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Environmental Toxicology and Chemistry, Vol. 26, No. 1, pp. 80–84, 2007

Printed in the USA
0730-7268/07 $12.00 + .00

ATRAZINE IS AN IMMUNE DISRUPTOR IN ADULT NORTHERN LEOPARD FROGS (*RANA PIPIENS*)

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(*Received* 18 *January* 2006; *Accepted* 29 *June* 2006)

Abstract—Atrazine, the most widely used herbicide in the United States, has been shown in several studies to be an endocrine disruptor in adult frogs. Results from this study indicate that atrazine also functions as an immune disruptor in frogs. Exposure to atrazine (21 ppb for 8 d) affects the innate immune response of adult *Rana pipiens* in similar ways to acid exposure (pH 5.5), as we have previously shown. Atrazine exposure suppressed the thioglycollate-stimulated recruitment of white blood cells to the peritoneal cavity to background (Ringer exposed) levels and also decreased the phagocytic activity of these cells. Unlike acid exposure, atrazine exposure did not cause mortality. Our results, from a dose–response study, indicate that atrazine acts as an immune disruptor at the same effective doses that it disrupts the endocrine system.

Keywords—Atrazine Immune disruption Frog *Rana pipiens*

INTRODUCTION

The global decline in amphibian populations has been under close examination for more than 20 years; more recently, the role played by environmental pollutants in this decline has received increased attention. It is well documented that some environmental pollutants disrupt the function of the endocrine system in many animal species [1]. Therefore, these substances are called endocrine disruptors and are defined by the Environmental Protection Agency as ''external agents that interfere in some way with the role of natural hormones in the body'' (http://www.epa.gov/scipoly/oscpendo/index.htm). Aspelin [2; http://www.epa.gov/oppbead1/pestsales/] estimated that by 2000, there will be over 20,000 herbicides and pesticides used in the United States and that these chemicals will have an estimated 900 active ingredients.

A growing body of evidence shows that pesticides and herbicides reduce the ability of frogs to resist parasitic infection. These compounds also suppress the humoral immune response of *Rana pipiens* and may have species-specific effects on the cellular immune response [3–5]. Atrazine is one of the most heavily used agricultural herbicides in the United States and can reach 40 ppb both in aquatic ecosystems and in precipitation [6]. Atrazine is the most common pollutant found in groundwater [7]. In *Xenopus laevis* and *Rana pipiens*, atrazine exposure to concentrations of 0.1 ppb causes endocrine disruption manifested as gonadal abnormalities and hermaphroditism in males [6,8] and thereby may disrupt their ability to reproduce. Atrazine has also been shown to cause immune deficiency in ranids during the larval stage of development [5].

The Environmental Protection Agency's maximum contaminant load for atrazine in drinking water is 3 ppb [9]. This concentration is 30 times higher than the 0.1 ppb that Hayes et al. [6,8] showed to cause feminization of male frogs in the laboratory. We wanted to investigate the effects atrazine has on the innate immune response in adult *R. pipiens* at doses similar to those that disrupt endocrine function. We hypothesized that atrazine, even at as low a concentration as 0.1 ppb, may act as an immune disruptor in similar ways to mild acid exposure, that is, by disrupting the innate immune response of adult *R. pipiens* (SETAC Globe, 2004, 5:48–49; [10]). To assess the innate immune response, we stimulated frogs with thioglycollate, a widely used inflammatory mediator [11–15]. Inflammatory response is characterized by an influx of leukocytes to the site of infection or injury. We measured the inflammatory response and phagocytic activity of white blood cells (WBCs) as indicators of innate immune response. To measure the phagocytic activity of WBCs, in vivo, the inoculation medium contained 1-micron beads impregnated with fluorescein isothiocyanate.

MATERIALS AND METHODS

Experimental animals and conditions

We conducted two experiments. In both experiments, *R. pipiens* of both sexes (average length ~7 cm and average mass ~30 g) were purchased from Amphibians of North America (Nashville, TN, USA). Frogs were caught in the northeastern United States by licensed collectors in September or October. Since these frogs were caught in the wild, we cannot control for previous exposure to environmental contaminants. The experiments were conducted shortly after the animals arrived and were acclimated to the laboratory for at least 5 d. The Widener University Institutional Animal Care and Use Committee approved all protocols for the experiments described in this paper.

