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# SENSITIVITY OF SELECTED IMMUNOLOGICAL, HEMATOLOGICAL, AND REPRODUCTIVE PARAMETERS IN THE COTTON RAT (*SIGMODON HISPIDUS*) TO SUBCHRONIC LEAD EXPOSURE

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ABSTRACT: The effect of lead exposure on cellular immunity, hematology, and reproductive and body condition in mature cotton rats (*Sigmodon hispidus*) was examined. Two groups of 36 cotton rats each were exposed to 0, 100, or 1,000 ppm lead in drinking water for either 7 or 13 weeks, between 31 August and 2 December 1990. Specific and non-specific cell-mediated immunity was assessed by measuring splenocyte proliferative responses to polyclonal mitogens (Concanavalin A and Pokeweed mitogen), in vivo 24-hr delayed-type hypersensitivity, metabolic activity of peritoneal macrophages, spleen mass and cellularity, and immune organ development. General physiological condition was assessed from hematological, morphological, and reproductive measures. Immune function was sensitive to lead exposure based on depressed proliferative responses of cultured splenocytes, smaller popliteal lymph nodes, and larger spleens among cotton rats receiving 1,000 ppm lead. Spleen mass was reduced in cotton rats receiving 100 ppm lead. Total leukocytes, lymphocytes, neutrophils, eosinophils, total splenocyte yield, packed cell volume, hemoglobin, and mean corpuscular hemoglobin were sensitive to lead exposure. Effects of lead exposure on general condition and reproductive parameters included reduced mass of liver, seminal vesicles, and epididymes in males following a 7-week exposure. Histopathologic changes reflected lead toxicity and included altered renal proximal tubular epithelium, renal intranuclear inclusions, and in some cases, lowered numbers of sperm and developing follicles. In general, lesions were more pronounced with increased lead concentration and longer exposure.

*Key words:* Cotton rat, *Sigmodon hispidus*, heavy metal, lead acetate, immunotoxicity, wildlife toxicology, ecotoxicology, bioindicator.

## INTRODUCTION

There has been increasing interest in the development of reliable *in situ* bioindicators for predicting potential hazards at the population, community, and ecosystem level. Numerous studies have been conducted to assess the toxicity of heavy metals, with lead as a principal compound of concern. The hazardous effects of lead on wildlife populations were recognized as early as 1874 when mortality of waterfowl and pheasants (*Phasianus colchicus*) in Texas and North Carolina (USA) was attributed to ingestion of spent lead shot (Hoffman et al., 1990). Although lead occurs naturally in the environment, human activities such as combustion engines and industrial emissions are responsible for most environmental lead contamination (Goyer, 1991). Inhalation of lead or consumption of lead-contaminated food and water comprise the principal routes of exposure. In addition to influencing the central nervous, hematopoietic, and renal systems (Goyer, 1991), lead also is a significant immunotoxin (Koller, 1990).

Although the data base on the immunotoxic properties of lead is growing, most studies have focused on inbred laboratory animals (Koller, 1990) and humans. In general, little information exists on the response of wildlife to environmental immunotoxicants. Examples of some exceptions include recent studies of the effects of lead shot on waterfowl immunity in which sensitivity of the immune system to lead was observed (Trust et al., 1990; Rocke and Samuel, 1991).

To adequately address the utility of wildlife species as environmental indicators, controlled laboratory studies are re-



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quired to assess their sensitivity to selected environmental contaminants. Our principal objective was to assess the sensitivity of the immune and hematopoietic systems of wild cotton rats (*Sigmodon hispidus*) to subchronic effects of exposure to inorganic lead. In addition, we were interested in documenting the effects of lead exposure on general morphological and reproductive condition of animals. We hypothesized that immune response parameters of cotton rats would be sensitive indicators of lead exposure.

## MATERIALS AND METHODS

Seventy-two adult cotton rats, with an initial mean (±SE) weight of 169.8 ± 5.0 g, were used from our outbred laboratory colony at Oklahoma State University, Stillwater, Oklahoma (USA). Animals within treatments were grouped by gender in triplicate and housed in polystyrene cages (43 by 27 by 19 cm) with wire lids and pine-chip bedding. Water and Purina Rodent Chow (Purina 5001, St. Louis, Missouri, USA) were provided ad libitum under natural light and dark conditions.

Cotton rats (36 males and 36 females) were randomly assigned to either a 7- or 13-wk subchronic exposure period, between 31 August and 2 December 1990. Within each exposure period, males and females further were assigned to one of three lead dose groups. This design resulted in a total of 12 animals (six male, six female) per lead dosage group for each exposure period. Lead exposure groups included lead acetate [$(CH_3COO)_2Pb \cdot 3H_2O$)] (Sigma Chemical Company, St. Louis, Missouri) administered at 0, 100, and 1,000 ppm in distilled drinking water provided ad libitum. Nitric acid (0.025%) was added to all three concentrations to maintain solubility of lead (Blakley and Archer, 1981). Daily intake of lead was estimated by measuring water consumption over a 24-hr period on four occasions. Total water consumption per cage was divided by the number of animals per cage and used to estimate daily lead consumption per individual.

