ASSESSING IMMUNOTOXICITY RISKS FROM EXPOSURE TO COMPLEX MIXTURES OF ENVIRONMENTAL CONTAMINANTS

PROJECT REPORT
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ABSTRACT

Wild juvenile cotton rats (Sigmodon hispidus) were used to examine the effects of exposure to Cyclophosphamide (CY) and differing levels of benzene on selected measures of cellular immunity following dietary protein restriction. Benzene caused marginal immunotoxicity as indicated by suppressed splenocyte proliferation and total circulating neutrophils. Cyclophosphamide and also crude protein restriction induced severe immune lesions manifested as thymus and spleen atrophy, depressed delayed hypersensitivity response, reduced proliferative capacity of splenocytes, and reduced numbers of total leukocytes, lymphocytes, and splenocytes. Although severe immune modulation resulted from the individual effects of CY exposure and dietary protein restriction, there appeared to be little toxicant by diet interaction.

STATEMENT OF THE PROBLEM

Development of biomarkers to assess exposure of organisms to environmental contaminants is increasing, and studies utilizing wild rodent models are becoming more common in the literature (McBee et al. 1987, Rattner et al. 1993). Numerous approaches including biochemical, physiological, and histological changes in organisms have proven useful for documenting exposure to xenobiotics. Use of these biomarkers in populations of wild animals possess new problems that can be difficult to address, due to the natural environmental-induced variation routinely observed in wildlife populations. Wildlife populations are continually bombarded by single and multiple stressors. Of all natural stressors, nutrition ranks as one of the most important in determining overall condition and physiologic integrity of individuals within wildlife populations. Routine temporal and spatial variation in the nutritional quality of forage resources can influence behavior, reproduction, and survival in animal populations (White 1978, Flowerdew 1987).

Immune response is one of several physiological markers of environmental contamination that has been extensively studied in laboratory rat and mouse models and more recently in wildlife species. The immune system is an interactive and dynamic network of cells and soluble mediators which are sensitive to intrinsic and extrinsic stressors such as age, sex, reproductive condition, nutrition, and chemical stress (Dean et al. 1986). Adequately assessing the potential hazards of environmental contaminants in wildlife populations will eventually require a clear understanding of possible interactions between normally encountered stressors and xenobiotic contaminants. Wild populations of animals routinely encounter temporal shifts in the nutritional quality of their habitat, which can lead to malnutrition and eventually alterations in immunocompetence and xenobiotic metabolism. Although much information exists on the sensitivity of the immune system to nutritional and chemical stress, less work has been done to explore possible interactions between these two stessors on immunocompetence (Kalamegham et al. 1981).

OBJECTIVES OF THE PROJECT

The present study was designed to explore the effects of acute xenobiotic exposure and severe protein restriction on selected measures of cell-mediated immunity in juvenile cotton rats (Sigmodon hisnidus). In addition, possible synergistic or antagonistic influences of protein restriction on contaminantinduced immune modulation were explored.

METHODOLOGY

A total of forty-one 47-day old juvenile cotton rats (mean weight at termination = 52.7 g ± 2.9(SE); range = 25.5 g to 91.0 g), representing 4 litters of 9 to 12 neonates each, were used in this study. All litters were conceived in the wild and born in our laboratory animal facility. Litters were weaned at 18 days post partum and at 21 days of age, litters were split and maintained on one of two isocaloric diets containing either 4% or 16% crude protein for a 26 day experimental period. Litters were further divided among 5 toxicant treatments resulting in a 2 X 5 factorial design. Diets and water were provided ad libitum and animals were maintained under natural light:dark conditions.

