TOXICITY RISKS ASSOCIATED WITH PETROCHEMICAL WASTES; BASED ON RESIDENT COTTON RATS (*SIGMODON HISPIDUS*)

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CHAPTER I

SENSITIVITY OF THE CFU-GM ASSAY TO MYELOTOXICANTS IN A WILD RODENT MODEL, *SIGMODON HISPIDUS*

ABSTRACT

Bone marrow is extremely sensitive to toxicants and *in vitro* culture of bone marrow progenitor cells has been shown to be a sensitive indicator of bone marrow injury in laboratory rodents. We explored the ability of a bone marrow progenitor cell assay to detect myelotoxicity in a wild rodent model (cotton rat, *Sigmodon hispidus*) that inhabits many contaminated ecosystems in the southern United States. Responsiveness of progenitor cells to recombinant murine GM-CSF and cotton rat lung-conditioned medium (LCM) was determined to optimize culture conditions for cotton rats. Myelotoxicity was induced in cotton rats by treating animals with either cyclophosphamide (8 or 80 mg/kg) or dexamethasone (500 ug/kg) over a five-day period. Exposure to the high dose of cyclophosphamide caused nearly total suppression of colony formation of granulocyte-macrophage progenitor cells (CFU-GM). Marked histological changes in both the bone marrow and spleen were also observed in cotton rats exposed to the high dose of cyclophosphamide. Although histological lesions were not apparent, the number of CFU-GM in the bone marrow of low-dose cyclophosphamide- and dexamethasone-treated

cotton rats was significantly suppressed compared to controls. The number of CFU-GM was consistently higher using LCM than recombinant murine GM-CSF. This reproducible, quantitative, *in vitro* bone marrow progenitor cell culture system was a sensitive indicator of myelotoxicity in wild cotton rats and should be useful for monitoring chronic exposures to low levels of environmental toxicants in wild rodent populations.

INTRODUCTION

Many of the contaminants that exist in the environment are potent immunotoxicants and exist as a diverse array of compounds (McMurry et al., 1994). Wild vertebrate models have proved useful as biomonitors of the deleterious effects of environmental contaminants for both human and wildlife populations (Elangbam et al., 1989; Faith et al., 1997; McBee et al., 1987; McMurry, 1993; Paranjpe et al.,1994). We have been particularly interested in using the hispid cotton rat (*Sigmodon hispidus)* as a biomonitor of the potential health effects from exposure to environmental contaminants associated with petrochemical wastes containing mixtures of heavy metals and hydrocarbons. Unfortunately, many of the biomarkers currently used to monitor exposure effects in wild vertebrate models like the cotton rat lack sufficient sensitivity to detect changes associated with chronic exposures to low levels of environmental contaminants (Hong et al., 1992). Thus, we have been exploring the use of tissues that tend to be more susceptible to toxic insults as biomarkers in wild rodent models.

Bone marrow has been shown to be one of the most susceptible tissues in the body to toxic insults, and *in vitro* culture of bone marrow progenitor cells has been used successfully as a sensitive indicator of bone marrow injury in laboratory mice (Chapin et al., 1989; Germolec et al., 1989; Hong et al., 1992). Assays of colony formation of hematopoietic cells often detect exposure to contaminants before one can measure actual hematological changes (Luster et al., 1985; Schurig et al., 1985). Evidence has accumulated with laboratory animals that low-level exposure to certain mixtures of environmental chemicals can produce myelotoxicity in the absence of other manifestations of toxicity in the parenchymal organs (Boorman et al., 1982). In particular, granulocyte-macrophage progenitor cells are often affected at lower-levels of exposure to chemical mixtures than other hematopoietic cell lines (Hong et al., 1992).

Although bone marrow cell cultures for colony-forming units-granulocyte-macrophage (CFU-GM) progenitor cells have been studied extensively in laboratory mice and humans, they have not been studied in wild rodents such as the cotton rat. The objective of this study was to develop a reproducible assay for evaluating responsiveness of bone marrow progenitor cells of the cotton rat to known myelotoxicants. Our primary interest was to develop an assay that could be used for a more comprehensive analysis of toxicity in wild cotton rats undergoing natural exposure to low levels of environmental petrochemicals on waste sites. Specifically, we defined the optimal conditions necessary for short-term culture, in semi-solid media, of CFU-GM from cotton rat bone marrow. Various sources of colony stimulating factor (CSF) were evaluated, including recombinant murine GM-CSF (Courtesy of Immunex, Seattle, WA) and lung-conditioned

media (LCM) from endotoxin-treated cotton rats and laboratory rodents. Validation of the assay was documented using the myelotoxicants cyclophosphamide and dexamethasone.

MATERIALS AND METHODS

Preliminary Experiment

We used a series of preliminary experiments to optimize culture conditions for use with cotton rat bone marrow. Preliminary exposure of cotton rats to cyclophosphamide at 100 mg/kg and dexamethasone at 500 ug/kg for five days was used to determine general sensitivity of the assay to severe myelosuppression. For CFU-GM cultures, we determined responsiveness of bone marrow cultures to recombinant murine GM-CSF at 100 and 200 ng/ml (Courtesy of Immunex, Seattle, WA) and media conditioned with the lungs of endotoxin-treated CD-1 laboratory mice, F-344 laboratory rats, and wild cotton rats (Sheridan and Metcalf, 1972).

Animals and Experimental Design

The sensitivity of the CFU-GM assay to known myelotoxicants was evaluated in an experimental trial using 32 male cotton rats weighing between 100 and 150 g (estimated average age approximately 3-6 mo) that were captured from the wild. Cotton rats were captured with live-catch traps (Sherman Traps Inc., Tallahassee, FL) baited with rolled oats. All cotton rats were taken to the laboratory animal facility, housed in polycarbonate cages with hardwood bedding, and provided food and water *ad libitum* under 12 light : 12 dark conditions at 23-24 °C during a 2-week acclimatization and 6-

day experimental period. Two groups of eight cotton rats each were given intraperitoneal (i.p.) injections of 0.5 ml cyclophosphamide (Sigma, St. Louis, MO) in saline at 8 mg/kg or 80 mg/kg for five consecutive days. Another group of eight cotton rats received daily i.p. injections of 0.5 ml dexamethasone (Sigma, St. Louis, MO) in saline at 500 ug/kg for five consecutive days. Eight cotton rats were used as controls, which received daily i.p. injections of 0.5 ml saline over the 5-day period. All injections were given between 9:00 AM and 10:00 AM.

Hematology and Histopathology

Twenty-four hours after the last exposure, cotton rats were anesthetized to a surgical plane with Metofane® (Pitman-Moore, Mundelein, IL). Whole blood was collected by periorbital sinus puncture. A heparinized blood sample (40 ul) was prepared for automated hematology analysis as previously described for cotton rats (Robel et al., 1996). Blood smears were prepared and differential cell counts were made by classifying 100 leukocytes. Animals were killed by cervical dislocation while still anesthetized. A complete necropsy was performed and major lymphoid organs and bone marrow were collected in 10% neutral-buffered formalin and embedded in paraffin for histopathological examination. Histopathological examination of bone marrow, spleen, and mesenteric lymph node was performed to evaluate the immunotoxic potential of the tested chemicals.

CFU-GM Assay

Bone marrow cells were collected from cotton rats by aseptically dissecting both femurs from the surrounding tissue, cutting them at the epiphysis, and flushing the shaft of the femur with 3.0 ml of RPMI-1640 (Sigma, St. Louis, MO). Single-cell suspensions were made by progressively passing the cells through a 22-gauge needle. Bone marrow cells were counted on an automated hematology analyzer (Serono-Baker Diagnostics, Allentown, PA) that was previously calibrated for cotton rats. White blood cell counts were determined and the cells adjusted to 1×10^5 cells/ml in growth medium (RPMI-1640 with 20% fetal bovine serum, 5% human AB serum, 200mM L-glutamine, 50 ug/ml garamycin, 1.5% methylcellulose, and 10% LCM or 100 ng recombinant murine GM-CSF). Recombinant murine GM-CSF and endotoxin-treated cotton rat LCM were used at optimal concentrations to reach the best growth conditions. Six-well flat-bottom (35 mm) tissue-culture plates in triplicate were incubated for 3 days at 37°C in a 5% CO_2 humidified incubator. After incubation, plates were examined under an inverted microscope and the total number of colonies (>50 cells/colony) per well were counted.

Preparation of Lung-Conditioned Medium

To prepare cotton rat LCM, 250 ug/100g of lipopolysaccharide endotoxin (LPS from *E. coli* serotype 0127:B8, Sigma, St. Louis, MO) was injected i.p. into each cotton rat. After one hour, the lungs were aseptically removed, chopped, and incubated in 15 ml of serum-free MEM (Sigma, St. Louis, MO) with antibiotics for 48 hours in a 5% CO_2 humidified incubator at 37°C. After incubation, the medium was filtered through both

0.45 um and 0.2 um filters that were pretreated with 5 ml of 0.05% polyethyleneglycol (PEG). The media were pooled, mixed, and frozen in aliquots at -70°C.

Statistical Analysis

All data were tested for normality (Proc Normal; SAS, 1994) and homogeneity of variances (Levene's test) prior to analysis (Steel et al., 1997). Data not meeting these assumptions were transformed (square-root or arcsine) prior to further statistical analysis. We used square-root (eosinophil count, CFU-GM using cotton rat LCM) and arcsine of square-root (CFU-GM using recombinant murine GM-CSF) transformations prior to statistical analyses, but we report untransformed means in the text. Statistical significance was determined by one-way analysis of variance (ANOVA; PROC GLM, SAS, 1994). When a significant main factor effect was indicated, Duncan's multiple-range test was used to determine significant differences among treatment means at $p < .05$. All values are presented as means ± SD.

RESULTS

Preliminary laboratory experiments were conducted to optimize the CFU-GM assay for use with bone marrow cells of cotton rats. Since recombinant cotton rat GM-CSF was not commercially available, we examined the responsiveness of bone marrow cultures of the cotton rat to various sources of GM-CSF. We evaluated CFU-GM development in bone marrow cultures of cotton rats using lung-conditioned media obtained from laboratory mice, F-344 rats, and cotton rats as a source of GM-CSF.

Cotton rat CFU-GM responsiveness was evident with both LPS-treated rat-lung and LPS-treated mouse-lung conditioned media. Mouse LCM showed about 62% cross-species reactivity of GM-CSF activity with cotton rat LCM. Colony formation of cotton rat bone marrow cells was highest in cultures with cotton rat LCM. In another preliminary experiment, we determined the optimal concentration of recombinant murine GM-CSF for use in bone marrow cultures of cotton rats. Optimal numbers of CFU-GM were obtained with an approximate final concentration of recombinant murine GM-CSF of 100 ng/ml culture media.

The number of CFU-GM was enumerated directly off the plates using an inverted microscope. Most colonies were at the surface of the semi-solid media and the size and shape of CFU-GM colonies varied markedly (Figure 1). The number of cells in each CFU-GM colony varied from 50 to several hundred cells/colony. Morphologically, the most common types of CFU-GM colonies were spherical with tight centers surrounded by peripheral areas of dispersed cells (Figure 2). Another type of colony was a dispersed colony which was larger in size but cells were loosely arranged with minimal adherence (Figure 3). Variation in size and shape of CFU-GM colonies is associated with the differentiation of the cell population comprising the colonies (Deldar et al., 1988; Dunn et al., 1978; Metcalf, 1970). Small, compact colonies consist mostly of immature monocytes, while dispersed colonies are mainly composed of mature granulocytes.

Treatment had no appreciable impact on body mass, relative mass of spleen, and hematology of cotton rats, with exception of lymphocyte counts (Tables 1 and 2). Lymphocyte counts showed a significant ($p < .0087$) treatment effect, where high-dose cyclophosphamide-treated cotton rats showed significantly decreased (60%) lymphocyte

counts compared to controls. Four of eight cotton rats treated with 80 mg/kg cyclophosphamide had numerous neutrophils containing *Hepatozoon spp.* organisms.

The number of CFU-GM using cotton rat LCM was 63% higher compared to recombinant murine GM-CSF (Table 3). A significant ($p < .0001$) treatment main effect was evident for the number of CFU-GM using both cotton rat LCM and recombinant murine GM-CSF. A dose-related reduction in colony formation was observed in cyclophosphamide-treated cotton rats. Using LCM, both cyclophosphamide groups and dexamethasone group showed a significant decrease in CFU-GM formation compared to controls. The number of CFU-GM using murine GM-CSF was significantly decreased in both cyclophosphamide-treated groups, but there were no differences between the dexamethasone-treated group and controls.

Histopathological changes were observed in cotton rats exposed to 80 mg/kg cyclophosphamide, including hypocellularity of bone marrow which was nearly void of developing hematopoietic cells (Figures 4 and 5). Spleens of cotton rats exposed to 80 mg/kg cyclophosphamide showed marked changes in lymphoid follicles which were sharply demarcated from red pulp with no germinal centers (Figures 6 and 7). Mesenteric lymph node showed similar changes where lymphoid follicles were obscure with no visible germinal centers (Figures 8 and 9). Cotton rats treated with cyclophosphamide at 8 mg/kg or dexamethasone showed no obvious histopathological changes in these immune tissues.

DISCUSSION

Success in culturing a colony of bone marrow cells is highly dependent on the use of media supplemented with CSF (Robinson et al., 1967), which has been demonstrated in a variety of tissues (Foster et al., 1968; Robinson et al., 1967, 1969; Sheridan and Stanley, 1971). Injection of mice with bacterial lipopolysaccharide (LPS) elicits acute rises in serum CSF levels and a marked granulocytopenia (Metcalf, 1971; Quesenberry et al., 1972). Medium conditioned by lung tissue from LPS-injected mice has been shown to be a rich source of CSF with high activity for stimulating colony formation in semi-solid cultures of mouse bone marrow cells (Sheridan and Metcalf, 1973). GM-CSF is of mesenchymal or macrophage origin and is produced at an early stage of the host response after the initial inflammatory stimulus is encountered (Bilyk and Holt, 1993) by endothelial cells, fibroblasts, osteoblasts, tracheal epithelial cells, and macrophages (Churchill et al., 1992; Horowitz et al., 1989; Seelentag et al., 1987; Smith et al., 1990; Thorens et al., 1987; Zucali et al., 1987). GM-CSF has the ability not only to induce proliferation and differentiation of hematopoietic progenitor cells but to enhance proliferation and survival of neutrophils, eosinophils, and macrophages (Lin et al., 1989; Lopez et al., 1986; Sonoda et al., 1989). In our study, optimal colony formation was obtained when cotton rat lung-conditioned medium was used as a source of GM-CSF.

We used both cyclophosphamide and dexamethasone to validate the CFU-GM assay in cotton rats because they both have myelotoxic properties. In our study hypocellularity of bone marrow and significantly suppressed lymphocyte counts were evident in cotton rats treated with 80 mg/kg cyclophosphamide for five days, in agreement with previous reports (Mackova and Suliova, 1986; Suliova et al., 1986). McMurry et al. (1994) reported that cotton rats exposed to 50 mg/kg cyclophosphamide

for 3 days had significantly lower neutrophil counts than controls, without a change in relative weight of spleen. A plausible explanation for the discrepancies between our study and theirs could be due to variations in experimental conditions including route of dosage, vehicle, duration of exposure, and dosage. It is plausible that exposure to low-dose cyclophosphamide and dexamethasone was not sufficient to cause sustained eosinopenia and lymphocytopenia but was sufficient to cause early regenerative activity of bone marrow. Cyclophosphamide is a chemotherapeutic immunosuppressive agent with toxic effects on production of bone marrow progenitor cells (Fried and Johnson, 1968; Hill, 1975; Pannacciulli et al., 1977). It also affects other lymphoid organs such as lymph nodes, spleen, and thymus. It has been reported that cyclophosphamide dosed to cotton rats three days/wk at 50 mg/kg can induce immunosuppression (Johnson et al., 1982; McMurry et al., 1991, 1994). Result is a marked decrease in the number of lymphocytes in the thymus and spleen which are the major targets for the cyclophosphamide action (Suliova et al., 1986). Erythroid cells in the bone marrow and in the spleen are the next most susceptible cell population. Ninkov and piletic (1974) and Berger (1982) observed that repeated doses (5 mg/kg and 15 mg/kg) of cyclophosphamide caused suppression of erythropoiesis but not granulopoiesis in laboratory rats. Laboratory rats given a single dose of cyclophosphamide of 120 to 150 mg/kg i.p. have a profound decrease in eosinophils from the bone marrow (Ottolenghi and Barnett, 1974). Suppression of hematopoiesis is proposed to be due to the injury of the stem cell compartment which is very sensitive to cytostatic agents (Fried and Barone, 1980; Hellman and Grate, 1971). Laboratory mice receiving > 30 mg/kg cyclophosphamide experience a progressive reduction in bone marrow cellularity and

number of colony-forming cells similar to our observations in cotton rats (Brown and Carbone, 1971; Fried et al., 1973).