Prior to the experiments, frogs were allowed to acclimate to the laboratory in 38-L tanks, filled 3 cm deep with aged tap water, and supplied with a Styrofoam (Dow Chemical, Midland, MI, USA) raft to allow the animals to climb out of the water. Water was changed daily, and frogs were fed three to four crickets (Fluker Farms, Port Allen, LA, USA) per frog 4 d a week. During the 8-d experiment individual frogs were kept in autoclaved sterile Tupperware (Tupperware, Orlando, FL, USA; 30 × 20 × 9 cm) containers with lids (treated with

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70% ethanol) filled with 500 ml of the appropriate solution (i.e., aged tap water or atrazine supplemented water). The solutions were changed daily. During experimentation, the Tupperware containers were placed in an environmental chamber at 25°C with 12:12-h light:dark cycle.

Experiment 1: Atrazine exposure at 21 ppb

Twenty-four adult *R. pipiens* were randomly allocated to four experimental groups: an atrazine-exposed group at 21 ppb followed by injection with Ringer solution (RA), an atrazine-exposed group at 21 ppb followed by thioglycollate stimulation (TA), a group exposed to aged tap water—no atrazine followed by injection with Ringer solution (RN), and a group exposed to aged tap water—no atrazine followed by stimulation with thioglycollate (TN).

Experiment 2: Atrazine dose response

Thirty-six adult *R. pipiens* were randomly allocated into six experimental groups with six frogs in each group: four experimental groups exposed to atrazine and two control groups exposed to aged tap water. The atrazine (ChemService, West Chester, PA, USA) groups were exposed to nominal doses of 10, 1, 0.1, and 0.01 ppb. All atrazine-exposed groups received thioglycollate stimulation and therefore were labeled TA groups. One control group exposed to aged tap water (no atrazine) was labeled RN and received injection with Ringer solution (Carolina Biological Supply, Burlington, NC, USA), and another control group exposed to aged tap water (no atrazine) received thioglycollate stimulation and was labeled the TN group.

White blood cell counts

White blood cells (WBCs) from the lavage fluid were counted using a Hausser hemocytometer (Fisher Scientific, Pittsburg, PA, USA). Aliquots of lavage fluid were added to a hemocytometer, and all four fields from the upper chamber were counted, and the average number was reported for each animal.

Phagocytic activity

The frogs were injected on the seventh day with 2 ml of either physiological Ringer solution or thioglycollate each containing 1-micron fluorescent beads. The frogs were euthanized on day 8 by ether asphyxiation, and a peritoneal lavage was performed with 10 ml of physiological Ringer solution. Blood was collected, and livers were excised and placed in −70°C storage for later characterization. Fluorescein isothiocyanate–labeled beads (Polysciences, Warrington, PA, USA) were diluted into the inoculation medium to a final concentration of 2.5×10^7 beads per ml and injected intraperitoneally on day 7. The number of beads used was carefully titrated, and cells were washed to avoid the occurrence of background beads associated with but not engulfed by peritoneal WBCs. The number of beads phagocytosed by each WBC was counted using a Leitz Diaplan fluorescence microscope (Leica Microsystems, Wetzlar, Germany). For both experiments, 100 or more WBCs were counted per frog. Based on the number of beads phagocytosed, cells were placed into the following categories: 0 beads (nonphagocytic cells), 1 to 3 beads, 4 to 6 beads, 7 to 9 beads, and >10 beads (highly phagocytic cells). Data from experiment 1 are expressed as the total percentage of phagocytic cells, and data from experiment 2 are expressed as one of two categories: nonphagocytic or highly phagocytic.

