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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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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 after 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 after 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). Because 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-dichloropropionanalide (propanil) are commonly used herbicides marketed as a chemical mixture under the product names of NOX-D and Herbanil 368 (Meister and Sine, 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 (Barnett and Gandy, 1989; Blakley, 1997; de la Rosa *et al.*, 2003, 2005). 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

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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. Because of 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. In addition, 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.*, 1999, 2000).

Our laboratory focuses on studying potential synergistic effects on the immune system after exposure to a mixture of propanil and 2,4-D. Previous studies have shown 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 ($B220^+CD43^+IgM^{low}$) cells and IgM^+ B ($B220^+CD43\text{-}IgM^{hi}$) 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, 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-h light-dark cycle and allowed to acclimate to the facility for 1 week. Food and water was provided *ad libitum*. 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, 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 h 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 650 nm of 0.4. Bacteria were heat killed for 4 h 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 (ip) 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 ip injection within 1 h 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. The 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 had 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 after 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 medium 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 μg/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 CD8α-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 μl of antibodies at the appropriate concentrations for 25 min 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 medium. 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 Institution, 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, Uniformed Services University of the Health Sciences) 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 h 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 h at 37°C in a 5% CO_2 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 1/2000 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 and then blocked with 3% BSA + PBS at 37°C for 2 h. 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 h at 37°C and washed. AP conjugated antibodies (100 μl/well) were added for 1 h 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 was read at 405 nm on a μQuant spectrophotometer (Bio-Tek instruments, Winooski, VT) using KCJunior software (Bio-Tek instruments). To determine the titer, a standard pooled serum sample was diluted and plated on each ELISA plate. The titer for each sample was determined by comparison to the standard sera when the OD 405 nm 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% CO_2 at a concentration of 5×10^5 cells/ml in 500 μl complete medium 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 by means ofa partial factorial design. A mixture interaction was defined as the sum of the responses of the individual components of the mixture being 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 three 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 whether 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 peaked at day 6–7 after HKSP vaccination (Wu *et al.*, 1999, 2000). 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, Fig. 1A) and peak 5 to 7 days post-exposure (144 ± 73 ASC and 252 ± 42 ASC, respectively, Fig. 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 twofold (83 ± 35 ASC). The predominant isotypes produced were IgM, IgG2b, and IgG3, and all had similar kinetics (Fig. 1A, 1B, and 1C, respectively). Comparable results were obtained when the number of ASC per spleen were determined (Fig. 1D, 1E, and 1F). IgG1 and IgG2a were below the limit of detection by ELISPOT at all time points.

After exposure to propanil or the mixture of propanil and 2,4-D, the number of PC-specific IgM (Fig. 1A, 1D), IgG2b (Fig. 1B, 1E), and IgG3 (Fig. 1C, 1F) ASC were significantly increased 5 days after immunization (3–4-fold over vehicle control). Similar fold increases were determined for the

