. Results are expressed as the ratio of spleen weight to body weight. Propanil and mixture treatments are significantly different from vehicle control on days 5, 7, 10, $p \leq .05$.

Table 1. Spleen cell populations 7 days after herbicide exposure and HKSP vaccination.

Treatment ^a	Total cells ^b	B220 ⁺ B cells ^c	Marginal zone B cells	Follicular B cells	CD4 ⁺ T cells	CD8 ⁺ T cells
Vehicle	80 ± 13.6	30 ± 3.6 ^d	1.9 ± 0.25	20 ± 3.8	18 ± 5.1	11 ± 3.7
Propanil	108 ± 31.8	34 ± 6.4	2.1 ± 0.31	23 ± 4.2	22 ± 5.9	15 ± 3.5
2,4-D	76 ± 17.6	33 ± 7.7	1.8 ± 0.42	22 ± 4.9	18 ± 5.0	12 ± 3.5
Propanil/2,4-D	143 ± 35.7 ^e	40 ± 5.4	2.1 ± 0.43	26 ± 2.4	27 ± 3.1	17 ± 0.9

^a C57BL/6 mice (5/group) were vaccinated with HKSP and treated with vehicle, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mg/kg mixture of propanil and 2,4-D. Spleens were harvested on day 7.

^b Total number of spleen cells ($\times 10^6$) $\pm$ SD.

^c Individual cell populations were determined by flow cytometric analysis as described in the Methods.

^d Data represents the total number of cells ($\times 10^6$) $\pm$ SD.

^e $p \leq 0.05$ versus vehicle control.

Figure 3. Propanil increases the number of splenic ASC in a dose dependent manner.

C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control (0), 25 mg/kg, 50 mg/kg, and 150 mg/kg propanil. Spleens were removed at day 7. Numbers of PC-specific IgM (A, D), IgG2b (B, E), and IgG3 (C, F) ASC were determined by ELISPOT assay. All values represent the mean $\pm$ SD of ASC per 1×10^6 B cells (A, B, and C) or per spleen (D, E, F). The asterisk (*) represents a significant difference ($p \leq .05$) from vehicle control.

Figure 4. Propanil and mixture exposure increases ex vivo antibody production by splenocytes following HKSP vaccination.

C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture.

Spleens were harvested on day 7 and cultured *in vitro* for 5 days. Antibody supernatants were collected and analyzed by ELISA. *De novo* PC-specific IgM (A) and IgG (B) production was determined by subtracting cycloheximide treated samples from the total antibody produced. Values represent the mean *de novo* antibody produced $\pm$ SD.

Propanil and mixture treatments are significantly different from vehicle control at dilutions 1:2, 1:4, and 1:8 for IgM and IgG, $p \leq .05$.

Figure 5. 2,4-D and mixture exposure decreases the number of PC-specific bone marrow ASC.

C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture. Bone marrow was harvested on days 3, 5, 7, 10. Numbers of PC-specific IgM (A, C) and IgG (B, D) ASC were determined by ELISPOT assay. Values represent the mean $\pm$ SD of ASC per 1×10^6 bone marrow cells (A, B) or per total bone marrow (D, E). 2,4-D and mixture treatments are significantly different from vehicle control on days 5, 7, and 10 for IgM and IgG, $p \leq .05$.

Figure 6. 2,4-D and mixture exposure decreases PC-specific titers.

C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture.

Serum was collected on days 3, 5, 7, 10. The titers of PC-specific IgM (A), IgG2b (B), and IgG3 (C) were determined by ELISA. Values represent the mean $\pm$ SEM. 2,4-D and mixture treatments are significantly different from vehicle control at day 10 for IgM and IgG, $p \leq .05$.

Figure 7. Herbicide exposure has no effect on the number of splenic PspA-specific ASC.

C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture.

Spleens were harvested on day 14. Numbers of PspA-specific IgM (A, C) and IgG (B, D) ASC were determined by ELISPOT assay. Values represent the mean $\pm$ SD of ASC per 1×10^6 B cells (A, B) or per spleen (C, D).

**CHAPTER 4: FURTHER CHARACTERIZATION OF THE
EFFECT OF PROPANIL EXPOSURE ON THE HUMORAL
IMMUNE RESPONSE**

INTRODUCTION

Previous studies demonstrated that propanil enhances the antibody response to the TI-2 antigen PC when presented on the surface of killed whole bacteria (de la Rosa, 2005). However, propanil did not increase the number of ASC to the TD antigen PspA on day 14 in mice that were vaccinated and treated with herbicides on day 0 (de la Rosa, 2005). In addition, the increased number of PC-specific ASC in propanil-treated animals did not cause an elevated response in PC-specific serum titers. The following experiments were performed to answer a number of questions raised from these findings that are important in determining the immune-based mechanisms involved in enhancing the humoral response.

The context in which the antigen is presented to the immune system can alter the response in a number of ways. For example, *S. pneumoniae* contains a number of pathogen associated molecular patterns (PAMPS) that are recognized by Toll-like receptors (Sen *et al.*, 2005). TLR have been shown to be important for enhanced antibody responses (Sen *et al.*, 2006). Processing and presentation by the APCs differs in mice immunized with particulate or soluble antigens (Ramachandra *et al.*, 1999). An example of a soluble antigen that induces a TI-2 PC-specific response independent of TLR activation is PC-Ficoll. The following experiments utilized PC-Ficoll to evaluate the importance of soluble versus particulate antigens in the ability of propanil to enhance the immune response.

The inability of propanil to increase the number of PspA-specific ASC suggests that propanil may induce changes on a specific subset of B cells responsible for TI-2 responses and the effect is independent of T-cell help. Alternatively, propanil may

enhance the antibody response only within a limited timeframe following herbicide exposure. For example, immunization of mice with DNP-Ficoll 3 days following propanil exposure suppresses the antibody response on day 7 (Barnett *et al.*, 1992). Given that the PspA response is not measurable until day 6 and peaks around 10-14 days, it is possible that propanil, which is eliminated from the body within 2 days, may not remain in the animal long enough to enhance the PspA response (Izmerov, 1984). Experiments were also performed to determine the effect of exposure to herbicides at different time points following HKSP immunization on the PspA response.

The inability of propanil to increase serum titers despite a robust enhancement of the splenic response and increased *ex vivo* antibody production suggest a number of explanations. Antibody catabolism has been discussed in Chapter 3. Alternatively, the increased antibodies may not exit the spleen into the serum due to binding of the antibodies to HKSP in the spleen. Finally, the serum antibody titers may be increased at a later time point. Experiments were performed to examine these possibilities.

METHODS

Animals

Six to eight week old C57Bl/6 female mice were purchased from Hill Top Lab Animals (Scottdale, PA). Mice were housed in microisolator cages in pathogen free conditions at West Virginia University's animal facility. Mice were kept on a 12 hr light-dark cycle and allowed to acclimate to the facility for 1 week. These studies were conducted in accordance with all federal and institutional guidelines for animal use and

were approved by the West Virginia University Institutional Animal Care and Use Committee.

Reagents

Propanil (3,4-dichloropropionanilide, 99% pure) was purchased from Chem Service (West Chester, PA). PC-Ficoll containing 6 PC molecules per molecule of Ficoll was purchased from Biosearch Technologies (Novato, CA). PC-KLH containing 15 PC molecules per molecule of Ficoll was purchased from Biosearch Technologies.

Bacterial preparation and immunization with R36A

S. pneumoniae strain R36A, an avirulent, nonencapsulated strain, was grown to mid-log phase in Todd-Hewitt broth (Becton Dickinson, Sparks, MD) + .05% yeast extract (Becton Dickinson) and stored at -80°C . For immunization, stock was cultured in a candle jar for 18 hrs at 37°C on blood agar plates (Becton Dickinson). A few characteristic colonies were selected and suspended in 200 ml Todd-Hewitt broth + .05% yeast extract. Bacteria were grown at 37°C to an absorbance reading at 650nm of 0.4 and heat killed for 4 hours in a 60°C water bath. A final concentration of 10^9 CFU/ml was established in PBS based on colony counts. Sterility was confirmed by culture. Heat-killed stock was stored at -20°C in 1 ml aliquots. Mice were immunized ip with either 5×10^6 or 2×10^8 CFU.

Animal exposures

Propanil was dissolved in peanut oil for a final concentration of 15 mg/ml for all experiments. Mice (5/group) were treated ip with either 150 mg of propanil/kg of body weight (mg/kg) or vehicle on the same day as immunization. For PC-Ficoll, mice were immunized i.p. with 50 μg of PC-Ficoll in 200 μl PBS as in a previous study (Wu *et al.*,

1999). For PC-KLH immunizations, mice were immunized i.p. with 100µg of PC-KLH in 200 µl PBS.

Preparation of splenocytes

Mice were euthanized with 100 µl Nembutal Sodium Solution (50 mg/ml, Abbott Laboratories, North Chicago, IL) vaccination. Spleen wet weights were recorded. Spleens were mechanically dissociated through Spectra nylon mesh (Spectrum Labs, Rancho Dominguez, CA) in complete cell media containing RPMI-1640 (BioWhitaker, Walkersville, MD), 10% heat inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc, Logan, UT), 10 mM HEPES (Sigma), 1 mM L- glutamine (Gibco, Rockville, MD), 5×10^{-5} M 2-mercaptoethanol (Sigma), 100 U/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). Red blood cells in the spleen were lysed with Tris-buffered ammonium chloride. Cell suspensions were washed twice and counted using a hemacytometer. Viability was determined using Trypan blue dye exclusion.

Measurement of antibody secreting B cells (ASC) in the spleen

Acrowell™ 96 well filter plates (Pall Life Sciences, Ann Arbor, MI) were coated with 50 µl PC-BSA (Biosearch Technologies, Novato, CA) (10 µg/ml) or 50 µl PspA (10 µg/ml) (PspA was a generous gift from Clifford Snapper, USUHS) overnight at 4°C. In all subsequent steps, plates were washed with PBS + .01% Tween-20. Plates were blocked with 200 µl/well complete medium + 25% FBS for 2 hours at 37°C. Plates were washed and cells (100 µl/well) were then added at a concentration of 5×10^6 cells/ml or 1×10^6 cells/ml. All samples were plated in triplicate. Plates were incubated for 4-6 hours at 37°C in a 5% CO₂ incubator. After washing, goat anti-mouse alkaline phosphatase (AP) conjugated IgG, IgG1, IgG2a, IgG2b, IgG3, or IgM antibodies (Southern

Biotechnology Associates, Birmingham, AL), diluted 1/250 in PBS + 1% BSA + .05% Tween-20, were added to the appropriate wells (100 μ l/well). Plates were incubated overnight at 4°C and washed. SIGMAFAST 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma-Aldrich, St. Louis, MO) were dissolved in distilled water and added at 100 μ l/ well. Color development was stopped by washing with distilled water. The number of spots/well was counted using a dissection microscope (Olympus Optical Co., Melville, NY). The number of ASC was calculated by using the mean number of spots from triplicate wells. The number of ASC was normalized to 1×10^6 B cells. Comparable fold increases were noted when normalized to whole spleen.

Flow cytometric analysis

Splenic cells were stained with the appropriate combinations of rat anti-mouse B220-APC (RA3-6B2). All steps were performed in PBS supplemented with 1% FBS and 0.04% sodium azide (Sigma). Briefly, 1×10^6 cells were stained in a total volume of 25 μ l of antibodies at the appropriate concentrations for 25 minutes on ice in the dark. After incubation, cells were washed twice and fixed in 0.04% paraformaldehyde overnight at 4°C (Fisher Scientific, Pittsburgh, PA). The following day cells were washed twice to remove the paraformaldehyde and resuspended in 1 ml of staining media. For each sample, 10,000 cells were collected for analysis on a Becton-Dickinson FACScan (Becton Dickinson Immunocytometry Systems, Mansfield, MA). Analysis was performed using WinMDI software (Joseph Trotter, Scripps Institute, San Diego, CA). Population percentages, obtained from flow cytometric analysis, were used to calculate the absolute cell number by multiplying the percentage of cells in a population by the total number of cells harvested per organ.