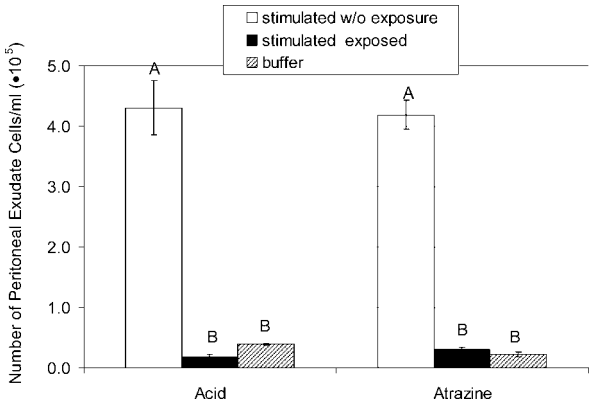

Fig. 1. Eight days of exposure to pH 5.5 or to atrazine (21 ppb) reduce the mean number ($n = 6$) of white blood cells ± standard error in the peritoneal exudates of adult *Rana pipiens* stimulated with thioglycollate to background levels. Stimulated groups were injected with thioglycollate to induce an inflammatory response. Control groups were injected with an iso-osmotic Ringer solution. Acid exposure data are from a previous experiment [10]. Statistical significance ($p < 0.05$) is indicated by different letters.

Statistical analysis

Peritoneal WBC counts and phagocytic activity were analyzed by a Kruskal–Wallis nonparametric analysis of variance followed by a nonparametric multiple comparisons using the Q stat test [16]. The percent of phagocytic activity was transformed by taking the arcsine of the square roots to make a normal distribution and then analyzed using a two-way analysis of variance with repetition with an alpha level of $p < 0.05$. The percentages of nonphagocytic cells and highly phagocytic cells were analyzed using a Mann–Whitney *U* test.

RESULTS

Experiment 1: WBC counts

Frogs exposed to atrazine at 21 ppb followed by thioglycollate stimulation showed a statistically significant diminished innate immune response when compared to frogs that had not been exposed to atrazine but injected with thioglycollate. These results are very similar to frogs that were exposed to acid (Fig. 1); acid exposed results are from a previous experiment [10] and are presented here only for comparison between environmental contaminants. We further analyzed the phagocytic activity by calculating the percentage of phagocytic cells (all cells that contained beads) and compared this among the groups (Fig. 2). The average percent ± standard errors (SE) of the thioglycollate-stimulated group with no atrazine exposure (TN) was 22.4 ± 4.8 compared to 3.3 ± 0.8 in the thioglycollate group exposed to atrazine (TA). The average percent of the Ringer-stimulated group with no atrazine exposure (RN) was 2.5 ± 0.9 compared to 9.6 ± 1.5 in the Ringer-stimulated group exposed to atrazine (RA). The two-way analysis of variance showed that the difference between the treatments (thioglycollate vs Ringer) as well as between the groups (atrazine exposure vs no atrazine exposure) were significant ($p = 0.006$ and $p = 0.014$, respectively) as well as the interaction term ($p = 1.03 \times 10^{-5}$).

Experiment 2: Dose response

WBC count. Thioglycollate-stimulated (TN) frogs exhibited the greatest number of peritoneal exudate cells ± SE (5.1 ± 0.95, $\times 10^5$; Fig. 3). Frogs exposed to atrazine at 10, 1, 0.1,

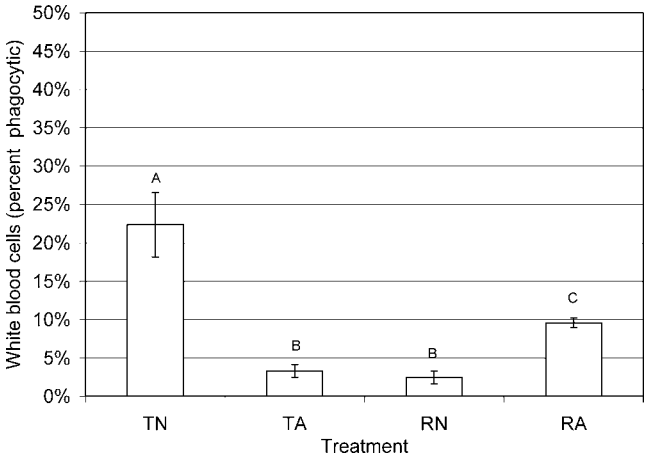

Fig. 2. Eight days of exposure to atrazine (21 ppb) reduced the mean percent ($n = 6$) of peritoneal phagocytic cells ± standard error in thioglycollate-stimulated frogs. The experimental groups were no atrazine exposure stimulated with thioglycollate (TN) and atrazine exposed and thioglycollate-stimulated (TA). The control groups were no atrazine exposure stimulated with Ringer solution (RN) and atrazine-exposed and stimulated with Ringer solution (RA). Data were analyzed using a two-way analysis of variance, and statistical significance ($p < 0.05$) is indicated by different letters.