At week 5 or 11 for exposure periods of 7 or 13 wk, respectively, delayed-type hypersensitivity response (DTH) was measured in cotton rats sensitized 8 days previously with a single percutaneous application of 100 μl of 3% 4-Et hoxy-methylene-2-phenyl-oxazol-5-one (oxazolone, Sigma Chemical Co.) in absolute alcohol to shaved abdomens (Jones, 1984). Animals were challenged 7 days post-sensitization with 50 μl of 3% oxazolone solution and absolute alcohol vehicle to the left and right ear, respectively. Twenty-four hours later, ear thickness was measured to the nearest 0.0254 mm using a Fowler micrometer (Model 52-222-001, Mitutoyo/MTI Co., Elk Grove Village, Illinois, USA). Hypersensitivity response was calculated as the percent increase in ear thickness of the challenged (left) ear relative to the control (right).

At the end of the experimental period of 7 or 13 wk, animals were anesthetized by Metofane inhalation (Methoxyflurane, Pitman-Moore, Mundelein, Illinois) until abdominal breathing was observed. Approximately 1 ml of whole blood was collected using heparinized microhematocrit tubes (75 mm by 1.1 mm inside diameter, Fisher Scientific, Pittsburgh, Pennsylvania) as a conduit, from the retro-orbital sinus plexus into 3-ml hematology vials (Becton Dickinson, Rutherford, New Jersey, USA) containing ethylenediaminetetraacetic acid (EDTA). Each blood sample was evaluated in a Serono-Baker automated hematology analyzer (Serono-Baker Diagnostics, Allentown, Pennsylvania, USA) and white blood cell count (WBC), red blood cell count (RBC), platelet count, packed cell volume (PCV), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) determined using standard settings for laboratory rats. Whole blood smears were prepared and differential leukocyte counts performed on 100 Wright-Giemsa-stained cells. Nucleated erythrocytes per 100 leukocytes were enumerated and the presence of basophilic stippling on erythrocytes was scored on a scale of 0 to 4: (0 = 0%, 1 = 1 to 2%, 2 = 3 to 5%, 3 = 6 to 15%, and 4 = >15% of erythrocytes with basophilic stippling).

Body length was measured to the nearest 1 mm from the point of the nose to the base of the tail; anesthetized cotton rats then were euthanized by cervical dislocation and body mass was recorded to the nearest 0.1 g. Necropsies included determining the mass to the nearest 0.1 mg of the following tissues: thymus gland, liver, spleen, uterus, paired adrenals, kidneys, popliteal lymph nodes, testes, epididymes, seminal vesicles, and ovaries. Mass of liver, thymus, kidneys, adrenals, spleen, and popliteal nodes was expressed relative to body mass (tissue mass divided by body mass, times 100). A general condition index was determined for each animal as the ratio of body mass to total body length (McMurry et al., 1994). In addition, the following tissues were stored in 10% neutral buffered formalin for histopathologic examination: adrenals, bone (femur), brain, epididymis, heart, kidney, liver, lung, lymph node, muscle, ovary, spleen, and testicle. All tissues (except bone) were embedded in paraffin wax, sectioned at 5 to 6

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μm and stained with hematoxylin and eosin (H&E). Sections of liver and kidney also were stained with Ziehl-Neelsen's and Fite's Ferraco to detect the acid fast inclusions (Luna, 1968). Bones were demineralized in 10% formic acid before being prepared for histopathology.

We used MEM-H medium, which consisted of 500 ml Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 5.125 ml of L-glutamine (Sigma, 200 mM solution), 5.0 ml of penicillin-streptomycin solution (Sigma, penicillin 10,000 U/ml and streptomycin 10 mg/ml), and 10,000 units of heparin (Sigma, 1,000 U/ml phosphate-buffered saline, PBS). Prior to necropsy, peritoneal macrophages were aseptically removed by reflecting the abdominal skin and injecting 20 ml of sterile ice-cold MEM-H into the peritoneum and vigorously palpating the abdomen for approximately 1 min. The cell suspension was removed with a 20 ml syringe and decanted into a 16 × 125 mm screw-cap culture tube. The cell suspension then was centrifuged for 8 min at 250 × *G* and 4 C, and the supernatant was decanted. The pellet was resuspended in 5 ml Tris-buffered 0.83% ammonium chloride (Tris/Cl, Sigma), incubated at 25 C for 5 min, underlayed with 1 ml sterile fetal bovine serum (FBS, Sigma), incubated an additional 2 min, and centrifuged as before. After decanting the supernatant, the pellet was resuspended in 5 ml of cold MEM-H-F (MEM-H with 10% FBS) and centrifuged. This wash step was performed twice. Total recovery of viable cells was determined by counting an aliquot of cells stained with Trypan blue (Sigma) using a hemacytometer (Fisher Scientific, Pittsburgh, Pennsylvania) and counting the four corner and center squares.

Spleens were aseptically removed, placed in preweighed 15 × 60 mm sterile petri dishes containing Roswell Park Memorial Institute medium containing supplements (RPMI-S) and weighed to the nearest 0.1 mg. Supplemented medium was prepared by supplementing 100 ml of RPMI 1640 medium (Sigma) with 1.025 ml L-glutamine, 1.0 ml sodium pyruvate (Sigma, 100 mM solution), 1.0 ml non-essential amino acids (Sigma, 100× solution), 1.0 ml penicillin-streptomycin solution, 100 μl 2-mercaptoethanol (Sigma, 50 μM prepared solution), and 11.5 ml horse serum (Sigma) (Lochmiller et al., 1993). Spleens were cut into three or four pieces and gently disrupted in 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 the supernatant was decanted into sterile 16 × 125 mm screw-cap test tubes. Cells were centrifuged for 7 min at 10 C and 275 × *G*, the supernatant was decanted, and the pellet was resuspended in 5 ml of RPMI-S; this wash step was performed three times. Viable cell counts were performed with a hemacytometer after lysing erythrocytes in Tris/Cl and staining with Trypan blue.