Measurement of PC- and PspA- specific titers

Serum samples were prepared via blood collected from the heart. Immulon 2 plates (for PC) or Immulon 4 plates (for PspA) (ThermoLabsystems, Franklin, MA) were coated overnight at 4°C with 5 µg/ml PC-BSA or 5 µg/ml PspA (50 µl/well). Plates were washed, blocked with 3% BSA + PBS at 37°C for 2 hours. Plates were washed and 100 µl/well of twofold dilutions of sera in PBS +1% BSA were added starting at 1/400 for the IgG and IgM ELISAs and 1/250 for the IgG subclasses. Plates were then incubated for 1 hour at 37°C and washed. AP conjugated antibodies (100 µl/well) were added for 1 hour at 37°C. Plates were washed and phosphatase substrate tablets (Sigma-Aldrich) were dissolved in PNPP (p-Nitrophenyl Phosphate, Disodium Salt) substrate buffer. Plates were developed and absorbance read at 405nm on a µQuant spectrophotometer (Bio-Tek instruments, Winooski, VT) using KCJunior software (Bio-Tek instruments).

Statistics

ANOVA was performed for all statistical analyses using a Tukey-Kramer t-test to perform multiple comparisons between all treatment groups. A significance level of $p \leq 0.05$ was used for all tests. Statistical analysis was performed using JMP software (SAS Institute Inc., Cary, NC). All experiments were performed 3 or more times with similar results. The figures are representative data from one experiment.

RESULTS

Propanil increases the number of PC-specific ASC following PC-Ficoll immunization.

Previous studies have demonstrated that propanil increases the antibody response to PC following immunization with the particulate antigen HKSP. To determine if the propanil-induced antibody enhancement requires immunization with whole bacteria, mice were vaccinated with the soluble TI-2 antigen PC-Ficoll and treated on the same day with propanil or vehicle. The number of splenic PC-specific ASC and the PC-specific serum titers were determined 7 days later by ELISPOT and ELISA, respectively. Propanil increased the number of PC-specific IgG2b, IgG3 and IgM ASC 3-4 fold in PC-Ficoll immunized animals (Figure 1). The fold increase in ASC in propanil-treated and PC-Ficoll immunized mice was similar to propanil-treated HKSP immunized mice. However, the baseline PC response to PC-Ficoll was 6-10 fold lower than after vaccination with HKSP (Figure 1). However, PC-specific serum titers in propanil-treated mice were not significantly increased following either PC-Ficoll or HKSP immunization compared to the controls (Figure 2). This data suggests that propanil induces a similar effect on the immune response to a soluble TI-2 antigen.

Propanil does not enhance the antibody response to a suboptimal HKSP immunization.

These experiments were performed to determine if the propanil-induced enhancement of the antibody response has a minimum threshold for antigen stimulation. To determine if propanil exposure can increase the antibody response to a suboptimal immunization to the same level as an optimal immunization, mice were immunized with 5×10^6 or 2×10^8 CFU of HKSP and treated on the same day with propanil or vehicle. The number of PC-specific ASC was determined at day 7. The number of PC-specific ASC in vehicle-treated mice immunized with 5×10^6 CFU HKSP is decreased 4-5-fold from

mice immunized with 2×10^8 CFU HKSP (Figure 3). In addition, propanil does not significantly affect the number of PC-specific ASC in mice immunized with 5×10^6 CFU HKSP. This data suggests that propanil enhancement of the antibody response has a minimum threshold of antigen stimulation to induce the effect.

Propanil increases secondary antibody responses to PC and PspA.

Previous data demonstrated that propanil enhances a primary TI-2 antibody response but not a primary TD response. These experiments were performed to determine if propanil enhances a TI-2 or TD secondary antibody response and if multiple exposures to propanil can produce either greater enhancement or prolonged enhancement of the antibody response. Mice were immunized with 2×10^8 HKSP on day 0 and day 14. Mice were exposed to propanil on either day 0, day 14, or days 0 and 14. PC and PspA-specific antibody responses were measured on day 21. The number of PC-specific ASC from mice treated with propanil once on day 0 and mice treated with propanil twice on days 0 and 14 were not significantly different from vehicle controls (Figure 4A). However, PC-specific ASC from mice exposed to propanil once on day 14 were increased 2-3 fold compared to controls (Figure 4A). None of the propanil exposure treatments significantly affected PC-specific serum titers (Figure 5A).

The effect of propanil on the PspA-specific response was similar to the PC-specific response. The number of PspA-specific ASC from mice exposed to propanil once on day 14 was increased 2-fold compared to vehicle-treated mice (Figure 4B). Propanil exposure on day 0 or days 0 and 14 did not significantly affect the number of PspA-specific ASC (Figure 4B). None of the propanil exposure treatments significantly affected PspA-specific serum titers (Figure 5B). Altogether, these data suggest that

propanil enhances the antibody response to both protein and polysaccharides. However, enhanced splenic ASC did not increase serum titers on day 21.

Delayed propanil exposure enhances a primary antibody response to PspA.

The previous findings demonstrating that propanil enhances the secondary antibody response to PspA when mice are exposed on day 14 suggest that the enhancement of the antibody response is dependent on the time of propanil exposure relative to the developing antibody response. To determine if propanil can increase the number of PspA-specific ASC during a primary response, mice were immunized with HKSP on day 0 and treated with propanil on day 9. The PspA and PC antibody responses were measured on day 14. The number of PspA-specific ASC was increased approximately 2-fold compared to vehicle-treated mice (Figure 6A). In addition, the number of PC-specific ASC was also increased 4-6-fold in propanil-treated mice compared to vehicle controls (Figure 6B). This data demonstrates that the enhancement of the antibody response is dependent on the condition of the antibody response when mice are exposed to propanil.

Propanil does not enhance the antibody response following PC-KLH immunization.

To determine if propanil requires the TD antigen to be presented in a particulate form such as HKSP to enhance the antibody response, mice were immunized with PC-KLH or HKSP and treated with propanil or vehicle on day 0. The number of PC-specific ASC was determined on day 7. Both high affinity and low affinity PC-specific ASC were also determined by using a high and low conjugation ratio of PC-BSA. To detect high affinity ASC, ELISPOT plates were coated with 2PC-BSA. Low affinity ASC were detected by 15PC-BSA coated plates. PC-KLH immunization produced a 2-fold greater

number of ASC in vehicle-treated animals compared to vehicle-treated animals immunized with HKSP (Figure 7A). Interestingly, propanil did not enhance the PC-specific response following PC-KLH immunization (Figure 7A). Analysis of the affinity of PC-specific antibodies demonstrates that the number of low affinity ASC is 20%, 65%, and 220% greater than high affinity IgM, IgG3, and IgG2b ASC, respectively (Figure 7A vs. Figure 7B). This data indicates that high and low affinity ASC are detectable with this assay. The number of low affinity PC-specific ASC in propanil-treated animals was increased approximately 4-fold for IgG2b and 5-fold for IgG3 and IgM (Figure 7A). The number of high affinity PC-specific ASC in propanil-treated animals were increased approximately 6-fold, 5-fold, and 4-fold for IgG2b, IgG3, and IgM, respectively (Figure 7B). Altogether, this data suggests that propanil does not preferentially increase either high or low affinity ASC.

DISCUSSION

This set of experiments addressed a number of questions concerning the effect of propanil on the antibody response. Effects of propanil on humoral responses to particulate and soluble antigens also provide important information on any putative effects on antigen processing. As mentioned previously, the role of T cells in the amplification of the antibody response can be assessed through TI-2 and TD comparison studies. Finally, determining when during the time of the response the immune system is susceptible to the effects of propanil exposure was also addressed. The results from these initial experiments may help to focus experiments studying the immunological mechanisms regulating the enhanced antibody response.

These data suggest that the increased antibody response is not due to a nonspecific polyclonal increase in ASC. Propanil increased the PC-specific response following HKSP and PC-Ficoll immunization. However, propanil does not increase the PC-specific response following PC-KLH immunization, despite a robust antibody response at day 7. In addition, propanil induction of nonspecific polyclonal activation would predict a greater increase in low affinity PC-specific B cells. However, the data indicates that both high and low affinity PC-specific ASC are increased equally. Previous reports demonstrated that the low affinity PC-specific serum titers are increased 120% compared to high affinity PC-specific serum titers in samples collected on days 6 or 9 (Wu and Ward, 1993). However, the affinity of individual isotypes was not determined (Wu and Ward, 1993). Our study supports the previous affinity analysis however precise comparisons are difficult due to the different time points and antigen employed. Altogether, these data suggest an antigen-specific increase in antibody production.

Another unresolved finding in the previous chapter was the discrepancy between increased ASC and increased ex vivo antibody production in propanil-treated animals, but unchanged serum antibody titers. The results from Chapter 3 indicate that antibody serum titers are more sensitive to changes in the number of BM ASC. Since the number of BM ASC are not increased in propanil-treated animals despite an increase in splenic ASC, propanil treatment may not increase the antibody serum titers since the increased number of plasma cells do not survive in the BM.

Propanil failed to amplify the antibody response exposure following a suboptimal HKSP immunization. This data provided an interesting finding that propanil exposure

increased the antibody response to a weak PC-Ficoll response but not to a weak HKSP immunization. The low conjugation ratio of the PC-Ficoll used in this experiment (6 PC-Ficoll) was lower than that used in previous experiments (25 PC-Ficoll) which generated serum titers comparable to optimal HKSP immunization (Wu *et al.*, 1999). A possible explanation for the discrepancy between responses is that the low PC-concentration on PC-Ficoll activates high affinity B cells. Propanil is able to amplify the PC response to low PC-Ficoll immunization because the concentration of PC-Ficoll molecules is in excess, allowing for receptor engagement by the increased number of responding PC-specific B cells. In contrast the low dose of HKSP would limit the number of individual PC expressing bacterium thus fewer BCR are able to bind to HKSP. This hypothesis would suggest that propanil cannot increase the number of PC-specific ASC in suboptimally vaccinated mice because the increased number of responding B cells require antigen stimulation to differentiate to plasma cells. Altogether, these data suggest that propanil enhancement of the antibody response is not dependent on particulate or soluble antigen presentation by the APC.

A second question addressed by these set of experiments was whether the enhancement of the antibody response by propanil was specific for TI-2 responses. Given the major differences in the processes leading to B cell activation in TD and TI-2 responses as noted in the General Introduction, addressing this issue is important for determining the mechanisms responsible for the increased numbers of ASC. These data demonstrate that enhancement of the antibody response is dependent on when the animal is exposed to propanil during the antibody response. Propanil increased the antibody response to a TD antigen when it is given 5 days prior the peak antibody response in

HKSP immunized mice. Although PC-KLH elicits a robust TD PC-specific response at day 7, previous reports have demonstrated that the T15⁺ antibody response following PC-KLH immunization is maximal on day 13 in Balb/c mice (Wiens *et al.*, 2003). The data suggests that propanil exposure to PC-KLH on day 8 would amplify the anti-PC response.

These data indicate that the amplification of the antibody response by propanil is not a direct effect on the B cells and may require T cell involvement. This hypothesis is supported by the observation that enhancement of the PC-specific response is not observed in HKSP immunized animals until day 5, despite actively differentiating and proliferating B cells by day 3. Similarly, PC-KLH immunization produces a greater number of PC-specific ASC on day 7 than a HKSP immunization. This also indicates robust B cell differentiation and proliferation. One of the differences between TD and TI-2 responses is the nature of T cell help and other costimulation molecules early on during B cell activation. For instance, T cell help is required for the first 3-4 days of the PC response and the first 5-6 days for the PspA response (Wu *et al.*, 2002). B7-dependent costimulation is required for the first 1-2 days during the PC response in contrast to the first 5-6 days for the PspA response (Wu *et al.*, 2000). It is possible that propanil may affect T cell dependent costimulation, which could explain the delayed enhancement of the PC response until day 5 and the requirement of delayed propanil exposure to enhance TD responses.

Altogether these data eliminate a number of possible immunological mechanisms by which propanil amplifies the TI-2 and TD humoral responses. Antibody enhancement does not require a particulate antigen on a whole killed bacterium. Increased antibody

responses are antigen specific and not polyclonal. However, more experimentation is required to resolve the discrepancy between ASC and serum titers and to determine the importance of T cell help in the amplified response.