Blenkinsopp and Blenkinsopp (1967) reported that single or continuous administration of dexamethasone reduced the number of eosinophils in blood, bone marrow, and tissue. Dexamethasone causes stimulation of bone marrow erythroid cells through stimulation of kidney erythropoietin production (Malgor et al., 1974). Dexamethasone also inhibits the proliferation of bone marrow stromal cells, which influence the differentiation of hematopoietic stem cells and their progeny (Locklin et al., 1995).

The presence of *Hepatozoon* spp. protozoa in neutrophils of cotton rats exposed to 80 mg/kg cyclophosphamide was observed, which may be caused by immunosuppression (Pannacciulli et al., 1977).

Marked reduction (> 90%) of CFU-GM was observed in cotton rats exposed to 80 mg/kg cyclophosphamide compared to controls. Histopathological examination revealed significant changes in bone marrow, spleen, and lymph node characterized by hypocellularity and lymphoid follicles containing no visible germinal centers as observed by Mackova and Suliova (1986). Among these organs, bone marrow was the most sensitive. Although a reduction of 22 to 48 % of CFU-GM was observed in cotton rats exposed to low-dose cyclophosphamide and dexamethasone, we did not observe any significant changes in other biomarkers such as body and organ weights, hematology, and histopathology.

Results from this study indicate that bone marrow of the cotton rat is as sensitive as laboratory rodents (Chapin et al., 1989; Germolec et al., 1989; Hong et al., 1992).

Quantitative evaluation of colony formation of the bone marrow progenitor cells following exposure to cyclophosphamide and dexamethasone detected myelotoxicity before we could detect histopathological or hematological changes at lower doses (Luster et al., 1985; Schurig et al., 1985). Our findings demonstrated that bone marrow of cotton rats could serve as a more sensitive indicator for detecting adverse health effects of certain toxic chemicals than other more traditional biomarkers. We suggest that CFU-GM assays may provide a sensitive indicator for detecting environmental contamination in field studies investigating the health effects from long-term exposure to hazardous wastes generated by the petroleum industry.

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Table 1. Mean (± SD) body weight (BW) and relative weight of the spleen in cotton rats treated with cyclophosphamide (CY; 8 or 80 mg/kg) or dexamethasone (500 ug/kg) for five consecutive days (n = 8 per group)

Experimental group	Body wt. (g) (before dose)	Body wt. (g) (after dose)	Spleen (g/kg)
Control	114.0 ± 23.9[a]	112.4 ± 19.9[a]	1.6 ± 0.3[a]
Dexamethasone	116.1 ± 32.6[a]	116.4 ± 29.0[a]	1.5 ± 0.5[a]
CY (low dose)	113.4 ± 24.7[a]	114.1 ± 23.6[a]	1.3 ± 0.6[a]
CY (high dose)	113.3 ± 26.0[a]	108.3 ± 21.2[a]	1.1 ± 0.5[a]

Note. Means within a column followed by the same letter are not significantly different at $p < .05$.

Table 2. Mean (± SD) white blood cell (WBC) and differential counts ($x10^3/mm^3$) of cotton rats treated with cyclophosphamide (CY; 8 or 80 mg/kg) or dexamethasone (500 ug/kg) for five consecutive days (n = 8 per group)

Experimental group	WBC	Neutrophil	Lymphocyte	Eosinophil	Monocyte
Control	6.90 ± 2.67[a]	2.24 ± 0.89[a]	4.21 ± 1.95[a]	0.28 ± 0.19[a]	0.17 ± 0.15[a]
Dexamethasone	9.39 ± 4.80[a]	3.72 ± 2.55[a]	4.86 ± 2.48[a]	0.40 ± 0.36[a]	0.38 ± 0.30[a]
CY (low dose)	11.29 ± 5.12[a]	4.70 ± 2.76[a]	5.55 ± 2.73[a]	0.42 ± 0.48[a]	0.61 ± 0.38[a]
CY (high dose)	6.19 ± 6.51[a]	4.09 ± 5.08[a]	1.67 ± 1.21[b]	0.10 ± 0.09[a]	0.32 ± 0.53[a]

Note. Means within a column followed by the same letter are not significantly different at $p < .05$.

Table 3. Mean (± SD) number of CFU-GM/10^5 bone marrow cells of cotton rats treated with cyclophosphamide (CY; 8 or 80 mg/kg) or dexamethasone (500 ug/kg) for five consecutive days using recombinant murine GM-CSF and cotton rat lung-conditioned medium (LCM) (n = 8 per group)

Experimental group	Lung-conditioned medium	Murine GM-CSF
Control	76.3 ± 15.5^{a}	48.1 ± 9.9^{a}
Dexamethasone	44.7 ± 11.8^{b} (58.6%)	37.4 ± 16.4^{ab} (77.8%)
CY (low dose)	52.8 ± 5.5^{b} (69.2%)	25.4 ± 6.9^{b} (52.9%)
CY (high dose)	6.4 ± 7.8^{c} (8.4%)	3.2 ± 4.8^{c} (6.7%)

Note. Means within a column followed by the same letter are not significantly different at $p < .05$. Number in parentheses indicates the percentage of the control value.

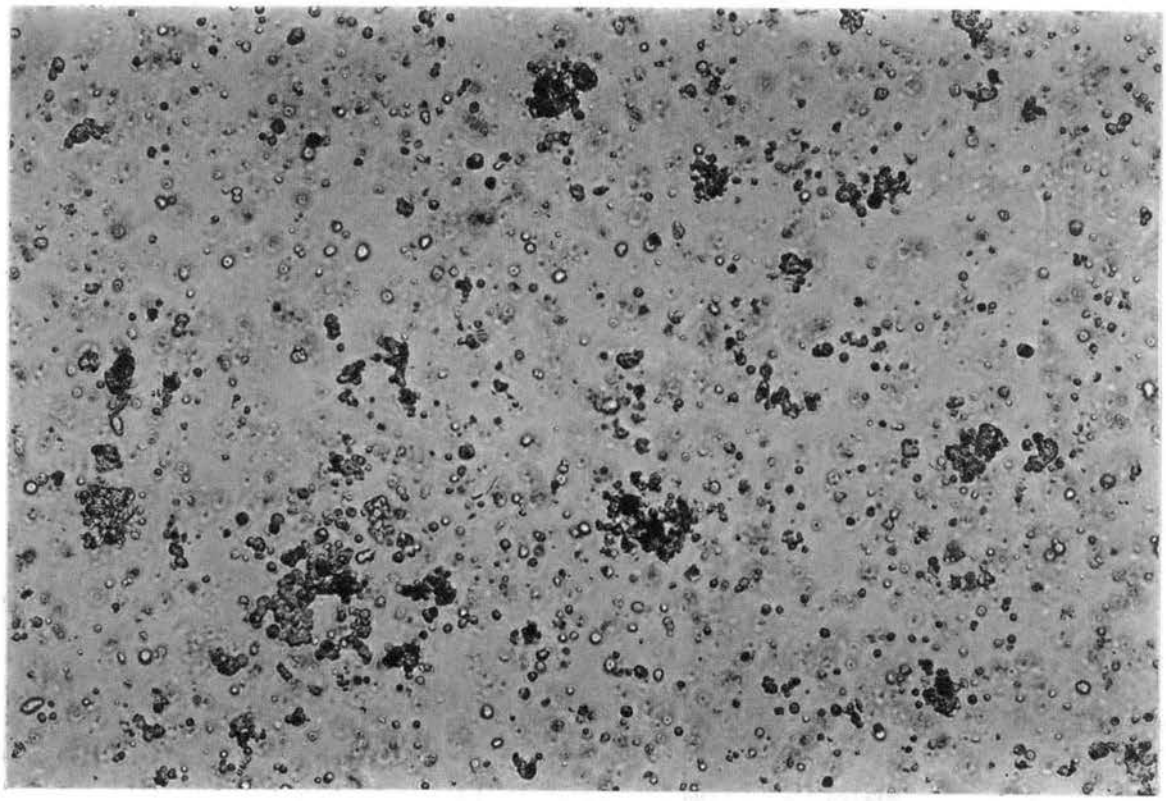

FIGURE 1. CFU-GM colonies from a cotton rat. The size and shape of colonies varied markedly. ×40.

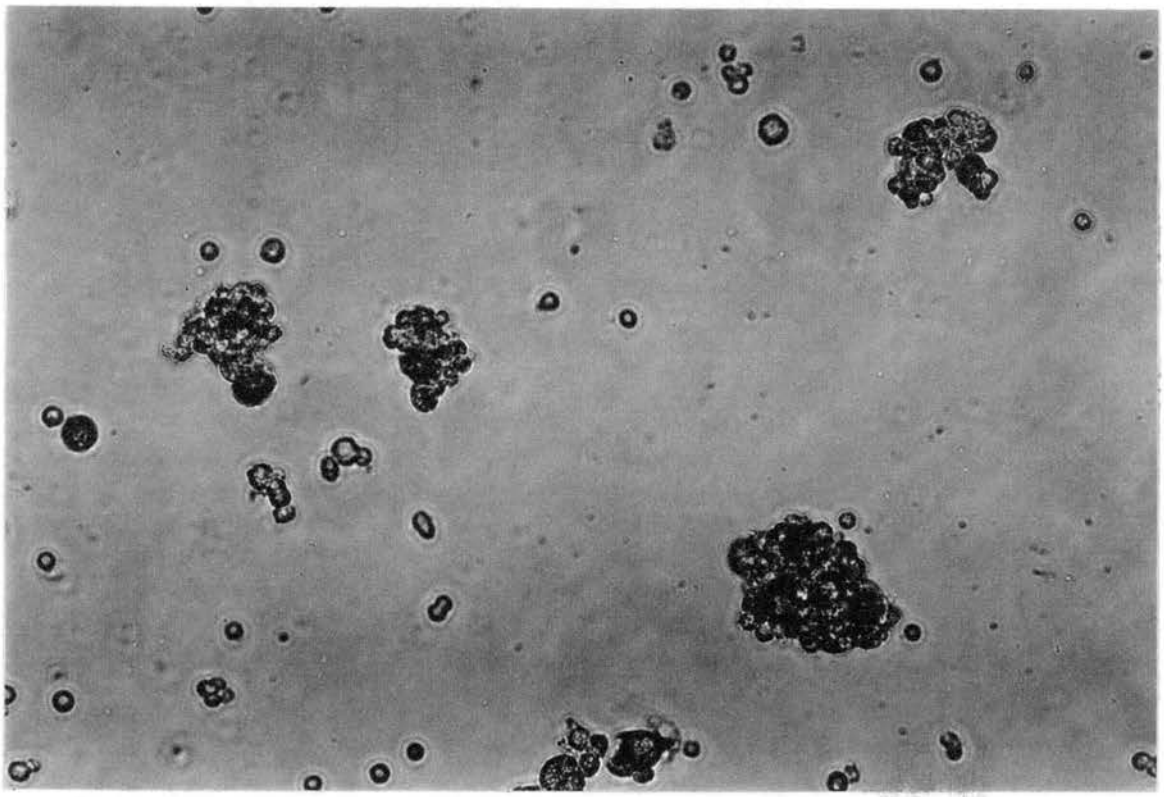

FIGURE 2. CFU-GM colonies from a cotton rat. The compact colony is small and spherical with tight center. ×200.

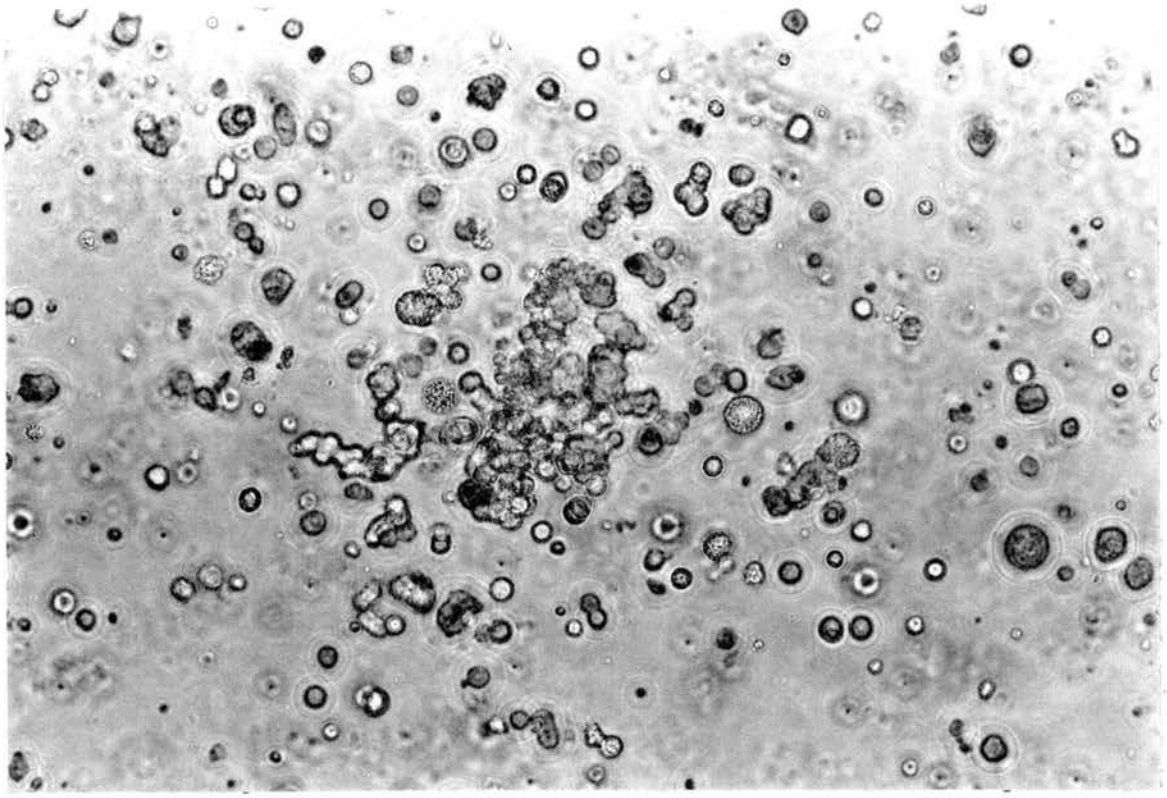

FIGURE 3. CFU-GM colonies from a cotton rat. The dispersed colony is larger in size and loosely arranged with minimal attachment to other cells. ×200.

