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Environmental Contamination and Toxicology

Immunological Responses of Weanling Cotton Rats (*Sigmodon hispidus*) to Acute Benzene and Cyclophosphamide Exposure

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Many environmental contaminants are potent immunotoxicants and capable of altering host resistance to pathogens and compromising overall immunological integrity (for reviews see; Vos, 1977; Faith et al., 1980). Environmental contaminants typically occur as a diverse group of compounds and are difficult to individually identify. The hazardous potential of individual toxicants or limited mixtures in laboratory studies is difficult to correlate with complex multichemical exposures found in situ (Rowley et al., 1983). Consequently, development of wild mammalian species as bioindicators of environmental contamination may provide an alternative approach to assessing the hazardous potential of immunotoxicants for both human and wildlife populations.

Fetal and newborn animals are potentially severly limited in their ability to biotransform xenobiotics (Sipes and Gandolfi, 1986) and most studies examining effects of exposure to heavy metals or hydrocarbons on immunity use mice and rats 4-6 weeks of age (Wierda et al., 1981; Aoyama, 1986). Cytochrome P-450-catalyzed reactions in rats may reach maximum levels by 30 days of age then slowly decrease to 50-60% of maximum by 600 days of age. Snyder and Kocsis (1975) reported on a study which demonstrated that young rats displayed a high rate of benzene metabolism and were more susceptible to benzene toxicity than older rats which had a slower rate of benzene metabolism. However, high levels (about 50-70% for mice and rats) of benzene can be expired unchanged through the lungs (Snyder and Kocsis, 1975; Travis et al., 1990) and conceivably pose little or no risk of immunosuppression (Snyder, 1987).

Studies in our laboratory are directed toward developing sensitive methods to evaluate environmental immunotoxicity hazards to wildlife and risks to humans. The present study was designed to test the sensitivity of selected cell-mediated immune response assays in weanling cotton rats following acute exposure to a monocyclic aromatic hydrocarbon (benzene) and cyclophosphamide which are known immunotoxicants in laboratory rodents (Dean et al., 1979; Snyder, 1987).

MATERIALS AND METHODS

A total of forty 21-day old weanling (weaned at 16-18 days of age) cotton rats (mean weight at termination = 47.6g±1.0(SE)), representing 5 litters of 8

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neonates each, all conceived in the wild and born in our laboratory animal facility were used in this study. A random pair of siblings from each litter were assigned to each of 4 experimental treatments (10 animals/treatment). Animals were grouped 2 or 4 in polystyrene cages with pine chip bedding. Food (Purina Rodent Chow) and water were provided ad libitum under natural light:dark conditions during a 12-day acclimation and 11-day experimental period.

Experimental treatments included 2 benzene (Aldrich Chemicals, Milwaukee, WS) dosages (100 or 1000 mg/kg body weight), cyclophosphamide (CY, Sigma Chem. Co., St. Louis, MO) as a positive control (50 mg/kg body weight), and corn oil vehicle as a negative control, administered intraperitoneally (IP) for 3 consecutive days. Benzene was administered in corn oil vehicle on days 1-3 of the experimental period. Positive and negative controls received corn oil on days 1-3 at volumes equal to benzene doses. Cyclophosphamide in distilled water vehicle was administered to positive control animals on days 8-10. The trial was terminated and necropsies performed on day 12 and weights recorded for thymus gland, liver, spleen, and paired kidneys and adrenal glands.

Animals were anesthetized with an intramuscular injection of ketamine hydrochloride (Bristol Laboratories, Syracuse, NY) at 50 mg/kg body weight. Blood was collected from the retro-orbital sinus plexus into 3-ml vacutainers (Becton Dickinson, Rutherford, NJ) containing EDTA. Hematocrits were determined by the microcapillary-centrifuge method. White blood cell (WBC) counts were determined manually using a hemacytometer. Whole blood cell smears were prepared and differential cell counts made by classifying 100 leukocytes.