Figure 1. Propanil exposure increases the number of PC-specific ASC following PC-Ficoll immunization.

Female C57BL/6 mice (5/group) were treated with 150 mg/kg propanil or vehicle control and vaccinated with 2×10^8 CFU HKSP or PC(6)-Ficoll. Numbers of PC-specific IgG2b, IgG3, and IgM ASC were determined by ELISPOT assay on day 7. Values represent the mean $\pm$ SD of ASC/1x10⁶ B cells. # Significantly different from Vehicle + PC-Ficoll, $p \leq .05$. * significantly different from Vehicle + HKSP, $p \leq .05$.

Figure 2. Propanil does not affect PC-specific serum titers following PC-Ficoll immunization.

Female C57BL/6 mice (5/group) were treated with 150 mg/kg propanil or vehicle control and vaccinated with 2×10^8 CFU HKSP or PC(6)-Ficoll. IgG2b, IgG3, and IgM PC-specific serum titers were determined by ELISA on day 7. Values represent the mean titer $\pm$ SD. * significantly different from Vehicle + Ficoll , $p \leq .05$.

Figure 3. Propanil exposure does not affect PC-specific ASC in suboptimally HKSP immunized mice.

Female C57BL/6 mice (5/group) were treated with 150 mg/kg propanil or vehicle control and vaccinated with 2x10⁸ or 5x10⁶ CFU HKSP. Numbers of PC-specific IgG2b, IgG3, and IgM ASC were determined by ELISPOT assay on day 7. Values represent the mean $\pm$ SD of ASC/1x10⁶ B cells. # Significantly different from Vehicle 5x10⁶, $p \leq .05$. * Significantly different from Vehicle 2x10⁸, $p \leq .05$.

A. PC

B. PspA

Figure 4. Day 14 propanil exposure increases the number of PC-specific and PspA-specific ASC following a HKSP boost on day 14.

Female C57BL/6 mice (5/group) were treated with vehicle control or 150 mg/kg propanil on day 0, day 14 or day 0 and day 14 and vaccinated on day 0 and day 14 with 2×10^8 CFU HKSP. (A) Numbers of PC-specific IgG2b, IgG3, and IgM ASC were determined by ELISPOT assay on day 21. (B) Numbers of PspA-specific IgG and IgM ASC were determined by ELISPOT assay on day 21. Values represent the mean $\pm$ SD of ASC/1x10⁶ B cells. * Significantly different from vehicle control, $p \leq .05$.

Figure 5. Propanil exposure does not affect serum titers following secondary HKSP immunization.

Female C57BL/6 mice (5/group) were treated with vehicle control or 150 mg/kg propanil on day 0, day 14 or day 0 and day 14 and vaccinated on day 0 and day 14 with 2×10^8 CFU HKSP. (A) IgG2b, IgG3, and IgM PC-specific serum titers were determined by ELISA on day 21. (B) IgG and IgM PspA-specific ASC were determined by ELISA on day 21. Values represent the mean titer $\pm$ SD.

A. PspA

B. PC

Figure 6. Day 9 propanil exposure increases the number of PC and PspA-specific ASC.

Female C57BL/6 mice (5/group) were treated with vehicle control or 150 mg/kg propanil on day 9 and vaccinated on day 0 with 2×10^8 CFU HKSP. (A) Numbers of PspA-specific IgG1, IgG2a, and IgM ASC were determined by ELISPOT assay on day 14. (B) Numbers of PC-specific IgG2b, IgG3, and IgM ASC were determined by ELISPOT assay on day 14. Values represent the mean $\pm$ SD of ASC/1x10⁶ B cells. * Significantly different from vehicle control, $p \leq .05$.

A. PC15-BSA

B. PC2-BSA

Figure 7. Propanil exposure does not affect the number of PC-specific ASC following PC-KLH.

Female C57BL/6 mice (5/group) were treated with vehicle control or 150 mg/kg propanil and vaccinated on day 0 with 2×10^8 CFU HKSP or PC-KLH. Numbers of PC-specific IgG2b, IgG3, and IgM ASC were determined by ELISPOT assay on day 7. (A) Low affinity B cells were detected using PC(15)-BSA coated plates. (B) High affinity B cells were detected using PC(2)-BSA coated plates. Values represent the mean $\pm$ SD of ASC/ 1×10^6 B cells. * Significantly different from vehicle HKSP, $p \leq .05$.

**CHAPTER 5: EVIDENCE FOR A NOVEL ENDOCRINE
DISRUPTOR: THE PESTICIDE PROPANIL REQUIRES
THE OVARIES AND STEROID SYNTHESIS TO ENHANCE
HUMORAL IMMUNITY**

Evidence for a novel endocrine disruptor: The pesticide Propanil requires the ovaries and steroid synthesis to enhance humoral immunity

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ABSTRACT

Steroid hormones are known to affect the humoral immune response to a variety of antigens. However, the mechanisms regulating these effects are poorly understood. The immunotoxic chemical, propanil, and estrogen have similar effects on the immune system including augmentation of humoral immune responses. Propanil enhances the number of phosphorylcholine (PC)-specific IgG2b, IgG3, and IgM antibody secreting cells (ASC) in the spleen 4-6-fold 7 days after vaccination of female C57BL/6 mice with heat-killed *Streptococcus pneumoniae* (HKSP). Several experiments were performed to test the hypothesis that propanil increases the response via an estrogenic pathway. Ovariectomy abrogated the effect of propanil on the PC-specific ASC response. Both *in vitro* and *in vivo* assays indicate that propanil does not bind either ER α or ER β . Exogenous estradiol administration in ovariectomized mice failed to restore the effect of propanil on the PC response. Treatment of female mice with a pure ER antagonist, ICI 162,780, or the progesterone antagonist, RU-486, did not inhibit the increase in ASC. These data suggest that estrogen and progesterone do not regulate the effect of propanil. However, complete inhibition of steroid synthesis with the GnRH antagonist, antide, abrogated the increased response in propanil-treated mice, indicating a necessary role for steroid synthesis. Experiments in male mice demonstrated that propanil increased the number of ASC comparable to female mice. However, orchiectomy did not inhibit this effect, suggesting that androgens do not regulate the amplification of the humoral response. These data suggest a novel role for the ovarian hormones in the regulation of the PC-specific antibody response.

INTRODUCTION

Numerous studies have characterized the immunomodulating effects of the steroid hormone 17 β -estradiol (E2) on developing lymphocyte populations in the thymus and bone marrow and on the peripheral humoral immune response. Studies have demonstrated that E2 induces thymic atrophy through a Fas/FasL pathway dependent on both estrogen receptor (ER) α and β (Do *et al.*, 2002; Erlandsson *et al.*, 2001; Yao and Hou, 2004). E2 treatment induces thymic atrophy primarily by decreasing the number of CD4⁺CD8⁺ double positive thymocytes (Screpanti *et al.*, 1989). E2 also inhibits B lymphopoiesis in the bone marrow (Erlandsson *et al.*, 2003; Islander *et al.*, 2003). Specifically, E2 decreases pro-B cell, pre-B cell, and mature B cells of the bone marrow in an ER α and ER β dependent manner (Erlandsson *et al.*, 2003). Studies on the effects of E2 on specific immune responses have demonstrated that chronic exposure to E2 increases both the number of splenic antibody secreting cells (ASC) and the serum antibody titers to bacterial and autoantigens (Meyers and Petersen, 1985; Verthelyi and Ahmed, 1998). Recent data suggest that E2 skews the genetic expression profile of B cells to enhance survival and to lower tolerance, which may contribute to the enhanced antibody response after E2 exposure (Frasor *et al.*, 2003; Grimaldi *et al.*, 2002). It has been proposed that naturally occurring and synthetic compounds capable of acting through an estrogenic pathway may be able to induce the same immunomodulating effects as the endogenous hormone.

Reports on endocrine disrupting chemicals (EDC) that are known estrogen mimics demonstrate that these compounds induce similar immune enhancement as E2 (Sobel *et al.* 2005; Yurino *et al.*, 2004). One study demonstrated that *in vitro* exposure to

bisphenol A (BPA) and diethylstilbesterol (DES) increased IgM secretion from B1 cells (Yurino *et al.*, 2004). In addition, chronic DES exposure accelerated the onset of systemic lupus erythematosus (SLE) in BWF1 mice (Yurino *et al.*, 2004). In another study illustrating the ability of EDC to enhance an autoimmune disease, the organochlorine pesticides chlordane, methoxychlor, and DDT accelerated the progression of SLE in (NZB x NZW)F₁ mice (Sobel *et al.*, 2005). Additionally, the anti-DNA titers were also increased in chlordane treated animals (Sobel *et al.*, 2005). These reports suggest that EDC have the potential to impact the immune system and accelerate the progression of autoimmune diseases.

The amide class herbicide 3,4-dichloropropionanilide (propanil) is predominantly used for the control of grassy weeds in rice crops. Exposure to propanil causes a diverse array of immunotoxic effects that are similar to those seen in animals treated with E2. Specifically, *in vivo* exposure to propanil induces thymic atrophy, with reduced double positive and single positive thymocyte populations (Cuff *et al.*, 1996; de la Rosa *et al.*, 2005). The thymic atrophy is associated with an increase in glucocorticoid production (Cuff *et al.*, 1996; de la Rosa *et al.*, 2005). However, administration of the glucocorticoid receptor antagonist RU486 did not completely abrogate the atrophy suggesting that glucocorticoids are only partially responsible for inducing thymic atrophy (de la Rosa *et al.*, 2005). Propanil also decreases the number of immature IgM⁺ B cells and pre-B cells in the bone marrow 7 days post-exposure, however the mechanism is unknown (de la Rosa *et al.*, 2003).

Recently, propanil has been demonstrated to enhance the humoral immune response to a bacterial vaccine (Salazar *et al.*, 2005). Heat-killed *Streptococcus*

pneumoniae (HKSP) was used as the model for measuring the antibody response. HKSP elicits a robust IgG2b, IgG3, and IgM response to the immunodominant T-independent type 2 (TI-2) polysaccharide phosphorylcholine (PC) (Wu *et al.*, 1999). When propanil is administered to mice simultaneously with a HKSP vaccine, the number of splenic ASC specific for PC is increased 4-6 fold over control animals (Salazar *et al.*, 2005). The current studies were designed to investigate the mechanism for the increase in PC antibody production following exposure to propanil.

Only limited studies have reported the endocrine disrupting effects of propanil. Using an *in vitro* luciferase reporter assay, propanil was found to be weakly anti-androgenic and lacked estrogenic receptor α - and β -binding activity (Kojima *et al.*, 2004). Another report found that the major metabolite of propanil, 3,4-dichloroaniline (3,4-DCA), binds the androgen receptor *in vitro* (Bauer *et al.*, 1998). Given the paucity of information describing the potential endocrine disrupting effects of propanil *in vivo*, one of the goals of this study was to better characterize propanil as an EDC, using both *in vivo* and *in vitro* models.

As described above, propanil induces a number of immunotoxic effects also caused by E2. The hypothesis for the present studies was that propanil increased the antibody response to HKSP vaccination through an estrogenic mechanism. The results demonstrate that propanil requires the ovaries and steroid synthesis to induce an increase in the antibody response in female mice. However, the experiments demonstrate that the enhanced antibody response following exposure to propanil occurs independently of the estrogen pathway. These studies suggest that the ovaries may regulate the systemic

humoral immune response through a novel, estrogen-independent mechanism and that propanil modulates this pathway.

MATERIALS AND METHODS

Mice

Six to eight week old C57Bl/6 female and male mice were purchased from Hill Top Lab Animals (Scottsdale, PA). Ovariectomized (OVX), castrated (CAS), and sham-operated mice were surgically gonadectomized at Hill Top Lab Animals. Mice were housed in microisolator cages in pathogen free conditions at West Virginia University's animal facility. Mice were kept on a 12 hr light-dark cycle and allowed to acclimate to the facility for 1 week. Gonadectomized mice were housed at least 2 weeks following surgery to allow endogenous hormones to decline. Food and water were provided *ad libidum*. These studies were conducted in accordance with all federal and institutional guidelines for animal use and were approved by the West Virginia University Institutional Animal Care and Use Committee.

