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CHARACTERIZATION OF THYMIC ATROPHY AND THE MECHANISM OF THYMOCYTE DEPLETION AFTER IN VIVO EXPOSURE TO A MIXTURE OF HERBICIDES

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3,4-Dichloropropionanilide (propanil) and 2,4-dichlorophenoxyacetic acid (2,4-D) are two commonly used herbicides that are marketed as a chemical mixture. It was hypothesized that the interaction between these two herbicides, when administered as a mixture, would result in a greater effect on the immune system than the individual components of the mixture. The present study demonstrates in a murine model that a mixture of propanil and 2,4-D, when compared to single herbicide exposures, exacerbates decreases in thymocyte populations 2 d postexposure and inhibits the repopulation of T-cells in the thymus 7 d postexposure. Exposure to 150 mg herbicide/kg body weight of propanil or 2,4-D alone had no effect on thymus weight. In contrast, decreases in the ratio of thymus weight to body weight (TW:BW) occurred 2 d after treatment with the mixture of 150 mg propanil/kg body weight + 150 mg 2,4-D/kg body weight (150/150). Thymic atrophy was associated with a decrease in the double-positive thymocyte population ($CD4^+CD8^+$) and correlated with sera corticosterone levels from 600 to 1000 pg/ml. Therefore, the hypothesis was tested that glucocorticoids, induced after exposure to herbicides, were responsible for the thymic atrophy and depletion of thymocytes. However, similar levels of corticosterone were induced after exposure to 50, 100, or 150 mg propanil/kg body weight, and 50/50 or 100/100 mixture treatments, doses that did not produce thymic atrophy or cell loss. In addition, RU 486, a glucocorticoid receptor blocker, only partially abrogated the thymic atrophy in mice exposed to the 150/150 mixture of herbicides. These results suggest that glucocorticoids are only partially responsible for herbicide-induced thymic atrophy. This study demonstrates that the effects of exposure to a mixture of chemicals cannot always be predicted based on single exposure data and emphasizes the importance of mixture-based studies.

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The environment that we live in is a complex mixture of chemicals, both natural and man-made. Most toxicology research, however, is focused on single-chemical exposures and does not account for mixture interactions. An in vivo exposure model was developed to assess the effects on the immune system of a mixture of two herbicides, propanil and 2,4-D. Propanil and 2,4-D are heavily used by the agricultural industry and are commonly applied as a mixture under the product names of NOX-D and Herbanil 368 *(Farm Chemicals Handbook,1998).* One of the major goals of this research was to determine if interactions between the two herbicides in a mixture result in a greater effect on the immune system than the individual components of the mixture.

Propanil is an amide herbicide that induces thymic atrophy and splenomegaly (Barnett & Gandy, 1989). Exposure to propanil has been shown to produce several immunotoxic effects, including reductions in T-dependent antibody responses, contact hypersensitivity responses, and mixed lymphocyte responses (Barnett & Gandy, 1989). Production of several cytokines by mitogen-stimulated peritoneal macrophages and splenocytes is decreased after in vitro or in vivo exposure to propanil (Xie et al., 1997; Zhao et al., 1998). Propanil has also been demonstrated to be toxic to developing immune-cell populations, suppressing myeloid stem-cell colony formation in the bone marrow, and selectively decreasing the number of thymocytes in the thymus (Blyler et al., 1994; Zhao et al., 1995). The thymic atrophy was correlated with a decrease in the $CD4^{+}CD8^{+}$ population (Zhao et al., 1995). Research into the mechanisms induced by propanil toxicity has determined that the thymic atrophy is attributed, in part, to increased sera corticosterone levels (Cuff et al., 1996). The cellular loss was partially alleviated by adrenalectomy (Cuff et al., 1996).

2,4-D, a chlorinated phenoxy herbicide, is reported to be the most widely used herbicide globally (http://www.24d.org). The wide range of applications results in exposure to both rural and metropolitan populations (Munro et al., 1992; reviewed in World Health Organization, 1989). For example, a 1995 study of urinary metabolites of U.S. adults found 12% contained 2,4-D, while in another study, 20% of Arkansas children had detectable concentrations of 2,4-D in their urine (Hill et al., 1989, 1995). In 1992, a comprehensive, integrated review of epidemiological and toxicological data on 2,4-D concluded that the herbicide is of low toxicity and poses a negligible risk to the public (Munro et al., 1992). However, several studies demonstrated that 2,4-D has effects on the immune system, including decreased peripheral blood T-cell and natural killer (NK) cell numbers, and decreased T-cell proliferative response in humans (Faustini et al., 1996). In murine models, 2,4-D was found to differentially affect the antibody response depending on the route of exposure (Blakley, 1986; Blakley & Schiefer, 1986). In a mixture exposure model, subchronic oral exposure to Tordon 202C, a mixture of the herbicides picloram and 2,4-D, produced a dose-dependent decrease in the T-dependent primary humoral immune response (Blakley, 1997). That study, however, did not look at the immunotoxicity of the individual components of the Tordon 202C mixture. The addition of 2,4-D to a mixture of parathion and toxaphene increased liver

enzymes involved in the metabolism of the other two pesticides even though 2,4-D alone did not affect liver enzyme levels (Chaturvedi et al., 1991). Thus, these reports suggest that 2,4-D may be immunotoxic and that the addition of 2,4-D to a mixture of chemicals may alter the response to the mixture.

The present study determined the effects on the thymus of mice after in vivo exposure to the chemical mixture of propanil and 2,4-D. Our results demonstrate that the mixture of propanil and 2,4-D induced greater thymic atrophy and loss of thymocytes than the individual herbicides. The effects on the thymus correlate with increases in serum corticosterone levels. However, corticosterone alone was not responsible for the effects on the thymus as corticosterone levels were also increased at herbicide doses that did not induce thymic atrophy. This study demonstrates that the effects after in vivo exposure to a mixture of propanil and 2,4-D cannot always be predicted by the addition of the effects of the individual herbicides contained in the mixture. These results emphasize the importance of studying chemical mixtures.

MATERIALS AND METHODS

Animals

C57Bl/6 female mice, 6–8 wk of age, 18–20 g, were purchased from Charles River Farms (Wilmington, DE). Animals were housed in the AAALAC-accredited vivarium at the West Virginia University Health Sciences Center under the care of a full-time veterinarian and a professional staff. Mice were housed in groups of 4–5 mice/micro-isolator cages and allowed to acclimate for 1 wk after shipment and received food (Harlan brand 8604, regular, non-autoclavable) and tap water ad libitum.