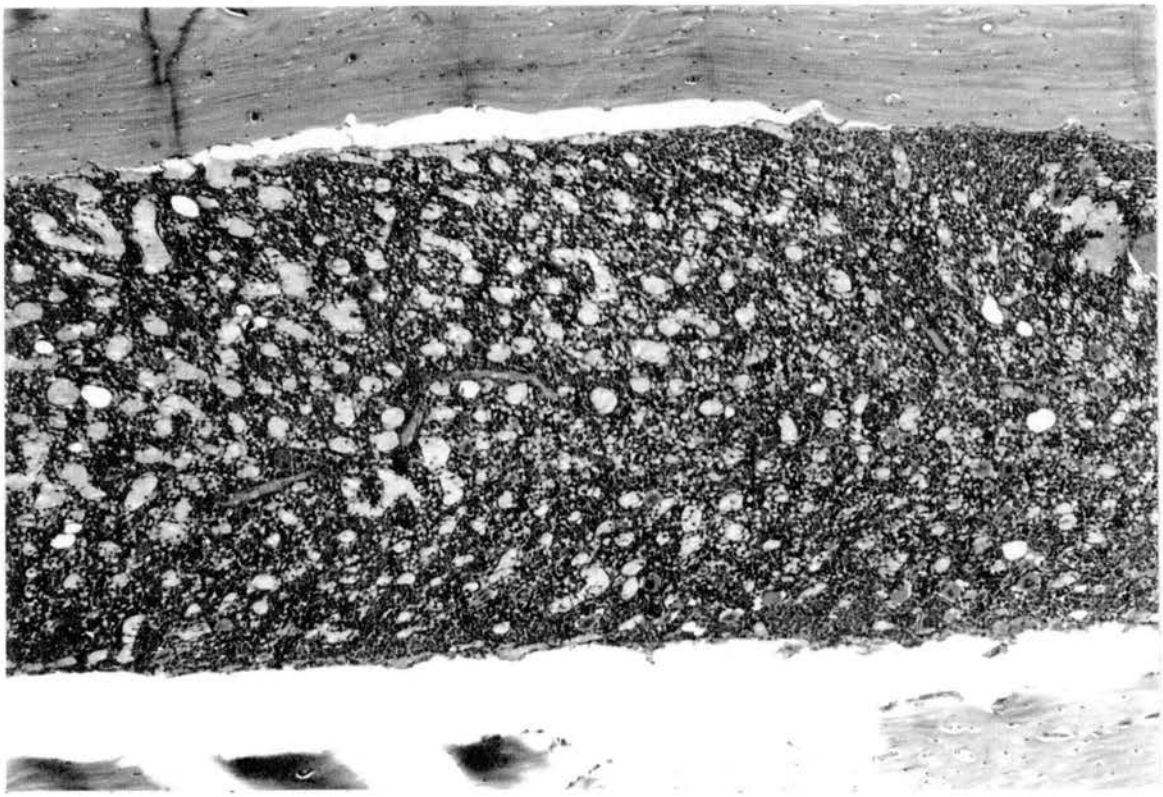

FIGURE 4. Histological section of bone marrow from a control cotton rat. Bone marrow is filled with hematopoietic cells. H&E. ×60.

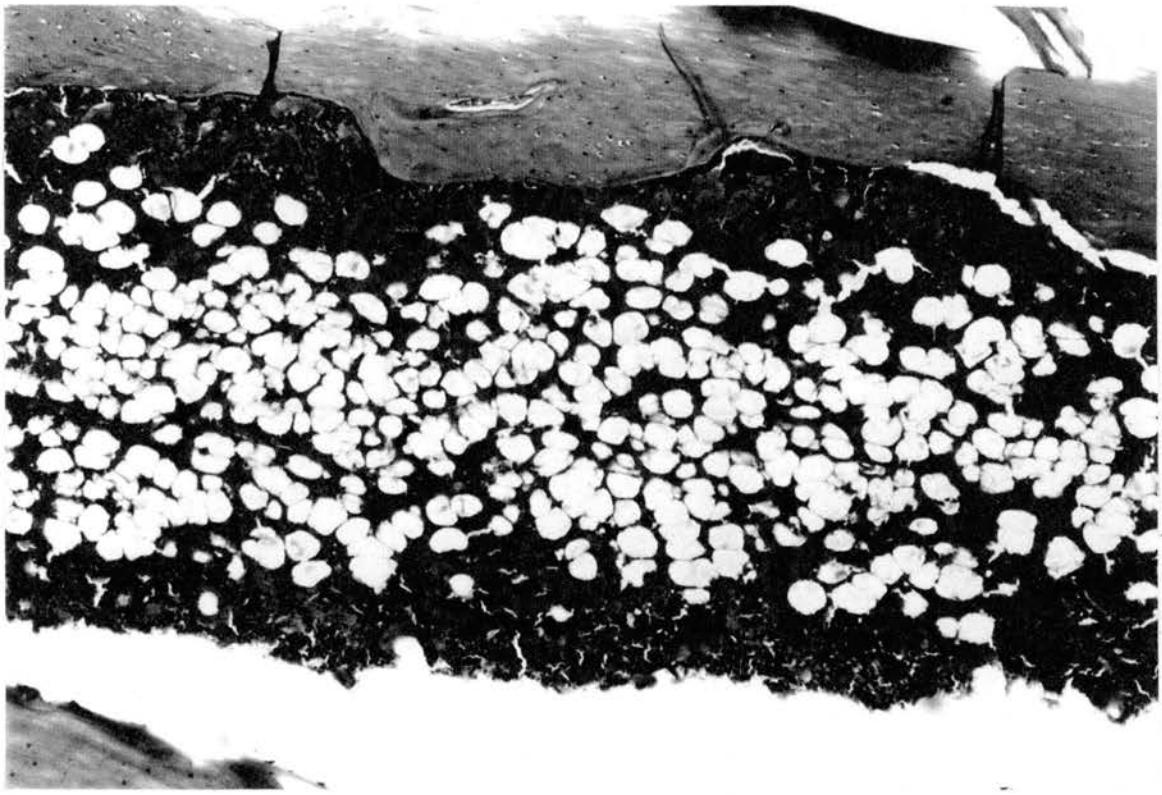

FIGURE 5. Histological section of bone marrow from a cotton rat exposed to 80 mg/kg cyclophosphamide for 5 days. Bone marrow is very hypocellular and nearly void of developing hematopoietic cells. H&E. ×60.

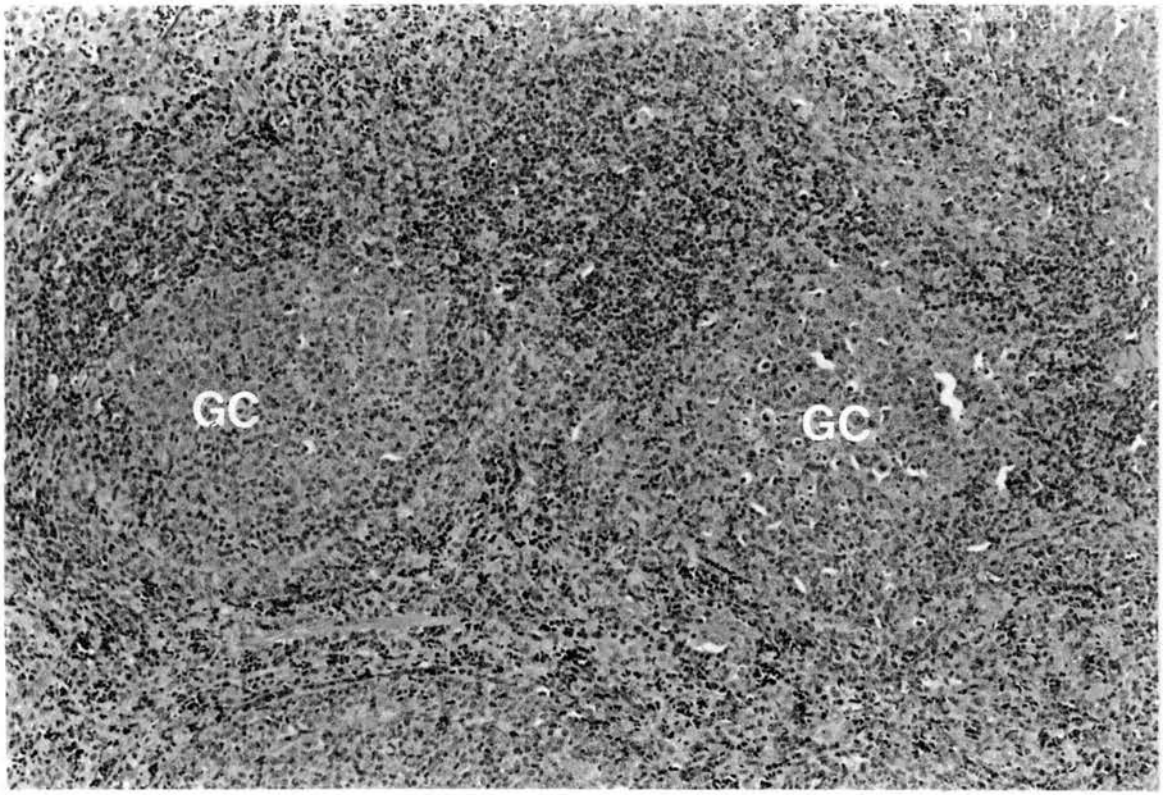

FIGURE 6. Histological section of spleen from a control cotton rat. Micrograph shows lymphoid follicles with less intensely stained germinal centers (GC). H&E. ×130.

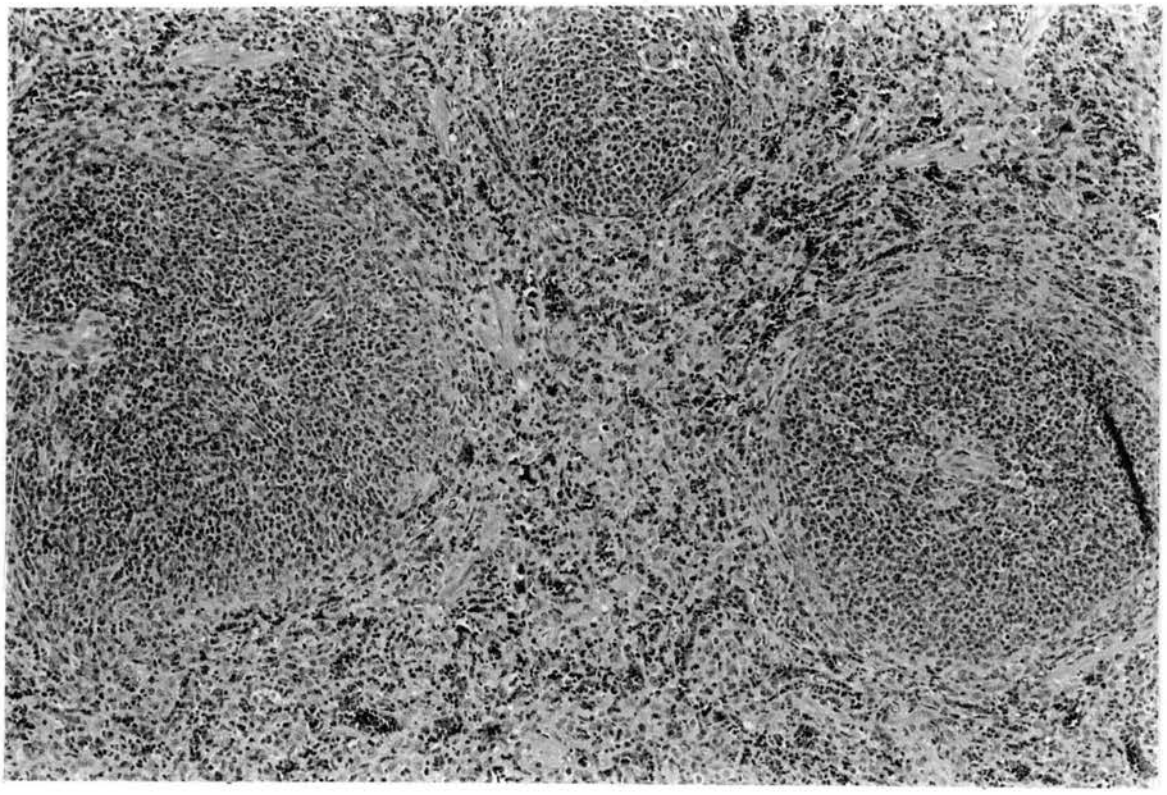

FIGURE 7. Histological section of spleen from a cotton rat exposed to 80 mg/kg cyclophosphamide for 5 days. Compare it to fig. 6. Note marked changes in lymphoid follicles with no germinal centers. H&E. ×130.

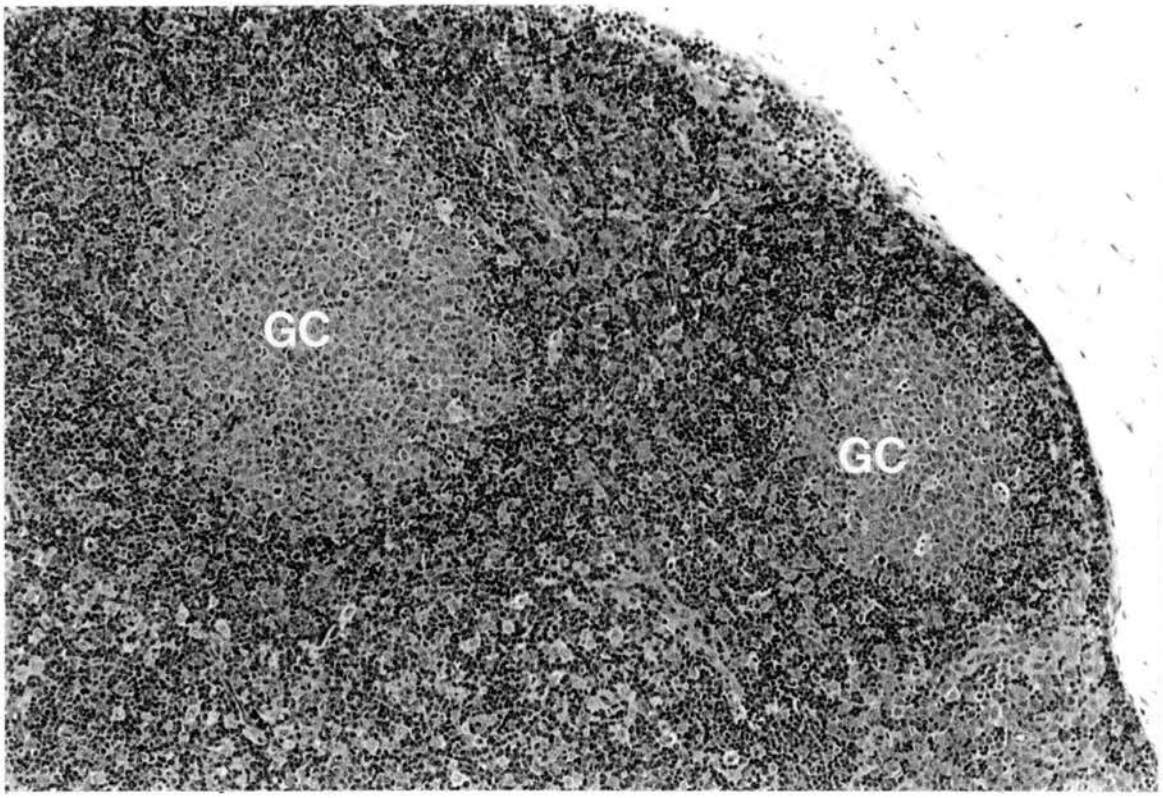

FIGURE 8. Histological section of mesenteric lymph node from a control cotton rat. Micrograph shows lymphoid follicles with less intensely stained germinal centers (GC). H&E. ×130.