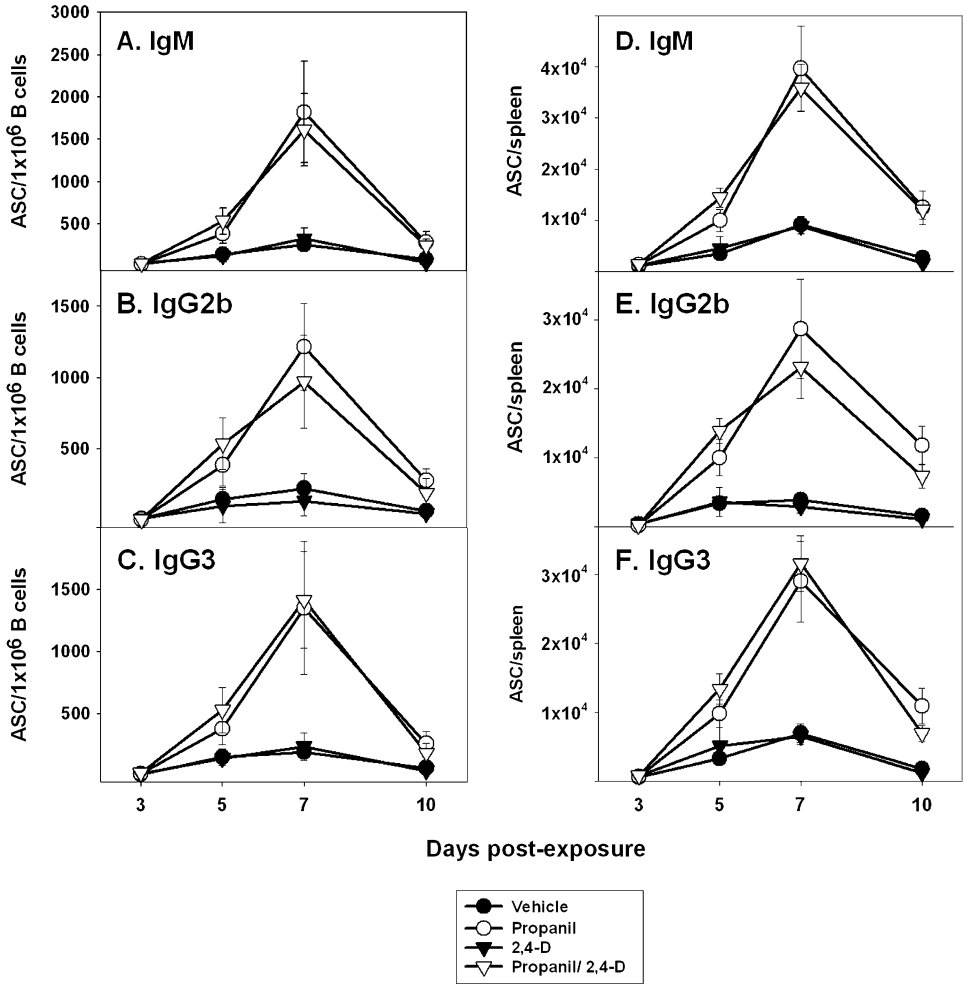

FIG. 1. Propanil and mixture exposure increases the number of PC-specific ASC in the spleen. C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture. Spleens were removed at days 3, 5, 7, and 10. Numbers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

number of ASC per whole organ (Fig. 1D, 1E, 1F) and for the number of ASC normalized to one million B cells (Fig. 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 with the mixture at day 7 post-exposure (Fig. 1A–1F). By day 10, the number of ASC was declining in all groups, but it was still significantly increased 3.5-fold in propanil and mixture-treated animals over control animals (Fig. 1A–1F). 2,4-D exposure alone did not alter the splenic ASC response in relation to the control at any of the days measured (Fig. 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 (Fig. 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

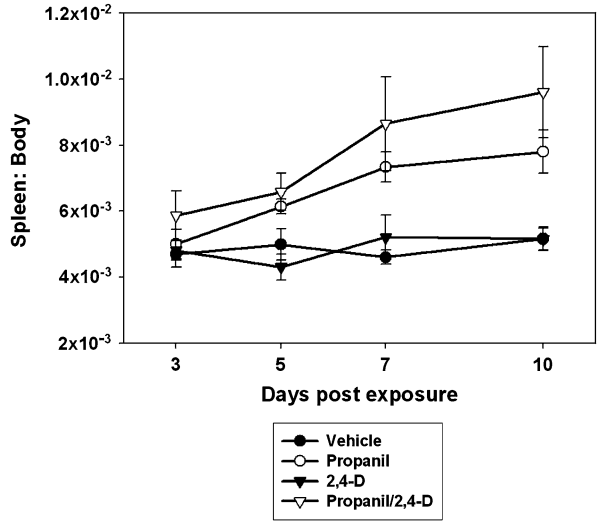

FIG. 2. Both propanil and mixture exposure increase spleen weight. C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture. Spleens were removed at days 3, 5, 7, and 10 and the wet weights determined. 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, $p \leq .05$.