Chemicals

Propanil (3,4-dichloropropionanilide, 99% pure) was purchased from Chem Service (West Chester, PA). 17 β -estradiol (E2) was purchased from Sigma Chemical Co. (St. Louis, MO) and diluted in 99% peanut oil (p.o.) and 1% EtOH. ICI 182,780 (ICI) was purchased from Tocris Bioscience (Ellisville, MO). ICI was diluted in 95% p.o. and 5% EtOH. The GnRH antagonist, antide, was purchased from Bachem (Torrance, CA) and dissolved in 20% propylene glycol and 0.85% saline. The progesterone antagonist, RU486, was purchased from Sigma Chemical Co. and dissolved in propylene glycol.

Bacterial preparation and immunization

S. pneumoniae strain R36A, an avirulent, nonencapsulated strain, was grown to mid-log phase in Todd-Hewitt broth (Becton Dickinson, Sparks, MD) + .05% yeast extract (Becton Dickinson) and stored at –80°C. For immunization, stock was cultured in a candle jar for 18 hrs at 37°C on blood agar plates (Becton Dickinson). A few characteristic colonies were selected and suspended in 200 ml Todd-Hewitt broth + .05% yeast extract. Bacteria were grown at 37°C to an absorbance reading at 650nm of 0.4 and heat killed for 4 hours in a 60°C water bath. A final concentration of 10^9 CFU/ml was established in PBS based on colony counts. Sterility was confirmed by culture. Heat-killed stock was stored at -20°C in 1 ml aliquots. Mice were immunized ip with 2×10^8 CFU. This dose of vaccine has previously been demonstrated to elicit an optimal PC-specific antibody response that peaks at day 7 post-vaccination (Wu *et al.*, 1999; Wu *et al.*, 2000).

Animal exposures

Propanil was dissolved in p.o. for a final concentration of 15 mg/ml or 20 mg/ml for all experiments. For the experiments measuring antibody responses, mice (5/group) were treated ip with either 150 mg of propanil/kg of body weight (mg/kg) or vehicle on the same day as vaccination with heat-killed *S. pneumoniae* (HKSP). The dose of propanil was chosen based on previous studies that demonstrate an increase in the antibody response to PC in vaccinated mice at 150 mg/kg (Salazar *et al.*, 2005).

In experiments using E2, ovariectomized mice were injected sc with a daily dose of E2 (10 µg/kg). Animals received the first dose of E2 1 hr prior to herbicide or vehicle

treatment and vaccination. Subsequent E2 treatments were given at the same time daily. Animals were sacrificed 24 hrs following the final E2 exposure.

In experiments using the ER antagonist, ICI 162,780 (10 mg/kg/day), was injected sc into female mice. Animals were treated with the first dose of ICI 1 hr prior to herbicide or vehicle exposure and vaccination. Subsequent ICI exposures were given at the same time daily throughout the course of the experiment.

In experiments using the GnRH antagonist, antide (60 µg/mouse), female mice were injected sc every 48 hours 14 days prior to propanil or vehicle exposure and vaccination (Couse *et al.*, 2003). Subsequent antide treatments were given at the same time every 48 hours throughout the course of the experiment.

For the uterotrophic assays, OVX mice were treated with either a single ip injection of the vehicle or propanil (150 mg/kg), or 3 daily sc injections of E2 (10 µg/kg/day). Mice were euthanized 24 hours after the last E2 exposure, and the wet weights of the uteri were recorded.

In experiments using the progesterone receptor antagonist, RU486 (12.5 mg/kg), female mice were injected sc 2 hours prior to propanil treatment, 12 hours following propanil treatment, followed by injections every 24 hours throughout the duration of the experiment. For thymic atrophy experiments, mice were injected with 200 mg/kg of propanil or vehicle control. The dose of propanil was based on previous studies on propanil-induced thymic atrophy (de la Rosa *et al.*, 2005). Thymic weights were recorded on day 3. For HKSP experiments, mice were treated with 150 mg/kg of propanil or vehicle control and vaccinated. Splenic antibody responses were measured on day 7.

Serum estradiol levels

Serum estradiol levels were determined for mice treated with antide for 14 days using the Estradiol EIA kit (Diagnostic Automation Inc., Calabasas, CA), according to the kit instructions. Briefly, serum samples collected from the saphenous vein or E2 standards were added to the provided 96-well microtiter plates coated with anti-E2 antibody. Anti-E2 antibody conjugated to peroxidase was added, and the wells were incubated for 90 min at room temperature. The wells were washed and substrate was added to each well for 20 min. The reaction was stopped and read at 450 nm with a μ Quant spectrophotometer (Bio-Tek Instruments, Winooski, VT). The level of detection for the assay was 1 pg/ml.

Preparation of splenocytes

Mice were euthanized with 100 μ l Nembutal Sodium Solution (50 mg/ml, Abbott Laboratories, North Chicago, IL) on days 5 or 7 following herbicide exposure and vaccination. Spleen wet weights were recorded. Spleens were mechanically dissociated through Spectra nylon mesh (Spectrum Labs, Rancho Dominguez, CA) in complete cell media containing RPMI-1640 (BioWhitaker, Walkersville, MD), 10% heat inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc, Logan, UT), 10 mM HEPES (Sigma), 1 mM L- glutamine (Gibco, Rockville, MD), 5×10^{-5} M 2-mercaptoethanol (Sigma), 100 U/ml penicillin (Gibco), and 100 μ g/ml streptomycin (Gibco). Red blood cells in the spleen were lysed with Tris-buffered ammonium chloride. Cell suspensions were washed twice and counted using a hemacytometer. Viability was determined using Trypan blue dye exclusion.

Measurement of antibody secreting B cells (ASC) in the spleen

Acrowell™ 96 well filter plates (Pall Life Sciences, Ann Arbor, MI) were coated with 50 µl PC-BSA (Biosearch Technologies, Novato, CA) (10 µg/ml) overnight at 4°C. In all subsequent steps, plates were washed with PBS + .01% Tween-20. Plates were blocked with 200 µl/well complete medium + 25% FBS for 2 hours at 37°C. Plates were washed and cells (100 µl/well) were then added at a concentration of 5×10^6 cells/ml or 1×10^6 cells/ml. All samples were plated in triplicate. Plates were incubated for 4-6 hours at 37°C in a 5% CO₂ incubator. After washing, goat anti-mouse alkaline phosphatase (AP) conjugated IgG, IgG2b, IgG3, or IgM antibodies (Southern Biotechnology Associates, Birmingham, AL), diluted 1/250 in PBS + 1% BSA + .05% Tween-20, were added to the appropriate wells (100 µl/well). Plates were incubated overnight at 4°C and washed. SIGMAFAST 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma-Aldrich, St. Louis, MO) were dissolved in distilled water and added at 100 µl/ well. Color development was stopped by washing with distilled water. The number of spots/well was counted using a dissection microscope (Olympus Optical Co., Melville, NY). The number of ASC was calculated by using the mean number of spots from triplicate wells. The number of ASC was normalized to 1×10^6 B cells. Comparable fold increases were noted when normalized to whole spleen (Salazar *et al.*, 2005).

Flow cytometric analysis

Splenic cells were stained with the appropriate combinations of rat anti-mouse B220-APC (RA3-6B2), rat anti-mouse CD4-FITC (GK1.5), or rat anti-mouse CD8α-PE (53-6.7) (BD PharMingen, San Diego, CA). All steps were performed in PBS supplemented with 1% FBS and 0.04% sodium azide (Sigma). Briefly, 1×10^6 cells were

stained in a total volume of 25 μ l of antibodies at the appropriate concentrations for 25 minutes on ice in the dark. After incubation, cells were washed twice and fixed in 0.04% paraformaldehyde overnight at 4°C (Fisher Scientific, Pittsburgh, PA). The following day cells were washed twice to remove the paraformaldehyde and resuspended in 1 ml of staining media. For each sample, 10,000 cells were collected for analysis on a Becton-Dickinson FACScan (Becton Dickinson Immunocytometry Systems, Mansfield, MA). Analysis was performed using WinMDI software (Joseph Trotter, Scripps Institute, San Diego, CA). Population percentages, obtained from flow cytometric analysis, were used to calculate the absolute cell number by multiplying the percentage of cells in a population by the total number of cells harvested per organ. Previous experiments have determined that the number of CD4⁺ T cells, CD8⁺ T cells, and B cells is not changed after propanil exposure (Salazar *et al.*, 2005).

ER binding assay

ER competitive binding assays were performed using purified recombinant ER α and ER β (Panvera, Madison, WI). Reactions were performed in 10 mM Tris, 1.5 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol at pH 7.4. Reactions containing 1 nmol ER, 10 nmol [³H]-E2, and the indicated concentrations of competing unlabeled E2 or propanil were performed in reaction buffer at 4°C for 18 to 20 h. [³H]-E2 bound to ER was separated from free [³H]-E2 by adding a 60% hydroxylapatite solution followed by repeated centrifugation and washing. The amount of [³H]-E2 bound to E2 was determined by liquid scintillation.

Ishikawa cells and alkaline phosphatase activity

Ishikawa cells (a generous gift from Richard B. Hochberg, Yale University Medical School, New Haven, Connecticut) were maintained in MEM medium (Sigma), 10% fetal bovine serum, as described (Littlefield *et al.*, 1990). Ishikawa cells were treated as described to assess the effect of propanil on estrogen-inducible alkaline phosphatase (Littlefield *et al.*, 1990). Briefly, cultures were rinsed twice with estrogen-free basal medium and maintained in this medium for 24 hours. Cells were then trypsinized and plated into 96 well plates (1×10^4 cells/well) in the presence or absence (negative control) of indicated concentrations of estrogen (positive control) or propanil. All cell treatments were performed in quadruplicate. After 72 hr, cultures were treated as described in detail, and alkaline phosphatase activity was determined by measuring the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol. MTT Cell Proliferation Kit (Roche Applied Science, Indianapolis, IN) was used as specified by the manufacturer to assess effects on cell numbers. Propanil had no effect on the proliferation of Ishikawa cells (data not shown).

Statistics

One-way analysis of variance (ANOVA) was performed for all statistical analyses using a Tukey-Kramer t-test to perform multiple comparisons between all treatment groups. A significance level of $p \leq 0.05$ was used for all tests. Statistical analysis was performed using JMP software (SAS Institute Inc., Cary, NC). All experiments were performed 3 or more times with similar results. The figures are representative data from one experiment.

RESULTS

Ovariectomization abrogates the antibody increase induced by propanil.

To test the hypothesis that propanil increases the antibody response after vaccination with HKSP through an estrogenic mechanism, OVX or sham-operated mice were vaccinated with HKSP and treated with propanil or the vehicle. All sham-operated mice exposed to propanil had a significantly increased number of PC-specific ASC compared to sham-operated vehicle-treated mice (Figure 1). PC-specific IgM and the dominant PC isotypes, IgG2b and IgG3, were significantly increased in propanil-treated animals (Figure 1). Spleen cells from these mice were also cultured *in vitro* for 5 days and the production of antibody measured by specific ELISA. There was an increase in antibody production that corresponded with the increase in ASC (data not shown). Ovariectomy abrogated the ability of propanil to increase the number of PC-specific ASC in the spleen (Figure 1). Comparable results were obtained when the data were normalized to 1×10^6 splenic B cells (Figure 1) or to the total spleen (data not shown). The number and percentage of B cells, CD4⁺ T cells and CD8⁺ T cells in the spleen was similar for all groups as determined by flow cytometry (data not shown). These data indicate that the ovaries are required for propanil to induce increases in the PC antibody response.

Propanil does not bind to estrogen receptors *in vivo* or *in vitro*.

To determine if propanil increased the PC antibody response by binding directly or indirectly with ER α , *in vivo* uterotrophic assays were performed. OVX mice were treated once with 150 mg/kg of propanil. Positive control animals were treated daily with E2 (He *et al.*, 2003). Uterine weights were recorded 3 days following the first exposure.