Experimental treatments included 3 benzene dosages (100, 500, or 1000 mg/kg body weight), Cyclophosphamide (CY) as a positive control (50 mg/kg body weight), and corn oil vehicle as a negative control, administered intraperitoneally (IP) for 3 consecutive days. None of the litters had exactly 10 individuals. In two litters with only 9 neonates, the 500 mg benzene dose was not replicated. In remaining litters with 11 and 12 neonates, the 500 mg benzene dose was preferentially ran in triplicate followed by triplicate runs of the negative controls. The first benzene dosage was administered in corn oil vehicle on days 15 to 17 of the experimental period. Positive and negative control animals concurrently received corn oil vehicle of equal volumes to benzene dosages. Cyclophosphamide (distilled water vehicle) was administered to positive control animals on days 22 to 24.

Animals were terminated and necropsies performed on day 27 and weights recorded for body, liver, paired adrenals, thymus gland, and spleen. Animals were anesthetized with an intramuscular injection of ketamine hydrochloride at 50 mg/kg body weight. Blood was collected from the retro-orbital sinus plexus into 3 ml vacutainers containing EDTA. White blood cell (WBC) counts were determined manually using a hemacytometer. Whole blood smears were prepared and differential cell counts made by classifying 100 Wright-Geisma stained leukocytes.

Animals were sacrificed by cervical dislocation while under ketamine anesthesia. Spleens were aseptically removed to a tared 15 x 60 mm sterile petri dish containing RPMI-S and weighed to the nearest 0.1 mg. RPMI-S was prepared by supplementing 100 ml of Roswell Park Memorial Institute (RPMI) 1640 medium with 1.025 ml L-glutamine (200 mM solution), 1.0 ml Na pyruvate (100 mM solution), 1.0 ml non-essential amino acids (100x solution), 1.0 ml penicillin/streptomycin solution (penicillin 10,000 U/ml and streptomycin 10 mg/ml), 100 ul 2-mercaptoethanol (50 uM prepared solution) and 11.5 ml normal horse serum. Spleens were cut into several pieces and gently disrupted in a sterile glass-on-glass homogenizer (0.15 mm clearance) containing 5 ml ice-cold RPMI-S. Cells were allowed to settle for 10 minutes and supernatant decanted into a sterile

16 x 125\m Xscrew cap culture tubes. Cells were centrifuged for 7 minutes at 10°C and 275 x g, supernatant decanted, and pellet resuspended in 5 ml of RPMI-S (this wash step performed 3 times). Viable cell counts were performed with a hemacytometer after lysing erythrocytes in tris-buffered 0.83% ammonium chloride (Tris/Cl) and staining with Trypan blue.

Lymphocyte proliferation was assessed by reduction of tetrazolium salt after in vitro mitogenic stimulation with concanavalin A (Con A) and pokeweed mitogen (PWM). Spleen cell suspensions were 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 hours at 37°C in a humidified incubator (5% Co2). After 69 hours, 10 ul of MTT (tetrazolium salt, 5 mg/ml in PBS) were added to each well, incubated the remaining 3 hours, and 160 ul of acid-isopropanol (176 ul concentrate HCl in 49.824 ml isopropanol) added to each well. Contents of each well were thoroughly mixed and absorbances at 570/630 nm were recorded against unstimulated controls as blanks.

Cotton rats were sensitized on day 19 with a percutaneous application of 100 ul of 3% 4-ethoxy-methylene-2-phenyl-oxazol-5 one (oxazolone) in absolute alcohol to shaved abdomens. Animals were challenged on day 26 with 50 ul of 3% oxazolone and absolute alcohol to the left afnd right ear, respectively. Immediately after sacrifice (24 hours post-challenge), both ears were removed and weighed to the nearest 0.1 mg. Delayed-type hypersensitivity response (DTH) was measured as percent weight change of the challenged (left) ear relative to the control (right).

A two-way analysis of variance (toxicant and diet level) with interaction was performed followed by a least-significant difference multiple range test for comparisons between means when main effects were significant. Apriori planned comparisons (single degree of freedom contrasts) were used in all analysis of variance procedures to compare means of major treatment components (benzene vs. negative control, CY vs. negative control, and 100 mg benzene vs. 1,000 mg benzene). Statistical differences between treatment and control groups were determined at the 10% level of significance.