Macrophage metabolic activity was assessed by in vitro cellular reduction of nitroblue tetrazolium dye (NBT, Sigma) using the method of Southwick and Stossel (1986), with the following modifications. Peritoneal cell suspensions were adjusted to $10^6$ macrophages/ml in MEM-H-F and a reaction mixture prepared by combining 500 μl of cell suspension with 300 μl of prewarmed (37 C) NBT (1 mg/ml in PBS) in 16 × 100 mm borosilicate culture tubes. Reaction mixtures were mixed thoroughly and incubated for 15 min at 37 C in a humidified incubator with 5% $CO_2$. Metabolic activity was terminated by adding 8 ml of 0.5 N HCl, mixing the suspension and then centrifuging for 8 min at 10 C and 1,250 × *G*. The supernatant was aspirated off, and 1 ml of pyridine (99+% pure, Sigma) held at 25 C was added to each pellet. Culture tubes were placed in a dry bath and the reaction mixture refluxed for 15 min at 110 to 114 C prior to centrifugation as before. Absorbance was recorded at 515 nm against a pyridine blank using a Bauch and Lomb Spectronic 501 spectrophotometer (Milton Roy Company, Rochesterlaan, Belgium).

Lymphocyte proliferation, after in vitro mitogenic stimulation with Concanavalin A (Con A, *Canavalia ensiformis*; Sigma) and Pokeweed mitogen (PWM, *Phytolacca americana*; Sigma), was assessed by cellular reduction of tetrazolium salt (Mosmann, 1983). Spleen cell suspensions were adjusted to a final concentration of 500,000 cells/90 μl in RPMI-S. Five concentrations of each mitogen were added (10 μl volumes) to duplicate aliquots (90 μl) of the final cell suspension in 96-well, flat bottom microtiter plates. Five concentrations of each mitogen (μg/ml of culture) were used: 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 with 5% $CO_2$. After 69 hr, 10 μl of MTT (tetrazolium salt, 5 mg/ml in PBS; Sigma) were added to each well, incubated the remaining 3 hr and terminated by thoroughly mixing 160 μl of acid-isopropanol (176 μl concentrate HCl in 49.824 ml isopropanol) to each culture. Absorbances at 570 and 630 nm were recorded on a Titertek Multiskan Plus MK II (Flow Laboratories, McLean, Virginia, USA) compared to unstimulated cultures (Lochmiller et al., 1993).

Data were tested for homogeneity of variances (Levene's test) prior to analysis of variance (PROC GLM) (SAS Institute Inc., 1985). A three-way analysis of variance with lead dose group, exposure period, and sex as main factor effects

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was used to detect significant differences. Protected least significant difference (LSD) multiple range tests were used for mean comparisons when main effects differed (Steel and Torie, 1980). Single degree of freedom contrasts were used to test main effects of control versus lead, control versus 1,000 ppm lead, and control versus 100 ppm lead. In several cases, interactions between dose group and exposure period were evident but were not statistically significant due to variation. Under these circumstances, interactions were considered biologically significant and the single effect of lead dose was examined for each exposure period.

## RESULTS

There was no difference in estimated mean (±SE) daily water consumption by cotton rats between the 7-wk (24.0 ± 1.4 ml) and 13-wk (24.6 ± 1.1 ml) exposure periods ($P > 0.05$). However, daily water consumption was significantly ($P = 0.0001$) greater for cotton rats receiving 1,000 ppm lead (29.8 ± 2.0 ml) compared to 100 ppm (21.6 ± 0.9 ml) and control groups (22.2 ± 0.9 ml). Estimated daily lead consumption for cotton rats consuming 100 ppm lead was 2.1 ± 0.2 mg and 2.2 ± 0.1 mg for the 7- and 13-wk trials, respectively. Estimated daily lead consumption in the 1,000 ppm lead dosage group was approximately 15-fold greater at 28.8 ± 3.2 mg and 30.4 ± 2.6 mg during the 7- and 13-wk trials, respectively. Two animals died during the study from causes other than lead toxicity. No abnormal behavioral responses were observed during the course of the experiment.

Although glomeruli appeared normal under light microscopic examination of H&E stained sections, we observed uniform alterations in the renal proximal tubular epithelium of all rats given 1,000 ppm lead in both exposure periods. Most epithelial cells were enlarged and had irregular apical borders encroaching upon the lumina of the tubules with occasional necrosis of tubular epithelium and sloughing of cells into the lumina of tubules. Although not present in livers, intra-nuclear inclusions were present in kidneys of all cotton rats receiving 1,000 ppm lead for 7 and 13 wk. Inclusions were typically pink to red solitary spheres, 2 to 7 μm in diameter, and usually found in enlarged nuclei with marginated chromatin in the straight segments of proximal tubules. Inclusions were not acid-fast with Ziehl-Neelsen's method, but were acid-fast with Fite's Ferraco method. Animals receiving 100 ppm lead for 7 and 13 wk had these same tubular changes but not the renal lead inclusions. Control animals were free of inclusions and had no tubule lesions.