As expected, E2 treatment significantly increased uterine weight (Figure 2). Propanil, however, did not increase the uterine weights compared to vehicle treated animals (Figure 2). This data suggests that propanil does not directly or indirectly activate ER α , which is primarily responsible for the E2-induced uterine proliferation (Weihua *et al.*, 2000).

To establish if propanil interacts directly with the ER α or ER β , an *in vitro* competitive ER binding assay was performed. Propanil did not bind either ER α or ER β over a wide range of concentrations (Figure 3), confirming a previous report (Kojima *et al.*, 2004). In addition, propanil was administered to Ishikawa cells, which express both ER α and ER β . Propanil did not increase estrogen-inducible alkaline phosphatase activity compared to controls, further supporting that propanil does not bind ER α or ER β (Figure 4).

Administration of exogenous E2 does not restore the enhanced antibody response in propanil-treated OVX mice.

To determine if propanil requires E2 to enhance the antibody response to HKSP, OVX mice were exposed once to either propanil or vehicle control. In addition, half of the propanil-treated and vehicle-treated OVX mice received daily sc E2 for 7 days. All groups were vaccinated with HKSP on day 0. The PC-specific responses from the OVX mice were compared with sham-operated mice. Uterine weights and PC-specific splenic ASC were measured on day 7. Uterine weights of OVX mice were significantly lower than sham-operated vehicle mice (Figure 5A). Exogenous E2 administration restored uterine weights of OVX mice comparable to uterine weights of sham-operated mice (Figure 5A, OVX Vehicle + E2 vs. Vehicle, OVX Propanil + E2 vs. Vehicle). The

addition of exogenous E2 to propanil-treated OVX mice failed to restore the increase in PC-specific ASC (Figure 5B, OVX Propanil + E2 vs. Propanil). All ovariectomized mice had a similar number of PC-specific ASC as vehicle control animals (Figure 5B). These results suggest that the inability of propanil to enhance the antibody response in OVX mice was not due to the absence of E2.

An ER antagonist does not inhibit the ability of propanil to enhance the antibody response.

The previous experiment demonstrated that the addition of exogenous E2 did not restore the ability of propanil to enhance the antibody response in OVX mice. However, it is possible that the dose of exogenous E2 given does not accurately mimic the level of E2 in mice with intact ovaries. To determine if signaling through the estrogen receptor is required for the increased immune response, mice were treated with the ER α and ER β antagonist ICI 182,780. Normal female mice were vaccinated and treated with propanil or vehicle on day 0, and given daily treatments with ICI 182,780. Uterine weights were measured to confirm the efficacy of the ICI treatment and the PC-specific antibody response was determined. The uterine weights of all animals receiving the ER antagonist were significantly reduced compared to control animals (Figure 6A). Treatment with the ER antagonist did not affect the basal PC-specific ASC response in vehicle-treated mice (Vehicle vs. Vehicle + ICI, Figure 6B). Propanil-exposed mice treated with ICI had an increased ASC response comparable to propanil alone (Propanil vs. Propanil + ICI, Figure 6B). These results demonstrate that the ER antagonist did not inhibit the increase in PC-specific ASC in propanil-treated animals, which suggests that signaling through the estrogen receptor pathway is not required for propanil to enhance the antibody response.

Inhibition of steroid synthesis in propanil-treated mice abrogates the increase in ASC.

Chemical inhibition of steroid synthesis was conducted to determine if a gonadal steroid other than estradiol regulates the propanil amplification of the humoral immune response. Animals were treated with the GnRH antagonist antide for 14 days prior to propanil exposure and HKSP vaccination to ensure complete suppression of ovarian steroid synthesis (Couse *et al.*, 2003). Serum estradiol levels were determined prior to propanil treatment on day 14. All mice had non-detectable levels of estradiol, which is indicative of the efficacy of the antide treatments (data not shown). Uterine weights and the immune response to PC were determined 7 days post-vaccination. Antide-treated animals had significantly decreased uterine weights (Figure 7A). Treatment with antide did not affect the basal PC-specific ASC response (Vehicle vs. Vehicle Antide, Figure 7B). The number of PC-specific ASC in mice treated with antide and exposed to propanil were not significantly different from the vehicle controls (Vehicle vs. Propanil Antide, Figure 7B), but were significantly different from the propanil-treated control animals that had an increased ASC response (Propanil vs. Propanil Antide, Figure 7B). These results demonstrate that steroid synthesis is necessary for the enhanced immune response in the spleens of mice exposed to propanil.

A progesterone receptor antagonist does not inhibit antibody enhancement by propanil.

Progesterone is another steroid hormone that has been shown to affect antibody production. The progesterone antagonist, RU486, was utilized to determine if

progesterone signaling is required for the increased antibody response. As previously stated, propanil induces thymic atrophy which is partially inhibited by RU486 (de la Rosa *et al.*, 2005). To determine the efficacy of the RU486 treatments, mice were treated with RU486 2 hours prior to propanil or vehicle treatment, and at 12 and 24 hours post-exposure. RU486 partially abrogated thymic atrophy as determined by measurement of the thymic weights and by quantification of the CD4⁺CD8⁺ thymocyte population (Figure 8A and 8B), demonstrating that RU486 is efficacious via the sc route.

To determine if blockade of the progesterone receptor inhibits the increased antibody response, mice were injected with RU486 2 hours prior to propanil exposure and vaccination. Mice were subsequently injected with RU486 12 hours post-exposure and every 24 hours following propanil treatment. Treatment with the progesterone antagonist did not affect the basal PC-specific ASC response in vehicle-treated mice (Vehicle vs. Vehicle + RU486, Figure 8C). Propanil-exposed mice treated with RU486 had an increased ASC response comparable to propanil alone (Propanil vs. Propanil + RU486, Figure 8C). These results demonstrate that the progesterone antagonist did not inhibit the increase in PC-specific ASC in propanil-treated animals, which suggests that progesterone is not required for propanil to enhance the antibody response.

Propanil enhances the antibody response in both normal and orchietomized males.

To determine if propanil increased the antibody response in a gender specific manner, male mice were vaccinated and treated with propanil or vehicle (Figure 9). The PC-specific splenic ASC were measured after 7 days. Propanil increased the IgG2b, IgG3 and IgM PC-specific splenic ASC approximately 3-fold (Vehicle vs. Propanil, Figure 9). Castration did not significantly affect the propanil-induced increase in PC-

specific ASC. Castrated males treated with propanil produced approximately the same fold increase in PC-specific ASC as normal propanil-treated male mice (Propanil vs. Propanil Castrated, Figure 9). These results suggest that androgens are not involved in the amplification of the immune response after propanil exposure.

DISCUSSION

Gonadal steroid regulation of the immune system is poorly understood. The majority of the research into the effects of sex steroids on the humoral immune response involves the hormones estradiol, progesterone, and testosterone. Estradiol has been demonstrated to increase the number of antigen-specific splenic ASC several fold (Verthelyi and Ahmed, 1998). The other major gonadal hormones progesterone and testosterone are generally considered to be immunosuppressive. The data presented here suggest a novel role for the amplification of the TI-2 humoral immune response by the ovary that is independent of estrogen or progesterone. This report also demonstrates the novel use of propanil exposure to study the mechanism for gonadal steroid regulation of antibody responses.

Previous reports demonstrated that propanil induces a number of immunotoxic effects that suggest a manipulation of the estrogenic pathway (de la Rosa *et al.*, 2003; de la Rosa *et al.*, 2005; Salazar *et al.*, 2005). Propanil has been classified by two studies as non-estrogenic and weakly anti-androgenic, as determined by receptor binding assays (Bauer *et al.*, 1998; Kojima *et al.*, 2004). However, given the heavy usage of propanil and the importance of studying endocrine disruption, extensive characterization of propanil as a potential EDC is warranted. Synthetic chemicals can manipulate the estrogenic pathway in a number of ways including 1) direct binding of ERs, 2) indirect ER activation by stimulating ER phosphorylation, 3) altered expression of the ER, 4) changing the levels of hormone expression, or 5) modifying the intracellular estrogenic signaling pathway (Borgest *et al.*, 2002).

The uterotrophic assay is the classic method to determine the *in vivo* estrogenic activity of a chemical. Measuring uterine weights after 3 days of chemical exposure is a sensitive assay for detecting weak ER agonists (O'Connor *et al.*, 1996). *In vivo* exposure to propanil did not induce an increase in uterine weight. Disadvantages of the uterotrophic assay include the inability to decipher antagonistic or agonistic activity in different organs, a lack of specificity, and the inability to measure ER β binding activity (O'Connor *et al.*, 1996; Weihua *et al.*, 2000). For instance, chemicals such as tamoxifen act either as ER antagonists or agonists within different organs or at different concentrations (Watanabe *et al.*, 1997). Therefore, the ER α and ER β binding activity of propanil were determined using *in vitro* assays. Propanil did not compete for E2 binding to either ER α or ER β nor did it increase estrogen-inducible alkaline phosphatase activity in ER-responsive cells. The results of the uterotrophic assay, ER binding studies, and the ER-inducible alkaline phosphatase assay establish that propanil is not a classic EDC.

Increasing ER expression levels might also affect antibody activity since the ER is found on many immune cells including B cells (Grimaldi *et al.*, 2002). Removal of E2 from circulation would eliminate the ability of ER expressing cells to respond. However, subcutaneous administration of E2 to OVX mice failed to restore the stimulatory effect of propanil on the antibody response to HKSP, which indicates that propanil does not require the presence of E2 to exert its effect on the splenic antibody response. This also suggests that propanil exposure does not induce antibody stimulation by increasing the ability of cells to bind E2. In addition, the data shows that high concentrations of E2 for a short period of time (7 days) do not affect the TI-2 antibody response.

Chronic low E2 exposure increases splenic ASC (Myers and Petersen, 1985; Verthelyi and Ahmed, 1998). However, high levels of E2, such as during the third trimester of pregnancy, is immunosuppressive (Confavreux *et al.*, 1998). Measuring E2 serum levels is compounded by a number of factors including estrous cycling and the selection of several time points for hormone analysis. An alternative approach to directly measuring estradiol serum levels is to inhibit the effects of E2 by blocking the ER. The ER antagonist ICI 182,780 has been demonstrated to completely attenuate the ability of ER α and ER β to regulate transcription by 1) impairing receptor dimerization, 2) increasing receptor degradation, thereby lowering ER expression, and 3) disrupting nuclear localization of the ER/ligand complex (Wakeling, 2000). Several reports have shown that administration of ICI 182,780 1 hr prior to EDC exposure is sufficient to inhibit the effect of an EDC (Ciana *et al.*, 2001; Jung *et al.*, 2005; Papaconstantinou *et al.*, 2001). Propanil does not appear to trigger antibody stimulation by acting on the estrogenic signaling pathway, as indicated by the failure of ICI 182,780 to inhibit the increased ASC response following propanil treatment. These data strongly suggest that propanil does not affect the TI-2 antibody response via an estrogenic mechanism.

The GnRH antagonist antide was utilized to determine if gonadal steroids regulate the ovarian-dependent stimulation of the antibody response. Antide treatment abrogated the effect of propanil similar to ovariectomy, suggesting that propanil stimulates the antibody response via a gonadal dependent mechanism in females. Antide treatment not only blocks the effects of GnRH, but also decreases the production of LH and FSH from the pituitary and eliminates the secretion of gonadal hormones (Couse *et al.*, 2003; Falset *et al.*, 1995). The hypothalamic hormone, GnRH, and the pituitary hormones, LH and

FSH, have all been demonstrated to regulate the antibody response (Athreya *et al.*, 1993; Jacobson and Ansari, 2004). Since ovariectomized animals have elevated levels of LH and FSH compared to antide-treated animals, but have similar antibody responses following propanil exposure, it can be inferred that LH and FSH do not participate in the increase in the number of PC-specific ASC (Wakeling *et al.*, 1991; Couse *et al.*, 2003).

Other non-estrogenic hormones in the steroid synthesis pathway can regulate the antibody response. Androgens can induce immunosuppression (Brick *et al.*, 1985). As mentioned previously, propanil has some anti-androgenic activity. However, antibody studies evaluating the effects of anti-androgens indicate that they are not immunostimulatory (O'Connor *et al.*, 2002). In addition, removal of the primary source of testosterone in males via castration did not affect the ability of propanil to enhance the antibody response. Altogether, this data does not support an androgenic mechanism.