Animals were sacrificed by cervical dislocation while under ketamine anesthesia. Spleens were aseptically removed, placed in preweighed 15x60 mm sterile petri dishes containing supplemented RPMI 1640 medium (RPMI-S, Sigma Chem. Co., St. Louis, MO) and weighed to the nearest 0.1 mg. RPMI-S was prepared as previously described (McMurry et al., 1991) by supplementing RPMI with L-glutamine, Na pyruvate, non-essential amino acids, penicillin/streptomycin, 2-mercaptoethanol, and normal horse serum. Spleens were cut into 3-4 pieces and gently disrupted using a sterile glass-on-glass tissue homogenizer (0.15 mm clearance) containing 5 ml ice-cold RPMI-S. Cells were allowed to settle for 10 min. and supernatant decanted into sterile 16x125mm screw-cap culture tubes. Cells were centrifuged for 7 min. at 10°C and 275xg, supernatant decanted, and pellet resuspended in 5 ml of RPMI-S (this wash step was performed 3 times). Viable cell counts were performed with a hemacytometer after lysing erythrocytes in tris-buffered 0.83% ammonium chloride (Tris/Cl, Sigma) and staining with Trypan blue (Sigma).

Lymphocyte proliferation, after in vitro mitogenic stimulation with concanavalin A (Con A) and pokeweed mitogen (PWM), was assessed by cellular reduction of tetrazolium salt (Mosmann 1983). Spleen cell suspension was adjusted to a final concentration of 500,000 cells/90 ul in RPMI-S. Four concentrations of each mitogen were added (10 ul volumes) to duplicate aliquots (90 ul) of the final cell suspension in 96 well, flat bottom microtitre plates. We used five concentrations of each mitogen (ug/ml of culture): Con A (0, 2.5, 5, 10, and 20) and PWM (0, 0.156, 0.313, 0.625, and

1.25). Cells were incubated for 72 hr at 37°C in a humidified incubator (5% CO_2). After 69 hr, 10 ul of MTT (tetrazolium salt, 5 mg/ml in PBS, Sigma) were added to each well, incubated the remaining 3 hr, and 160 ul of acid-isopropanol (176 ul concentrate HCl in 49.824 ml isopropanol) added to each well. Absorbances at 570nm/630nm were recorded on a Titertek Multiskan Plus MK II (Flow Laboratories, McLean, VA) against unstimulated controls as blanks.

Cotton rats were sensitized on day 4 with a percutaneous application of 100 ul of 3% 4-ethoxy-methylene-2-phenyl-oxazol-5-one (oxazolone, Sigma) in absolute alcohol to shaved abdomens. Animals were challenged on day 11 with 50 ul of 3% oxazolone and absolute alcohol to the left and right ear, respectively. Immediately after sacrifice (24 hr post-challenge), both ears were removed and weighed to the nearest 0.1 mg. Delayed-type hypersensitivity responsiveness (DTH) was measured as percent weight change of the challenged (left) ear relative to the control (right).

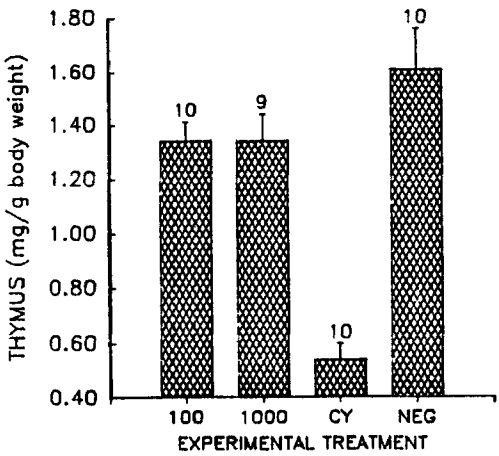
A probability level of $P \leq 0.100$ was used to determine statistical differences between treatment and control groups. Differences in organ morphology and immune responsiveness were determined by one-way analysis of variance (SAS, 1985). Duncans multiple range test was used for comparisons among treatment means when main effects were different. Specific contrasts (single degree of freedom) were used in all analysis of variance procedures to compare differences between major treatment components (benzene vs. negative control, negative vs. positive control, and 100mg benzene vs. 1000mg benzene).