Testicular sections from three cotton rats consuming 1,000 ppm lead for 13 wk had marked reductions in sperm number (<40% of controls) and one animal in the 7-wk trial had no spermatogenesis. Likewise, during the 13-wk trial, three animals consuming 100 ppm lead had reduced sperm numbers (<60% of controls) with no spermatogenesis in one animal. Female reproductive organs also were affected, as ovaries from four animals on 1,000 ppm lead (three in 13-wk and one in 7-wk trial) consisted primarily of corpora lutea with few developing follicles. Only two animals receiving 100 ppm lead (7-wk trial) had the ovarian lesion. In comparison, control animals had numerous developing follicles with interspersed corpora lutea.

Centrilobular hepatocytes from treatment and control animals had sharp, clear vacuoles representative of fat within the cytoplasm resulting in peripheral displacement of the nucleus (fatty change). Other tissues including adrenals, brain, femur, epididymis, heart, lung, lymph node, spleen, and skeletal muscle appeared normal.

Initial and terminal body masses and condition scores of cotton rats did not differ ($P > 0.05$) among experimental treatments in either time exposure period. Condition scores were greater for males (119.1 ± 3.7) than for females (100.6 ± 3.2, $P = 0.0001$). Relative liver mass (g/g body mass times 100) was significantly influenced by experimental treatment ($P = 0.043$) and sex ($P = 0.0001$) with liver mass heavier in male (3.3 ± 0.2) compared to female

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cotton rats (2.4 ± 0.05). Based on specific contrasts, liver mass of cotton rats in the control group was significantly ($P = 0.018$) heavier compared to animals exposed to lead (Table 1). This effect was most prominent in the 7-wk trial ($P = 0.032$) compared to the 13-wk trial ($P > 0.05$). Other measures of general condition such as paired kidney and adrenal gland masses (mg/g body weight times 100) and total serum protein concentration did not differ ($P > 0.05$) by experimental treatment (Table 1). However, female cotton rats did have significantly heavier adrenals (33.5 ± 1.8, $P = 0.0001$) and kidneys (0.64 ± 0.01, $P = 0.002$) compared to male cotton rats (18.5 ± 0.8 and 0.58 ± 0.02, respectively).

Thymus, spleen, and paired popliteal node masses were included as gross measures of lead toxicity on primary (thymus) and secondary lymphoid organs (Table 1). Relative thymus mass was not significantly ($P > 0.05$) influenced by lead treatment, although there was a treatment by time interaction ($P = 0.071$). During the 13-wk trial, thymus mass was significantly ($P = 0.029$) heavier in control animals compared to those given 1,000 ppm lead. Spleen mass, but not popliteal node mass, was significantly influenced by lead treatment ($P = 0.0001$, Table 1) and there was a significant ($P = 0.014$) treatment by sex interaction. Spleen mass of control males was heavier ($P = 0.006$) than males in the 100 ppm lead group, and spleen mass of control females was lighter ($P = 0.0002$) compared to females on 1,000 ppm lead. Popliteal nodes were heavier ($P = 0.035$) in control animals compared to those receiving lead; this was most apparent in the 7-wk exposure group (Table 1).

Mass of seminal vesicles and epididymes were not significantly ($P > 0.05$) influenced by experimental treatment. However, mass of seminal vesicles was heavier ($P = 0.035$) in controls compared to lead-treated cotton rats (Table 1). In addition, seminal vesicles and epididymes were heavier ($P = 0.026$) in controls compared to those animals receiving 1,000 ppm lead. Differences for seminal vesicles and epididymes mass were restricted to the 7 wk- ($P = 0.020$) as opposed to the 13-week trial ($P > 0.05$). Masses of testes, coagulating gland, uterus, and ovaries were not influenced by experimental treatment ($P > 0.05$).

White blood cell counts and lymphocyte and eosinophil counts were significantly ($P = 0.040$) influenced by lead exposure with a significant ($P = 0.029$) treatment by time interaction for WBC (Table 2). Additionally, lymphocyte ($P = 0.009$) and eosinophil ($P = 0.008$) counts were higher for males (5.4 ± 0.8 and 0.53 ± 0.08 × $10^3/\mu l$, respectively) compared to females (3.0 ± 0.3 and 0.29 ± 0.06 × $10^3/\mu l$, respectively). During the 7-wk trial, WBC counts were higher ($P = 0.015$) in control animals compared to those on 100 ppm lead. During the 13-wk trial, WBC were higher ($P = 0.013$) in animals exposed to 1,000 ppm lead. Elevated lymphocyte counts were a significant ($P = 0.016$) contribution to the difference in WBC in cotton rats given 1,000 ppm lead compared to controls. Total eosinophil counts followed a similar trend as WBC, being higher ($P = 0.020$) in control animals than lead-exposed cotton rats during the 7-wk trial. Although neutrophil counts were not significantly ($P > 0.05$) affected by lead exposure, neutrophil counts were higher ($P = 0.038$) for control animals compared to those receiving lead during the 7-wk trial (Table 2). Total monocytes and basophils were not influenced ($P > 0.05$) by lead exposure.

Total splenocyte yield, but not splenocytes per mg spleen, was significantly ($P = 0.032$) influenced by lead exposure (Table 2). Total splenocyte yield from cotton rats exposed to 100 ppm lead was significantly reduced compared to control ($P = 0.046$) or 1,000 ppm lead ($P = 0.013$) groups.