and 0.01 ppb showed a significantly reduced number of peritoneal exudate cells compared to the TN group: 0.2 ± 0.1, 0.71 ± 0.15, 0.5 ± 0.05, and 3.1 ± 0.91, × 10^5, respectively ($p < 0.05$ for all). The number of peritoneal exudate cells at 0.01, 0.1, 1, and 10 ppb atrazine are significantly different from each other ($p < 0.05$). However, the number of peritoneal exudate cells at 10 ppb and RN are not significantly different from each other ($p > 0.2$).

Experiment 2: Dose response

WBC phagocytosis. Thioglycollate-stimulated (TN) frogs had the highest percentage of highly phagocytic cells ± SE (cells that engulfed >10 beads, 9.8% ± 1.5%). Peritoneal WBCs from frogs stimulated by thioglycollate and exposed to atrazine showed suppressed levels of phagocytic activity at doses of 1 ppb (5.1% ± 1.0%), 0.1 (3.96% ± 1.69%), and 0.01 ppb (6.5% ± 1.7%) when compared to the TN group.

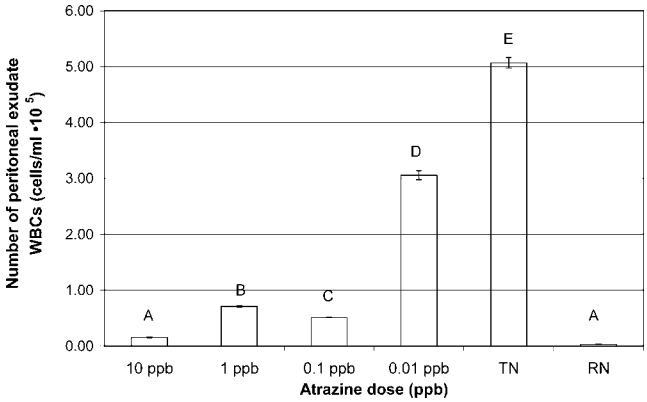

Fig. 3. Atrazine exposure reduced the total number of peritoneal exudate white blood cells (WBCs) following thioglycollate stimulation in a dose-dependent fashion. The minimal effective dose was 0.01 ppb. TN represents the thioglycollate-stimulated group without atrazine exposure, and RN represents the Ringer solution–stimulated group without atrazine exposure. Statistical significance ($p < 0.05$) is indicated by different letters.

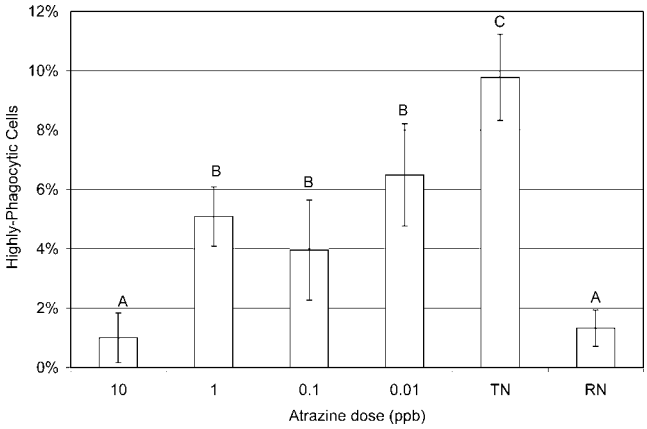

Fig. 4. Atrazine exposure reduced the number of highly phagocytic cells (cells that engulfed >10 beads) in a dose-dependent fashion. The minimal effective dose was 0.01 ppb. TN represents the thioglycollate-stimulated group without atrazine exposure, and RN represents the Ringer solution–stimulated group without atrazine exposure. Statistical significance ($p < 0.05$) is indicated by different letters.

