Evidence for a Novel Endocrine Disruptor: The Pesticide Propanil Requires the Ovaries and Steroid Synthesis to Enhance Humoral Immunity

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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 (ASCs) in the spleen four- to sixfold 7 days after vaccination of female C57BL/6 mice with heat-killed Streptococcus pneumoniae. 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 estrogen receptor (ER) α or 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 182,780, or the progesterone antagonist RU486 did not inhibit the increase in ASCs. These data suggest that estrogen and progesterone do not regulate the effect of propanil. However, complete inhibition of steroid synthesis with the gonadotropin and progesterone do not regulate the effect of propanil. These data suggest 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 (Scarpenti et al., 1989). E2 also inhibits T 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 ERα- and ERβ-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 (ASCs) and the serum antibody titers to bacterial antigens and autoantibodies (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 (EDCs) 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 and diethylstilbestrol (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 EDCs to enhance an autoimmune disease, the organochlorine pesticides chlordane, methoxychlor, and DDT accelerated the progression of SLE in (NZB × 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 EDCs 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 IgMþ 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 ASCs specific for PC is increased four-to sixfold 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 antiandrogenic 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 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. They were kept on a 12-h 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 libitum. These studies were conducted in accordance with all the federal and institutional guidelines for animal use and approved by the West Virginia University Institutional Animal Care and Use Committee.

**Chemicals.** Propanil (3,4-dichloropropionanilide, 99% pure) 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.** Streptococcus pneumoniae strain R36A, an avirulent, nonencapsulated strain, was grown to mid-log phase in Todd-Hewitt broth (Becton Dickinson, Sparks, MD) + 0.05% yeast extract (Becton Dickinson) and stored at −80°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 + 0.05% yeast extract. Bacteria were grown at 37°C to an absorbance reading at 650 nm of 0.4 and heat killed for 4 h in a 60°C water bath. A final concentration of 10⁶ 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 × 10⁵ 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, 2000).

**Animal exposures.** Propanil was dissolved in p.o. for a final concentration of 15 or 20 mg/ml for all experiments. For the experiments measuring antibody responses, mice (five per 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 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, OVX mice were injected sc with a daily dose of E2 (10 µg/kg). Animals received the first dose of E2 1 h prior to herbicide or vehicle treatment and vaccination. Subsequent E2 treatments were given at the same time daily. Animals were sacrificed 24 h following the final E2 exposure.

In experiments using the ER antagonist, ICI (10 mg/kg/day) was injected sc into female mice. Animals were treated with the first dose of ICI 1 h 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 per mouse), female mice were injected sc every 48 h 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 h 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 three daily sc injections of E2 (10 µg/kg/day). Mice were euthanized 24 h 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 h prior to propanil treatment, 12 h following propanil treatment, followed by injections every 24 h 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 anti-14 days using the Estradiol ELISA 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 μQuant 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 μl 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, Logan, UT), 10mM HEPES (Sigma), 1mM L-glutamine (GIBCO, Rockville, MD), 5 × 10⁻⁵M 2-mercaptoethanol (Sigma), 100 U/ml penicillin (GIBCO), and 100 μg/ml streptomycin (GIBCO). RBCs 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 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 + 0.01% Tween 20. Plates were blocked with 200 μl per well complete medium + 25% FBS for 2 h at 37°C. Plates were washed, and cells (100 μl per well) were then added at a concentration of 5 × 10⁶ or 1 × 10⁶ cells/ml. All samples were plated in triplicate. Plates were incubated for 4–6 h 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 + 0.05% Tween 20, were added to the appropriate wells (100 μl per 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 per well. Color development was stopped by washing with distilled water. The number of spots per well was counted using a dissection microscope (Olympus Optical Co., Melville, NY). The number of ASCs was calculated by using the mean number of spots from triplicate wells. The number of ASCs was normalized to 1 × 10⁶ 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-allophycocyanin (RA3-6B2), rat anti-mouse CD4-fluorescein isothiocyanate (GL1.5), or rat anti-mouse CD8α-phycocerythrin (53-67) (BD PharMingen, San Diego, CA). All steps were performed in PBS supplemented with 1% FBS and 0.04% sodium azide (Sigma). Briefly, 1 × 10⁶ 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 0.4% 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 ERα and ERβ (Panvera, Madison, WI). Reactions were performed in 10mM Tris, 1.5mM EDTA, 1.0mM 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 18–20 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 AP activity.** Ishikawa cells (a generous gift from Richard B. Hochberg, Yale University Medical School, New Haven, CT) were maintained in minimum essential medium (Sigma), 10% FBS, as described (Littlefield et al., 1990). Ishikawa cells were treated as described to assess the effect of propanil on estrogen-inducible AP (Littlefield et al., 1990). Briefly, cultures were rinsed twice with estrogen-free basal medium and maintained in it for 24 h. Cells were then trypsinized and plated into 96-well plates (1 × 10⁵ cells per 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 h, cultures were treated as described in detail, and AP 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 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 ≤ 0.05 was used for all tests. 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**