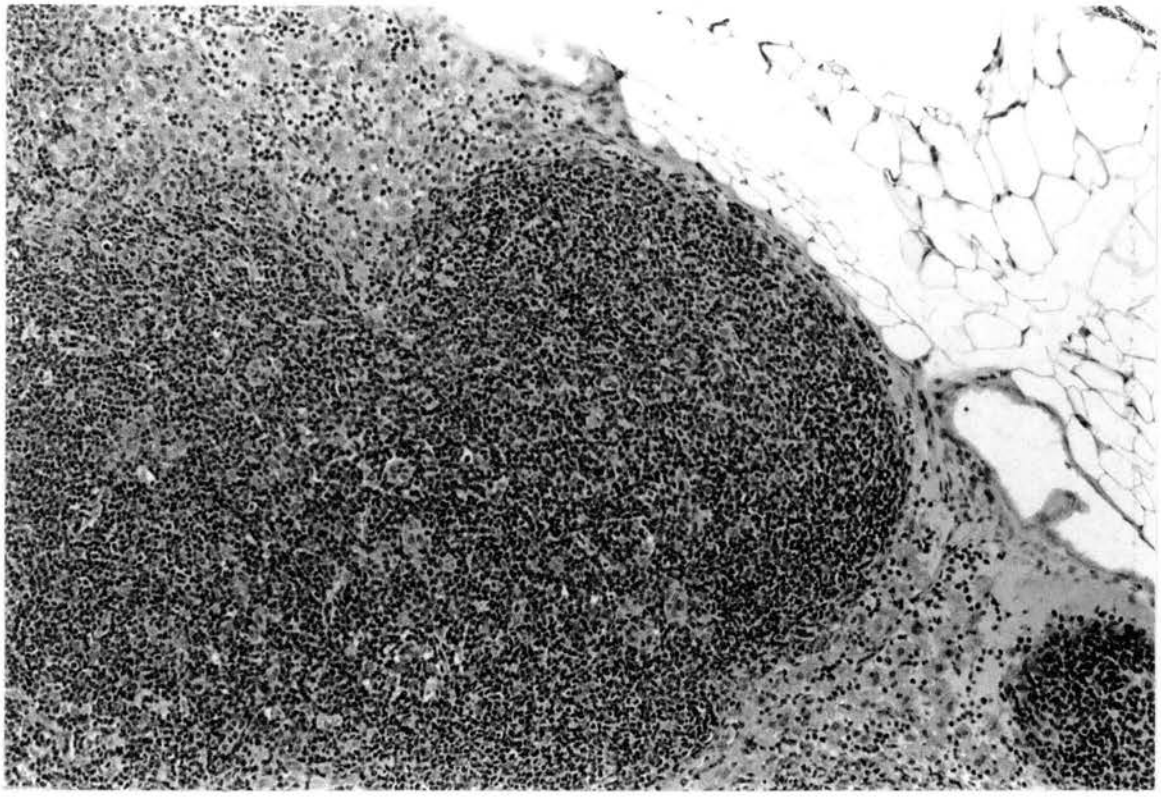

FIGURE 9. Histological section of mesenteric lymph node from a cotton rat exposed to 80 mg/kg cyclophosphamide for 5 days. Compare it to fig. 8. Note lymphoid follicles with no visible germinal centers. H&E. ×130.

CHAPTER II

EVALUATION OF MYELOTOXICITY IN COTTON RATS (*SIGMODON HISPIDUS*) RESIDING IN HABITATS CONTAMINATED WITH PETROLEUM INDUSTRIAL WASTES

ABSTRACT

A variety of chemical mixtures exist in soil contaminated with petrochemical wastes, yet no comprehensive assessment of their impact on terrestrial ecosystems has been conducted. The purpose of this study was to evaluate hematotoxicity risks to wild populations of cotton rats (*Sigmodon hispidus*) residing in habitats previously contaminated by petroleum industrial wastes. Resident cotton rats were monitored on nine contaminated sites and ecologically matched reference sites in Oklahoma. The possible toxicological interaction of petrochemical wastes on bone marrow was investigated by using the assay of colony formation of granulocyte-macrophage progenitor cells. There was a consistent 21 to 39 % decreases in the number of colony-forming units-granulocyte-macrophage (CFU-GM) in cotton rats from petrochemical-contaminated sites compared to matched reference sites, with no changes in hematological or histopathological parameters. These results suggest that bone marrow

progenitor cell culture is a sensitive indicator for the assessment of ecotoxicity risks associated with petrochemical wastes that are generated by the oil refining industry. Long-term exposure to hazardous wastes associated with the petroleum industry may represent a subtle risk to the hematopoietic system in humans.

INTRODUCTION

Hematopoiesis is a highly dynamic, closely regulated process designed for the daily production of large numbers of circulating blood cells from lineage-specific precursors in the bone marrow. Partly because of its consistently high rate of cell turnover, hematopoietic tissue is uniquely sensitive to a wide variety of toxic agents, including environmental pollutants (Boorman et al., 1984a; Hong et al., 1989; Luster et al., 1985). With the isolation and purification of a number of marrow-stimulating cytokines ("colony stimulating factors") within the last few decades, quantitative *in vitro* culture of the various hematopoietic precursors has become a powerful research tool used to study the events of hematopoiesis as well as the effects of various chemicals on that process. In this regard, the quantitative evaluation of lineage-specific hematopoietic precursors (colony forming units) in culture has proven to be a very sensitive indicator of myelotoxicity. It is routinely used to determine the myelotoxic potential of therapeutic agents and to evaluate potential methods of ameliorating these effects (Gallicchio et al., 1992, 1993, 1994; Luster et al., 1984; Reagan 1993). It has also been shown that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an environmental pollutant produced as a contaminant in the synthesis of 2,4,5-trichlorophenoxyacetic acid, inhibits bone marrow

hematopoiesis by a direct inhibition of proliferating stem cells (Luster et al., 1985). Lead, cadmium, ethylene glycol monomethyl ether (EGMME), ochratoxin A, benzo(a)pyrene and other polycyclic aromatic hydrocarbons, and allyl isovalerate (AIV) have all been reported to affect bone marrow progenitor cells in rodents (Boorman et al., 1984b; Goyer, 1996; Hong et al., 1988a, 1988b; Legraverend et al., 1983; Shore and Douben, 1994).

Evidence has accumulated that exposure to many chemicals found in the soil of petrochemical-contaminated waste sites (e.g. halogenated aromatic hydrocarbons, polycyclic aromatic hydrocarbons, and heavy metals) can alter immune function in laboratory animals at chemical exposure levels where other toxic alterations are not observed (Koller, 1980; Dean et al., 1986; Luster et al., 1987). Germolec et al.(1989) reported that exposure to a complex chemical mixture consisting of 25 common groundwater contaminants frequently found near toxic waste dumps can produce changes in hematopoietic and immune functions, including suppression of hematopoietic stem cells and antigen-induced antibody-forming cells. Recent field studies by our research laboratory have raised serious concerns regarding immunotoxicity risks to wild rodents inhabiting petrochemcial-contaminated sites (McBee et al., 1987; McBee and Bickham, 1988; McMurry et al., 1999; Propst et al., 1999; Rafferty, 1998). We hypothesized that the mechanism of immunotoxic effect observed in those field studies was related to suppression of bone marrow progenitor cells. We explored this hypothesis by monitoring the degree of myelotoxicity in wild cotton rats (*Sigmodon hispidus*) from populations residing in replicated petrochemical-contaminated waste sites in Oklahoma.

MATERIALS AND METHODS

Experimental Design and Study Sites

We selected nine study areas that had previously received petroleum wastes generated from the refining of crude oil. Each of the 9 study areas (herein referred to as units 1 through 9) consisted of a contaminated site and a matched reference site (located from 1 to 16 km from its paired contaminated site). Reference sites were selected based on their similarities of vegetation structure and composition, soil types, and topography with their matched contaminated sites. All contaminated sites were characteristic of a disturbed tall grass prairie ecosystem and were chosen based on accessibility to the site, historical use for the disposal of petroleum industrial wastes, and adequate vegetation for supporting a resident small mammal population. The contaminated site on unit 1 was used for the disposal of asphalt wastes in impoundment. The contaminated site on unit 3 was used for the disposal of tank-bottom sludges. The contaminated sites of the other seven units were abandoned land-treatment facilities where oil refinery wastes were tilled into the soil for eventual biodegradation. Recent studies reported that surface soil of land-treatment facilities contained elevated concentrations of heavy metals (lead, cadmium, and zinc), fluoride, and polycyclic aromatic hydrocarbons (Rafferty, 1998; Schroder et al., 1999). No historical information was available on the levels and mixtures of contaminants that were disposed on these nine units.

Units 1 through 4 were sampled in September of 1997 and units 5 through 9 in 1998. Twelve cotton rats (6 males and 6 females) were collected from each contaminated and matched reference site by live-trapping with Sherman live-catch traps (Sherman Traps Inc., Tallahassee, FL) baited with rolled oats. Captured cotton rats were taken to

the laboratory animal facility and placed individually in polycarbonate cages with wire tops and wood-chip bedding. Food (Purina 5001 laboratory rodent chow, Purina Mills, St. Louis, MO) and tap water were provided *ad libitum*. Animals were temporarily housed under a 16L : 8D light-dark illumination cycle at 23-24 °C and 50 % relative humidity before anesthetizing with Metofane® (Pittman-Moore, Mundelein, IL) and killing by cervical dislocation within 48 hours of their capture.

Whole blood was collected prior to sacrifice by periorbital sinus puncture. A heparinized blood sample (40 ul) was prepared for automated hematology analysis (Serono-Baker Diagnostics, Allentown, PA).

CFU-GM Assay

In vitro culture of granulocyte-macrophage progenitor cells to assess the ability of colony formation was performed as previously described (Kim 1999). Briefly, bone marrow cells from one femur were aseptically collected and single-cell suspensions were prepared by passing the cells through a 22-gauge needle. Nucleated cells were enumerated using a previously calibrated automated hematology analyzer (Serono-Baker Diagnostics, Allentown, PA). Aliquots of 10^5 cells were suspended in 1 ml of RPMI-1640 media containing 20 % fetal bovine serum, 5 % human AB serum, 200mM L-glutamine, 50 ug/ml garamycin, 1.5 % methylcellulose, and 10% cotton rat lung-conditioned medium (LCM). Aliquots were placed in six-well flat-bottom (35 mm in diameter) tissue-culture plates in triplicate and incubated for 3 days at 37 °C in a 5% CO_2 humidified incubator. Following incubation, colonies containing ≥ 50 cells were counted using an inverted microscope.

Statistical Analysis

All data were tested for normality (Proc Normal; SAS, 1994) and homogeneity of variances (Levene's test) prior to analysis (Steel et al., 1997). We used a randomized complete block design in a 2 × 9 factorial format with two treatments (contamination and reference) and nine study units (units 1-9) to evaluate differences in colony numbers between treatments and among study units. Comparisons were made using PROC MIXED (SAS, 1994) with sources of variation distributed among the main factor effects and the interaction term (study unit by treatment interaction). Significant interaction term effects were compared using the SLICE option for the LSMEANS statement. Satterthwaite's approximation was used in calculating degrees of freedom for the error term. Statistical significance for all hypothesis tests was set a priori at $p < .05$ and all values are presented as means ± SD.

RESULTS

A significant ($p < .0001$) treatment main effect indicated that the mean number of CFU-GM in cotton rats differed between petrochemical-contaminated and reference sites. Overall, the mean number of CFU-GM ($83.6 \pm 41.2/10^5$ vs $114.2 \pm 53.2/10^5$ bone marrow cells) in cotton rats from petrochemical waste sites was considerably lower than reference sites (Table 1).

A statistically significant ($p < .0043$) study unit by treatment interaction was evident for the average number of CFU-GM in cotton rats. Least square means analysis

revealed that the average number of CFU-GM was significantly lower ($p < .05$) in cotton rats from eight of nine contaminated sites when compared to their matched reference sites (Table 1). The only exception was unit 4 where the average number of CFU-GM was not significantly ($p < .29$) different between the contaminated and reference sites. While there was considerable variability in colony numbers among treatment units, the relative degree of suppression in number of CFU-GM in cotton rats from contaminated sites was relatively consistent. The average number of CFU-GM in cotton rats from contaminated sites was suppressed 61.1 to 79.1 % of reference animals for these eight study units. There was little variability in average CFU-GM numbers within and between animals from the reference sites on units 5 through 9, where values ranged between 98.4 and 119.5 CFU-GM/10^5 bone marrow cells.

DISCUSSION

Environmental contamination induced by petrochemical wastes has been known to induce immunotoxicity and fluorosis in resident cotton rats (Elangbam et al., 1989, 1991; McBee et al., 1987, 1988; McMurry et al., 1999; Paranjpe et al., 1994; Propst et al., 1999; Rafferty, 1998; Thompson et al., 1988). Our understanding of how complex mixtures of environmental contaminants impact terrestrial ecosystems is generally poor and inadequate for ecological risk assessment. This is due in part to a general lack of well-defined and sensitive assay systems for evaluating the effects and risks of contaminant exposure on organisms, populations, and communities in the ecosystem. Our study examined myelotoxicity of cotton rats inhabiting petrochemical waste sites by

using *in vitro* culture of bone marrow progenitor cells to predict impacts of contamination on assemblages of organisms in terrestrial environments.

The hematopoietic system is a well-defined, highly organized, and regulated process responsible for maintenance and renewal of granulocytes, monocytes, lymphocytes, erythrocytes, and platelets. These blood cells differentiated from pluripotential stem cells, bipotential or monopotential progenitor cells, and differentiated precursor cells, which lose their capacity for proliferation and self-renewal during differentiation. The differentiation and maturation of hematopoietic cells is regulated by endogenously produced glycoproteins known as cytokines or growth factors (Cline and Golde, 1979). These various growth factors include interleukins, colony stimulating factors (CSF), erythropoietin, thrombopoietin, as well as nutritional factors (iron, vitamins). Abnormalities in blood cell production may be restricted to a single lineage, such as granulocyte or erythrocyte precursors, or may involve several hematopoietic lines. Thus, a variety of stages of hematopoietic cell differentiation may serve as targets of chemical damage, which can ultimately result in clinically recognizable disease syndromes.

It has been reported that prolonged exposure to a mixture of groundwater contaminants in mice causes suppression of bone marrow granulocyte-macrophage progenitor cells and erythroid precursors and of antigen-induced antibody-forming cells without changes in other histopathological or hematological parameters (Chapin et al., 1989; Germolec et al., 1989; Hong et al., 1992). Among those changes, only suppression of granulocyte-macrophage progenitors occurred at lower-level exposures of the mixture of groundwater contaminants. Such sensitivity is especially important in the study of

environmental contaminants where exposure levels are likely to be low and the frequency of overt parenchymal toxicity in a study population may be low and occur only after extended exposure.

The present study demonstrated that the number of CFU-GM was reduced 61 to 79 % of controls in cotton rats from contaminated sites. While there was a statistically significant decrease in the number of CFU-GM, there was some inter-assay (assays done at different times) variations of progenitor counts probably due to variation in activity of LCM and L-glutamine. However, the numbers of CFU-GM within animals on a particular day (assays done at the same time) were consistent. Although our study demonstrated significant myelotoxicity in cotton rats from petrochemical-contaminated sites, the degree of myelosuppression among cotton rats within each contaminated site was not consistent. Several factors could have contributed to the variation in the number of CFU-GM among cotton rats from petrochemical-contaminated waste sites. These factors may include sampling errors, avoidance of contaminated food or soil, variation in concentration of contaminants through the sites, and individual sensitivity to exposure to contaminants. Even though the cotton rat has a small home range averaging 0.5 ha (Nowak and Paradiso, 1991), it is possible to trap cotton rats that recently immigrated to a contaminated site.

Although exposure risks to animals or humans from environmental contaminants usually involve a variety of chemicals rather than individual chemicals, information on the effects and mechanisms of chemical interactions for complex mixtures is limited. The degree of myelosuppression we observed in cotton rats appeared to be of insufficient magnitude to alter host immune function. It has been stated that immune functions can

generally be suppressed by at least 30 % prior to observing changes in resistance to infectious agents (Germolec et al., 1989). Our previous study demonstrated that cotton rats exposed to cyclophosphamide at 8 mg/kg for 5 days expressed a 31% decrease in colony formation of bone marrow progenitors, but without changes in total WBC, lymphocyte, neutrophil, and eosinophil counts. This would account for the absence of histopathological and hematological changes in our study except for myelosuppression which was usually less than 30% reduction in bone marrow colony formation.

Hong et al. (1991) found that myelotoxic effects of chemical treatment were amplified with subsequent whole body irradiation compared to irradiated vehicle controls. This suggests a residual marrow damage and an increased sensitivity of hematopoietic system to subsequent irradiation stress. Yang et al. (1989) suggested that toxicity caused by environmental chemical mixtures would not involve acute toxic responses. It is more likely an insidious effect disrupting the homeostasis of the organism. Even though exposed animals may appear clinically normal, final toxicity may be expressed by the enhancement or potentiation of other acute exposure of chemical, physical, and/or biological agents. Thus, it is possible that subclinical myelotoxicity caused by environmental contaminants might be exacerbated by subsequent acute exposure to drug or radiological therapy in humans.

In conclusion, we detected significant suppression of bone marrow granulocyte-macrophage progenitor cells in cotton rats exposed to petroleum industrial wastes without any morphological or histopathological changes. These results show that *in vitro* culture of bone marrow progenitor cells is one of the most sensitive indicators for detecting adverse health effects of certain environmental contaminants. Our study provides the first

examination of myelotoxicity of cotton rats to evaluate ecotoxicity risks associated with chronic exposure to petrochemical waste products that are generated by the oil refining industry. Whether the observed changes were sufficient in magnitude to affect disease resistance following exposure to the toxic insults was not determined. Not only is this information useful in assessing risks to organisms in terrestrial ecosystems, but it may also assists those in making predictions regarding human health risks.

