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Lack of immunotoxic effects of repeated exposure to atrazine associated with the adaptation of adrenal gland activation

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ABSTRACT

T cell-dependent IgM antibody production and natural killer cell (NKC) activity were assessed in SD rats orally administered atrazine for 28 days to males (0, 6.5, 25, or 100 mg/kg/day) or females (0, 3, 6, or 50 mg/kg/day), or 30 or 500 ppm in diet (3 or 51 mg/kg/day). Anti-asialo GM1 antibodies (NKC) and cyclophosphamide (antibody-forming cell assay [AFC]) served as positive controls. Pituitary (ACTH, prolactin), adrenal (corticosterone, progesterone, aldosterone), and gonadal (androgens, estrogens) hormones were assessed after 1, 7, and/or 28 days of treatment. Food intake and body weights were significantly reduced in the highest dosed males, and transiently affected in females. Urinary corticosterone levels were not increased in atrazine-treated groups in either sex at any time point measured (10, 22, or 24 days). Corticosterone and progesterone were elevated in males after a single atrazine dose ≥6.5 mg/kg/day, but not after 7, 14, or 28 doses. There were no effects on adrenal, pituitary, or gonadal hormones in females. Atrazine did not suppress the AFC response or decrease NKC function after 28 days in males or females. Atrazine had no effect on spleen weights or spleen cell numbers in males or females, although thymus weights were elevated in males receiving the highest dose. The lack of immunotoxic effect of atrazine was associated with diminished adrenal activation over time in males, and no effects on adrenal hormones in females.

1. Introduction

Atrazine [(2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine)] is a chlorotriazine herbicide that is used to control annual grasses and some broadleaf weeds (Gianessi and Marcelli, 2000). The herbicidal action of triazines is based upon inhibition of photosynthesis by blocking electron transfer at the reducing site of chloroplast complex II. Although the toxicity of atrazine has been well characterized (Breckenridge et al., 2008; Simpkins et al., 2011), there have been relatively few systematic investigations of its immunotoxic potential. The results from *in vitro* studies suggest that atrazine may adversely affect cytokine production (Devos et al., 2003; Hooghe et al., 2000); the mitogenic activation and survival of lymphocytes (Chen et al., 2015; Lee et al., 2016; Pistl et al., 2003); mast cell degranulation (Mizota and Ueda, 2006); macrophage function (Karrow et al., 2005); the cytolytic activity of NKC (Rowe et al., 2007; Whalen et al., 2003); and the functional integrity of dendritic-like cells (Pinchuk et al., 2007; Thueson et al., 2015). However, there are only a limited number of studies on the effect of atrazine on immune function *in vivo* (Fournier et al., 1992; Karrow et al., 2005). Studies have also been conducted on the immunotoxicity potential of atrazine during development (Filipov et al., 2005; Rooney et al., 2003; Rowe et al., 2006, 2008), but as noted by the USEPA (EPA, 2010), the results from these investigations have been inconsistent.

Acute doses of atrazine, when administered by gavage to male (Laws et al., 2009; Pruett et al., 2008) or female rats (Fraites et al.,

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2009) or female mice (Pruett et al., 2003, 2009) activate the hypothalamic-pituitary-adrenal (HPA) axis, as indicated by elevated plasma concentrations of ACTH and/or corticosterone. The adrenal response to acute stressors has been used to predict changes in immunological parameters (Pruett, 2001; Pruett et al., 1999, 2000, 2003; Pruett et al., 2003). Measurement of immunological parameters in relation to adrenal activation in atrazine-treated animals has only been reported once previously and was performed in female mice administered 150 mg/kg of atrazine intraperitoneally (Pruett et al., 2009). Thus, the current study was conducted to evaluate the adrenal response following repeated doses of atrazine administered orally over time and to determine if atrazine induces immunotoxicity using standard 28-day exposure protocols developed to assess such effects (USEPA, 1998).

In order to assess the effects of atrazine exposure on the HPA axis and immune function, young adult male and female Sprague Dawley rats were administered atrazine for up to 28 days (males: at 6.5, 25, or 100 mg/kg via gavage; females: at 3, 6, or 50 mg/kg/day via gavage, or at 30 or 500 ppm in the diet). Furthermore, considering the rapid phase 1 metabolism and pharmacokinetic clearance of atrazine and its mono-dealkylated metabolites (de-ethyl atrazine [DEA] and de-isopropyl atrazine [DIA]) from plasma following bolus dose administration (Campbell et al., 2016), additional groups of female rats that were administered atrazine in diet for 28 days were included in the study. This permitted an assessment of the impact of pharmacokinetics on immunotoxicity parameters and urinary corticosterone levels after 4 weeks of dosing in the diet compared with animals that were administered equivalent doses by gavage. The results indicated that the HPA axis was activated after a single dose of atrazine in males, however, the adrenal response habituated over time and was not evident by day 7 of treatment. No effects of treatment were noted on the integrity of splenic antibody production (AFC assay) or NKC activity after 28 days of treatment.