Source of Xenobiotics

Propanil was purchased from Chem Service (West Chester, PA) and has a purity of 99%. 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). The concentration of 2,4-D used for all experiments was calculated based on the amount of active 2,4-D. The inert ingredients in the commercial formulation are proprietary information.

Exposure of Mice to Herbicide Mixtures

Mice were treated with either a single herbicide (propanil or 2,4-D) or a 1:1 mixture of both herbicides. Propanil was dissolved in peanut oil (Planters; Nabisco, Inc.) and 2,4-D amine was dissolved in phosphate-buffered saline (PBS) to the appropriate concentration. Mice were weighed prior to intraperitoneal (ip) herbicide injection. Single herbicide doses contained 50, 100, 150, or 200 mg herbicide/kg body weight of either propanil (P50, P100, P150, P200) or 2,4-D (D50, D100, D150, D200). Mixture treatments were given by

TABLE 1. Herbicide Treatment

Single-herbicide treatment[a]		
Propanil (mg/kg)	2,4-D (mg/kg)	Mixture treatment,[b] propanil (mg/kg)/ 2,4-D (mg/kg)
Peanut oil alone (0)	PBS alone (0)	Peanut oil/PBS (0/0)
50	50	50/50
100	100	100/100
150	150	150/150
200	200	200/200

[a] Mice received either the vehicle, propanil, or 2,4-D, ip, at the indicated dose of herbicide (mg) to body weight (kg).

[b] Mice received either the vehicle or propanil and 2,4-D, ip, at the indicated dose of herbicide (mg) to body weight (kg). Propanil and 2,4-D were given in two consecutive injections.

two consecutive ip injections, in a 1:1 ratio of propanil and 2,4-D. Treatment doses were administered as outlined in Table 1. The doses were chosen based on previous research demonstrating the doses of propanil that produced thymic atrophy (Cuff et al., 1996; Zhao et al., 1995) and preliminary studies in our laboratory that determined the dose of 2,4-D that induced thymic atrophy (data not shown).

Cell Isolation

Thymuses were weighed and single-cell suspensions were prepared by teasing the thymuses apart and passing them sequentially through 18- and 26-gauge needles attached to 3-ml syringes. Red blood cells were lysed with Tris-buffered ammonium chloride, pH 7.2. Single-cell suspensions were washed and resuspended in PBS supplemented with 1% fetal bovine serum (FBS, Hyclone, Logan, UT) and 0.04% sodium azide (Sigma, St. Louis, MO) (staining media). Thymus to body weight was calculated as the percent of the individual thymus weight to the total body weight determined on d 2 or 7 post-exposure.

Flow Cytometric Analysis of Thymocyte Populations

Isolated cells were stained with anti-CD4-FITC (GK1.5) and anti-CD8α-PE (53-6.7) (BD Pharmingen, San Diego, CA). Thymocytes were separated into the 4 major developmental populations, which progress from double-negative thymocytes ($CD4^-CD8^-$), to double-positive thymocytes ($CD4^+CD8^+$), and finally to the single-positive ($CD4^+CD8^-$ and $CD4^-CD8^+$) T-cell populations. Briefly, 1×10^6 cells were stained in a total volume of 25 μl of staining media with antibodies at the appropriate concentration, 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 media. For analysis, 10,000 cells were analyzed on a Becton-Dickinson FACScan (Becton Dickinson Immunocytometry Systems, Mansfield,

MA). Analysis was performed using WinMDI software (Joseph Trotter, Scripps Institute, San Diego, CA). Representative dot plots from a vehicle-treated animal and an herbicide-treated animal are shown in Figure 2A. Population percentages, obtained from the flow cytometric analysis, were used to calculate the absolute cell numbers of a thymocyte population by multiplying the ratio of cells in a population by the total number of cells harvested per organ.

Serum Corticosterone Levels

Mice used in serum corticosterone experiments were housed two per cage. Mice were injected ip with propanil, 2,4-D or a mixture of the 2 herbicides between 9 a.m. and 10 a.m. Serum was collected at 1, 4, 12, 24, or 48 h after injection using 4 mice in each treatment group per time point. All efforts were made to reduce stress on these animals by minimizing noise and movement. Blood was collected from the retro orbital plexus within 30 s of handling. Serum corticosterone levels were measured using a ^{125}I radioimmunoassay kit for rats and mice from ICN Biomedicals, Inc. (Costa Mesa, CA).

RU 486 Treatment

Mice were given 100 mg RU 486/kg body weight by gavage (RU 486, Sigma-Aldrich) 2 h prior to, and 12 h after, ip administration of 150 mg propanil + 150 mg 2,4-D herbicide/kg mixture treatment or 75 mg corticosterone/kg body weight given by gavage (Sigma-Aldrich). RU 486 has a reported initial half-life of approximately 2 h in the serum with high concentrations in adipose tissue in the rat (Heikinheimo et al., 1994). Thymuses were harvested, weighed, and single-cell suspensions of thymocytes prepared. Thymocytes were stained at 48 h after corticosterone or herbicide exposure. The 75-mg/kg oral dose of corticosterone was chosen since it has been shown to functionally mimic the action of endogenous glucocorticoid increases (Burns et al., 1994). The second dose of RU 486 was administered 12 h after exposure to the herbicide mixture or corticosterone to ensure that all receptors remained blocked for the remainder of the time course (Burns et al.,1994; Flint et al., 2000).

Statistics

Comparison of the experimental groups to vehicle control: One-Way analysis of variance (ANOVA) was done on thymocyte population percentages and total cell numbers. Post hoc determination of significant differences in the response of treated animals to vehicle control was done using a Dunnett's *t*- test. An alpha value $< .05$ was used to establish statistical significance.

A Tukey HSD analysis was used to compare the changes in thymus weight to body weight ratio (TW:BW) of the 100/100 mixture to 200 mg/kg single herbicide treatment and $CD4^+CD8^+$ total cell number response of the 50/50 mixture to those of the 100-mg/kg single-herbicide-treated groups with a significance level of $p \leq .05$.