**Ovariectomy 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, O VX 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 ASCs compared to sham-operated, vehicle-treated mice (Fig. 1). PC-specific IgM and the dominant PC isotypes IgG2b and IgG3 were significantly increased in propanil-treated animals (Fig. 1). Spleen cells from these mice were also cultured in vitro for 5 days, and the production of antibody was measured by specific enzyme-linked immunosorbent assay. There was an increase in antibody production that corresponded with the increase in ASCs (data not shown). Ovariectomy abrogated the ability of propanil to increase the number of PC-specific ASCs in the spleen (Fig. 1). Comparable results were obtained when the data were normalized to 1 × 10⁶ splenic B cells (Fig. 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 were 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 ERs In Vivo or In Vitro

To determine if propanil increased the PC antibody response by binding directly or indirectly with ERα, 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 (Fig. 2). Propanil, however, did not increase the uterine weights compared to vehicle-treated animals (Fig. 2). These data suggest that propanil does not directly or indirectly activate ERα, which is primarily responsible for the E2-induced uterine proliferation (Weihua et al., 2000).

To investigate if propanil interacts directly with ERα or ERβ, an in vitro, competitive ER-binding assay was performed. Propanil did not bind either ERα or ERβ over a wide range of concentrations (Fig. 3), confirming a previous report (Kojima et al., 2004). In addition, propanil was administered to Ishikawa cells which express both ERα and ERβ. Propanil did not increase estrogen-inducible AP activity compared to controls, further supporting that propanil does not bind ERα or ERβ (Fig. 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 the propanil-treated and vehicle-treated OVX mice received sc E2 daily 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 ASCs were measured on day 7. Uterine weights of OVX mice were significantly lower than those of sham-operated vehicle mice (Fig. 5A). Exogenous E2 administration restored uterine weights of OVX mice comparable to uterine weights of sham-operated mice (Fig. 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 ASCs (Fig. 5B, OVX propanil + E2 vs. propanil). All OVX mice had a similar number of PC-specific ASCs as vehicle control animals (Fig. 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 ER is required for the increased immune response, mice were treated with the ER\(\alpha\) and ER\(\beta\) antagonist ICI. Normal female mice were vaccinated and treated with propanil or vehicle on day 0 and given daily treatments with ICI. 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 those of control animals (Fig. 6A). Treatment with the ER antagonist did not affect the basal PC-specific ASC response in vehicle-treated mice (vehicle vs. vehicle + ICI, Fig. 6B). Propanil-exposed mice treated with ICI had an increased ASC response comparable to propanil alone (propanil vs. propanil + ICI, Fig. 6B). These results demonstrate that the ER antagonist did not inhibit the increase in PC-specific ASCs in propanil-treated animals, which suggests that signaling through the ER pathway is not required for propanil to enhance the antibody response.

**Inhibition of Steroid Synthesis in Propanil-Treated Mice Abrogates the Increase in ASCs**

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 undetectable 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 (Fig. 7A). Treatment with antide did not affect the basal PC-specific ASC response (vehicle vs. vehicle antide, Fig. 7B). The number of PC-specific ASCs in mice treated with antide and exposed to propanil were not significantly different from the vehicle controls (vehicle vs. propanil antide, Fig. 7B) but significantly different from the propanil-treated control animals that had an increased ASC response (propanil vs. propanil antide, Fig. 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 h prior to propanil or vehicle treatment and at 12 and 24 h postexposure. RU486 partially abrogated thymic atrophy as determined by measurement of the thymic weights and by quantification of the CD4+CD8+ thymocyte population (Figs. 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 h prior to propanil exposure and vaccination. Mice were subsequently injected with RU486 12 h post-exposure and every 24 h 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, Fig. 8C). Propanil-exposed mice treated with RU486 had an increased ASC response comparable to propanil alone (propanil vs. propanil + RU486, Fig. 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 (Fig. 9). The PC-specific splenic ASCs were measured after 7 days. Propanil increased