Progesterone has been demonstrated to be primarily immunosuppressive in pregnant women (Beagley and Gockel, 2003). Observations in pregnant women with autoimmune diseases such as multiple sclerosis have demonstrated that progesterone and estradiol levels are at the highest during the third trimester when the severity of disease decreases (Confavreux *et al.*, 1998). However, some studies have demonstrated that the number of antigen-specific ASC in the vaginal tissue is increased following mucosal immunization and progesterone treatment (Johansson *et al.*, 1998). Blockade of the progesterone receptor with RU486 did not have any effect on the stimulation of the antibody response in propanil-treated mice. This suggests that antibody enhancement does not require a progesterone-dependent mechanism.

Loss of the increased PC response following ovariectomy or inhibition of steroid synthesis may be the result of an indirect effect caused by the loss in homeostatic steroid hormone levels. Immunomodulatory hormones, such as GnRH and E2, are produced locally in lymphoid organs. Studies have shown that the gonads regulate these locally produced lymphoid hormones, suggesting another potential mechanism for ovarian regulation of the humoral immune response. For instance, GnRH has been demonstrated to have immunomodulating effects independent of other hormones. Reports have shown that GnRH receptor mRNA and GnRH mRNA are expressed in murine spleen cells and human B lymphocytes (Jacobson *et al.*, 1998; Khan *et al.*, 2003; Silveira *et al.*, 2002). In addition, the spleen has been demonstrated to have a higher affinity for GnRH peptide compared to other endocrine and nonendocrine organs including the testes and liver (Khan *et al.*, 2003). Three weeks of exogenous GnRH treatment increases both the total IgG serum levels in female ovariectomized mice and the proliferation of B and T lymphocytes in response to LPS and ConA stimulation, respectively (Jacobson and Ansari, 2004).

The classically studied source of GnRH is the hypothalamus, although it is unlikely that hypothalamic produced GnRH can directly affect spleen function since the concentration of GnRH in the portal blood is low and rapidly metabolized (Eskay *et al.*, 1975). Evidence suggests that the ligand for GnRH receptors on splenic lymphocytes is likely to be derived from another endogenous source and may act in an autocrine or paracrine fashion in non-pituitary organs (Chen *et al.*, 1999). Both GnRH mRNA and GnRH receptor mRNA are altered in splenocytes throughout the estrous cycle, however, the exact mechanism of this regulation is unknown (Jacobson *et al.*, 1998).

It is possible that the ovaries are involved in regulating the local GnRH production in the spleen, which contributes to the increased antibody response in propanil-treated animals. This hypothesis might also explain the contrasting results between castrated and ovariectomized animals. There is data which indicates that male and female discrepancies in immune function exist in response to GnRH treatment following gonadectomy (Jacobson *et al.*, 1999). These data suggest that the sexual disparities exist due to a residual effect caused by hormonal differences experienced early in development.

Additional evidence exists suggesting gonad-mediated hormone regulation in lymphocytes and the spleen. A recent report demonstrated that removal of the ovaries alters the activity of the steroid hormones aromatase and 17 β -hydroxysteroid dehydrogenase locally in the spleens and T cells of mice (Samy *et al.*, 2001). In addition, castration had the opposite effect on the activity of these enzymes (Samy *et al.*, 2001). The role of these steroidogenic pathways in the spleen and T cells on the humoral immune response has not been evaluated. Propanil may act by disrupting the ovarian control of the steroidogenic enzymes in the spleen leading to altered B cell activation. Analyzing the local spleen-specific and ovary-specific hormone receptor mRNA, hormone mRNA, and the activity of steroid enzymes would yield valuable clues to the mechanism regulating this effect.

In contrast to the results presented here, previous studies have demonstrated that exposure to propanil can decrease the response to a T dependent and a T-independent antigen (Barnett *et al.*, 1992; Barnett and Gandy, 1989). In the previous studies, C57Bl/6 female mice were exposed i.p. to propanil on day 0, immunized i.v. on day 3

with the TI-2 antigen DNP-Ficoll, or the T dependent antigen, SRBC, and the plaque-forming cell response in the spleen determined on day 7. In the present studies, propanil was administered i.p. on day 0 and the mice immunized i.p. with HKSP on day 0. The PC-specific ASC response was determined on day 7. It is possible that the different results between the studies in the PC antibody response is due to the nature of the antigen, purified antigen versus whole heat-killed bacteria. However, preliminary studies in the laboratory have demonstrated a similar enhancement of the ASC response to the TI-2 antigen PC-Ficoll when propanil is administered at the time of PC-Ficoll immunization. Therefore, the timing of propanil exposure and immunization may be the critical factor in the ultimate effect that propanil has on the humoral immune response. Preliminary studies demonstrate that propanil can be given 3 days after HKSP immunization and still enhance the PC immune response. In addition, preliminary experiments demonstrate that propanil can also enhance the ASC response in the spleen to a T dependent antigen on HKSP, PspA. The PspA-specific serum antibody titers peak approximately 14 days post-immunization. Propanil must be given on day 9 after immunization to enhance the PspA response. If the animals are exposed to propanil at the time of immunization on day 0 there is no effect on the immune response to PspA on day 14. Taken together, these studies suggest that propanil exposure must occur near the time of the peak antibody response to increase the response. It is not yet known if the ovaries are required for the enhancement of the immune response to PC-Ficoll or PspA.

Altogether, these data support two novel findings. First, propanil enhances the antigen-specific antibody response in females in an ovary-dependent, estrogen-independent manner unlike other EDC reported to date. Second, the data suggests a

novel role for antibody stimulation by the ovaries following chemical exposure. Future studies will utilize propanil as a unique tool to examine interactions between the immune and endocrine systems with an emphasis on how the ovaries contribute to regulation of humoral immune responses.

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Figure 1. Ovariectomy abrogates the propanil-induced increase in PC-specific splenic ASC.

OVX and sham-operated female C57BL/6 mice (5/group) were treated with 150 mg/kg propanil or vehicle control and vaccinated with 2×10^8 CFU HKSP. Numbers of PC-specific IgG2b, IgG3, and IgM ASC were determined by ELISPOT assay on day 7.

Values represent the mean $\pm$ SD of ASC/1x10⁶ B cells. * Significantly different from all other groups, $p \leq .05$.

Figure 2. Propanil does not increase uterine weights in OVX mice.

C57BL/6 mice (5/group) were ovariectomized and treated with vehicle control (open bar), 150 mg/kg propanil (hatched bar), or 10 µg/kg/day of E2 (black bar). Uterine weights were recorded after 3 days. Values represent the mean $\pm$ SD. * Significantly different from vehicle, $p \leq .05$.

Figure 3. Propanil does not bind to ER α or ER β .

Competitive binding assays determined the binding of propanil to ER α or ER β in the presence of [3 H]-E2. Values represent the percent of [3 H]-E2 bound to ER in the presence of either propanil (open circles) or unlabeled E2 (closed circles).

Figure 4. Propanil does not increase estrogen-inducible alkaline phosphatase activity in Ishikawa cells.

Ishikawa cells were treated with propanil (1 μ M, 3 μ M, 10 μ M, or 100 μ M) or E2 (10 pM or 100 pM). Alkaline phosphatase activity was measured after 72 hours. Values represent the mean absorbance recorded in the propanil or E2 treated cells. * Significantly different from vehicle, $p \leq .05$.

Figure 5. Addition of exogenous E2 does not restore the increase in PC-specific ASC of propanil-treated animals.

OVX and sham-operated female C57BL/6 mice (5/group) were treated with 150 mg/kg propanil or vehicle control and vaccinated with 2×10^8 CFU HKSP. OVX mice were treated with 10 μ g/kg/day of E2 or the vehicle. Spleens and uteri were removed after 7 days. (A) Uterine weight. * Significantly different from vehicle control, $p \leq .05$. (B) Numbers of PC-specific IgG2b, IgG3, and IgM ASC. Values represent the mean $\pm$ SD of ASC/1x10⁶ B cells. * Significantly different from vehicle control, $p \leq .05$.

Figure 6. Addition of the ER antagonist ICI 182,780 does not block the increase in ASC in propanil-treated animals.

C57BL/6 mice (5/group) were vaccinated with 2×10^8 CFU HKSP, and treated with 150 mg/kg propanil or vehicle control, with or without ICI 182,780 (10 mg/kg/day) or vehicle. Spleens and uteri were removed after 5 days. (A) Uterine weight. *

Significantly different from vehicle control, $p \leq .05$. (B) Numbers of PC-specific IgG2b,

IgG3, and IgM ASC. Values represent the mean $\pm$ SD of ASC/1x10⁶ B cells. *

Significantly different from the respective vehicle control, $p \leq .05$.

Figure 7. Antide treatment abrogates the propanil-induced increase in PC-specific ASC.

C57BL/6 female mice were treated with antide (60µg/mouse) or vehicle for 14 days as described in Methods. Mice were vaccinated on day 14 with 2×10^8 CFU HKSP and treated with 150 mg/kg propanil or vehicle. Spleens and uteri were removed 7 days post-vaccination. (A) Uterine weight. * Significantly different from vehicle control, $p \leq .05$. (B) Numbers of PC-specific IgG2b, IgG3, and IgM ASC. Values represent the mean $\pm$ SD of ASC/1x10⁶ B cells. * Significantly different from vehicle control, $p \leq .05$.

Figure 8. Treatment with the progesterone antagonist RU486 does not inhibit the increase in ASC in propanil-treated animals.

C57BL/6 female mice were treated with RU486 as described in the Methods. Thymic wet weights and thymocyte populations were determined on day 3. (A) Thymus to body weight ratio. * Significantly different from the vehicle control and vehicle + RU486, $p \leq .05$. (B) CD4⁺CD8⁺ cells. * Significantly different from the vehicle control and vehicle + RU486, $p \leq .05$. # Significantly different from vehicle, propanil, and vehicle + RU486, $p \leq .05$. (C) Numbers of PC-specific IgG2b, IgG3, and IgM ASC were determined by ELISPOT assay on day 7 post-vaccination. Values represent the mean $\pm$ SD of ASC/ 1×10^6 B cells or per spleen. * Significantly different from the respective vehicle control, $p \leq .05$.

Figure 9. Propanil enhances the antibody response in both normal and orchiectomized males.

Sham-operated and castrated C57BL/6 male mice were vaccinated with 2×10^8 CFU HKSP and treated with 150 mg/kg propanil or vehicle control. Spleens were removed after 7 days. Numbers of PC-specific IgG2b, IgG3, and IgM ASC were determined by ELISPOT assay. Values represent the mean $\pm$ SD of ASC/1x10⁶ B cells or per spleen. * Significantly different from the respective vehicle control, $p \leq .05$.

**CHAPTER 6: PROPANIL EXPOSURE DOES NOT
ENHANCE SURVIVAL TO A VIRULENT *S. PNEUMONIAE*
CHALLENGE FOLLOWING A SUBOPTIMAL
IMMUNIZATION**

INTRODUCTION

The previous experiments demonstrated that propanil enhances the number of PC-specific ASC in the spleen, but does not affect serum antibody titers. Previous reports have shown that PC-specific antibody is protective against a lethal challenge with *S. pneumoniae* (Briles *et al.*, 1992; Wallick *et al.*, 1983). However, protection is correlated with circulating antibodies in the serum (Fischer *et al.*, 1995). These experiments were conducted to determine if propanil enhances the survival of HKSP immunized animals lethally challenged with *S. pneumoniae*.

Previous studies evaluating the survival of mice following HKSP immunizations have been performed using an immunization schedule of one ip injection on day 0 and a second immunization on day 14 followed by lethal challenge on day 21 (Wallick *et al.*, 1983; Briles *et al.*, 1992; Fischer *et al.*, 1995). However, PC-specific titers peak by day 7 and subsequent immunizations do not significantly increase the PC antibody titers (Wu *et al.*, 2002; Wu *et al.*, 1999). Analysis of the idiotypes produced in response to HKSP immunization demonstrates that the high affinity T15⁺ is increased as much as 20-fold (2 µg/ml to 40 µg/ml) following the second immunization in C57BL/6 mice (Ruppert *et al.*, 1980). These studies suggest that the second immunization enhances survival by increasing the percent of high affinity PC-specific antibody produced in C57BL/6 mice rather than increasing the total amount of PC-specific antibodies in the serum.