FINDINGS

Initial mean body weight of all cotton rats included in the trial was 32.6 g $\pm$ 0.57 (SE). All cotton rats maintained on the 4% crude protein diet had significantly ($P < 0.001$) lower body weight at the end of the trial compared to the 16% crude protein group. Animals on the 4% protein diet essentially maintained their initial

body weight during the trial compared to those on the 16% diet, which approximately doubled their body mass. Mean body mass was not influenced by toxicant treatment ($\underline{P} > 0.100$). Relative liver weight, but not paired adrenal glands, was significantly ($\underline{P} < 0.001$) heavier in cotton rats on the 16% protein diet. Liver and paired adrenal glands were not significantly ($\underline{P} > 0.100$) influenced by toxicant treatment. Specific contrasts revealed relative liver weights to be heavier ($\underline{P} < 0.051$) in cotton rats dosed with 1000 mg benzene compared to those dosed with 100 mg benzene. Additionally, paired adrenal gland weights were heavier ($\underline{P} < 0.068$) for animals dosed with benzene compared to the negative control group.

Relative weights of thymus ($\underline{P} < 0.011$) and spleen ($\underline{P} < 0.066$) were significantly influenced by toxicant and there was a significant ($\underline{P} < 0.096$) toxicant by diet interaction for spleen weight. Both thymus and spleen weights were heavier ($\underline{P} < 0.003$) in 16% protein groups compared to 4% protein groups. Specific contrasts indicated that cotton rats exposed to CY had smaller thymus glands than negative controls ($\underline{P} < 0.004$). The same was true for spleen weights in animals consuming a 16% crude protein but not 4% protein diet ($\underline{P} < 0.017$). Cotton rats given benzene while on the 4% protein diet had heavier spleens than those in the control group ($\underline{P} < 0.032$).

Peripheral blood leukocyte counts ($\underline{P} < 0.001$) and absolute numbers of lymphocytes ($\underline{P} < 0.001$) were significantly influenced by toxicant. Mean number of lymphocytes and neutrophils varied significantly ($\underline{P} < 0.037$) between diets, with neutrophils more abundant and lymphocytes less abundant in animals on a 16% crude protein diet compared to a 4% protein diet. Total leukocytes and lymphocytes were significantly depressed by CY treatment compared to negative controls ($\underline{P} < 0.002$ and $\underline{P} < 0.001$, respectively). Total neutrophils were significantly ($\underline{P} < 0.096$) higher in cotton rats receiving benzene compared to negative control groups. Splenocyte yield, expressed as total splenocytes and splenocytes per mg of spleen, was significantly influenced by toxicant treatment ($\underline{P} < 0.003$ and $\underline{P} < 0.069$, respectively) and diet ($\underline{P} < 0.001$). Cotton rats fed 16% crude protein exhibited higher total and relative splenocyte yields compared to animals receiving 4% crude protein diets. Specific contrasts indicated that both measures of splenic cellularity were lower in CY exposed cotton rats than negative controls ($\underline{P} < 0.008$).

Optimum doses of Con A and PWM for splenocyte proliferation were 20 and 0.625 ug/ml of culture, respectively. No significant effects of toxicant or diet were observed at the optimum mitogen doses ($\underline{P} > 0.100$). However, specific contrasts showed that splenocyte proliferation in Con A (20 ug/ml) stimulated cultures were significantly ($\underline{P} < 0.066$) enhanced by CY treatment compared to negative controls. Splenocyte proliferation to a suboptimal dose of Con A (2.5 ug/ml) was significantly ($\underline{P} < 0.031$) influenced by experimental treatment, but not diet. Splenocytes from cotton rats treated with CY ($\underline{P}<0.005$) exhibited significantly less proliferative capacity than negative controls.

In vivo measurement of cellular immunity as measured by delayed-type hypersensitivity was significantly influenced by toxicant treatment ($P < 0.040$) and diet ($P<0.010$). Animals maintained on a 4% crude protein diet had greater DTH responses than those on the 16% diet. Specific contrasts indicated that cotton rats dosed with CY had a lower DTH response compared to negative controls ($P < 0.013$).