Partial Factorial Analysis Interactions between the individual herbicide components of a mixture were determined by ANOVA analysis using a partial factorial design with dosages defined as nominal factors. The factorial design is specific to the statistical analysis of the combined effects of two types of treatments (Schoen, 1996). For our experiments, a partial two-factor design was used. The null hypothesis for this model specifies additivity in that the response to the mixture is equal to the total response of the mixture components. A mixture interaction defines a response that is above or below the line of additivity.

A multiple regression analysis was used to correlate total cell numbers across all treatments at 2 and 7 d posttreatment with increases in glucocorticoid hormones at all time points. Statistical analysis was performed using JMP software (SAS Institute, Inc., Cary, NC). Experiments were repeated three to five times with three to four animals per group. Data are expressed as means $\pm$ the standard deviation.

RESULTS

Exposure to Herbicides Causes Thymic Atrophy

Herbicide treatment did not affect the body weight of the mice at any dose (data not shown). Thymic atrophy was measured as the percentage of thymus weight to body weight (TW:BW). Thymus to body weight ratios were not affected by single herbicide treatment of P50 to P150 or D50 to D150 (Figure 1). Mixtures of either 50/50 or 100/100 did not affect TW:BW ratios (Figure 1). Treatment with the 150/150 mixture, however, produced a statistically significant decrease in the % of TW:BW as compared to vehicle control (Figure 1). In addition, TW:BW ratios of P200 and D200 single herbicide, and 200/200 mixture treated mice, were also significantly decreased from vehicle control (Figure 1). For clarity, only the PBS + peanut oil vehicle control is shown. All vehicle controls were similar and did not have any effects on the thymus compared to untreated animals (data not shown). Significant thymic atrophy remained for at least 7 d posttreatment in the 150/150 and 200/200 mixture group (data not shown). Single herbicide treatment of P200 and D200 also resulted in significant thymic atrophy by 7 d posttreatment (data not shown).

An analysis was done to compare the TW:BW of P200 and D200 with mixtures containing the same total herbicide concentration (100/100). Based on the molecular weight of the compounds, these are similar molar equivalent doses, 0.919 m*M*/kg (0.458 m*M*/kg propanil and 0.452 m*M*/kg 2,4-D) for the 100/100 mixture dose versus 0.916 m*M*/kg for P200 and 0.904 m*M*/kg for D200. TW:BW ratios after single herbicide treatments of P200 and D200 were significantly decreased as compared to the 100/100 mixture (Figure1). Furthermore, the 100/100 mixture response was similar to the responses by the single 100 treatments and the vehicle control. These results demonstrate that the

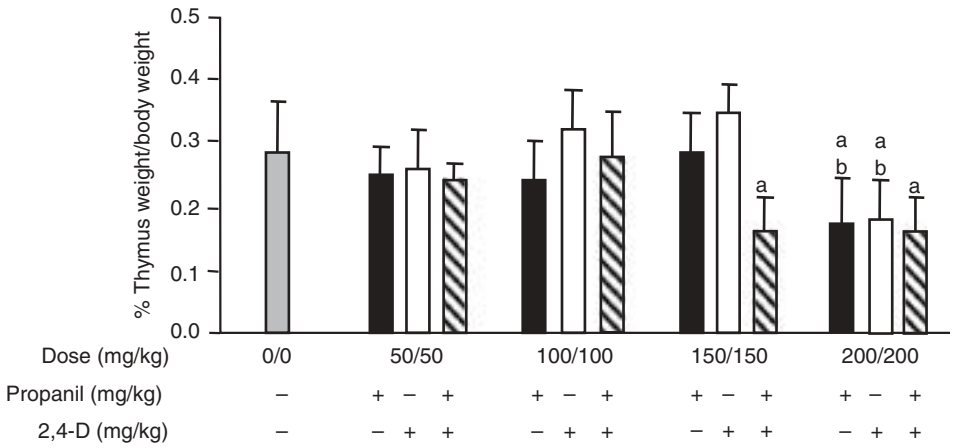

FIGURE 1. Thymic atrophy after exposure to herbicides. Mice were treated with vehicle (gray bars), propanil (black bars), 2,4-D (open bars), or propanil and 2,4-D (striped bars) and thymuses were removed at 2 d posttreatment as described in Materials and Methods. Thymus to body weight ratio is expressed as a percentage. The letters above the bars represent significant differences from the vehicle control (a) and the 100/100 group (b) ($p < .05$).

effects are not simply due to the total herbicide concentration at this dose but specific to the individual herbicides.

Exposure to Propanil and 2,4-D Decreases the $CD4^+CD8^+$ Population in the Thymus

The effects of herbicide treatment on the thymocyte populations in the thymus were assessed by flow cytometric analysis at 2 and 7 d postexposure. Representative dot plots from a vehicle-treated animal and an herbicide-treated animal are shown in Figure 2A. The P100, P150, P200, and D200 single-herbicide treatments significantly decreased the absolute cell number of the $CD4^+CD8^+$ thymocyte population relative to the vehicle control at 2 d posttreatment (Figure 2B). Mixture treatments of 100/100, 150/150, and 200/200 also significantly decreased $CD4^+CD8^+$ absolute cell number (Figure 2B). In addition, there was a statistically significant interaction that resulted in greater than additive decreases in the $CD4^+CD8^+$ population after exposure to the 150/150 mixture (Figure 2B, denoted by ψ). In contrast, the factorial analysis indicated that there was a less than additive mixture interaction after exposure to the 200/200 mixture treatment (Figure 2B, denoted by ψ).