2. Materials and methods

2.1. Animals

The protocols used for the in-life phase of these studies were approved by the Animal Care and Use Committee of WIL Research, Ashland, OH. Animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011) and with NIH and AAALAC guidelines. Rats were used as the test system on this study. This animal model is recognized as appropriate for toxicity studies with atrazine. Rooney et al. (2003) reported that the rat is responsive to the effects of atrazine on immune system parameters (Rooney et al., 2003). In addition, the use of rats removed the need to pool samples to perform hematology tests and hormone assays in individual animals in each treatment group. In addition, the majority of previous studies describing atrazine activation of the HPA have been performed in the rat.

Young adult females were approximately 8–9 weeks of age upon receipt and a minimum of 12.5 weeks at initiation of treatment. Males were 10 weeks of age at arrival and 11 weeks of age at initiation of treatment. All male and female Sprague-Dawley rats [Crl:CD (SD)] were purchased from Charles River Laboratories (Raleigh, NC) and were housed individually in suspended wire-mesh cages under controlled temperature (22 ± 3 °C) and humidity (50± 20%). Females were maintained on a 14-hr light (0500 h–1900 h)/10-hr dark photoperiod, whereas males were maintained on a 12-hr light (0600 h–1800 h)/12-hr dark cycle. All animals had *ad libitum* access to food (LabDiet® 5002, PMI Nutritional, LLC) and water (reverse osmosis-purified) throughout the duration of the acclimation period and study. Males in the pair-fed (PF) control group were given the average amount of feed consumed on the prior day by animals administered the highest dose of atrazine (100 mg/kg/day). All animals were deprived of food during the urine collection phase of the study.

Male studies were confined to the assessment of adrenal hormones and immune function. Female treatments were performed in conjunction with other female animals designed to determine the effects of repeated atrazine treatments on estrous cyclicity, luteinizing hormone (LH) surge, and metrics of fertility. In females, atrazine was delivered in bolus doses via gavage or distributed doses via atrazine incorporated in animal chow. The results of repeated atrazine treatment on female rat fecundity can be found in a previous publication (Foradori et al., 2014); the immunosuppressive effects of atrazine on females were consolidated with male studies in the present report. Immunotoxicity studies commonly use 28 days of treatment to assess possible effects. In the current studies, tissues from separate male treatment groups were examined for atrazine's proposed effects on adrenal and gonadal hormones on 1, 7, 14, or 28 days of treatment. After it was determined that atrazine had little or no effect on adrenal and gonadal hormones levels after 1–7 days of treatment, female animals were treated for 28 days to determine immunotoxic effects.

2.2. Test and control materials

Atrazine (97.5% pure), as provided by Syngenta Crop Protection, LLC, was admixed with PMI Nutrition International Certified Rodent LabDiet® 5002 in the dietary subgroups or was administered by oral gavage as an aqueous suspension in 1% methylcellulose (Spectrum Chemical Manufacturing Corporation). The Rodent LabDiet® 5002 (female feeding study) or the suspension of 1% methylcellulose in deionized water (gavage studies) was administered to vehicle control animals. Cyclophosphamide (CPS), obtained from the Sigma-Aldrich Chemical Company (St. Louis, MO), was diluted in phosphate-buffered saline (PBS) prior to use. Rabbit anti-asialoganglioside GM1 antibody (AA), obtained from Wako Chemicals (Richmond, VA), was reconstituted as a 1:10 dilution in sterile water prior to use.

2.3. Test article homogeneity, stability, and formulation concentration

It was determined that atrazine was homogeneously distributed in the 1% methylcellulose suspensions and in powdered Rodent LabDiet prior to the commencement of dosing. Aqueous and feed samples were determined to be stable for 11–15 days when stored at either room temperature or −20 °C. The concentration of atrazine in each dose formulation was assessed by HPLC and was found to be within acceptable limits of target concentrations in the study in males (Supplemental Table 1) and females (Supplemental Table 2). Atrazine was not detected in any vehicle control samples. These analyses assured that the intended dose of atrazine was administered.

2.4. Good laboratory practice standards

The *in vivo* studies, which were conducted at WIL Research Laboratories (Ashland, OH), and the immunotoxicity assays performed by ImmunoTox® Inc. (Richmond, VA) were conducted in compliance with Good Laboratory Practice standards (USEPA, 1983). ELISA and RIA hormone analyses were conducted at the University of Arizona, Phoenix, AZ using good scientific practices, and the LC/MS/MS analyses were performed by NMS Labs, Willow Grove PA according to the Clinical Laboratory Improvement