RESULTS AND DISCUSSION

We observed no gross behavioral changes; neuromuscular abnormalities or mortality during the trial in any of the cotton rats receiving benzene. One animal from the 1000 mg/kg benzene group died during the experiment from non-treatment related causes. Neurological distress (piloerection, excitation, tremors) as well as some mortality has been observed in mice receiving 440-660 mg/kg benzene (Wierda et al., 1981).

Relative weights of thymus gland ($P < 0.001$) and liver ($P < 0.063$) were significantly influenced by experimental treatment (Fig. 1). Weanling cotton rats exposed to CY ($P < 0.001$) or benzene ($P < 0.032$) had significantly depressed thymus weights compared to negative controls. Conversely, liver and kidney weights were greater among CY-treated animals compared to negative controls ($P < 0.009$, $P < 0.029$), respectively (Fig. 1). Relative weights of spleen and adrenal glands did not differ ($P > 0.100$) among experimental treatments.

WBC counts, hematocrits, and viable splenocyte yields were significantly ($P < 0.001$) influenced by experimental treatments with significant depression induced by CY compared to benzene and negative control groups (Table 1). Cotton rats treated with CY had significantly lower lymphocyte ($P < 0.001$) and neutrophil ($P < 0.019$) counts than negative control animals (Table 1). Specific contrasts also indicated that cotton rats exposed to CY had significantly lower eosinophil counts compared to negative control ($P < 0.046$) animals.



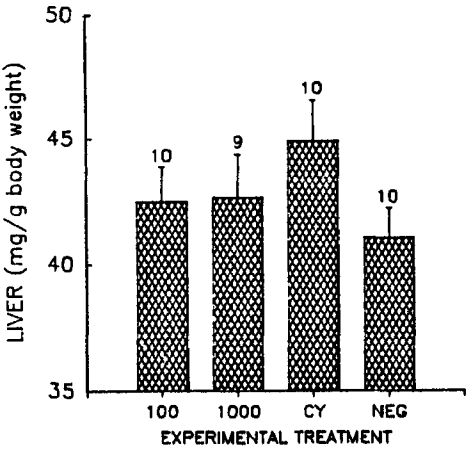
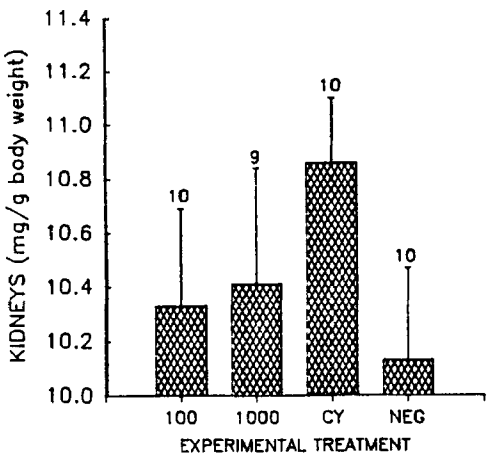


Figure 1. Differences in mean thymus, liver, and paired kidney weights of weanling cotton rats dosed intraperitoneally with benzene (100 and 1000 mg/kg body weight), cyclophosphamide (CY, 50 mg/kg body weight), or corn oil (NEG) for 3 consecutive days. Values above standard error (SE) bars represent sample size (n).



Optimum doses for Con A and PWM stimulation were 10 and 1.25 ug/ml culture, respectively. Lymphoproliferative response to PWM demonstrated significant ($P < 0.015$) treatment variation although the same was not true for Con A ($P > 0.10$) (Fig. 2). Response to PWM was depressed in CY-dosed cotton rats compared to negative controls ($P < 0.004$).

Cellular immunity as measured by delayed-type hypersensitivity response to a percutaneously applied antigen, was significantly ($P < 0.076$) influenced by experimental treatment. Specific contrasts showed a significant ($P < 0.036$) depression in DTH responsiveness of cotton rats exposed to CY ($5.53 \pm 1.9\%$) but not benzene (12.18 ± 3.3 and 17.21 ± 5.7) compared to negative controls (15.78 ± 3.9).