To determine if the effects of the mixture on the $CD4^+CD8^+$ population were the result of exposure to the total herbicide concentration of the mixture, rather than due to effects that are specific to the individual herbicides, the responses were compared 2 d after treatment to either P100 or D100 to a mixture containing an equivalent concentration of herbicide (50/50). Based on the molecular weight of the compounds, these are similar molar equivalent

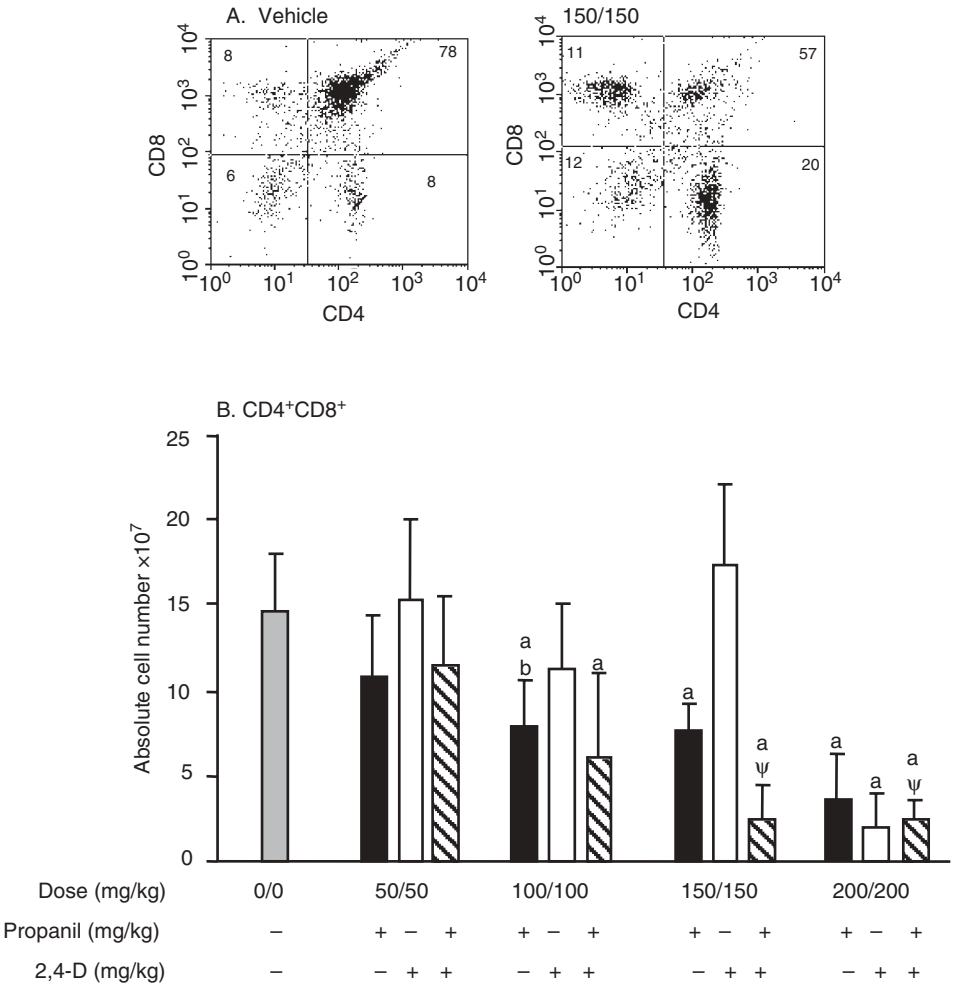

FIGURE 2. Loss of the $CD4^+CD8^+$ thymocyte population after exposure to herbicides. Mice were treated with vehicle or herbicides and thymuses were removed 2 d posttreatment. Thymocytes were stained with anti-CD4 FITC and anti-CD8 PE and total cell numbers calculated as described in Materials and Methods. (A) Representative dot plots from a vehicle-treated mouse, Vehicle, or a propanil and 2,4-D-treated mouse, 150/150. Numbers in each quadrant represent the percent of positive cells. (B) Absolute cell numbers of $CD4^+CD8^+$ thymocytes from mice treated with vehicle (gray bars), propanil (black bars), 2,4-D (open bars), or propanil and 2,4-D (striped bars). Bars represent the mean ± the standard deviation. The letters above the bars represent significant differences from the vehicle control (a) and the 50/50 group (b) ($p < .05$); ψ represents a mixture interaction ($p \leq .05$).

doses, 0.455 m*M*/kg (0.229 m*M*/kg propanil and 0.226 m*M*/kg 2,4-D) for the 50/50 mixture dose versus 0.458 m*M*/kg for P100 and 0.452 m*M*/kg for D100. The 50/50 mixture response was similar to vehicle treatment and to the individual P50 and D50 single herbicide treatments but was significantly different

from the single P100 treatment (Figure 2B). This indicates that the decrease in the $CD4^+CD8^+$ population by P100 was not due simply to the total dose of 100 mg/kg herbicide but specific to propanil. In contrast, the 100/100 mixture response was significantly different from the response to the vehicle and statistically similar to the P200 or D200 response, suggesting that the mixture and the single 200-mg/kg herbicide treatments are equally toxic to the $CD4^+CD8^+$ population at this concentration.

The $CD4^-CD8^+$ absolute cell number was decreased as compared to vehicle control for mixture groups exposed to 100/100, 150/150, and 200/200 (Figure 3). Single-herbicide treatments of P200 or D200 were also significantly decreased compared to the vehicle control (Figure 3). In the 150/150 mixture-treated group, there was a mixture interaction indicating a greater than additive response to the mixture (Figure 3, denoted by ψ). In contrast, 200/200 treatment resulted in a mixture interaction that indicates a less than additive response (Figure 3, denoted by ψ). $CD4^-CD8^+$ absolute cell number after treatment with the 50/50 mixture was similar to vehicle and P50 or D50 single herbicide treatments, but was significantly different from the P100 and D100 single-herbicide treatments (Figure 3). The $CD4^+CD8^-$ and $CD4^-CD8^-$ populations were not significantly affected by single-herbicide and mixture treatment as compared to vehicle control at any of the doses tested (data not shown).