Previous experiments primarily concentrated on the effect of a single exposure of propanil on a primary antibody response. Therefore, the following experiments utilized a lethal challenge exposure model following a primary immunization. A single primary immunization with HKSP was found to induce a suboptimal level of protection against a

lethal challenge. Addition of propanil to the primary immunization did not affect mortality outcomes.

METHODS

Animals

Six to eight week old C57Bl/6 female mice were purchased from Hill Top Lab Animals. Mice were housed in microisolator cages in pathogen free conditions at West Virginia University's animal facility. Mice were kept on a 12 hr light-dark cycle and allowed to acclimate to the facility for 1 week. These studies were conducted in accordance with all federal and institutional guidelines for animal use and were approved by the West Virginia University Institutional Animal Care and Use Committee.

Reagents

Propanil (3,4-dichloropropionanilide, 99% pure) was purchased from Chem Service.

Bacterial preparation

S. pneumoniae strain R36A, an avirulent, nonencapsulated strain, was grown to mid-log phase in Todd-Hewitt broth + .05% yeast extract and stored at -80°C. For immunization, stock was cultured in a candle jar for 18 hrs at 37°C on blood agar plates. A few characteristic colonies were selected and suspended in 200 ml Todd-Hewitt broth + .05% yeast extract. Bacteria were grown at 37°C to an absorbance reading at 650nm of 0.4 and heat killed for 4 hours in a 60°C water bath. A final concentration of 10⁹ CFU/ml was established in PBS based on colony counts. Sterility was confirmed by culture.

Heat-killed stock was stored at -20°C in 1 ml aliquots. Mice were immunized ip with 2×10^8 CFU.

S. pneumoniae strain D39, an encapsulated virulent strain from which R36a was derived, was grown on blood agar plates for 18 hr at 37°C. A few colonies were added to Todd-Hewitt broth + 10% FBS and grown to mid-log phase (approximately 1×10^8 CFU/ml). Bacterial concentration was confirmed by plating. Bacteria was stored in 10% glycerol at -80°C. For lethal challenge, bacteria was thawed, washed in PBS, and resuspended at the appropriate concentration (approximately 2×10^7 CFU/ml).

Animal exposures

Propanil was dissolved in peanut oil for a final concentration of 15 mg/ml for all experiments. Mice (4-8/group) were treated ip with either 150 mg of propanil/kg of body weight (mg/kg) or vehicle on the same day as immunization. On day 7, mice were challenged either ip or iv with D39. Survival was monitored for 7 days, at which time the experiment was terminated. An LD₅₀ dose of *S. pneumoniae* was experimentally determined prior to these studies.

RESULTS

IP injected oil enhances survival to a lethal challenge to D39.

To determine if ip injection of the vehicle affects survival against a lethal challenge of *S. pneumoniae*, 3 groups of mice were vaccinated ip with HKSP and treated with saline, vehicle or propanil. Immunized mice treated with either vehicle or propanil had an increased survival rate (60%) compared to immunized alone animals only immunized with HKSP (0%) (Figure 1). It is possible that oil may increase survival by

inducing a general inflammatory reaction. Previous research demonstrated that ip oil exposure increases the number of inflammatory cells in the spleen (data not shown). These data suggest that interpretations from studies using an ip challenge model is complicated by the effects of the vehicle.

IP injected oil does not affect survival to an iv D39 challenge.

The previous experiment demonstrated that evaluation of the effects of propanil on survival to a lethal challenge requires altering the route of exposure to the virulent bacteria. Previous studies have shown that ip and iv routes of challenge have approximately the same degree of virulence (Briles *et al.*, 1992). Mice were treated with or without the vehicle, immunized ip with HKSP and challenged iv with virulent *S. pneumoniae*. Survival of immunized animals was not affected by ip vehicle treatment (Figure 2). Therefore, the iv route of challenge was chosen for future experimentation.

Propanil treatment does not increase survival in suboptimally immunized mice.

To determine if the increased number of PC-specific ASC induced by propanil affects survival, vaccinated mice were treated with propanil or vehicle and challenged with 2×10^6 CFU of D39 on day 7. Results were compared to naïve animals treated with propanil or vehicle. The CFU of D39 used was determined to be a LD₅₀ in HKSP vaccinated mice. Immunization with HKSP did not consistently increase survival compared to naïve animals (Figures 3A and 3B). In addition, propanil exposure in naïve animals did not affect survival compared to vehicle treated naïve animals (Figure 3A). Finally, propanil-treatment did not increase survival in immunized mice compared to either naïve mice or vehicle-treated immunized mice (Figures 3A and 3B).

DISCUSSION

These experiments begin to address the significance of the increased number of PC-specific ASC in the spleen. The experiments also address the question of whether antibody serum titers or ASC in the spleen are more important for virulent bacterial challenges. These results demonstrate that one vaccination of HKSP is not sufficient to increase survival to a lethal challenge. Furthermore, propanil was not able to enhance survival following a suboptimal immunization.

As previously mentioned, a second immunization with HKSP increases the percent of T15⁺ antibodies in the serum. The inability to protect mice following one HKSP injection is likely due to the relatively low concentration of T15⁺ antibodies. Previous reports have shown that T15⁺ idiotype antibodies are more effective at protecting against a lethal challenge than other idiotypes produced to PC (Nicoletti *et al.*, 1993; Briles *et al.*, 1982a; Briles *et al.*, 1984). Experiments have shown that passive immunization with T15⁺ antibodies increases protection from a lethal ip *S. pneumoniae* challenge in a dose dependent fashion (Briles *et al.*, 1982a). Given the importance of T15⁺ antibodies for protection, future experiments should evaluate the effect of propanil on PC-specific idiotypes in both the spleen and serum.

Alternative strategies to examine the effect of propanil on mortality include studying the effect of propanil on a secondary immunization. Our previous results demonstrate that propanil treatment at the time of the second immunization results in an increased PC-specific ASC response in the spleen. Challenging propanil and vehicle-treated mice that have been immunized twice would determine if propanil increases survival following an optimal immunization.

Altogether, this data begins to address relevance of an increased local antibody response in resolving a virulent challenge. Immunization with an alternative antigen that may increase serum titers such as PC-Ficoll or PC-KLH would be valuable in exploring the necessity of a corresponding increase in serum titers. In addition, passive immunization with pooled antibody collected from *ex vivo* splenic cultures from propanil-treated animals would determine the efficacy of the antibodies produced from the amplified response. Altogether, these studies support previous reports demonstrating the importance of serum titers to protect against a lethal challenge.

Figure 1. IP oil injection increases survival to a lethal ip *S. pneumoniae* challenge.

Female C57BL/6 mice (5-7/group) were immunized ip with 2×10^8 CFU HKSP and treated ip with saline, 150 mg/kg propanil or vehicle. Mice were challenged ip with a 2×10^6 CFU D39 *S. pneumoniae* on day 7. Values represent the percent of surviving mice.

Figure 2. IP oil injection does not affect survival to a lethal iv *S. pneumoniae* challenge.

Female C57BL/6 mice (5/group) were immunized ip with 2×10^8 CFU HKSP and treated ip with saline or vehicle. Mice were challenged iv with a 2×10^6 CFU D39 *S. pneumoniae* on day 7. Values represent the number of surviving mice.

Figure 3. Propanil exposure does not increase survival to a lethal challenge following a suboptimal immunization.

(A) Female C57BL/6 mice (4-8/group) were immunized ip with 2×10^8 CFU HKSP or saline and treated ip with 150 mg/kg propanil or vehicle. Mice were challenged iv with a 2×10^6 CFU D39 *S. pneumoniae* on day 7. (A) Values represent the percent of surviving mice. (B) Values represent the number of surviving mice.

CHAPTER 7: GENERAL DISCUSSION

Objective

These studies were designed to 1) examine the effect of an exposure to a mixture of herbicides on the humoral response utilizing the HKSP immunization model, 2) determine the mechanism regulating the propanil-induced enhancement of the PC-specific response induced by propanil, and 3) determine if propanil acts as an adjuvant that enhances the efficacy of an immunization.

Endocrine regulation of the humoral response

Propanil was found to require ovarian steroid synthesis to enhance the PC-specific response following HKSP immunization. However, the data demonstrates that propanil enhances the humoral response independent of an estrogenic mechanism. In addition, evidence was presented suggesting that progesterone is not involved in the increased response. Castration of male mice did not affect the propanil-induced response in male mice, suggesting an androgenic independent mechanism. This data eliminates the vast majority of published mechanisms by which the gonads directly regulate the antibody response.

A valid hypothesis must account for the two primary observations: 1) the ovaries are required for enhancement, and 2) the testes are not required. These observations suggest that the unknown factor produced by the ovaries required for antibody enhancement is also produced outside of the male gonads, assuming a similar mechanism for antibody enhancement in male and females. There are two hypotheses that may explain the data. 1) Propanil exposure induces the ovaries to produce or suppress protein(s) with a novel function that stimulates the antibody response. The site of protein

synthesis in males is outside of the gonads. 2) Alternatively, when mice are ovariectomized or steroid synthesis is inhibited, this disrupts local splenic hormone regulation. Propanil may act directly on the splenic hormones. However, disruption of ovarian regulation interferes with the ability of the spleen to respond to propanil. Both hypotheses represent novel roles for the ovarian regulation of the humoral immune response.

The first hypothesis suggests that propanil exposure alters ovarian function either directly or indirectly. There are several difficulties with this hypothesis. Presently, the existing literature has not characterized a protein or hormone associated with ovarian regulation of the antibody response that induces a similar response as observed in the propanil exposure model. Identification of the responsible protein(s) will be difficult since there is not a small group of likely candidates and detection would require a large screen of proteins and mRNA via such techniques such as proteomics and RNA microarrays. Additionally, the data indicates that the protein(s) would be synthesized independent of the testes in males. Locating the responsible protein in males would be more difficult since a target site has not been identified.

The second hypothesis proposes that propanil affects the local endocrine-regulated genes in the spleen. As mentioned in Chapter 5, the spleen contains a number of sex hormone mRNAs as well as sex hormone receptor mRNAs and steroidogenic enzymes. The ovaries and testes contribute to regulation of these hormones, receptors, and enzymes (Samy *et al.*, 2001; Jacobson *et al.*, 1998; Jacobson *et al.*, 1999). Notably, the ovaries and testes differentially regulate these local endocrine systems. The significance of global hormones on the immune response is well established. However,

the impact of local production of endocrine mediators in the spleen and lymphocytes is poorly understood. It is possible that propanil does not affect ovarian function, but instead exerts its effects on the spleen. Removal of the ovaries may disrupt local splenic hormone production, thereby preventing “normal” enhancement of the antibody response by propanil. Addition of the ovarian hormones such as E2 and progesterone to OVX mice prior to propanil exposure would address whether the ovarian hormones act in a supplementary fashion. Although E2 treatments in OVX mice did not restore the enhanced response following propanil exposure, studies suggest that both progesterone and E2 are required for accurate mimicry of ovarian hormone production. For example, complete GNRH and LH feedback regulation in OVX mice requires both progesterone and E2 (Dalkin *et al.*, 1990). Failure of E2 and progesterone supplements in OVX mice to restore the propanil enhancement of the antibody response would suggest that propanil is exerting its effect on the ovaries and not the spleen.

The results gathered by testing either one of these hypotheses would yield novel findings. Since the discovery of endocrine-related hormones and receptors in lymphoid organs, their function is largely unknown. Propanil could be used as a model reagent to study these locally produced hormones and their contribution to immune regulation. Alternatively, discovery of a novel protein or a novel function of an ovarian protein could lead to a number of studies elaborating the role of this protein in normal immune function and disease.