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Table 1. Mean (± SD) number of CFU-GM/10^5 bone marrow cells of cotton rats from nine petrochemical waste sites and their matched reference sites in Oklahoma during summer, 1997 and 1998

Year	Location	Reference site	Contaminated site
1997	Unit 1	156.8 ± 34.5	95.9 ± 38.9[a] (61.1%)
	Unit 2	221.6 ± 43.6	166.0 ± 23.7[a] (75.0%)
	Unit 3	80.1 ± 19.3	55.4 ± 18.5[a] (69.1%)
	Unit 4	39.4 ± 10.0	29.0 ± 11.8 (73.5%)
1998	Unit 5	98.4 ± 14.5	77.6 ± 14.0[a] (78.8%)
	Unit 6	102.3 ± 13.0	74.5 ± 26.1[a] (72.8%)
	Unit 7	101.9 ± 12.8	79.0 ± 19.8[a] (77.5%)
	Unit 8	105.2 ± 17.3	83.2 ± 17.5[a] (79.1%)
	Unit 9	119.5 ± 27.9	88.7 ± 22.5[a] (74.2%)

Note. Number in parenthesis indicates the percentage of the control value.

[a] significantly different from reference site at $p < .05$.

CHAPTER III

WIDESPREAD RISKS OF DENTAL FLUOROSIS IN COTTON RATS (*SIGMODON HISPIDUS*) RESIDING ON PETROCHEMICAL WASTE SITES

ABSTRACT

Fluoride has been identified as a ubiquitous contaminant of soils where petrochemical wastes have been disposed. The purpose of this study was to assess how widespread toxicity risks are to resident vertebrates from chronic exposure to fluoride in the soil of petrochemical-contaminated waste sites. We seasonally examined wild cotton rats (*Sigmodon hispidus*) that were seasonally collected from 12 contaminated and 12 ecologically matched reference sites across Oklahoma. The risks of cotton rats exposed to fluoride were analyzed by means of gross examination, histopathology, and scanning electron microscopy of rat incisors. Cotton rats from reference sites showed no pathologic changes in incisors (98%). In comparison, 46% of cotton rats from contaminated sites had various degrees of dental lesions. Prevalence and severity of dental lesions in cotton rats from contaminated sites were significantly influenced by season. There was a 45% increase in prevalence and a 65% increase in severity of dental lesions from summer to winter. This study demonstrated that cotton rats are very

sensitive biomonitors for assessing toxicity risks from soils contaminated with fluoride and that such assessments should consider seasonal influences.

INTRODUCTION

Fluoride is ubiquitous in the environment and all animals ingest small amounts in their diet without any adverse effects. Although trace amounts of fluoride may be required for optimum tooth and bone development (McClure, 1970), excessive fluoride ingestion induces harmful damage (Kierdorf et al., 1996; Shupe et al., 1987; Suttie, 1980; Walton, 1987). Dental fluorosis is a hypomineralization of tooth enamel or dentin produced by exposure to excessive fluoride during the period of tooth formation (Horowitz, 1989). Because fluoride can accumulate in the body, chronic toxicosis is often seen in livestock from ingesting a diet contaminated by industrial fluoride effluents, water high in fluoride, vegetation grown on high-fluoride soil, or feed supplements and mineral mixtures with high levels of fluoride (Shupe, 1963). The major industrial sources of fluoride contamination are those associated with aluminum reduction, phosphate processing, steel manufacturing, petroleum refining, brick and tile production, and combustion of fossil fuels.

Fluoride-containing chemicals are frequently used in the refining of petroleum products. A major fluoride source is hydrofluoric acid which is used as a catalyst in gasoline production. Waste hydrofluoric acid is neutralized and often disposed in waste-sludge pits (Gary and Handwerk, 1984). Biodegradation of petroleum waste products in land treatment farms has been shown to result in an accumulation of inorganics such as

fluoride in the soil (Schroder et al., 1999). A survey of three petrochemical-contaminated sites on an abandoned oil refinery found resident small mammals to have a high frequency of fluoride-induced dental lesions (Paranjpe et al., 1994). Because of these earlier studies, we initiated a more extensive survey of petrochemical-contaminated waste sites to evaluate the extent of fluoride toxicity and the risks posed to resident cotton rat (*Sigmodon hispidus*) populations inhabiting these areas. A total of 24 cotton rat populations were surveyed to document the prevalence and severity of dental lesions induced by fluoride in the soil.

MATERIALS AND METHODS

Experimental Design and Study Sites

We seasonally surveyed 12 petrochemical waste disposal units that were distributed across Oklahoma. Each unit consisted of a petrochemical-contaminated study site and a matched reference study site in similar habitat located <16 km from the contaminated site. Reference sites were chosen based on a visual assessment of similarity of vegetation structure and composition with their paired contaminated sites; sites were also similar with respect to topography and soil types. All contaminated sites were characteristic of a disturbed prairie ecosystem, with early successional species dominating the vegetation community. These units were selected based on historical use for disposal of petrochemical wastes, presence of adequate vegetation to provide suitable cover to support a resident small mammal population, and accessibility to the site. The contaminated sites on units 1, 2, 3, 5, 6, and 11 were used for the disposal of tank-bottom

sludges. The contaminated site on unit 9 was used for the disposal of asphalt wastes in impoundment. The contaminated sites of the other five units were abandoned land-treatment facilities where oil refinery wastes were tilled into the soil for eventual biodegradation. Little historical information existed on what levels and mixtures of contaminants were incorporated into soils on these contaminated study sites.

Sampling occurred across three years (September 1995-February 1998); 4 study units were sampled during each of those years. All 12 units were sampled during two seasons (February and September) to evaluate the influence of season on occurrence or prevalence of dental fluorosis. Soil samples were collected from each contaminated- and reference site within each unit. Total soil fluoride was analyzed by using fusion methods as described by McQuaker and Gurney (1977). A total of 573 cotton rats were collected over the 3 years, and we attempted to obtain six male and six female cotton rats from each contaminated- and matched reference site within each season. All cotton rats were reproductively mature and weighed more than 50 g.

Sherman live traps (Sherman Traps, Inc., Tallahassee, FL) were used to collect cotton rats during each season. Animals were anesthetized with Metofane® (Pittman-Moore, Mundelein, IL) and killed by cervical dislocation. At necropsy, the skull was collected in 10% neutral-buffered formalin for further evaluation of the teeth. Upper and lower incisors were scored under a magnifying glass according to predetermined scoring criteria (Table 1). Both humeri were removed from each cotton rat, dried to constant weight, and digested in acid by a method adapted from Andrews et al. (1989). Bone samples were analyzed for fluoride concentration by using methods as described by Schroder et al. (1999).

Preparation for Light Microscopy

To prepare sections of decalcified enamel, we used Procion dye, reactive red 4 (Sigma Co., St. Louis, MO), fixation to preserve enamel during decalcification (Goland et al., 1965). The incisors were immersed in 1% solution of reactive red 4 for 24 hours. Following an initial fixation, the incisors were thoroughly washed in distilled water and reimmersed in 5% formic acid for 5 hours for partial decalcification. The incisors were washed in distilled water and fixed in 1% solution of reactive red 4 overnight. The incisors were washed and fixed in vials in 1.6% cacodylate buffered glutaraldehyde for 2 hours and rinsed in 0.1M cacodylate buffer. The specimens were postfixed in 1% cacodylate buffered osmium for 2 hours, rinsed 3 times in 0.1M cacodylate buffer for 20 min each, and kept in a third rinse overnight at 4°C. The specimens were dehydrated in ethanol and kept overnight in 1:1 100% ethanol/LR White at 4°C. The specimens were kept in 100% LR White, kept overnight, and embedded in paraffin molds. The incisors were sectioned longitudinally and 1-um-thick sections were stained with methylene blue.

Preparation for Scanning Electron Microscopy

Incisors were placed in 10% Clorox solution for 24 hours to remove superficial organic material. Incisors were rinsed in distilled water, dehydrated through an acetone series, and air-dried in a covered petri plate. All specimens were glued onto A1 stub and coated with gold/palladium.

Statistical Analysis

We used a randomized complete block design in a 2 × 2 × 12 factorial format with two treatments (contamination and reference), two seasons (summer and winter), and 12 study units (units 1-12) to evaluate whether petrochemical contamination was associated with increased risk of severity score of dental lesions for resident cotton rat populations. Comparisons were made using PROC MIXED (SAS, 1994) with sources of variation distributed among the main factor effects and the interaction terms (study unit by treatment interaction, season by treatment interaction, and study unit by season interaction). Significant interaction term effects were compared using the SLICE option for the LSMEANS statement. Satterthwaite's approximation was used in calculating degrees of freedom for the error term. The relationship between bone fluoride and dental score was determined using simple linear regression analysis. Seasonal differences in prevalence of dental lesions were determined using a z-test (EpiCalc 2000 Version 1.02). P-values of < .05 were considered to be significant, and all values are presented as means ± SD.

RESULTS

The Gross Lesions

A total of 287 cotton rats from contaminated sites and 286 from reference sites were scored for dental lesions (Table 2). Nearly all cotton rats captured from the 12 reference sites had upper and lower incisors that possessed a normal deep yellow-orange color (Figure 1). In comparison, cotton rats collected from contaminated sites had various degrees of dental pathology ranging from slight horizontal striation in the enamel to

chalkiness with discoloration of the enamel (Figures 2-6). Only seven cotton rats (2.0%) from the 12 reference sites had any evidence of dental lesions and these consisted of mild striations in the lower incisors. However, 133 out of 287 cotton rats (46.3%) from the 12 contaminated sites had various lesions in the lower and upper incisors. Cotton rats from habitats where petrochemical wastes were land-treated had a higher incidence (92/120; 77%) of dental lesions than other types of disposal sites.

Prevalence of dental lesions in cotton rats from contaminated sites was significantly ($p < .001$) influenced by season. There was a 45.3% increase in prevalence of lesions from summer to winter. Fifty-four of 143 cotton rats (37.8%) had various dental lesions in summer, whereas 79 of 144 cotton rats (54.9%) had dental lesions in winter. There was also a significant ($p < .001$) seasonal effect in concentration of fluoride in bone of cotton rats from contaminated sites. Concentration of fluoride in bone averaged 771.1 ± 1,010.7 mg/kg in summer compared to 1,936.0 ± 2,356.9 mg/kg in winter. The seasonal relationship between concentration of fluoride in bone and severity of dental lesions in cotton rats was significant ($p < .001$) for both summer and winter (Figures 7).

The frequency of animals with severity score for dental lesions of 5 was low in contaminated sites: 0 = 154, 1 = 29, 2 = 21, 3 = 44, 4 = 24, and severity score 5 = 15 cotton rats. There was a significant ($p < .001$) treatment main effect in severity of dental lesions for cotton rats. Cotton rats from reference sites had an average severity score of 0.03, whereas cotton rats from contaminated sites had an overall average score of 1.30, ranging from a mean of 0.00 to 3.92 per site (Table 3). A significant ($p < .0036$) study unit by treatment interaction was evident in severity of dental lesions in populations of

cotton rats. Least square means analysis revealed that severity of dental lesions was significantly greater on contaminated sites for units 4, 7, 8, 9, 10, 11, and 12.

Elevated levels of soil fluoride were detected on contaminated sites compared to reference sites. Average concentration of fluoride in soil of reference sites was 90 ± 53 mg/kg ranging from 24 to 176 mg/kg. Soils on contaminated sites had more than 19-fold higher levels of fluoride than reference sites, with a mean of 1,707 ± 2,089 mg/kg ranging from 60 to 5,257 mg/kg. There was a strong relationship between concentrations of fluoride in soil and bone of cotton rats from contaminated sites. The relationship ($p < .001$) between soil fluoride and the severity of dental lesions of cotton rats from both contaminated and reference sites was evident (Figure 8).

Microscopic examination of the labial surface of the incisor demonstrated marked alterations in incisors of cotton rats from contaminated sites (Figures 9 and 10). The normally tall columnar ameloblasts with a palisading arrangement and basally oriented nuclei were not present. Instead, the cells were flattened and irregularly arranged without basal orientation of nuclei. The enamel surface was very irregular and pale, and the underlying enamel prism was moderately thin as compared to normal teeth with the loss of Hunter-Schreger bands.

Scanning electron microscopic examination of cotton rat dental lesions was consistent with the descriptions of Nordlund and Lindskog (1986), where the enamel surface of the incisors of cotton rats from contaminated sites showed enamel hypoplasia with a rough surface covered with irregular depositions of mineralized material (Figures 11 and 12). The boundary between the hypoplastic area and the surrounding enamel was well demarcated.

DISCUSSION

This study demonstrated that cotton rats residing on petrochemical waste sites contaminated with fluoride had a substantial risk of developing dental lesions consistent with dental fluorosis. Dental fluoritic lesions are caused by exposure to excessive fluoride during the period of tooth formation and mineralization and reflect toxic damage to ameloblasts (Jubb et al., 1993). Ameloblasts (enamel-forming cells) are very sensitive to changes in mineral metabolism and reflect dental changes induced by excessive fluoride ingestion that is metabolized by the body (Massler et al., 1941). Dental fluorosis of the laboratory rat seems to result from alteration of proteolytic activity of enamel proteases, which is responsible for the breakdown of amelogenin protein from the enamel matrix (Den Besten and Heffernan, 1989). It is also possible that fluoride may alter the enamel proteins, thereby making enamel protenase difficult to degrade the enamel proteins (Robinson and Kirkham, 1990).

Fluoride-induced lesions in the enamel are irreversible and correlate with the quantity of fluorides ingested during tooth development. Black and McKay (1916) first described dental fluorosis induced by exposure to ingested fluoride as a mottling of the enamel. Pathological changes in the enamel organ have been reported in response to various agents, such as fluoride (Neiman and Eisenmann, 1975; Nordlund et al., 1986), cobalt, strontium (Neiman and Eisenmann, 1975), cadmium (Katsuta et al., 1996), phosphonoformic and phosphonoacetic acids (Caracatsanis et al., 1989), vitamin A (Harris and Navia, 1980), vitamin D (Berdal et al., 1989), tetracycline (Kallenbach, 1980;

Westergaard, 1980), and vinblastin (Moe and Mikkelsen, 1977). Dental fluoritic lesions may range from slight horizontal striation in the enamel (hypomineralization - mottling), moderate or marked mottling or striation, chalkiness with discoloration to hypoplasia (including small tooth size), pitting, and/or thinning and erosion of the enamel (Shupe et al., 1987). It has been reported that enamel hypoplasia induced by fluoride occurrs following formation of sub-ameloblastic cysts (Nordlund et al., 1986). The extent of the cystic lesions was age- and dose-related and resulted from cell degradation and fragmentation of the Tomes' processes of ameloblasts.

We observed that the lower incisors were more severely damaged than upper incisors in cotton rats. Developing teeth are more susceptible to toxic and metabolic changes. Rodent incisors grow continuously and are regarded as a delicate instrument for determining the changes in dental structures (Thoma, 1950). The normal rate of eruption in the laboratory rat is about 2.0 mm for the upper and 2.8 mm for the lower incisors per day. Because lower incisors proliferate faster than upper incisors, they should be more sensitive to toxic insults (Thoma, 1950).

Our study demonstrated a strong seasonal influence on bone fluoride and prevalence and severity of dental fluorosis in cotton rats from petrochemical-contaminated sites. Schroder et al. (1999) reported that total soil fluoride from land treatment waste sites (1,954 mg/kg) were more than 10-fold higher than those from reference sites (121 mg/kg). Total vegetation fluoride (total fluoride for johnson grass and brome grass) from land treatment sites was significantly elevated. Exposure pathway models developed by Schroder et al. (1999) indicated that the consumption of vegetation rather than incidental ingestion of soil was the primary pathway of exposure in winter.