Snyder (1987) reported on several studies which concluded that the immunotoxic properties of benzene are exerted only after it has been metabolically transformed. Bone marrow stem cells (Snyder and Kocsis, 1975), mitotic cells (Parmentier and Dustin, 1948), lymphocytes, especially B cells and suppressor T cells (Irons et al., 1979; Aoyama, 1986; Snyder, 1987),

Table 1. Total white blood cells and subpopulations, hematocrit (Hct) and total splenocytes for weanling cotton rats exposed to three intraperitoneal injections of either benzene, cyclophosphamide (CY) or corn oil.

Cell parameter	Experimental treatment[1] Benzene 100mg/kg BW	Benzene 1000mg/kg BW	CY 50mg/kg BW	Negative control
WBC[2]	5.00(0.69)	5.01(0.67)	1.99(0.32)	5.40(0.87)
Hct (%)	39.25(0.93)	39.38(0.85)	34.45(0.97)	39.60(0.64)
Splenocytes[3]	47.35(5.69)	54.28(10.4)	14.76(2.78)	58.85(15.9)
Lymphocytes	2.63(0.51)	2.19(0.37)	0.53(0.08)	2.84(0.55)
Neutrophils	2.13(0.37)	2.55(0.45)	1.38(0.32)	2.08(0.31)
Eosinophils	191(60)	199(75)	44(13)	350(192)

[1]n=10 for all treatments except 1000mg benzene where n=9 for all parameters except Hct (n=8), (Mean±SEM)
[2]WBC, lymphocytes, and neutrophils = (x 10^3/mm^3)
[3](x 10^6)

and hepatocytes (Abraham et al., 1986) are sensitive to benzene metabolites.

It is clear that immunocompetence of juvenile rodents is not directly comparable to adult rodents. Although cellular and humoral components neccessary for a mature immune response may be present in early juvenile life, overriding humoral and/or cellular suppressive factors and inefficient cellular interactions may interfere with a normal response (Murgita and Wigzell, 1981). In addition, differences in level and function of the enzymes responsible for detoxification in adults and juveniles complicate comparisons of their responses to toxicants.

Except for mean thymus weight, acute benzene exposure induced neither suppression or stimulation of the immune system in weanling cotton rats given the exposure regimen used in our study. These results contradict previous studies which demonstrated substantial benzene-induced immunotoxic lesions in laboratory rodents (Pfeifer and Irons, 1981; Wierda et al., 1981; Aoyama, 1986; Rosenthal and Snyder, 1987). Among these studies, functional impairment of cell-mediated immunity, depression of spleen and blood cellularity, decreased lymphoproliferative responsiveness, lowered IgM production, and depressed spleen and thymus weights were observed. Conversely, benzene and benzene metabolites have shown stimulatory effects on cell-mediated immunity (Aoyama, 1986) and when used in low doses (Pfeifer and Irons, 1981).

Plausible explanations for the discrepancies between our study and others include variations in experimental conditions (inhalation vs. IP injection) and dosage regimens. Previous studies examining immunotoxic effects of benzene exposure in rodents typically terminated experiments after the last dosage was administered (Weirda et al., 1981; Aoyama, 1986; Rosenthal and Snyder, 1987). Cotton rats in this study were sacrificed 9 days following benzene exposure in order to duplicate the time-lag that would be required for an in situ study utilizing the same set of immune assays. Because rodents can metabolize and clear benzene within 24 hr after exposure (Gerarde and Ahlstrom, 1966; Snyder and Kocsis, 1975) and may recuperate quickly from benzene-induced lesions, careful consideration must be given to the amount