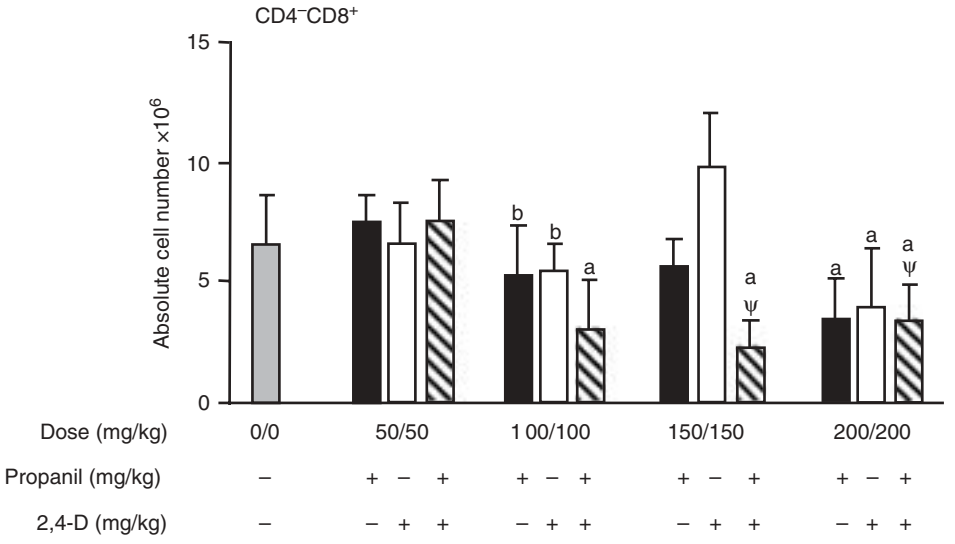

FIGURE 3. Loss of the $CD4^-CD8^+$ thymocyte population after exposure to herbicides. Mice were treated with vehicle (gray bars), propanil (black bars), 2,4-D (open bars), or propanil and 2,4-D (striped bars) and thymuses were removed at 2 d posttreatment. Thymocytes were stained with anti-CD4 FITC and anti-CD8 PE and total cell numbers calculated as described in Materials and Methods. Bars represent the mean ± the standard deviation. The letters above the bars represent significant differences from the vehicle control (a) and the 50/50 group (b) ($p < .05$); ψ represents a mixture interaction ($p \leq .05$).

The thymocyte populations were analyzed at 7 d posttreatment to determine if the effects on the thymus were persistent. The absolute cell numbers for all of the thymocyte populations remained significantly decreased by 150/150 and 200/200 mixture treatment (Figure 4, A, B, C, and D). Seven days after treatment with P150 or P200 the absolute number of $CD4^+CD8^+$ cells had recovered to vehicle control levels (Figure 4A). After the P150 treatment, the $CD4^-CD8^+$ population had recovered by 7 d; however, this population was still decreased by the P200 treatment (Figure 4C). Interestingly, all thymocyte populations were decreased by 7 d after treatment with D200 (Figure 4, A, B, C, and D).

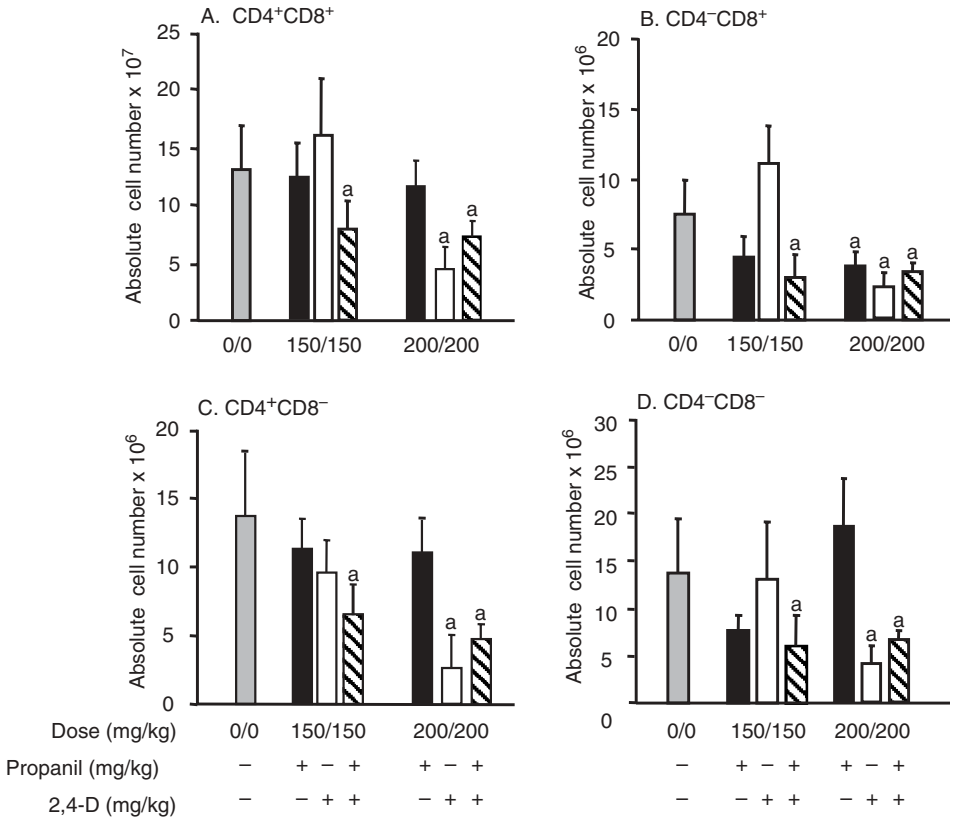

FIGURE 4. Changes in thymocyte populations seven days after exposure to herbicides. Mice were treated with vehicle (gray bars), propanil (black bars), 2,4-D (open bars), or propanil and 2,4-D (striped bars) and thymuses were removed at 7 d posttreatment. Thymocytes were stained with anti-CD4 FITC and anti-CD8 PE and total cell numbers calculated as described in Materials and Methods. Bars represent the mean ± the standard deviation. (A) $CD4^+CD8^+$ thymocyte population. (B) $CD4^-CD8^+$thymocyte population. (C) $CD4^+CD8^-$ thymocyte population. (D) $CD4^-CD8^-$ thymocyte population. The letter above the bars represents a significant difference from the vehicle control (a) ($p < .05$).