Cells from frogs exposed to 0.1 ppb had phagocytic activity (3.96% ± 1.69%) significantly different from that of nonstimulated (RN) resident peritoneal cells. However, peritoneal WBC from frogs stimulated by thioglycollate and exposed to 10 ppb had the same phagocytic activity (1.0% ± 0.8%) as nonstimulated (RN) resident peritoneal cells (1.3% ± 0.6%) but were significantly different from the TN-stimulated group (Fig. 4).

The thioglycollate-stimulated group (TN, no atrazine exposure) had the lowest level of nonphagocytic cells ± SE (70.7% ± 1.45%). At 10 ppb atrazine exposure with thioglycollate stimulation, the percent of nonphagocytic cells (94.7% ± 0.71%) was the same as the nonstimulated, background (RN) group (95.4% ± 0.61%). The percent of nonphagocytic cells in the thioglycollate-stimulated group (TA) at 1 ppb atrazine (84.2% ± 1.0%), 0.1 ppb atrazine (84.3% ± 1.69%), and 0.01 ppb atrazine (84.6% ± 1.73%) was reduced when compared to the 10 ppb atrazine-exposed group and the RN groups; however, they still appear to be greater than the TN group. The 10 ppb atrazine-exposed group (TA) was significantly different than the thioglycollate-stimulated group without atrazine exposure (TN; Fig. 5).

DISCUSSION

Our study indicates that atrazine acts as an innate immune system disruptor in addition to its endocrine disruption action as shown by Hayes et al. [6,8]. Atrazine (21 ppb) affects the innate immune response of adult *R. pipiens* in similar ways to acid (Fig. 1). That is, atrazine exposure suppressed the experimentally induced (thioglycollate-stimulated) recruitment of WBCs to the peritoneal cavity to background (Ringer control) levels. Unlike mild acid exposure, however, atrazine does not cause mortality during an 8-d exposure period. Therefore, it appears that both acid and atrazine function as immune disruptors. Immune disruptors may act on both innate and acquired immunity. A disruptor may suppress innate immunity by decreasing the inflammatory response, reducing phagocytic activity, and disrupting cytokine networks.

Thioglycollate-induced peritonitis is a commonly used model to study inflammation [17]. Previous studies of ours [10] support findings that phagocytosis of fluorescein isothiocyanate microspheres can be reliably used to assess the activity

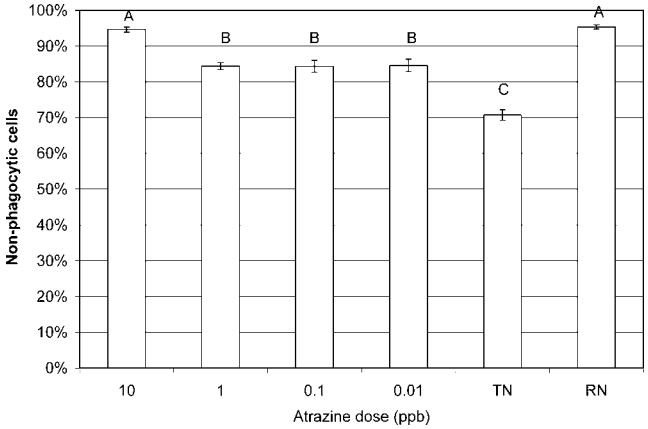

Fig. 5. Atrazine exposure increased the number of nonphagocytic cells (cells that did not engulf any beads). The minimal effective dose was 0.01 ppb. TN represents the thioglycollate-stimulated group without atrazine exposure, and RN represents the Ringer solution–stimulated group without atrazine exposure. Statistical significance ($p < 0.05$) is indicated by different letters.

of phagocytic WBCs. The inflammatory response elicited by intraperitoneal injection of thioglycollate varies among species [18] and among strains (of mice) used. Furthermore, the mechanisms by which this inflammatory response is elicited are complex, vary among species and strains, and are sensitive to a variety of endogenous and exogenous factors. However, this widely used model is a useful tool to study the innate immune response of vertebrate phyla.