Except for relative spleen mass, no statistical diet by toxicant interactions were observed. Visual examination of several variables (WBC, lymphocytes, splenocyte proliferation) showed a deviation from the monotonic relationship associated with noninteractive effects. However, variation in response was pronounced in several cases and may have masked most statistically significant interactions.

CONCLUSIONS

Benzene and CY require metabolic transformation, which in turn, yields metabolites that are toxic to mitotic cells and can potentially target specific lymphocyte subpopulations (Snyder 1987). We observed increased total neutrophils and relative adrenal gland weight in cotton rats following acute exposure to benzene. Although benzene typically inhibits both T and B lymphocyte proliferation in laboratory rats and mice (Aoyama 1986), splenocyte proliferation and delayed-hypersensitivity did not appear to be sensitive to benzene exposure given our experimental design.

The general lack of benzene-induced immunotoxicity could be due to a number of different factors including dosage regimen (inhalation vs. injection), dosage schedule, or a general lack of benzene sensitivity in cotton rats. Animals in the present study were sacrificed 9 days after the last benzene dose to duplicate the time-lag factors associated with using in situ biomonitors which may require prolonged immunization schedules for certain assays (delayed-type hypersensitivity response). Benzene is rapidly metabolized and the hematopoietic system can potentially recuperate from benzene-like lesions within 3 days (Osmond and Everett 1964). This demonstrates the neccessary precautions which should be taken when choosing immunoassays for use on rodents collected from contaminated sites.

Cyclophosphamide was administered immediately prior to termination of the experiment as a positive control of our ability to detect changes in immunocompetence. Cyclophosphamide resulted in substantial immunologic lesions. Like benzene, the effects of CY are relatively short-lived and animals can recuperate quickly after the last CY dosage (Shand 1979). Relative immune organ weights and cellularity, circulating leukocytes, and indices of cell-mediated immunity (Con A-induced splenocyte proliferation, DTH responsiveness) were all

negatively impacted by CY. These results parallel findings of a number of other rodent studies (Dean et al. 1979; McMurry et al. 1991), and highlight the sensitivity of cotton rat immune function to xenobiotic contamination.

Twenty-six days of dietary crude protein restriction had a significant impact on most measures of immunocompetence. Relative weights of spleen, thymus, and liver, as well as neutrophil and eosinophil counts and splenic cellularity, were significantly depressed in animals on the 4% crude protein diet. In contrast, delayed-type hypersensitivity was enhanced in the proteinrestricted group. Enhanced DTH response is consistent with findings of other studies dealing with protein restriction in cotton rats (Vestey et al. 1993).

In the wild, small mammal populations routinely encounter periods of reduced quality of the available nutrients (White 1978). The 4% protein diets in this study represent a severe crude protein restriction as indicated by the lack of growth observed in the protein restricted group. Specially prepared experimental diets may potentially be less palatable than natural forages, although, the experimental diets used in our study appeared to be readily consumed.

Porter et al. (1984) demonstrated the complicated nature of the effects of malnourishment, infection, and chemical stressor interactions on growth and reproduction in laboratory and wild mice. In part, their results showed the potential synergistic action of marginal malnourishment on chemically stressed animals. We expected that synergistic and/or antagonistic interactions between diet level and toxicant exposure would be evident in our study. Relative spleen weight resulted in the only indication of a significant interaction between diet restriction and contaminant exposure. It appears that some degree of splenic hypertrophy resulted from benzene toxicity in cotton rats on the 4% protein diet which was not evident in the 16% protein group. No other statistically significant interactions were observed due possibly to the severity of the dietary restriction. Cotton rats maintained on 4% protein diets may have been immunosuppressed to the point that additional immunotoxic insults were not measureable or biologically significant. Additionally, severe protein restriction is known to impair metabolic processess often required to produce toxic metabolites from many xenobiotic compounds (Kalamegham et al. 1981). However, this was a factor based on the results with CY.

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