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**FIG. 5.** Addition of exogenous E2 does not restore the increase in PC-specific ASCs of propanil-treated animals. OVX and sham-operated female C57BL/6 mice (five per group) were treated with 150 mg/kg propanil or vehicle control and vaccinated with 2 x 10^8 CFU HKSP. OVX mice were treated with 10 µg/kg/day of E2 or the vehicle. Spleens and uteri were removed after 7 days. (A) Uterine weight. *p < 0.05. (B) Numbers of PC-specific IgG2b, IgG3, and IgM ASCs. Values represent the mean ± SD of ASCs/1 x 10^6 B cells. *p < 0.05.
the IgG2b, IgG3, and IgM PC-specific splenic ASCs approximately threefold (vehicle vs. propanil, Fig. 9). Castration did not significantly affect the propanil-induced increase in PC-specific ASCs. CAS males treated with propanil produced approximately the same fold increase in PC-specific ASCs as normal propanil-treated male mice (propanil vs. propanil CAS, Fig. 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 ASCs 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, 2005; Salazar et al., 2005). Propanil has been classified by two studies as nonestrogenic and weakly antiandrogenic, 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 (Borgeest 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 ERβ-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 ERα- and ERβ-binding activity of propanil were determined using in vitro assays. Propanil did not compete for E2 binding to either ERα or ERβ nor did it increase estrogen-inducible AP activity in ER-responsive cells. The results of the uterotrophic assay, ER-binding studies, and the ER-inducible AP 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, sc 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.

FIG. 7. Antide treatment abrogates the propanil-induced increase in PC-specific ASCs. C57BL/6 female mice were treated with antide (60 μg per mouse) or vehicle for 14 days as described in the “Materials and Methods” section. Mice were vaccinated on day 14 with $2 \times 10^8$ 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, $p \leq 0.05$. (B) Numbers of PC-specific IgG2b, IgG3, and IgM ASCs. Values represent the mean ± SD of ASCs/1 x 10^6 B cells. *, Significantly different from vehicle control, $p \leq 0.05$. 

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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 show 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 ASCs (Myers and Petersen, 1985; Verthelyi and Ahmed, 1998). However, high levels of E2, such as during the third trimester of pregnancy, are 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 has been demonstrated to completely attenuate the ability of ERα and ERβ 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 1 h 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 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.

FIG. 8. Treatment with the progesterone antagonist RU486 does not inhibit the increase in ASCs in propanil-treated animals. C57BL/6 female mice were treated with RU486 as described in the “Materials and Methods” section. 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 ≤ 0.05. (B) CD4⁺CD8⁺ cells. *, Significantly different from the vehicle control and vehicle + RU486, p ≤ 0.05. #, Significantly different from vehicle, propanil, and vehicle + RU486, p ≤ 0.05. (C) Numbers of PC-specific IgG2b, IgG3, and IgM ASCs were determined by ELISPOT assay on day 7 post-vaccination. Values represent the mean ± SD of ASCs/1 × 10⁶ B cells. *, Significantly different from the respective vehicle control, p ≤ 0.05.
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 luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary and eliminates the secretion of gonadal hormones (Couse et al., 2003; Fallest 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 OVX 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 ASCs (Couse et al., 2003; Wakeling et al., 1991).

Other nonestrogenic 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 antiandrogenic activity. However, antibody studies evaluating the effects of antiandrogens 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, these data do 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 ASCs 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 OVX mice and the proliferation of...
B and T lymphocytes in response to lipopolysaccharide and concanavalin A 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 nonpituitary 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 CAS and OVX animals. There are data which indicate that male and female discrepancies in immune function exist in response to GnRH treatment following gonadectomy (Jacobson et al., 1999). Both GnRH mRNA and GnRH receptor mRNA are altered in splenocytes 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 17β-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 and Gandy, 1989; Barnett et al., 1992). In the previous studies, C57BL/6 female mice were exposed ip to propanil on day 0 and immunized iv on day 3 with the TI-2 antigen dinitrophenyl-Ficoll or the T-dependent antigen sheep red blood cells, and the plaque-forming cell response in the spleen was determined on day 7. In the present studies, propanil was administered ip on day 0, and the mice were immunized ip 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 are 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, pneumococcal surface protein A (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 EDCs reported to date. Second, the data suggest 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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REFERENCES


beta-estradiol and dexamethasone on subsets expressing T cell antigen receptor or IL-2 receptor. J. Immunol. 142, 3378–3383.