Immune mediators of the enhanced humoral response

Although it is known that the ovaries are required for the enhanced humoral response, the role of lymphocytes and costimulating molecules is unknown. As

mentioned in the General Introduction, there are a number of ways the PC-specific antibody response is enhanced. Both the innate and the adaptive immune system regulate the antibody response. Complement receptors enhance the antibody response by lowering the activation requirements of the B cell. APC such as FDC also enhance the antibody response via a CR2 dependent mechanism. As discussed in Chapter 4, propanil is not able to enhance the antibody response to a suboptimal immunization. In addition, affinity analysis suggests that lower affinity PC-specific B cells are not increased. This data suggests that a complement-mediated mechanism does not contribute to the enhanced response.

Other innate immune mechanisms such as MZ macrophages, cytokines and TLR regulate the TI-2 response via different mechanisms. MZ macrophages negatively regulate the PC-specific response. The data indicates that propanil did not affect the number of splenic macrophages. However, measurements of the MZ subpopulation was not performed, therefore this mechanism cannot be eliminated. Cytokines are important for early isotype switching. Since both the IgG isotypes and IgM are increased equally following propanil exposure, enhanced isotype switching is not likely to be responsible for the enhanced response. The available literature has shown that TLR stimulation increases the PC-specific response in a dissimilar manner compared to propanil exposure. However, TLR9 stimulation enhances polysaccharide specific antibody titers (Sen *et al.*, 2006). As previously shown, propanil does not enhance antibody titers. Furthermore, propanil enhancement of the number of splenic ASC is not further increased at later time points. Altogether, the data does not strongly indicate a specific innate immune mechanism for enhanced TI-2 responses following propanil exposure.

T cells possess a number of costimulating molecules that enhance the TI-2 antibody response. The inability of propanil to enhance the PC-KLH response on day 7 and the inability of a day 0 propanil-treatment to enhance the PC-specific response on day 3 suggests a role for T cells in the enhanced response. A direct method for evaluating the role of T cells is T cell deletion studies. As mentioned previously in the Discussion of Chapter 4, T cells are not required for normal PC-specific responses after 4 days. It has also been shown that delayed propanil exposure enhances the PC-specific response similarly to simultaneous vaccination and propanil treatment. Conflicting reports present evidence for a necessity of either CD4⁺ or CD8⁺ T cells in PC antibody responses (Wu *et al.*, 2002; Kobrynski *et al.*, 2005). An initial study could employ an anti-CD8, anti-CD4, or an anti-CD3 antibody on day 4 following HKSP immunization. Mice could then be exposed to propanil to determine the necessity of the different subpopulations of T cells in the amplified PC-response.

A possible mechanism for T cell mediated enhancement of the antibody response in propanil-treated mice is via a CD40 dependent pathway. As mentioned in the General Introduction, anti-CD40 treatment enhances the TI-2 antibody response with similar kinetics (Garcia *et al.*, 1999a). In addition, CD40 ligation is also important for TD responses. The number of TI-2 antigen-specific ASC is not increased until day 6 following anti-CD40 treatment (Garcia *et al.*, 1999a). Immunohistochemistry analysis suggests that the increase is the result of extended rounds of proliferation rather than an increase in recruitment of low affinity B cells to the response (Garcia *et al.*, 1999a). In addition, splenomegaly is observed in anti-CD40 treated animals with similar kinetics seen in propanil treated mice (Garcia *et al.*, 1999a). The splenomegaly observed in the

anti-CD40 treated animals are the result of an increase in white pulp and IgD⁺ cells (Garcia *et al.*, 1999a). Propanil treatment is hypothesized to increase splenomegaly via an influx in undifferentiated cells (de la Rosa, dissertation). Another discrepancy between the two treatments is the predominant IgG1 and IgG3 increase in anti-CD40 treated mice (Garcia *et al.*, 1999a). However, IgM was not measured in this report, making conclusions about the effects on isotype switching difficult. Altogether, these similarities in responses suggest a plausible T cell dependent mechanism to enhance the TI-2 response. However, a CD40 mechanism could still be plausible if T cells are not involved in the enhanced response. Some studies have shown a T cell independent source for CD40L ligation (Grammer *et al.*, 1999).

Studies exploring the immunological regulation of the antibody response may also aid in determining the mechanism for ovarian regulation by narrowing the search for enzyme or hormone mRNA. Identification of the affected immune cells important for the antibody enhancement will aid in a more precise analysis of the kinetics of activation of individual cells. Understanding the exact time of activation will further narrow the range of time for the mechanism of endocrine mechanism. Using both endocrine directed and immunological approaches will be necessary for efficient identification of the mechanisms involved.

Proliferation vs. polyclonal activation of B cells

Three primary methods for amplifying the number of ASC is by increasing the proliferation of activated B cells, by increasing the number of B cells recruited activated in the response, or increased differentiation. The evidence presented in Chapters 3 and 4 suggest that polyclonal activation is not the mechanism by which propanil enhances the

number of ASC. Polyclonal activation is characterized by increased lower affinity B cells, increased number of nonspecific antibodies, and increased recruitment of B cells into the follicles and red pulp of the spleen. An increase in proliferation is characterized by extended rounds of division, which leads to more B cells derived from the same progenitor cell. This results in an increased number of ASC with identical affinity and isotype. The third method for increasing the number of ASC is increased differentiation. This mechanism would suggest an increase in switching. Since all the predominant IgG isotypes are enhanced equally and IgM is also enhanced similarly to the IgG isotypes, this indicates that increased switching is not responsible for the increase in ASC.

Although our data suggests an increase in proliferation rather than polyclonal activation or increased differentiation, more specific experiments are necessary for a more definitive conclusion. More precise affinity analysis could be conducted using idiotype analysis since the affinities of the various idiotypes are known. Identifying the number of T15 and M167 idiotypes in propanil and vehicle-treated animals is another way of conducting affinity analysis. Determining if propanil increases the number of T15⁺ B cells similarly as other idiotypes will provide information on the polyclonal activation. In addition, T15 and M167 analysis provides information on the contribution of B1 B cells and MZ B cells since T15 plasma cells are predominantly derived from B1 cells and M167 are predominantly MZ B cells (Martin *et al.*, 2001). Finally, incorporating immunohistochemistry data and BrdU assays would provide information on the proliferation and recruitment of B cells into the antibody response. These studies would provide a more definitive assessment of polyclonal and proliferative activity following propanil exposure. In addition, determining the method of enhancement would

aid future experiments studying the immunological and endocrine mechanisms regulating the response.

Correlating serum antibody titers with ASC responses

An important question remaining from Chapters 3 and 4 is the lack of increased titers despite a several fold increase in the number of splenic ASC. Addressing this issue is important for future studies examining the effect of propanil exposure on a virulent challenge. The *ex vivo* antibody production from spleens of propanil-treated animals suggests that the increased number of splenic ASC produce antibodies normally. In addition, BM ASC were also demonstrated to contribute to the serum titers. However, splenic ASC in propanil-treated animals do not migrate to the BM in greater numbers. One explanation for the lack of increased titers could be that plasma cells from the spleen must migrate and survive in the BM in greater numbers to produce an increase in serum titers.

There are several possibilities for a lack of increased BM ASC in propanil-treated animals. The plasma cells may fail to migrate from the spleen to the BM. Alternatively, the BM may be unable to support the increased number of ASC. In order to address the issue of migration in propanil-treated animals, analysis of apoptosis could be performed. Previous studies indicate that splenic ASC that do not migrate to the BM will undergo apoptosis (Smith *et al.*, 1996). To determine if the increased number of splenic ASC are migrating to the BM or undergoing apoptosis, annexin V staining could be performed in splenic plasma cells and measured by flow cytometry. Increased levels of annexin expression from propanil-treated ASC compared to vehicle-treated animals would provide evidence suggesting that the increased ASC do not migrate in greater numbers

because the plasma cells undergo apoptosis. To address the impact of splenic plasma cell migration to the BM on antibody titers, *ex vivo* production of antibodies could be measured from the BM, spleen and peripheral blood. Comparing the quantity of antibody produced from ASC from each site would address the importance of successful migration of plasma cells from the spleen to the BM to could be measured. Splenic, BM and peripheral blood ASC could be cultured *ex vivo* and the amount of antibody produced into the supernatant could be quantitated. Directly comparing the amount of antibody produced by these three sources would provide evidence for or against the hypothesis that increased splenic ASC must migrate to the BM to increase serum titers. Altogether, these experiments would provide experimental evidence to address the correlation between serum titers and BM and splenic ASC.

The effect of propanil on a survival to a virulent *S. pneumoniae* challenge

Determining the effect of propanil exposure on the host's mortality following virulent challenge is important for assessing the relevance of the antibody enhancement model. Several additional experiments would provide more conclusive results concerning propanil's efficacy as an adjuvant. As mentioned in Chapter 6 Introduction, the T15 response is strongly linked to effective clearance of virulent *S. pneumoniae*. Two immunizations with HKSP are necessary for an optimal immunization. In addition, the T15 response following one injection of HKSP in Balb/c mice is approximately the same as double immunization in C57BL/6 mice (Ruppert *et al.*, 1980).

From this data, two experiments can be derived to evaluate the effect of propanil on an optimal immunization. As demonstrated in Chapter 4, propanil enhances a secondary PC response to HKSP. However, the effect of propanil on the T15 idiotype

has not been determined in C57BL/6 mice. C57BL/6 mice could be immunized twice with HKSP to elicit an optimum response. Challenging mice treated with propanil during a secondary response would determine if propanil enhances the optimal response. Alternatively, Balb/c mice could be substituted for C57BL/6 mice in the protection experiments since propanil enhances the response in Balb/c mice similarly to C57BL/6 mice (Schafer, personal communication). From these experiments, the protective effect of a propanil exposure on the optimal antibody response can be determined. Combined with the suboptimal data and serum antibody analysis, these studies would provide the requisite information to draw conclusions about the relevance of the enhanced splenic ASC response.

2,4-D as a model to study plasma cell homing and survival

The experiments in Chapter 3 demonstrated that 2,4-D reduces both the number of ASC in the BM and serum antibody titers. Although the mechanism is unknown as well as the antigen specificity of the effect, this discovery yielded a novel finding concerning the early importance of BM ASC to the primary TI-2 serum titers. Future studies could use 2,4-D as a model reagent to study BM plasma cells.

The mechanism regulating the 2,4-D induced decrease in BM ASC should be determined prior to employing 2,4-D as a model. Possible mechanisms include 1) defective migration out of the spleen, 2) failure to home properly to the BM, or 3) inability to support plasma cells upon homing to the BM due to disruption of the BM microenvironment. Upon establishing the mechanism of action, 2,4-D could then be used as a specific disruptor of long-lived BM plasma cells.

Summary

This dissertation presented findings that have implications for both the emerging studies of endocrine-mediated immune interactions and the field of endocrine disruption. As we begin to understand the purpose of the expression of endocrine-related genes in lymphocytes and lymphoid organs as well as the impact of these genes on basic immune function, new tools and models will be needed to address these basic questions. The novel effect of enhancing the number of splenic ASC in conjunction with ovarian steroid synthesis may yield valuable insight into this emerging field. Determining the mechanism by which propanil interacts with both the endocrine and immune system may not only yield new information into how these systems interact, but also, these experiments may reveal that propanil will serve as a useful tool for studying other endocrine-immune interactions.

Secondly, endocrine disruption is of recent concern in the field of toxicology. A large number of ubiquitously occurring compounds have the potential for endocrine disruption. The impact of these newly discovered endocrine disruptors on human health is currently being addressed. However, given the promiscuous binding nature of the ER and the need for rapid, large-scale screening, most studies have focused exclusively on evaluating the affinity of a chemical or compound to the ER. Rapid, comprehensive examinations of other potential nonestrogenic effects of compounds on the endocrine system are absent in the field of toxicology. Although propanil interacts with the endocrine system at high doses in our model, these experiments may be useful in discovering alternative nonestrogenic mechanisms for chemicals to interact with the endocrine system. Once the molecular targets are identified in this propanil exposure model, other chemicals could be rapidly screened for similar effects. As demonstrated in

our model as well as others, endocrine disruption can affect a number of other biological systems in the host organism. Given the impact of the endocrine system on the overall health of the organism as well as the dependence of the modern economy on the chemical industry, thorough evaluation of these compounds is necessary for sustained human and environmental health.

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