The Induction of Glucocorticoids After Herbicide Exposure

Administration of corticosterone has been demonstrated to selectively deplete the $CD4^+CD8^+$ thymocyte population in vivo (Reichert et al., 1986). Therefore, corticosterone levels in the serum, from mice exposed to propanil, 2,4-D, or a 1:1 mixture of the two, were measured at 1, 4, 12, 24, and 48 h posttreatment. Treatment with P50, P100, P150, and P200 and all mixture treatments (50/50, 100/100, 150/150, and 200/200) increased serum corticosterone to peak levels of 600–1000 ng/ml at 1 h posttreatment (Figure 5, A, B,

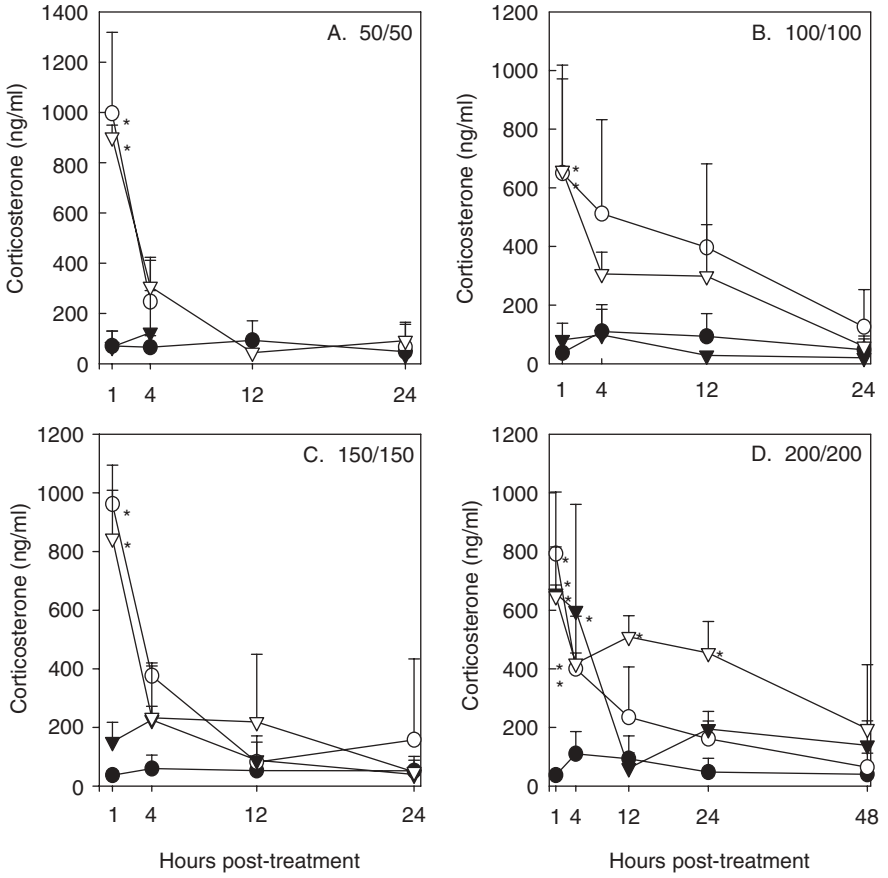

FIGURE 5. The induction of glucocorticoids after herbicide exposure. Sera corticosterone levels were measured at 1, 4, 12, 24, and 48 h (200 mg/kg dose only) posttreatment with vehicle (closed circle), propanil (open circle), 2,4-D (closed triangle), or propanil and 2,4-D (open triangle). (A) 50-mg/kg Single-herbicide treatments or 50/50 mixture treatment. (B) 100 mg/kg Single-herbicide treatments or 100/100 mixture treatment. (C) 150 mg/kg Single-herbicide treatments or 150/150 mixture treatment. (D) 200 mg/kg Single-herbicide treatments or 200/200 mixture treatment. Numbers represent the average of eight mice ± the standard deviation. The asterisk (beside the data points) represents a significant difference from the vehicle control ($p < .05$).

C, and D). 2,4-D only elevated corticosterone levels at the highest dose of D200 (Figure 5D). Corticosterone levels fell sharply during the initial 4 h after P50, P100, P150, and 50/50, 100/100, 150/150 treatments followed by gradual decreases until 24 h posttreatment when corticosterone levels had returned to baseline (Figure5, A, B, C). In contrast, corticosterone levels decreased gradually after 200/200 treatment and only returned to baseline levels after 48 h (Figure 5D). Additional experiments were performed with lower doses of propanil since the P50 dose had significantly increased corticosterone levels. Exposure to P5 did not increase glucocorticoid levels over vehicle control (58 ± 36 ng/ml vs. 67 ± 49 ng/ml, respectively). However, 1 h posttreatment with P10 (218 ± 165 ng/ml) or P25 (399 ± 119 ng/ml), corticosterone levels were increased. Similar increases were measured after exposure to mixtures of 10/10 (275 ± 195 ng/ml) or 25/25 (427 ± 209 ng/ml).

The Role of Glucocorticoids in Thymic Atrophy and $CD4^+CD8^+$ Thymocyte Depletion

A multiple-regression analysis was done to compare exposure-related total cell loss across all treatment groups with sera corticosterone increases. Decreases in the $CD4^+CD8^+$ thymocyte population after exposure to the 50/50–200/200 mixtures were correlated (slope = -8.1×10^{-4}) to glucocorticoid increases that occurred between 1 and 24 h posttreatment. Decreases at 7 d posttreatment for all of the thymocyte populations after 150/150 mixture treatment also had a correlation with the earlier 1–12 h increases in sera glucocorticoid levels. These results demonstrate that the cellular decreases in the thymus at 2 and 7 d posttreatment correlate with the sera corticosterone increases that occur early in the stress response. Therefore, glucocorticoid increases are a potential mechanism for thymocyte loss after mixture treatment and the effects of glucocorticoid hormones may last as long as 7 d posttreatment.

RU 486 was used to determine if increased glucocorticoid levels contribute to cell loss in the thymuses of mixture-treated animals. The 150/150 dose was chosen because a greater than additive decrease was noted in the $CD4^+CD8^+$ thymocyte population at this treatment level (Figure 2B). Corticosterone and the 150/150 mixture treatment significantly reduced thymus to body weight (Figure 6A) and the total number of $CD4^+CD8^+$ cells (Figure 6B). RU 486 alone had no significant effect on the thymus size (Figure6A) or total cell number of $CD4^+CD8^+$ cells (Figure 6B). Treatment with RU 486 rescued the $CD4^+$ $CD8^+$ population from the decreases associated with corticosterone exposure (Figure 6B). In animals exposed to the 150/150 mixture treatment, pretreatment with RU 486 partially abrogated the thymic atrophy (Figure 6A) and loss of the $CD4^+CD8^+$ population; however, they were still significantly decreased in comparison to the vehicle-treated mice (Figure 6A and 6B).