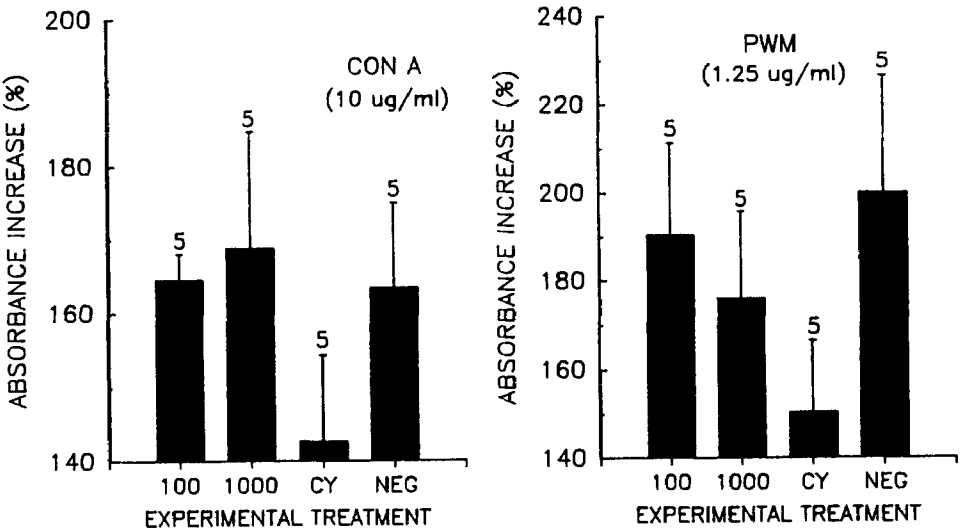


Figure 2. Differences in mean lymphoproliferative response (expressed as a percentage of unstimulated blanks) of splenocytes stimulated with Concanavalin A (Con A) and Pokeweed mitogen (PWM) from weanling cotton rats dosed intraperitoneally with benzene (100 and 1000 mg/kg body weight), cyclophosphamide (CY, 50 mg/kg body weight), or corn oil (NEG) for 3 consecutive days. Values above standard error (SE) bars represent sample size (n).

of time animals are maintained in the laboratory prior to analysis of immune function. If assays are used that require several days for immunization (eg., delayed-type hypersensitivity assays, plaque assays), animals may need to be housed on-site in enclosures that allow continued contact with the contaminated environment.

Cyclophosphamide was used in this study to document our ability to detect immunological lesions in the event that benzene-induced lesions were not apparent. Thereby, we could confirm that any inability to detect benzene-induced lesions was not simply due to a failed assay. Like benzene, CY also requires metabolic transformation to exert its toxic effects (Bach and Strom, 1985). Normally, metabolism occurs in liver microsomes through the MFO system although other pathways may yield toxic metabolites. Cyclophosphamide is quickly metabolized and targets mitotic cells (Bach and Strom, 1985) and its immunosuppressive capabilities are lost within a few days after challenge (Shand, 1979). Cyclophosphamide has been shown to suppress antibody production, lymphocyte proliferation, and DTH responses in laboratory mice (Dean et al., 1979), although DTH responsiveness may also be enhanced through selective elimination of suppressor T cells (Shand, 1979). Cyclophosphamide administered to cotton rats 3 days/wk at 50 mg/kg has been reported to be immunosuppressive (Johnson et al., 1982). In this study, final CY injection was administered 2 days prior to sacrifice and immunosuppression was indicated by decreased thymus weight, WBC, Hct, lymphocyte, neutrophil, and eosinophil counts, splenocyte yields, DTH response, and lymphoproliferative response to Con A and PWM compared to

negative controls. Additionally, relative liver and kidney weights increased in CY-treated animals.

Previous work with adult cotton rats dosed with benzene and CY (McMurry et al. 1991) found similar immunological response to benzene exposure as with weanling cotton rats. However, although significant immunosuppression was demonstrated in adult animals (depressed thymus and spleen weight, peripheral and tissue cellularity, and lymphoproliferative response to PWM) in response to CY, weanling cotton rats are considerably more sensitive to similar CY dosages. Increased sensitivity to CY may be a reflection of an immature immune system although weanling cotton rats appear equally capable of detoxifying and/or eliminating benzene compared to adults.

Variation in immunological response, possibly due to genetic heterogeneity with respect to immune (Biozzi et al., 1984) and metabolic responses to xenobiotic exposures, was observed in this study. In addition to genetic variation, wild populations of rodents are susceptible to inherent variation in immune response due to exogenous factors such as nutrition (Gershwin et al., 1985). Due to these and other sources of variation in immune response, experimental design of in situ studies will require rigorous attention to ecological similarity and close proximity between contaminated and reference sites in addition to adequate replication of sites. The positive results obtained with CY demonstrate the sensitivity of the immune system of wild cotton rats to an immunotoxicant. However, additional validation is warranted to more completely characterize the overall immunotoxic sensitivity of cotton rats.

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