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 (Fig. 3A–3F). A 25-mg/kg dose of propanil failed to increase the number of splenic ASC over control animals (Fig. 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 were 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 (Fig. 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 (Fig. 5A–5D). 2,4-D and mixture herbicide exposure decreased the number of PC-specific IgM and IgG ASC in the bone marrow approximately twofold by day 5 compared to the vehicle controls (Fig. 5A–5D). There was a significant decrease at days 7 and 10 (2–3-fold) in ASC in the bone marrow (Fig. 5A–5D). Propanil-treated animals had responses comparable to the vehicle controls at all time points (Fig. 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 (Fig. 6A, 6B, and 6C). These results demonstrate that the increased splenic ASC in propanil-treated mice do not

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 ($\times 10^6$) ± SD.
[c]Individual cell populations were determined by flow cytometric analysis as described in *Materials and Methods*.
[d]Data represents the total number of cells ($\times 10^6$) ± SD.
[e]$p \leq 0.05$ versus vehicle control.

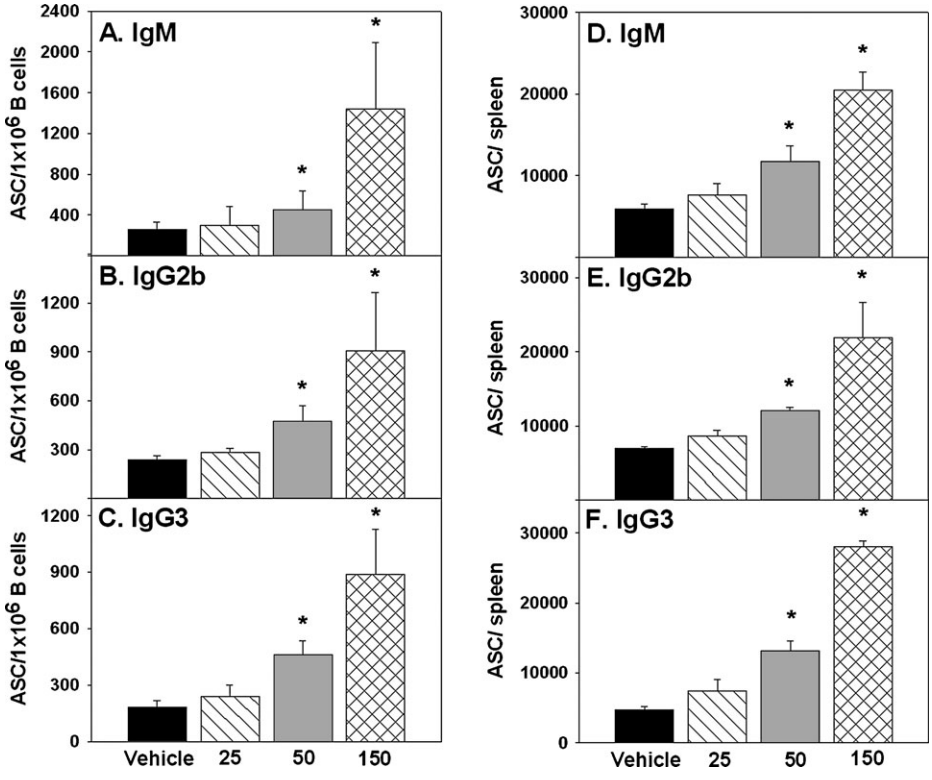

FIG. 3. Propanil increases the number of splenic ASC in a dose-dependent manner. C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control (0), 25 mg/kg, 50 mg/kg, and 150 mg/kg propanil. Spleens were removed at day 7. Numbers of PC-specific IgM (A, D), IgG2b (B, E), and IgG3 (C, F) ASC were determined by ELISPOT assay. All values represent the mean ± SD of ASC per 1×10^6 B cells (A, B, and C) or per spleen (D, E, and F). The asterisk (*) represents a significant difference ($p \leq .05$) from vehicle control.