Higher levels of fluoride in brome grass in winter (33.5 mg/kg) compared to summer (12.4 mg/kg) may have contributed to a greater risk of dental fluorosis. Nutritional differences across seasons may also have contributed to observed seasonal changes in the severity of dental lesions (Berdal et al., 1989; Harris and Navia, 1980). An additional factor may be that cotton rats collected in winter are relatively older than those trapped in the summer breeding season, which may contribute to a longer exposure period. Additionally, clearance rates of fluoride from plasma are retarded in older animals (Ekstrand and Whitford, 1984). Previous studies have reported age-related abnormalities in teeth of mice, characterized by hypoplastic, grooved, or undulating enamel (Humphreys et al., 1985).

In rodents, the timing of the exposure to fluoride is an important factor of the severity of dental lesion because the early maturation stage of enamel formation is most sensitive to the effects of fluoride (Den Besten and Thariani, 1992). The individual variance in gastric pH can affect the dental lesion because of the inverse relationship between gastric acidity and the rate of gastric absorption (Appleton, 1994). Also the level and the duration of exposure to fluoride affects the formation of fluorosed enamel (Den Besten and Thariani, 1992; Angmar-Mansson and Whitford, 1985). Higher levels of fluoride accumulate locally in the adjacent bony environment of the developing teeth and locally high fluoride is released over the period of time that is sufficient to cause dental fluorosis.

Few studies have reported dental lesions in wild rodents residing in waste sites contaminated with fluoride. Paranjpe et al. (1994) reported dental fluorosis in the incisors of wild cotton rats from an abandoned oil refinery sites but they did not classify the

severity of dental lesions nor note seasonal influences on dental fluorosis. Dental lesions induced by fluoride have been reported in many other species. Dental lesions have been reported in field voles, wood mice and moles from areas polluted by fluoride associated with aluminum reduction plants (Walton, 1987). Shupe et al. (1987) reported fluoride-induced incisor lesions in cattle, sheep, horses, deer, elk, and bison from areas where vegetation, water, or soils were abnormally high in fluorides. Fluorosed dental enamel was identified in red deer from a fluoride polluted region related to thermal power plants burning low quality brown coal (Kierdorf et al., 1996). Chronic fluoride toxicity in cattle is manifested by lameness consistent with fracture of the pedal bone; dental defects in incisor teeth consisted of discoloration, mottling, hypoplasia and increased wear; and reduced appetite (Shupe, 1963; Suttie, 1980).

It has been reported that several factors other than fluoride induced dental lesion and those lesions have to be distinguished with fluoride-induced lesions. Kallenbach (1980) described the enamel defect in teeth of laboratory rats after injection with tetracycline. Single administration of colchicine induced defective enamel characterized by white bands with rough surface (Hashimoto, 1990). Laboratory rats injected with cadmium developed dental lesions characterized by mild bleaching of incisors (Katsuta et al., 1996).

It has been shown that there is a direct relationship among the degree of dental fluorosis, bone fluoride level, and the concentration of fluoride in drinking water (Den Besten, 1994). A positive relationship between dental fluorosis and bone fluoride level was demonstrated in our study, however there was large variance in bone fluoride level and dental fluorosis. Variation of dental lesions and bone fluoride may be due to the

individual variations in fluoride susceptibility and the thickness of the already-formed enamel matrix which reflects the age of the animal. A positive relationship between bone fluoride and soil fluoride was evident in this study whereas soil fluoride did not directly reflect severity of dental lesions. It appears that examination of bone fluoride and soil fluoride levels should be accompanied with visual examination of dental lesions as a screening procedure to determine the risks for cotton rats exposed to fluoride in areas where petrochemical wastes were disposed.

We conclude that cotton rats are highly sensitive bioindicators of dental fluorosis risks associated with petrochemical wastes. Seasonal influence on the prevalence and severity of dental lesions and concentration of bone fluoride in cotton rats were evident in this study indicating that season of the year should be incorporated into any future population monitoring programs related to dental fluorosis. Factors responsible for these seasonal influences on dental fluorosis should be determined in future studies.

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Table 1. Scoring criteria for assessing severity of dental lesions induced by fluoride in cotton rats

Lesion score	Incisor characteristics
0 (Normal)	Smooth, glossy deep yellow-orange incisors
1 (Slight effect)	Slight striation (or mottling) in lower incisors
2 (Mild to moderate effect)	Prominent mottling or striation (white chalky) in lower incisors
3 (Moderate effect)	White chalky lower incisors; slight mottling in upper incisors
4 (Marked effect)	White chalky lower incisors; definite striation (or mottling) in upper incisors
5 (Severe effect)	White chalky lower and upper incisors

Table 2. Comparison of number of rats with dental lesions from twelve study units in Oklahoma during summer 95 and winter 98 determined by scoring system

Period of collection	Location	Number of animals	Lesion score					
			0	1	2	3	4	5
Sep. 95	Unit 1	12 (12)	12 (11)	0 (1)	0 (0)	0 (0)	0 (0)	0 (0)
	Unit 2	12 (12)	10 (12)	2 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Unit 3	12 (12)	12 (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Unit 4	12 (10)	2 (9)	2 (1)	1 (0)	4 (0)	3 (0)	0 (0)
Feb. 96	Unit 1	12 (12)	12 (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Unit 2	12 (12)	11 (12)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)
	Unit 3	11 (12)	10 (12)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Unit 4	12 (12)	0 (11)	0 (1)	0 (0)	5 (0)	3 (0)	4 (0)
Sep. 96	Unit 5	12 (12)	12 (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Unit 6	12 (12)	12 (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Unit 7	12 (12)	11 (12)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Unit 8	12 (12)	1 (11)	4 (0)	4 (1)	2 (0)	1 (0)	0 (0)
Feb. 97	Unit 5	12 (12)	12 (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Unit 6	12 (12)	11 (12)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Unit 7	12 (12)	1 (11)	5 (1)	4 (0)	1 (0)	1 (0)	0 (0)
	Unit 8	12 (12)	2 (11)	4 (1)	1 (0)	5 (0)	0 (0)	0 (0)
Sep. 97	Unit 9	11 (12)	3 (12)	2 (0)	1 (0)	2 (0)	2 (0)	1 (0)
	Unit 10	12 (12)	1 (12)	2 (0)	1 (0)	4 (0)	2 (0)	2 (0)
	Unit 11	12 (12)	3 (12)	1 (0)	1 (0)	3 (0)	3 (0)	1 (0)
	Unit 12	12 (12)	10 (12)	1 (0)	1 (0)	0 (0)	0 (0)	0 (0)

Feb. 98	Unit 9	12 (12)	0 (12)	0 (0)	1 (0)	4 (0)	3 (0)	4 (0)
	Unit 10	12 (12)	0 (12)	1 (0)	4 (0)	4 (0)	2 (0)	1 (0)
	Unit 11	12 (12)	5 (12)	1 (0)	0 (0)	3 (0)	2 (0)	1 (0)
	Unit 12	12 (12)	0 (11)	1 (1)	1 (0)	7 (0)	2 (0)	1 (0)

Note. Numbers outside parentheses indicate number of animals from contaminated site, whereas numbers in parentheses indicate number of animals from reference site.

Table 3. Comparison of seasonal differences in severity of dental fluorosis in cotton rats from twelve study units in Oklahoma across three years indicated by the average dental score (± SD)

Location	Treatment	Average dental score	
		Summer (September)	Winter (February)
Unit 1	Reference	0.08 ± 0.29[a]	0
	Contaminated	0	0
Unit 2	Reference	0	0
	Contaminated	0.17 ± 0.39	0.17 ± 0.58
Unit 3	Reference	0	0
	Contaminated	0	0.09 ± 0.30
Unit 4	Reference	0.09 ± 0.30	0
	Contaminated	2.33 ± 1.50[a]	3.92 ± 0.90[a]
Unit 5	Reference	0	0
	Contaminated	0	0
Unit 6	Reference	0	0
	Contaminated	0	0.08 ± 0.29
Unit 7	Reference	0	0.08 ± 0.29
	Contaminated	0.08 ± 0.29	1.67 ± 1.07[a]
Unit 8	Reference	0.17 ± 0.58	0.08 ± 0.29
	Contaminated	1.83 ± 1.11[a]	1.75 ± 1.22[a]
Unit 9	Reference	0	0
	Contaminated	2.09 ± 1.81[a]	3.83 ± 1.03[a]
Unit 10	Reference	0	0

	Contaminated	2.83 ± 1.59[a]	2.83 ± 1.11[a]
Unit 11	Reference	0	0
	Contaminated	2.42 ± 1.78[a]	1.92 ± 1.93[a]
Unit 12	Reference	0	0.08 ± 0.29
	Contaminated	0.25 ± 0.39	3.08 ± 0.99[a]
Overall	Reference	0.03 ± 0.20	0.02 ± 0.14
	Contaminated	0.98 ± 1.50[a]	1.62 ± 1.74[a]

[a] Significantly different from the scores of reference site at $p < .05$.

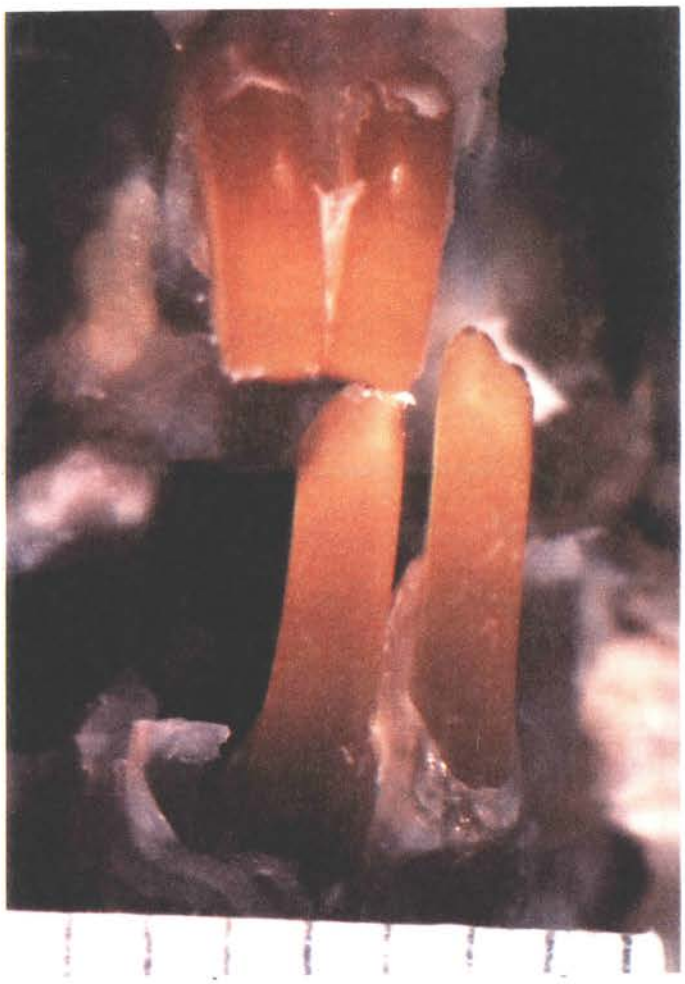


FIGURE 1. Incisors from a cotton rat. Note normal deep, glossy, yellow-orange color of the incisors.

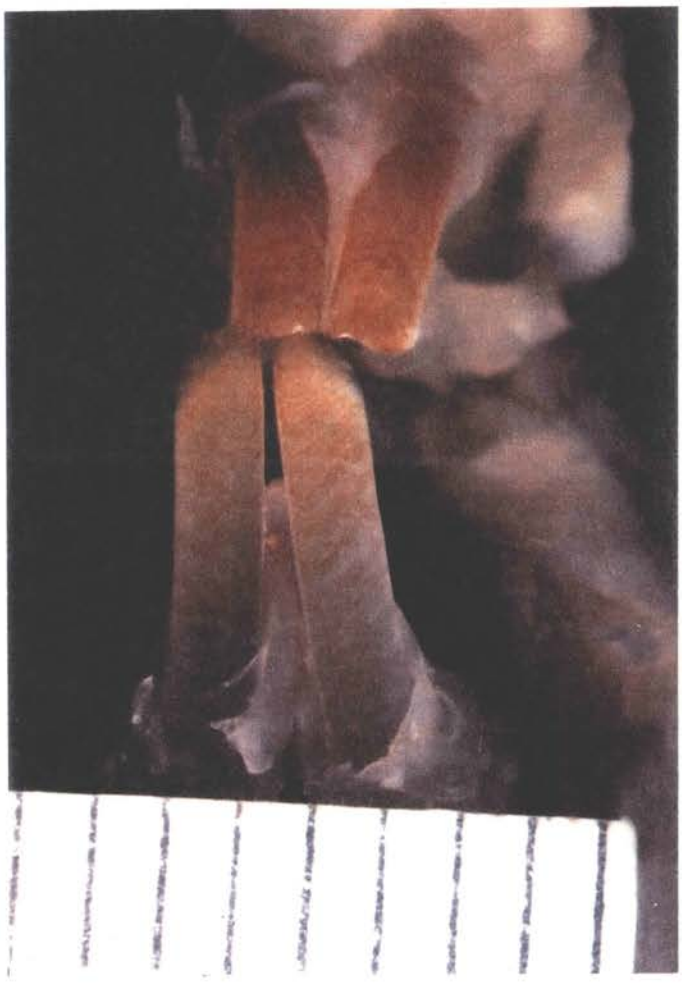


FIGURE 2. Incisors from a cotton rat with severity score for dental lesions of 1. Note slight striations in the lower incisors.

FIGURE 3. Incisors from a cotton rat with severity score for dental lesions of 2. Note prominent mottling and striations in the lower incisors.

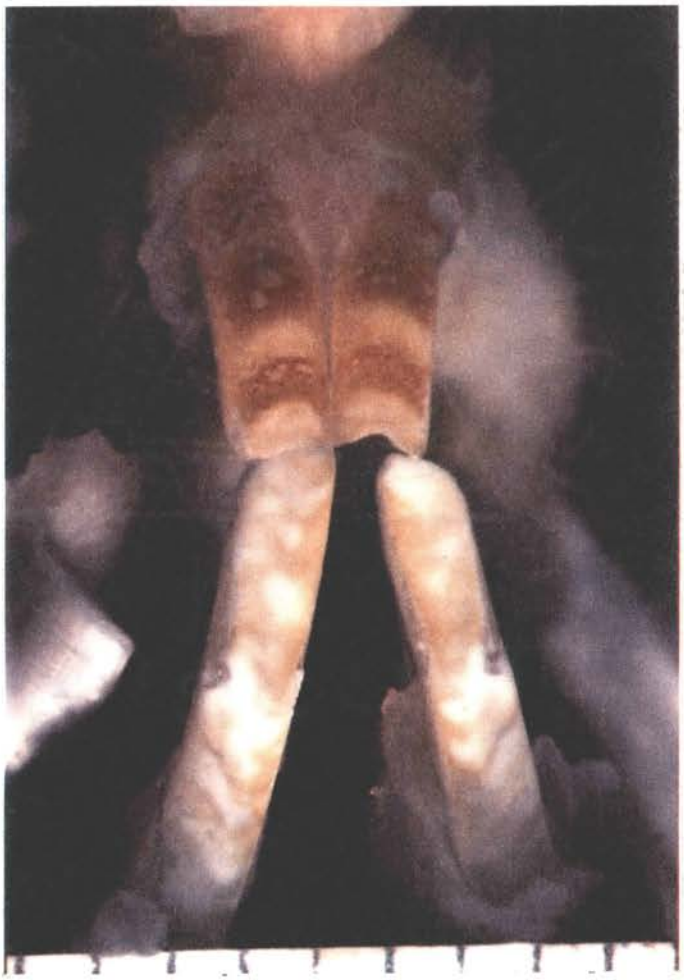


FIGURE 4. Incisors from a cotton rat with severity score for dental lesions of 3. The lower incisors are white chalky. The upper incisors have moderate striations.