Packed cell volume and HGB concentration were significantly ($P = 0.013$) influenced by lead exposure (Table 2) with depressed ($P = 0.024$) PCV and HGB values

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TABLE 1. Mean (SE) condition and reproductive parameters of cotton rats exposed to 0 ppm, 100 ppm, and 1,000 ppm lead acetate in drinking water for 7 or 13 weeks.

| Parameter | Lead concentration administered in water for 7 weeks | | | Lead concentration administered in water for 13 weeks | | |
|---|---|---|---|---|---|---|
| | 0 ppm | 100 ppm | 1,000 ppm | 0 ppm | 100 ppm | 1,000 ppm |
| Number sampled[a] | 11 | 12 | 12 | 12 | 11 | 12 |
| Liver (% g/g BW)[b] | 3.6 (0.4) | 2.8 (0.2)[c] | 2.7 (0.2)[c] | 2.9 (0.3) | 2.8 (0.3) | 2.5 (0.2) |
| Kidneys (% mg/g BW) | 655.0 (27.8) | 602.6 (24.6) | 660.7 (27.4) | 563.4 (23.4) | 570.8 (29.3) | 582.6 (10.4) |
| Adrenals (% mg/g Bw) | 30.9 (4.8) | 29.9 (3.4) | 27.2 (3.0) | 22.5 (1.6) | 22.8 (2.9) | 21.7 (2.3) |
| Total protein (g/dL) | 6.9 (0.2) | 7.0 (0.2) | 6.9 (0.1) | 7.0 (0.2) | 7.0 (0.1) | 6.7 (0.1) |
| Thymus (% mg/g BW) | 28.9 (6.5) | 42.9 (5.7) | 38.2 (9.2) | 43.3 (8.9) | 31.2 (4.3) | 20.2 (7.5)[c] |
| Spleen (% mg/g BW) | 97.7 (9.1) | 68.6 (4.7)[c] | 115.1 (11.4) | 103.5 (7.3) | 87.7 (7.6) | 170.3 (23.4)[c] |
| Popliteal nodes (% mg/g BW) | 3.4 (0.7) | 1.9 (0.2)[c] | 2.3 (0.4) | 2.5 (0.3) | 2.4 (0.4) | 2.3 (0.3) |
| Number sampled[c] | 6 | 6 | 6 | 6 | 6 | 6 |
| Testes (g) | 2.23 (0.13) | 2.11 (0.10) | 1.66 (0.31) | 1.56 (0.32) | 1.41 (0.34) | 1.40 (0.33) |
| Seminal vesicles (g) | 1.53 (0.23) | 1.22 (0.15) | 0.68 (0.16)[c] | 0.89 (0.31) | 0.64 (0.23) | 0.68 (0.25) |
| Epididymes (mg) | 550.6 (36.8) | 553.3 (37.6) | 375.7 (70.4)[c] | 376.7 (89.7) | 356.9 (70.2) | 324.0 (78.4) |
| Coagulating gland (mg) | 288.7 (53.0) | 249.0 (36.6) | 151.3 (46.2) | 57.1 (18.9) | 69.4 (22.9) | 54.4 (16.2) |
| Ovaries (mg) | 36.6 (7.3) | 32.6 (7.8) | 25.4 (4.5) | 35.9 (3.3) | 43.3 (7.0) | 36.1 (5.5) |
| Uterus (mg) | 264.3 (92.1) | 128.3 (35.0) | 133.1 (24.8) | 159.7 (36.0) | 179.9 (44.9) | 212.1 (33.2) |

[a] Exceptions include liver (0 ppm, 7 weeks, $n = 10$), total protein (100 ppm, 7 weeks, $n = 11$), and popliteal nodes (1,000 ppm, 7 weeks, $n = 9$).

[b] Mass of liver, kidneys, adrenals, thymus, spleen, and popliteal nodes expressed as: (organ mass/body mass) times 100.

[c] Values significantly ($P \leq 0.05$) different from controls (0 ppm).

[d] Exceptions include ovaries and uterus weights where $n = 5$ (0 ppm, 7 weeks and 100 ppm, 13 weeks).

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TABLE 2. Mean (SE) hematology parameters from cotton rats exposed to 0, 100, and 1,000 ppm lead acetate in drinking water for 7 or 13 weeks.