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 (Fig. 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

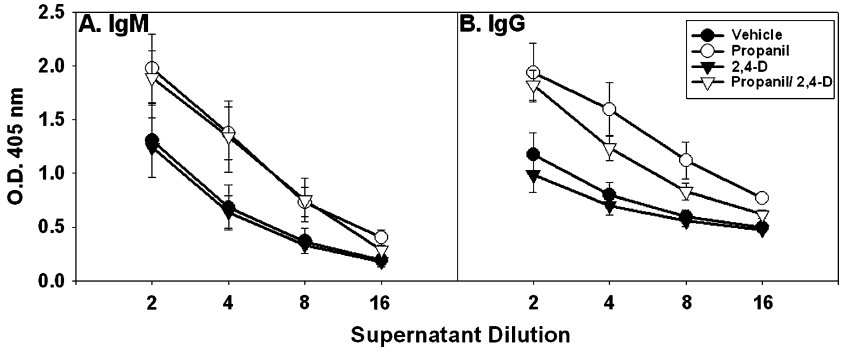

FIG. 4. Propanil and mixture exposure increases *ex vivo* antibody production by splenocytes after HKSP vaccination. C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture. Spleens were harvested 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 ± SD. Propanil and mixture treatments are significantly different from vehicle control at dilutions 1:2, 1:4, and 1:8 for IgM and IgG; $p \leq .05$.

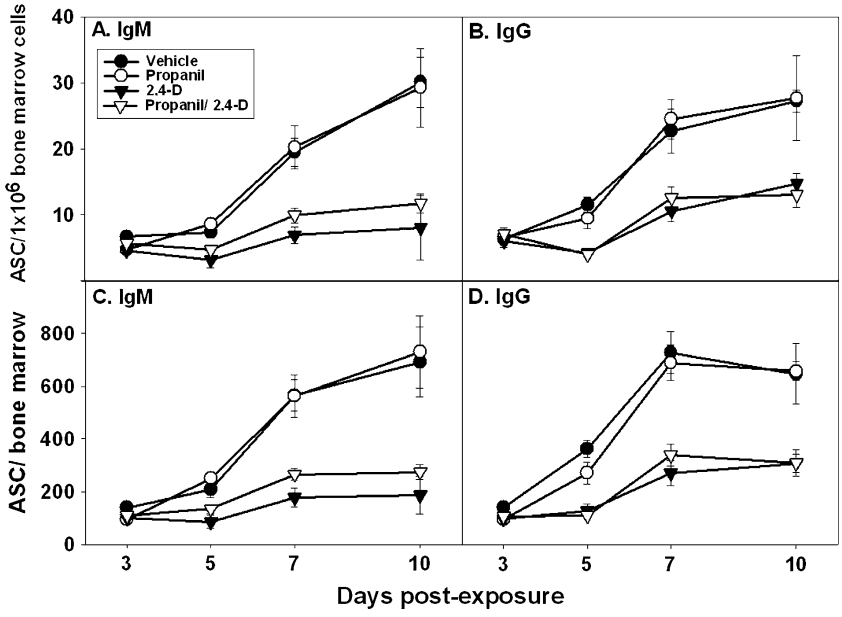

FIG. 5. 2,4-D and mixture exposure decreases the number of PC-specific bone marrow ASC. C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture. Bone marrow was harvested on days 3, 5, 7, and 10. Numbers of PC-specific IgM (A, C) and IgG (B, D) ASC were determined by ELISPOT assay. Values represent the mean ± 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; $p \leq .05$.

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 (Fig. 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 (Farenhorst and Prokopowich, 2003; Meister and Sine, 2003). Previous findings have