Our data indicate that atrazine at 21 ppb with Ringer stimulation (without thioglycollate) stimulates phagocytic activity of resident peritoneal cells but suppresses phagocytic activity of thioglycollate-recruited cells (Fig. 2). This difference may be an example of a nonmonotonic dose response to atrazine that has been reported in the literature [19].

We also used thioglycollate-induced peritonitis to study the dose response of atrazine's effects on the innate immune response in our frogs. Hayes et al. [6] showed that atrazine-induced endocrine disruption occurs at 0.1 ppb, a dose 30 times lower than the Environmental Protection Agency's maximum contaminant level. Therefore, we designed our dose–response experiment with similar atrazine doses to those used by Hayes et al. [8]. Our results are similar to those published by Hayes et al. [6,8] and indicate that immune disruption, like endocrine disruption, occurred at 0.1 ppb (Figs. 3 to 5). At 10 ppb atrazine exposure, both recruitment and phagocytic activity were reduced to the level of nonexposed control frogs, that is, to background levels (RN; Figs. 3 and 4). At doses between 0.1 and 1.0 ppb atrazine exposure, WBC recruitment to the inflammatory site was still reduced but to a lesser degree. However, recruited cells appear to have high phagocytic activity although less than stimulated nonexposed frogs (TN; Fig. 4). Therefore, atrazine appears to still function as an immune disruptor even at these low doses. Although not tested for, this result suggests that the mechanism underlying recruitment is more sensitive to atrazine than the mechanism regulating phagocytosis. This may be due to a differential effect of atrazine on different cytokine networks.

Gilbertson et al. [3] studied the effects of exposure to a mixture of pesticides on the innate, humoral, and cell-mediated immune response of adult *R. pipiens*. Overall, the results of that study suggest that pesticides can stimulate or suppress different aspects of the immune response. The activation of *R. pipiens* phagocytic cells in the Gilbertson et al. [3] study was measured by whole blood chemiluminescence assay, described in detail in Marnilla et al. [20]. Frogs exposed to pesticides had a lower chemiluminescence value (indicating lower phagocytic activity) than frogs from a pesticide-free population. Our results provide a measure of phagocytic activity in vivo using a single herbicide and also demonstrated a herbicide-induced suppression of the innate immune response. Christin et al. [4] examined the in vitro phagocytic activity of isolated splenocytes of adult *R. pipiens* exposed to a mixture of six pesticides and herbicides (including atrazine) at an ecologically relevant dose. They found that pesticide exposure did not have suppressive effects on phagocytosis and splenocyte numbers. Therefore, phagocytic cells present in the spleen may be less sensitive than phagocytic cells recruited to an inflammatory site. Recruited cells have responded to chemical signals and cell surface receptors, and therefore they are in a physiologically activated state. However, at the highest dose, animals that were exposed to the pesticides had the lowest resistance to infection by the lung worm, *Rhabdias ranae*, suggesting that agricultural herbicides and pesticides affect frogs' ability to deal with parasitic infection. In a field study, Kiesecker [5] found a synergistic relationship between trematode infection leading to limb deformities and pesticide exposure. Furthermore, in a laboratory study, Kiesecker [5] found supporting evidence to suggest an association between pesticide exposure and increased trematode infection. These results suggested a pesticide-induced decrease in immunocompetence.

A growing body of literature indicates that exposure to ecologically relevant mixtures of pesticides and herbicides cause immunosuppression [3,4,20]. Our study indicates that exposure to very low doses of atrazine alone also act as an immunosuppressor of the innate immune response. Immunosuppression affects host–pathogen interactions, and because all recently documented amphibian extinctions in Australia, Britain, North America, and Central America [21] are associated with pathogens, immunosuppression may play a role in the global amphibian decline.

Acknowledgement—This work was supported partially by Widener University Provost's and Faculty Development Grants, Sigma Xi Grants in Aid of Research, and Tribeta Foundation Research Scholarship. We greatly appreciate the support of our former Provost Larry Buck and our former Dean Larry Panek for their support of our work.

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