| Parameter[a] | Lead concentration administered in water for 7 weeks | | | Lead concentration administered in water for 13 weeks | | |
|---|---|---|---|---|---|---|
| | 0 ppm | 100 ppm | 1,000 ppm | 0 ppm | 100 ppm | 1,000 ppm |
| WBC ($\times 10^3/\mu l$)[b] | 7.55 (1.90) | 4.13 (0.64)[c] | 5.54 (0.71) | 5.98 (0.91) | 6.99 (1.07) | 13.98 (2.34)[c] |
| Lymphocytes ($\times 10^3/\mu l$) | 3.33 (0.36) | 2.50 (0.51) | 3.57 (0.54) | 3.16 (0.56) | 3.87 (0.76) | 8.80 (1.95)[c] |
| Neutrophils ($\times 10^3/\mu l$) | 3.58 (1.61) | 1.45 (0.23)[c] | 1.52 (0.24)[c] | 2.39 (0.45) | 2.60 (0.33) | 4.12 (1.05) |
| Eosinophils | 541 (145) | 109 (20)[c] | 341 (80) | 337 (72) | 429 (164) | 705 (151) |
| Splenocytes ($\times 10^6$) | 135 (18) | 91 (16)[c] | 162 (46) | 117 (10) | 98 (11)[c] | 150 (20) |
| Splenocytes/mg spleen ($\times 10^3$) | 927 (175) | 784 (80) | 767 (115) | 656 (67) | 628 (50) | 457 (32) |
| RBC ($\times 10^6/\mu l$) | 4.84 (0.21) | 5.03 (0.13) | 4.75 (0.13) | 4.78 (0.17) | 4.90 (0.13) | 4.56 (0.13) |
| PCV (%) | 33.3 (1.3) | 34.5 (0.7) | 32.4 (0.9) | 34.0 (1.0) | 33.9 (0.7) | 30.8 (0.7)[c] |
| HGB (g/dl) | 10.6 (0.4) | 10.7 (0.2) | 10.2 (0.3) | 10.4 (0.3) | 10.3 (0.1) | 9.4 (0.3)[c] |
| MCV ($\mu^3$) | 69.0 (1.2) | 68.9 (1.0) | 68.3 (1.1) | 71.5 (0.9) | 69.5 (1.2) | 68.0 (1.8) |
| MCH (pg) | 22.0 (0.4) | 21.4 (0.4) | 21.5 (0.5) | 22.0 (0.3) | 21.1 (0.5) | 20.6 (0.6)[c] |
| MCHC (%) | 31.9 (0.2) | 31.1 (0.3)[c] | 31.5 (0.3) | 30.7 (0.2) | 30.4 (0.4) | 30.3 (0.2) |
| PLT ($\times 10^3$) | 738 (44) | 718 (21) | 776 (30) | 863 (47) | 838 (53) | 895 (70) |
| Basophilic stippling | 0.18 (0.12) | 0.09 (0.09) | 1.67 (0.26)[c] | 0.17 (0.17) | 0.0 (0.0) | 1.75 (0.30)[c] |
| NRBC/100 WBC | 0.91 (0.34) | 2.0 (0.91) | 112.9 (73.7)[c] | 1.25 (0.55) | 1.27 (0.30) | 70.8 (33.0)[c] |

[a] Sample size ($n$) = 11 for 0 ppm in 7 weeks and 100 ppm in 7 and 13 weeks (exception is splenocytes and splenocytes/mg spleen where $n$ = 12 for 100 ppm in 7 weeks). Sample size = 12 for all other treatments.

[b] WBC = white blood cells; RBC = red blood cells; PCV = packed cell volume; HGB = hemoglobin; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PLT = platelets; NRBC = nucleated red blood cells.

[c] Values significantly ($P \leq 0.05$) different from controls (0 ppm).

in the 1,000 ppm lead group compared to control animals. Alterations in PCV and HGB were evident in the 13-wk trial ($P = 0.011$) but not the 7-wk trial ($P > 0.05$). Red blood cell counts, MCV, MCH, MCHC, and platelet counts were not significantly ($P > 0.05$) influenced by lead treatment, although RBC were higher for males ($P = 0.001$) and MCV ($P = 0.0003$) and MCH ($P = 0.003$) values were higher for females. Mean corpuscular hemoglobin was significantly ($P = 0.041$) depressed in cotton rats given 1,000 ppm lead compared to controls but the effect was evident in the 13-wk exposure group only ($P = 0.049$). Numbers of nucleated and basophilic stippled erythrocytes were significantly ($P < 0.001$) increased by lead treatment compared to controls (Table 2).

Lead exposure altered proliferative responses of splenocytes to mitogenic stimulation at all Con A ($P \leq 0.023$) and PWM ($P \leq 0.001$) doses (Fig. 1). Proliferative response of splenocytes to all doses of Con A ($P \leq 0.021$) and PWM ($P \leq 0.004$) was significantly reduced in cotton rats exposed to 1,000 ppm lead compared to animals receiving 0 and 100 ppm lead. The only exception was for 2.5 $\mu$g/ml Con A, where suppression in the 1,000 ppm lead group was less clear ($P = 0.05$). Splenocyte proliferation did not differ between animals receiving 0 and 100 ppm lead ($P > 0.05$) with the exception of 2.5 $\mu$g/ml Con A in the 7-wk trial ($P = 0.031$). Differences were more pronounced during the 13-wk trial compared to the 7-wk trial (Fig. 1).

No significant ($P > 0.05$) variation among treatments was observed for delayed-type hypersensitivity (DTH) response and metabolic activity of macrophages. Mean (±SE) hypersensitivity responses ranged from 20.7 (±2.8) to 40.9 (±4.8)% for animals on 1,000 ppm lead for 7 wk and 100 ppm lead for 13 wk, respectively. Relative metabolic activity (mean absorbance units ± SE) of macrophages ranged from 0.101 (±0.013) to

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0.258 (±0.056) for cotton rats on 1,000 ppm lead for 7 wk and 0 ppm lead for 13 wk, respectively. Mean (±SE) macrophage metabolic activity was significantly ($P = 0.032$) greater for males (0.201 ± 0.033) compared to females (0.112 ± 0.017).

## DISCUSSION

Based on our results, we conclude that exposure and severe lead-induced toxicity occurred on male reproductive organs, hematological parameters, lymphoid tissues, and cellular immune responses to polyclonal mitogens. Despite the comprehensive nature of these effects, we observed no gross indication of lead-induced toxicity in the cotton rats. Presence of acid-fast inclusion bodies is evidence of lead exposure (Zook, 1972); but we failed to observe central nervous system lesions or skeletal (femur) malformations with other histological results, presumably due to the use of adult animals (Goyer, 1991). Likewise, no behavioral anomalies were observed and, unlike Eyden et al. (1978), we observed no reduction in body mass or other condition indices in lead-exposed animals.