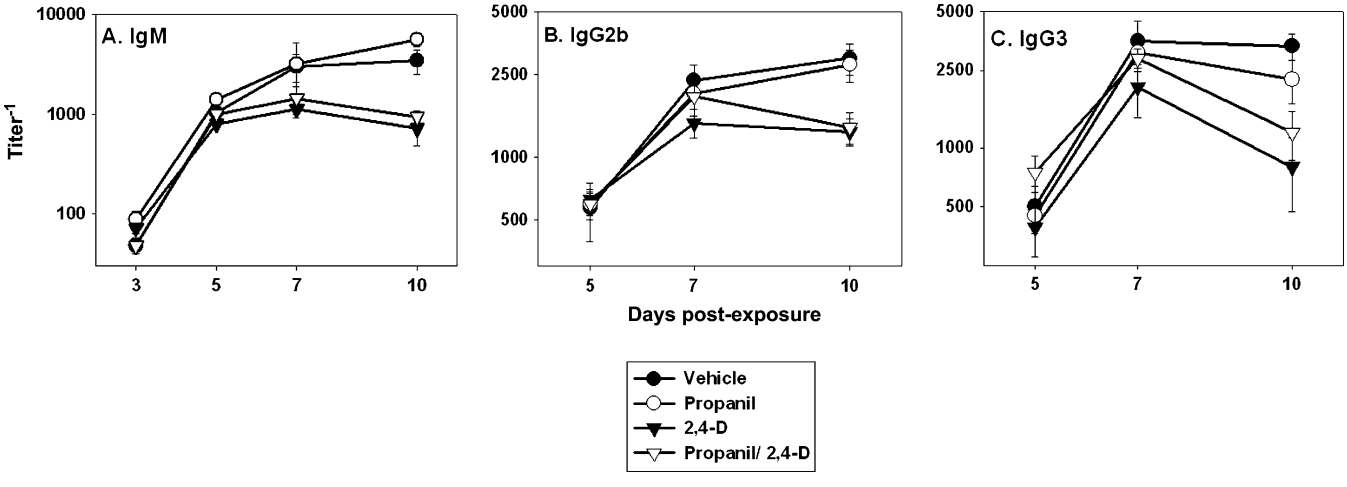

FIG. 6. 2,4-D and mixture exposure decreases PC-specific titers. C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture. Serum was collected on days 3, 5, 7, and 10. The titers of PC-specific IgM (A), IgG2b (B), and IgG3 (C) were determined by ELISA. Values represent the mean ± SEM. 2,4-D and mixture treatments are significantly different from vehicle control at day 10 for IgM and IgG; $p \leq .05$.

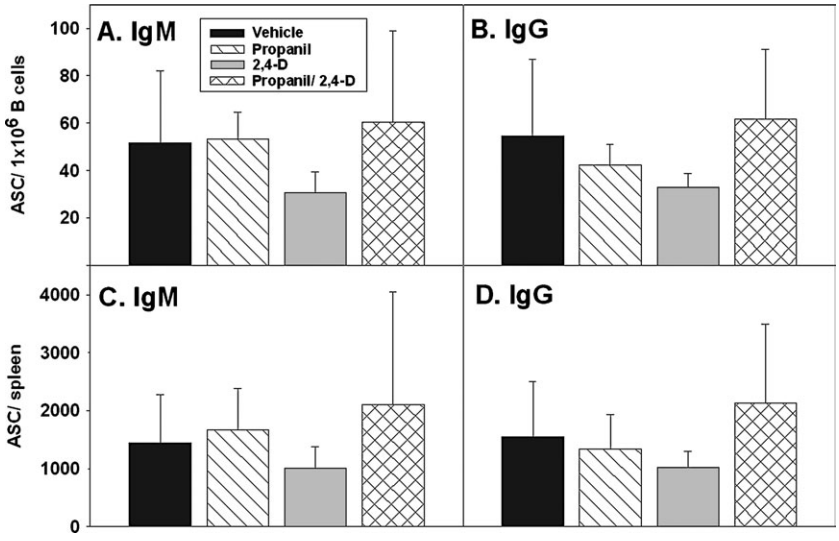

FIG. 7. Herbicide exposure has no effect on the number of splenic PspA-specific ASC. C57BL/6 mice (5/group) were vaccinated with HKSP (2×10^8 CFU/mouse) and treated with vehicle control, 150 mg/kg propanil, 150 mg/kg 2,4-D, or a 150/150 mixture. Spleens were harvested on day 14. Numbers of PspA-specific IgM (A, C) and IgG (B, D) ASC were determined by ELISPOT assay. Values represent the mean ± SD of ASC per 1×10^6 B cells (A, B) or per spleen (C, D).

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, 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 severalfold 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 antibody levels are at a saturated concentration; therefore no increase in serum titers would be detected. However, the second possibility is unlikely because 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 they demonstrate 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 knockout 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 were not affected. This further 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 exposure to propanil, 17β-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 17β-estradiol has been reported to increase ASC to bacterial and autoantigens in C57BL/6 mice (Verthelyi and Ahmed, 1998). In addition, exposure to 17β-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 (Guinamard *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 17β-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 as a result of 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 affect 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 occurred on the day of HKSP vaccination. The peak response to PC was determined 7 days post-exposure, whereas 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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