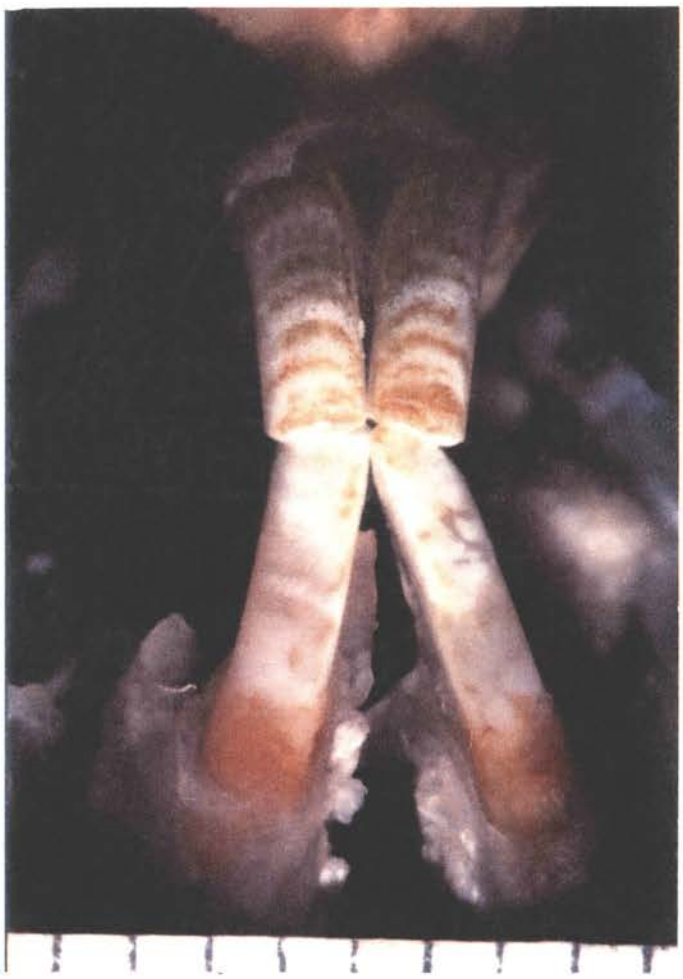


FIGURE 5. Incisors from a cotton rat with severity score for dental lesions of 4. The lower incisors are white chalky. The upper incisors have marked striations.

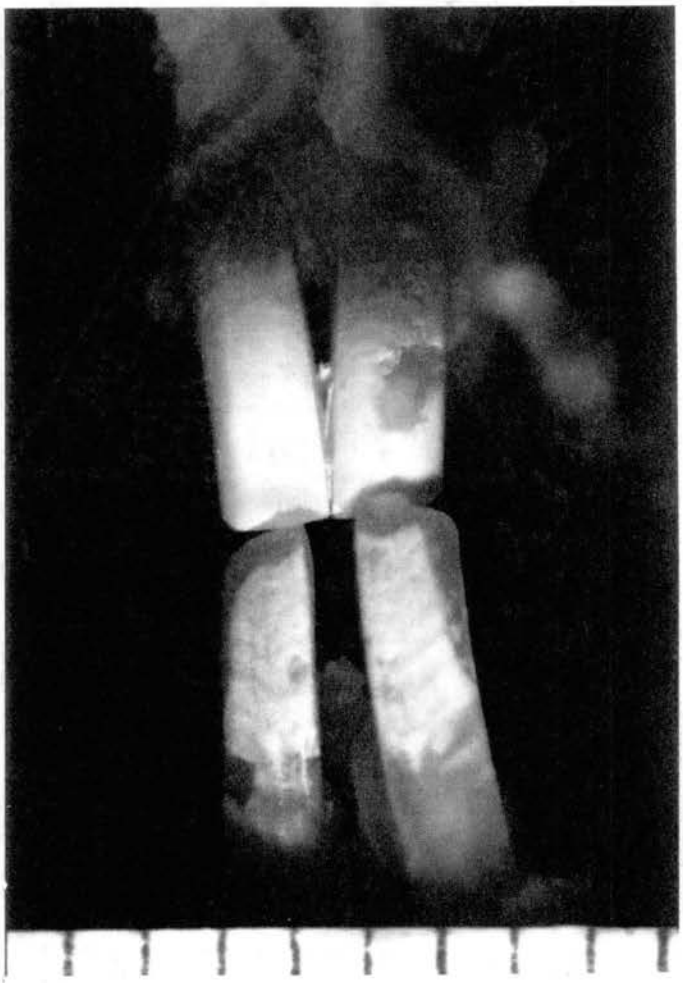
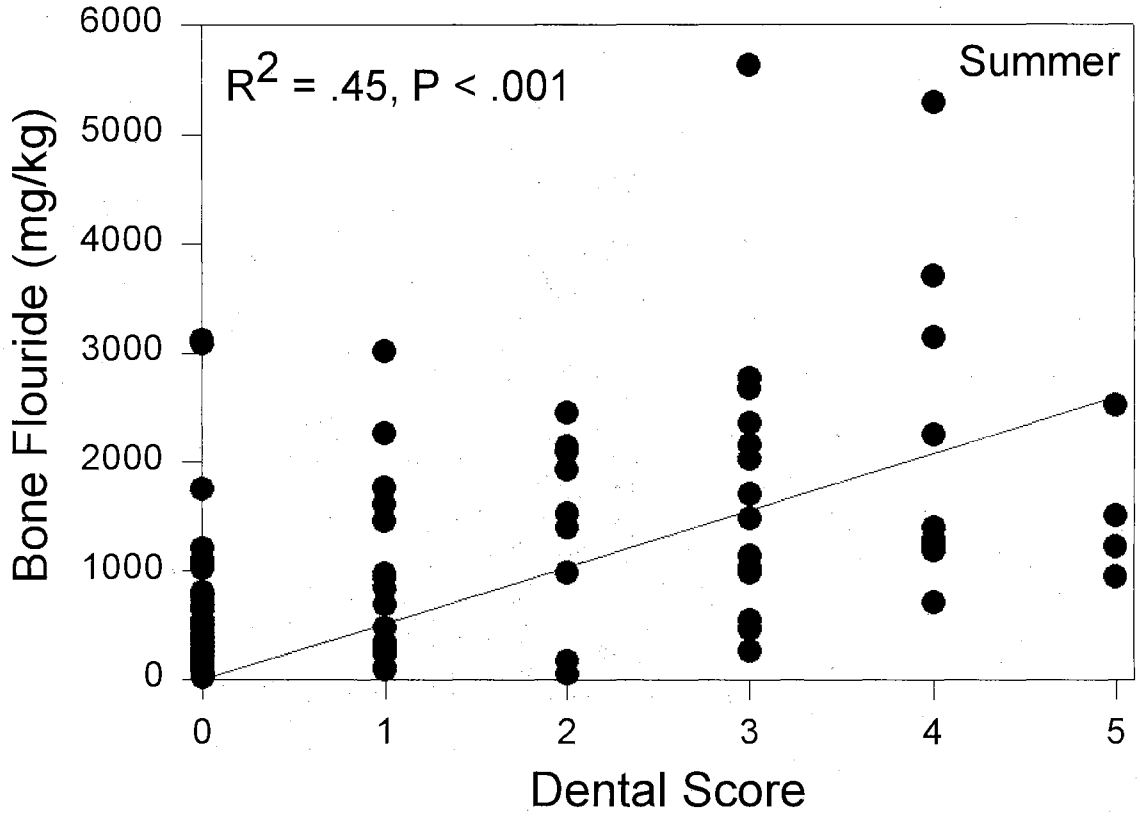


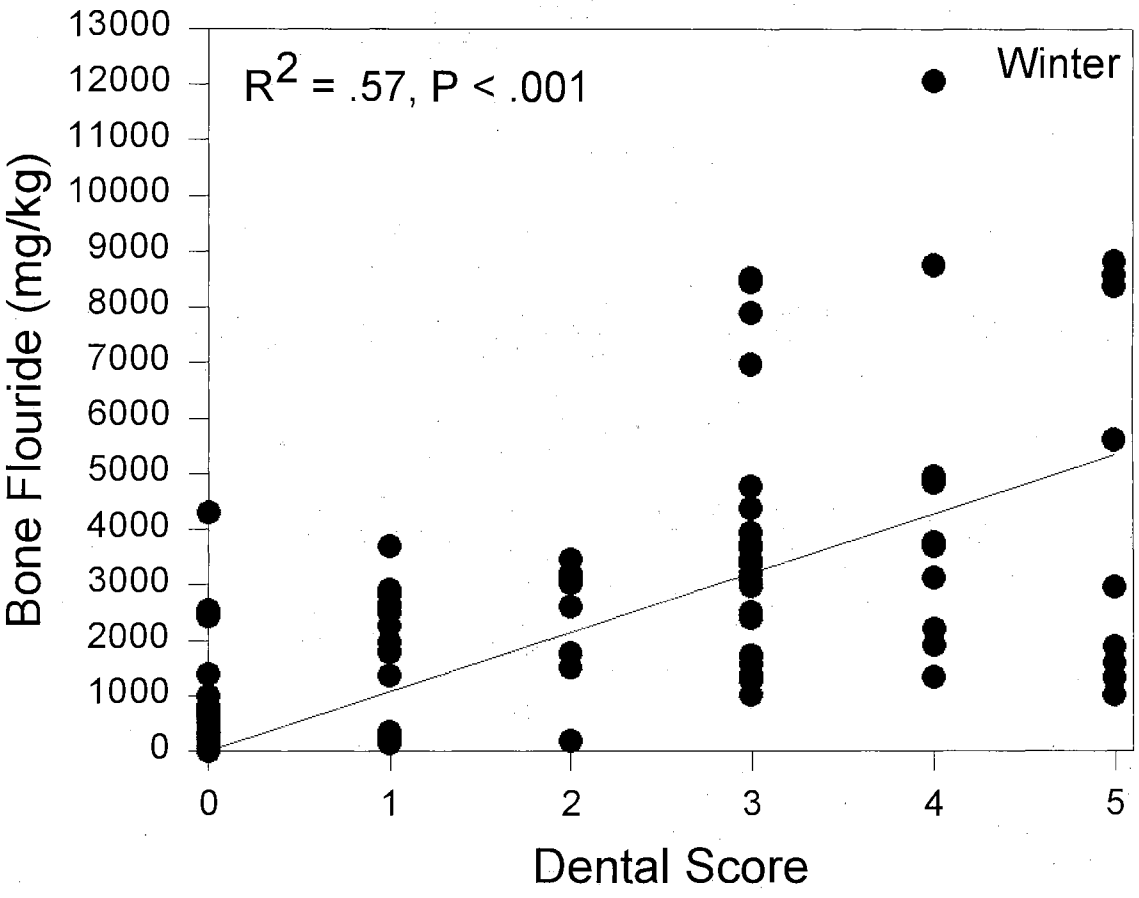
FIGURE 6. Incisors from a cotton rat with severity score for dental lesions of 5. Both upper and lower incisors are white chalky.

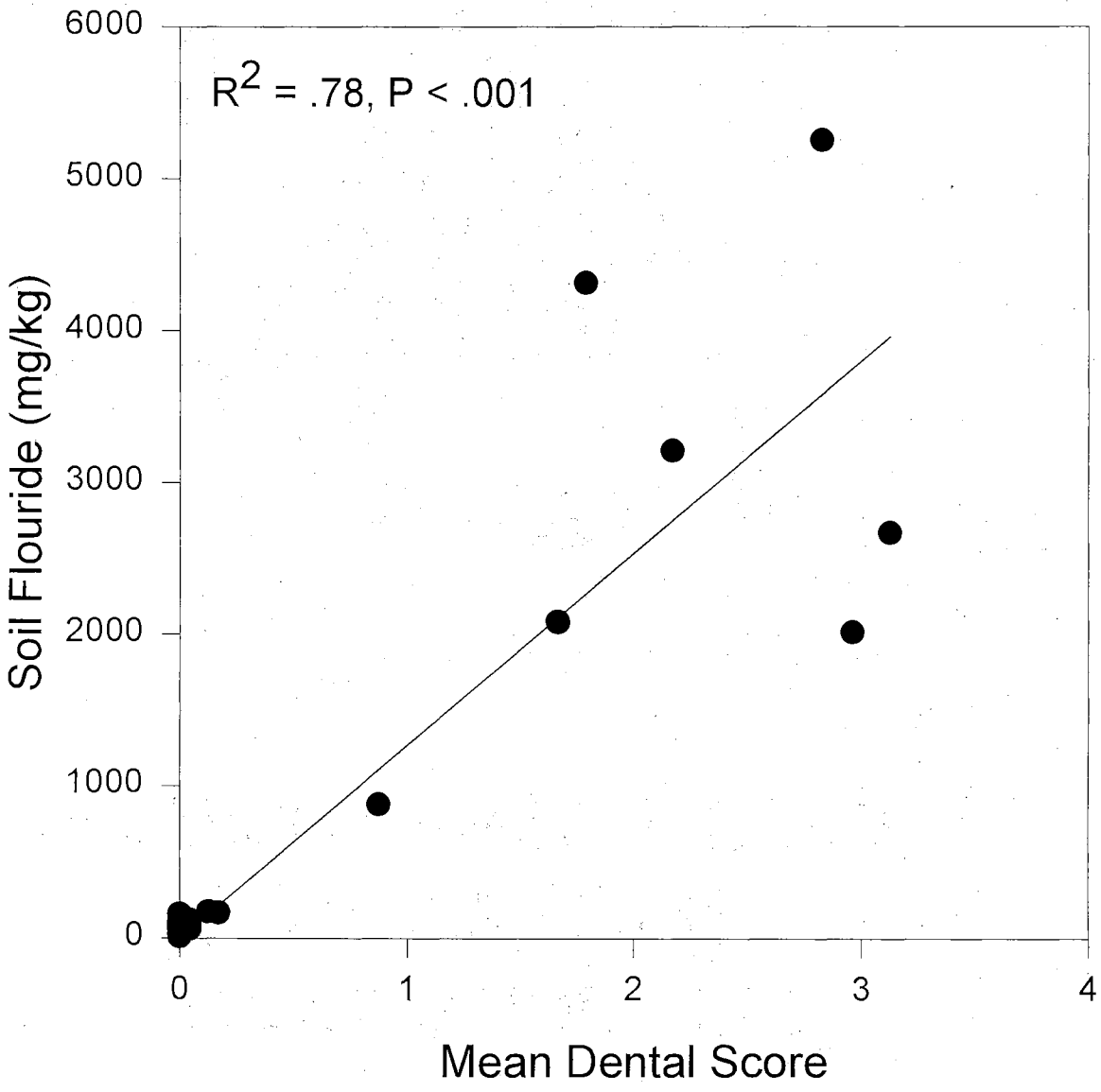
Figure Legends

FIGURE 7. Relationship between concentration of fluoride in bone tissue and severity of dental lesions in cotton rats collected from 12 contaminated sites and their matched reference sites in Oklahoma during summer and winter.

FIGURE 8. Relationship between mean concentration of soil fluoride and mean severity of dental lesions in cotton rats from 12 contaminated sites and their matched reference sites in Oklahoma.