The toxic potential of lead on mammalian reproduction has been recognized for some time (Bell and Thomas, 1980). From our results, we infer that reproductive organ mass of males was sensitive to lead exposure; however the same was not true for females. In contrast, in histological examination of both sexes, we noted lesions similar to those found by Stowe and Goyer (1971) and Zook (1972). Interestingly, mass of male reproductive organs was most sensitive in the 7-wk trial when animals were reproductively active, although histological lesions were evident during both exposure periods. Cotton rats were maintained on a natural photoperiod and we believe that the mass of male reproductive organs in the 13-wk trial declined with decreased day length; thus, this measure probably was not a sensitive indicator of lead exposure. Reproductive condition of the animal should be considered if organ mass is used as an index of lead toxicity.

Lead exposure also had a significant and dynamic impact on the hematological profile of cotton rats as measured by reductions in total leukocyte numbers after 7 wk of lead exposure and elevated levels after 13 wk. Neutrophil and eosinophil counts were decreased in lead-treated animals during the early stages of lead toxicity (7 wk), resulting in a concomitant decrease in total leukocytes. However, with a longer exposure to lead, eosinophil counts recovered and, along with lymphocytes, increased in cotton rats exposed to 1,000 ppm lead. Lead is a sulfydryl binding agent that could potentially interfere with normal activation and proliferation of leukocytes (Dean and Murray, 1991). Recovery of leukocyte populations in lead-exposed cotton rats after 13 wk may be attributable to toxicant-induced proliferation of quiescent pluripotent hematopoietic stem cells following depletion of circulating differentiated cells as demonstrated with other toxicants (Synder, 1987).

Lead is well known for its capacity to cause anemia and induce abnormal erythrocyte morphology (Goyer, 1991). Although RBC, MCHC, and platelet counts were not measurably affected by lead exposure, reductions in PCV, HGB, and MCH levels by 13 wk of exposure to 1,000 ppm lead were evidence of anemia, which may impair oxygen transport capacity. The presence of a large number of nucleated erythrocytes in the systemic circulation was evidence for a shortening of erythrocyte life span; this is in agreement with previous studies (Waldron, 1966). Lead-induced anemia is not due completely to increased hemolysis but may involve an imbalance of potassium and sodium in the cell leading to decreased osmotic fragility and cell volume (Karai et al., 1981).

Recognition of the immunotoxic properties of lead on immune function has led to a plethora of toxicokinetic studies in laboratory animals (Koller, 1990). In addition to focusing on the effects of lead on

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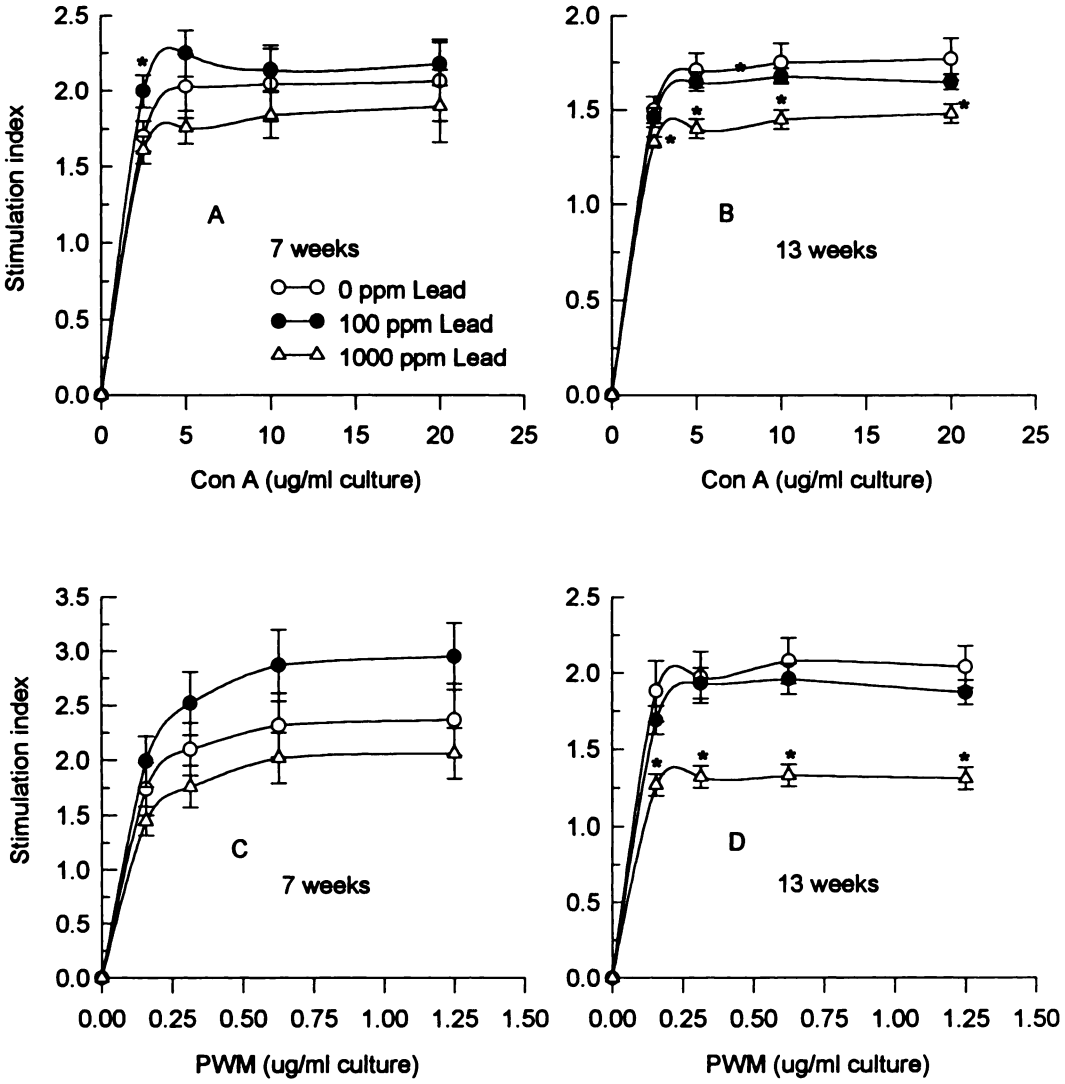