DISCUSSION

In this study a detailed analysis of the effects on the thymus after in vivo exposure to a mixture of propanil and 2,4-D is presented. In our studies, at 2 d

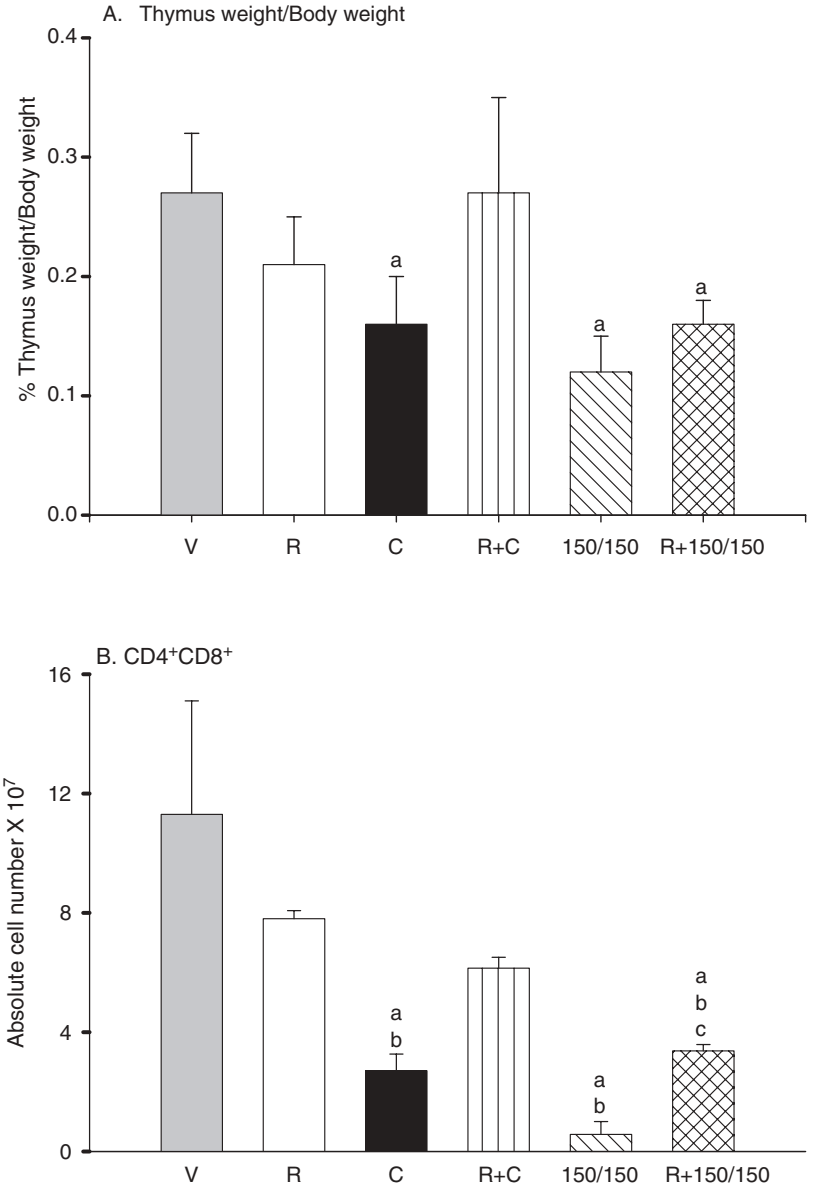

FIGURE 6. Thymus weight and $CD4^+CD8^+$ population changes after RU 486 pretreatment and herbicide exposure. Mice were treated with RU 486 2 h prior to and 12 h following herbicide mixture treatment. Treatment groups include: vehicle (V), RU 486 (R), corticosterone (C), RU 486 + corticosterone (R + C), 150/50 mixture treatment (150/150), or RU 486 + 150/150 mixture treatment (R + 150/150). Thymuses were removed at 2 d posttreatment and stained with anti-CD4 FITC and anti-CD8 PE and total cell numbers calculated as described in Materials and Methods. (A) Thymus to body weight ratio is expressed as a percentage. (B) Absolute cell numbers of $CD4^+CD8^+$ thymocytes. Numbers represent the mean ± the standard deviation. The letters above the bars represent significant differences from the vehicle control (a), the RU486 group (b), and the 150/150 group (c) ($p < .05$).

postexposure, the $CD4^+CD8^+$ and $CD4^-CD8^+$ thymocyte populations were decreased and by 7 d postexposure all thymocyte populations were lower. The simplest explanation for the loss in the $CD4^-CD8^+$ and $CD4^+CD8^-$ populations is that it is a downstream effect from the initial substantial loss of the $CD4^+CD8^+$ population, which supplies the pool of thymocytes that develop into the mature single positive populations. Similarly, the loss of the $CD4^-CD8^-$ thymocyte population may result from effects on the bone marrow since it was previously demonstrated that exposure to the herbicides decreases bone marrow populations (de la Rosa et al., 2003). Alternatively, exposure to the herbicides may alter cell-signaling pathways that contribute to thymocyte survival. It has been demonstrated that thymocyte populations express NF-κB, and it has been proposed that activation of NF-κB may regulate important survival signals during thymocyte development (Sen et al., 1995, Sen, 2001; Voll et al., 2000). Studies previously demonstrated that propanil decreases nuclear levels of NF-κB in macrophages and a human T-cell line (Frost et al., 2001; unpublished data). Alterations in NF-κB in the thymus after herbicide exposure could therefore affect thymocyte development and survival.

Hypocellularity in the thymus has been used as a tool to determine the extent of involvement of glucocorticoid hormones in the stress response (Han et al., 1993; Padgett et al., 2000). Glucocorticoids are immunoregulatory hormones known to produce thymic atrophy by apoptotic depletion of the $CD4^+$ $CD8^+$ thymocyte population (Cohen, 1992; Wyllie, 1980). Several studies have associated thymic atrophy with increases in glucocorticoid levels, induced by chemical exposure (reviewed by Pruett et al., 1993). Propanil exposure is associated with an increase in systemic glucocorticoid levels, and adrenalectomy partially relieves thymic atrophy in propanil-treated mice (Cuff et al., 1996). Therefore, it was hypothesized that glucocorticoids were a potential mechanism for the adverse effects on the thymus after exposure to a mixture of propanil and 2,4-D. To test our hypothesis, the glucocorticoid receptor blocker RU 486 was utilized. RU 486 is a glucocorticoid receptor II blocker that has been shown to effectively block apoptosis of thymocytes exposed to dexamethasone in vitro and to block thymic atrophy associated with increased sera levels of glucocorticoids after ethyl alcohol consumption in vivo (Xue et al., 1996). This block is specific to the stress reaction and does not interfere with the type I mineralocorticoid receptor responsible for regulation of homeostasis (Miller et al., 1998).