FIGURE 1. Mean (±SE) stimulation indices of splenocyte proliferation following in vitro stimulation with four concentrations each of Concanavalin A (Con A) (2.5, 5.0, 10.0, and 20.0 μg/ml culture; A and B) and Pokeweed mitogen (PWM) (0.156, 0.313, 0.625, and 1.25 μg/ml culture; C and D) in cotton rats consuming water containing 0, 100, and 1,000 ppm lead acetate for 7 or 13 weeks. Sample size ($n$) = 12 in all cases except 0 ppm lead for 7 weeks and 100 ppm lead for 13 weeks where $n$ = 11.

host resistance to pathogenic organisms (Dean and Murray, 1991), others also have evaluated the effect of lead on splenocyte proliferation, lymphoid tissue mass, humoral immunity, and macrophage function, with mixed results (Gaworski and Sharma, 1978; Faith et al., 1979; Koller et al., 1979; Blakley et al., 1980; Blakley and Archer, 1981). Although similar studies in wild rodent species are lacking, we infer from our initial results that, in general, immunotoxic responses (signs of toxicity and dose response) of cotton rats to lead ingestion are similar to a number of studies involving laboratory rodents.

We observed alterations in lymphoid organ mass, splenic cellularity, and proliferative capacity of splenic T- and B-lymphocytes to in vitro mitogenic stimulation; this varied with lead dose and exposure

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duration. Conversely, in vivo delayed hypersensitivity responses and metabolic activity of peritoneal macrophages remained unchanged regardless of lead dose or exposure period. Changes in lymphoid organ mass in cotton rats were similar to findings of Faith et al. (1979) who observed reductions in both actual and relative masses of thymus glands concomitant with increased spleen size in laboratory rats exposed to 25 and 50 ppm lead. Interestingly, spleen mass was reduced in cotton rats exposed to 100 ppm lead but increased in animals receiving 1,000 ppm lead. The mechanism for this is unclear, but may be related to lead-induced changes in extramedullary hematopoiesis in the spleen, enhanced by anemic conditions in animals receiving 1,000 ppm lead.

Lymphoproliferative responses to both Con A and PWM were the most sensitive indicators of lead-induced immunotoxicity. Comparisons with other studies were difficult because of the tremendous variation in experimental design among studies. Species, strain, and age factors undoubtedly act in combination with variations in dosage and duration and route of exposure to produce a variety of immune responses. Increased, decreased, or unaffected proliferative responses of T and B lymphocytes following lead exposure have been documented (Gaworski and Sharma, 1978; Faith et al., 1979; Koller et al., 1979; Blakley and Archer, 1982). Mixed responses among lymphocyte subpopulations also have been reported. For example, Koller et al. (1979), using 28-day-old CBA mice given 13 to 1,300 ppm lead in drinking water for 10 weeks, reported that Con A-induced T cell proliferation was minimally affected compared to significant suppression in B cell proliferation following stimulation with phytohemaglutinin.

Based on the depression of mitogen-induced proliferation of cotton rat splenocytes to both Con A and PWM, we believe that a functional impairment occurred for both T and B lymphocytes, especially among chronically exposed animals. Impairment of blastogenesis was not dependent on mitogen dose, as reduced proliferation was observed at all mitogen doses. Concanavalin A is a T-cell mitogen and stimulates T suppresser cells (Stites, 1984). Pokeweed mitogen stimulates B-cells and T-cells in a T-cell dependent fashion (Stites, 1984). Lead is a sulfydryl binding agent with a high affinity for subcellular sulfhydryl groups (Dean and Murray, 1991) which may block thiols associated with immune regulatory cells such as macrophages which are involved in lymphocyte activation, proliferation, and differentiation. Blakley and Archer (1981) provided support for this hypothesis in their study where the addition of 2-mercaptoethanol, an exogenous thiol reagent, restored normal plaque-forming cell activity in lead-exposed cultures. We included 2-mercaptoethanol in our culture media, yet lead treatment had no discernible impact on metabolic activity of macrophages. Thus our results differed from other studies which implicated macrophage function as pivotal to lead-induced immunotoxic lesions (Faith et al., 1979; Blakley and Archer, 1981).

Based on our results, cellular immune response, hematological response, and male and female reproductive organs of cotton rats were affected by lead exposure. As expected, lesions were more severe with increased dose and longer exposure to lead. Mean expectation of further life (average duration of residence) for cotton rats in wild populations ranges from approximately 8 to 14 wk (Peterson, 1973; Cameron, 1977); thus cotton rats inhabiting contaminated sites in the wild could encounter lead for periods of time shown to affect their physiological integrity.

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