Our results demonstrate that sera corticosterone levels were increased after exposure to all doses of propanil, D200, or the herbicide mixture. The corticosterone increases were correlated with atrophy of the thymus and loss of the $CD4^+CD8^+$ population. However, the data demonstrated that increases in corticosterone alone after herbicide exposure were not sufficient to induce loss of thymocyte populations and RU 486 only partially abrogated the effects on the thymus. For example, corticosterone levels were increased to maximal levels after exposure to P50 (888.53 ± 287.47) and the 50/50 mixture treatment (710.46 ± 202.61), but there were no decreases in thymus size or thymocyte

populations at these doses. Significant increases in sera corticosterone levels were also measured after P10 and P25 propanil treatment with no effects on the thymus (data not shown). Therefore, although it is possible that the RU 486 protocol used in our studies did not completely block the in vivo activity of corticosterone, these results suggest that additional mediators or mechanisms contribute to the effects on the thymus.

Glucocorticoids are known to complement the biological response to cytokines and other hormones (Wiegers & Reul, 1998; Wu & Pruett, 1996). Although corticosterone is the main hormone produced in the mouse during a stress reaction, additional mediators may contribute to the thymic atrophy associated with the herbicide mixture treatment (Pruett & Fan, 2001). Therefore, it is possible that glucocorticoids, together with other paracrine and endocrine factors, can selectively affect specific lymphocyte populations. If 2, 4-D induces additional hormones, the combination of 2,4-D and propanil, a compound known to increase glucocorticoid hormones, could result in a mixture response that is greater than the response to the individual herbicides.

Thymic atrophy is a commonly used indicator of acute chemical stress. However, the long-term significance of a transient effect on the adult thymus on peripheral T-cell populations and subsequent immune responses is not clear. Studies did not find any changes to the splenic $CD4^+$ or $CD8^+$ T-cell populations from 2 to 14 d after exposure (data not shown). Preliminary data in our laboratory indicate that mice exposed to the mixture of herbicides have, in fact, an increase in the number of antibody producing cells in the spleen in response to vaccination with heat-killed *Streptococcus pneumoniae*. This would suggest that the thymic atrophy in our model does not result in subsequent immune suppression of the humoral response. It is not known, however, if other peripheral T-cell functions, such as cytotoxic T-cell activity, are affected. It is possible that the effects of chemical exposure on the thymus versus peripheral immune organs may result from different mechanisms and have quite disparate effects. Thymic atrophy in an adult animal may simply serve as a biomarker for some chemical stressors. Glucocorticoids are also commonly induced after chemical stress and correlate with thymic atrophy. Therefore, measurement of sera glucocorticoid levels from peripheral blood may serve as a less invasive biomarker. The use of the area under the corticosterone concentration versus time curve as a predictor of the effects of chemical stress on immune parameters is an active area of research in other laboratories (Pruett & Fan, 2001; Pruett et al., 2003).

Mixture-specific effects occurred in the thymus after exposure to the mixture of propanil and 2,4-D. In this study, a partial factorial design was employed to determine the effects on the thymus that were specific to the mixture. A linear dose response to 2,4-D was not established over the five doses tested. Therefore, a nonlinear ANOVA analysis was performed using nominal variables as factor levels with the null hypothesis stating that the response to the mixture would be the addition of the response to the individual mixture components. The definition of an interaction is that the response

to the mixture significantly differs from the sum of the effects of the single components. Such a finding would be particularly important because the health risks that are associated with exposure to a mixture of chemicals are commonly calculated from models that assume an additive response to the individual components of the mixture (U.S. Environmental Protection Agency, 1986). Our results demonstrate that there are mixture interactions between propanil and 2,4-D that would not have been predicted based on an additive response model. Mixture interactions demonstrating greater than additive decreases occurred after exposure to 150/150 mixture treatment in the $CD4^+CD8^+$ and $CD4^-CD8^+$ populations (Figures 2B and 3).

Our results at the 200-mg/kg dose demonstrated that there were mixture interactions indicating less than additive decreases after 200/200 exposure in the $CD4^+CD8^+$ and $CD4^-CD8^+$ thymocyte populations (Figure 2B and 3). Cellular decreases occurring after exposure to mixtures that contained 200 mg/kg of either herbicide were not additive, but equal to the 200-mg/kg single herbicide response. These data suggest that the response to 200 mg/kg of either propanil or 2,4-D delineate a biological threshold or saturation point where all thymocytes that are sensitive to the effects of the chemicals have been affected. A linear dose response to 2,4-D exposure in the thymus may exist between a no-effect 150-mg/kg dose and the higher dose of 200 mg/kg. However, the reproducible although not statistically significant decrease that occurs in the $CD4^+CD8^+$ population after 100 mg/kg 2,4-D exposure allows for the possibility that the response to 2,4-D may be bimodal (Figure 2B). The 150/150 mixture then pairs either a no-effect or threshold dose of 2,4-D with a dose of propanil that has been determined to be in the linear range of the dose response. Therefore, within the range of our experiments, there appear to be biological effects that are specific to the mixture and support a nonlinear statistical analysis of the interaction. The 150/150 mixture exposure, for example, was also found to delay thymocyte recovery. In contrast to the 150-mg/kg single herbicide treated thymocytes, all populations remained significantly decreased at 7 days after 150/150 treatment (Figure 4). These data suggest that the 150/150 mixture increased the biological effects of the herbicides on the thymus.

An ip route of exposure was employed in this study at doses previously shown to induce immunotoxic effects. The most common routes of exposure to herbicides in humans, however, are by inhalation, ingestion, or dermal. Preliminary data using an aspiration route of exposure to propanil and 2,4-D demonstrate comparable effects on the thymus, suggesting that an environmental route of exposure will have similar effects as the ip exposure model. Future studies will determine the immunotoxicity of the mixture of herbicides using the aspiration route of exposure.

Overall, these studies suggest that in the complex, in vivo biological response to a mixture, nontraditional interactions may occur that would not be predicted by traditional statistical models. This emphasizes the need for additional studies for further comparisons between in vivo responses and statistical models of analysis of mixtures.

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