Summer
R^2 = .45, P < .001
Bone Flouride (mg/kg)
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
Dental Score


Winter
R^2 = .57, P < .001
Bone Flouride (mg/kg)
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
Dental Score


R^2 = .78, P < .001
Soil Flouride (mg/kg)
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
Mean Dental Score

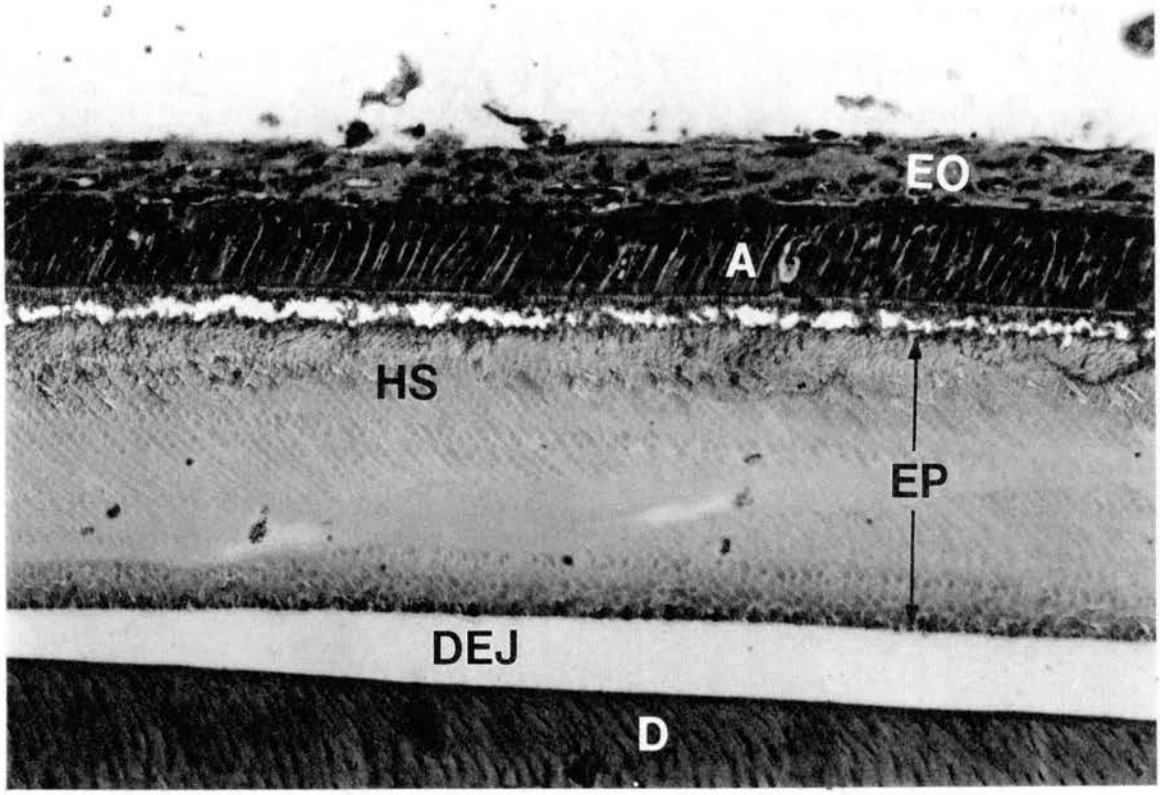


FIGURE 9. Incisor from a cotton rat from a reference site. Note enamel organ (EO), the palisading arrangement of tall columnar ameloblasts (A) with basally oriented nuclei, underlying enamel prism (EP) containing Hunter-Schreger bands (HS), dentin-enamel junction (DEJ), and dentin (D). Decalcified section. Methylene blue. ×280.

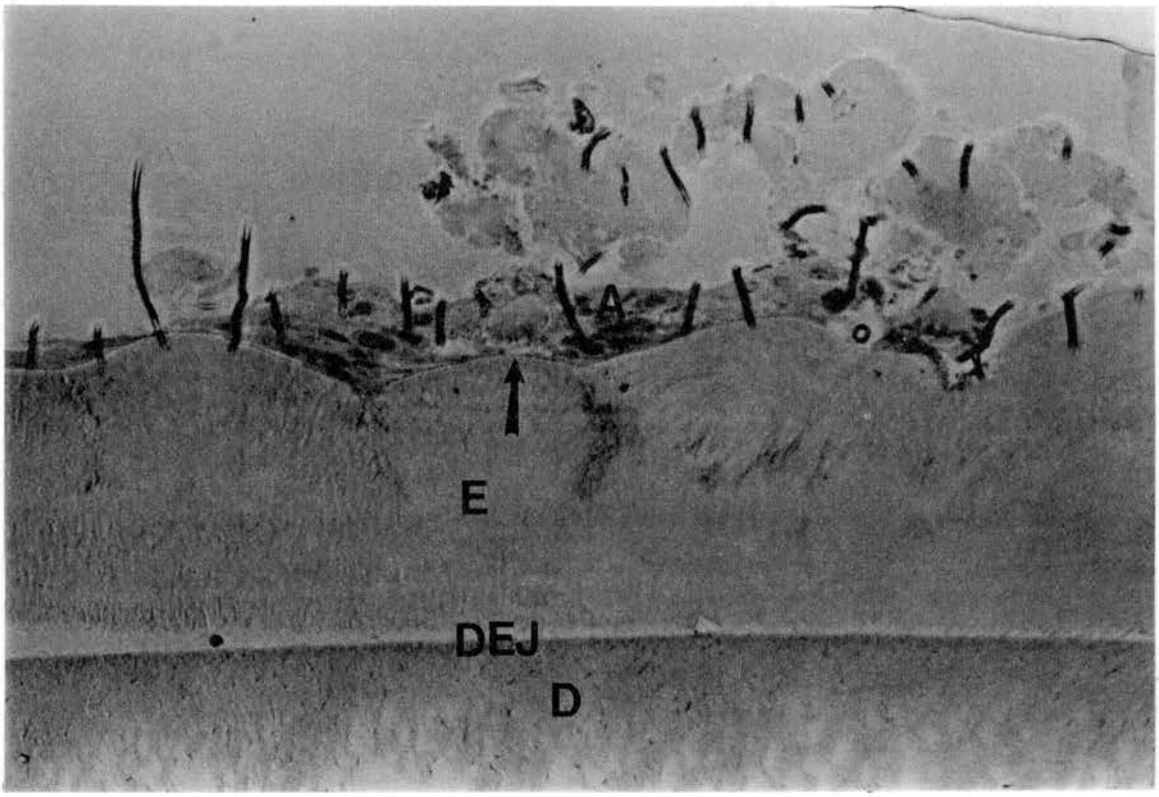


FIGURE 10. Incisor from a cotton rat from a contaminated site. Compare it to fig. 9. Note flattened and irregularly arranged ameloblasts (A) without basal orientation of nuclei, uneven surface of the enamel (arrow), and thinning of the enamel (E) with the loss of Hunter-Schreger bands. Decalcified section. Methylene blue. ×280.

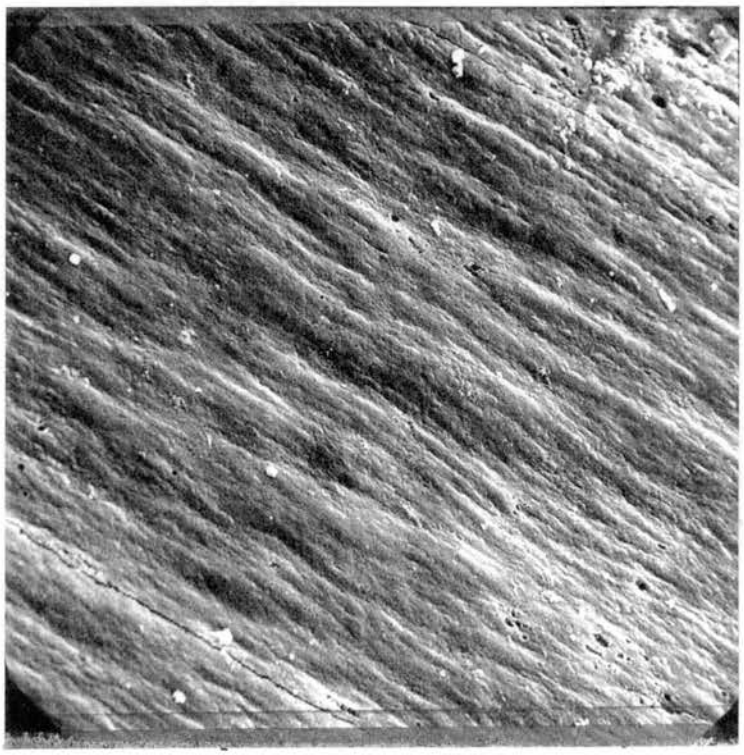


FIGURE 11. Scanning electron micrograph of the labial surface of the incisor from a cotton rat from a reference site. Note smooth enamel surface.

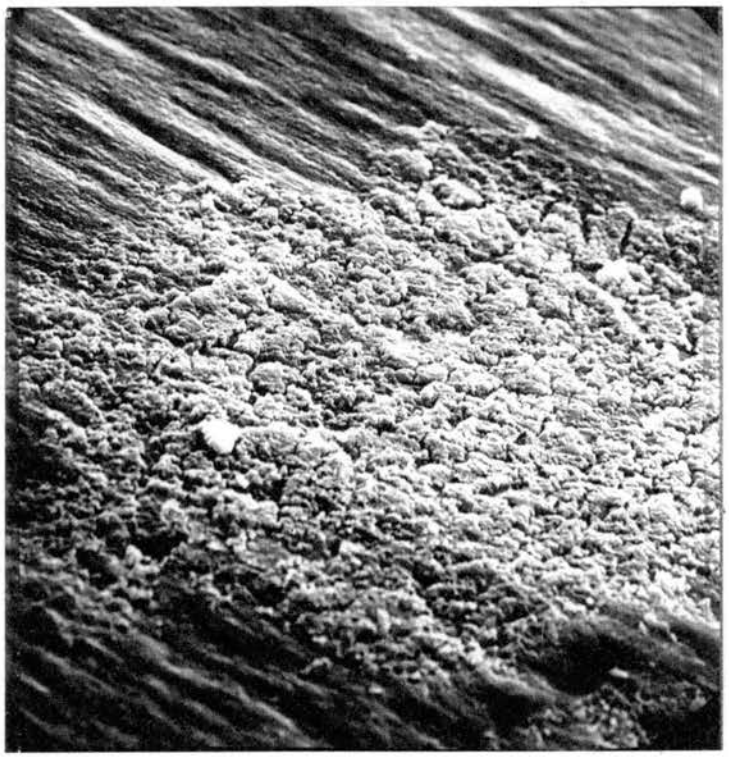


FIGURE 12. Scanning electron micrograph of the labial surface of the incisor from a cotton rat from a contaminated site. Note large area of hypoplasia with rough surface. The boundary between the hypoplastic area and the surrounding enamel is well demarcated.

CHAPTER IV

EFFICACY OF HISTOPATHOLOGY IN DETECTING PETROCHEMICAL-INDUCED TOXICITY IN WILD COTTON RATS (*SIGMODON HISPIDUS*)

ABSTRACT

A variety of chemical mixtures exist in the soil of petrochemical waste sites, and many of these compounds are known immunotoxicants that have been observed to induce immune alterations in wild rodents inhabiting many of these petrochemical waste sites. Conventional histopathological assessments have been widely used with considerable success to investigate immunotoxicity of various agents under laboratory conditions. We hypothesized that histopathologic assessments would be equally sensitive for detecting exposure to complex mixtures of toxicants in cotton rats (*Sigmodon hispidus)* residing in contaminated habitats. We examined histopathological parameters of cotton rats that were seasonally collected from 13 petrochemical-contaminated waste sites and 13 ecologically-matched reference sites in Oklahoma over a 3 year period. Histopathological examination did not reveal any lesion associated with exposure to petrochemical wastes except renal inclusion bodies. Prevalence and severity of histologic lesions in liver and kidneys of cotton rats were significantly influenced by season, where prevalence and severity were lower in winter than summer on all study sites. These results suggest that

the evaluation of toxicity from exposure to contaminants in the soil of industrial waste sites using histopathological assessments is not sensitive enough to detect exposure to the low levels of environmental contaminants present on most waste sites.

INTRODUCTION

The disposal of petrochemical wastes involves application of wastes onto or into soil to avoid adverse impacts to human health and the environment by using the assimilative capacity of the soil to treat and dispose of the applied wastes. The widespread disposal of petrochemical wastes into the environment has become a significant problem of concern in recent decades. A variety of chemical mixtures are usually found in soil of these types of waste sites and many of these compounds have known effects on immune function. Recent field studies have raised serious concerns regarding immunotoxicity and fluorosis risks to wild vetebrates, terrestrial ecosystems, and potential exposure of humans (Elangbam et al., 1989; McBee et al., 1987; McBee and Bickham, 1988; McMurry et al., 1993, 1999; Paranjpe et al., 1994; Propst et al., 1999; Rafferty, 1998; Schroder et al., 1999). Elangbam et al. (1989) reported significant differences in population characteristics of cotton rats residing in toxic waste disposal sites compared to those from reference sites. It has been reported that cotton rats residing in hazardous-waste sites associated with the petrochemical industry have significantly higher frequencies of chromosomal aberrations (McBee et al., 1987; McBee and Bickham, 1988; Thompson et al., 1988). Significant induction of hepatic cytochrome P-450 has been reported in cotton rats from petrochemical disposal sites (Elangbam et al.,

1989, 1991). Evidence has accumulated that cotton rats inhabiting petrochemical waste sites have higher risks of immunotoxicity (McMurry et al., 1993, 1999; Propst et al., 1999).

Histopathological alterations of fish tissues have been useful biomarkers of effect of exposure to environmental toxicants, which reflects prior alteration in physiological and/or biochemical function (Huggett et al., 1992). Histopathological examination of lymphoid organs has been deserved an extremely sensitive indicator for investigating immunotoxicity of various agents in laboratory rodents (Doi et al., 1996; Gopinath, 1996; Schuurman et al., 1994). Crevel et al. (1997) reported that histopathological evaluation of the immune system is sufficiently sensitive to identify potential immunotoxicants without recourse to immune function tests.

The primary aim of our research was to evaluate the sensitivity of histopathological assessments for detecting toxicity in wild rodents exposed to petrochemical wastes in their environments. We have used the wild cotton rat (*Sigmodon hispidus*) as an indicator species, which has been suggested as a useful biomonitor for studying the ecological impact of environmental pollutants (Elangbam et al., 1989, 1991; Faith et al., 1997; Paranjpe et al., 1994). We hypothesized that histopathological assessments would provide a sensitive approach to detecting exposure to complex mixtures of toxicants in wild populations of small mammals. To explore this hypothesis, we conducted histopathological examinations of various organs of cotton rats from replicated petrochemical waste sites and reference sites throughout Oklahoma.

MATERIALS AND METHODS

Site Selection

We selected 13 locations for study (herein referred to as "units") in Oklahoma with each unit consisting of a petrochemical-contaminated waste site and a matched reference site. Reference sites were located <16 km from the contaminated site and were chosen based on their similarity in vegetation structure and composition, soil types, and topography with their matched contaminated sites. All contaminated sites were characteristic of a disturbed prairie ecosystem, with early successional species dominating the vegetation community, and these sites had soil that historically received a variety of petrochemical wastes. These sites were also chosen based on the presence of adequate vegetation cover for supporting a resident small mammal population. No historical information was available on the actual amount, concentration, and mixtures of contaminants that were previously disposed of on these sites.

Experimental Design

Cotton rats were collected from 13 study units for histopathological assessment from September 1995 to February 1998. Four units were sampled each year (5 units were sampled in 1996) in February and September to evaluate seasonal influences on sensitivity of cotton rats to environmental contaminants. A total of 624 cotton rats were examined from these 13 units during the study. Six male and six female cotton rats were collected from each study site during each season. All cotton rats were reproductively mature and weighed more than 50 g.

Cotton rats were caught live in traps and taken to the laboratory animal facility. All cotton rats were placed individually in polycarbonate cages with food and tap water provided *ad libitum*, housed under a 16L : 8D light-dark illumination cycle at 23-24 °C and 50% relative humidity, and were processed within 48 hours of capture. Animals were anesthetized with Metofane® (Pittman-Moore, Mundelein, IL) and total body weight was recorded. Anesthetized animals were killed by cervical dislocation, and liver, kidneys, adrenal glands, gonad, and spleen were weighed at necropsy. The following tissues were collected in 10% neutral-buffered formalin: liver, kidneys, lung, mesenteric lymph node, adrenal glands, gonad, heart, duodenum, ileum, jejunum, cecum, and colon. All tissues were routinely processed, sectioned at 5-7 um, and stained with hematoxylin and eosin for histopathology. Histologic lesions of liver and kidney were graded for severity using a scoring system whereby 0 = no lesion, 1 = mild, 2 = moderate, 3 = severe (Table 1). Sections of kidney were stained with Fite's Ferraco to detect renal acid-fast intranuclear inclusions.

Statistical Analysis

We used a randomized complete block design in a 2 × 2 × 13 factorial format with two treatments (contamination and reference), two seasons (summer and winter), and 13 study units (units 1-13) to evaluate whether petrochemical contamination was associated with increased risk of severity score of histological lesions and morphological parameters of selected organs of resident cotton rat populations. Comparisons were made using PROC MIXED (SAS, 1994) with sources of variation distributed among the main factor effects and the interaction terms (study unit by treatment interaction, season by treatment

interaction, and study unit by season interaction). Significant interaction term effects were compared using the SLICE option for the LSMEANS statement. Satterthwaite's approximation was used in calculating degrees of freedom for the error term. Differences in prevalence of lesions were determined by using a z-test (EpiCalc 2000 Version 1.02). P-values of $< .05$ were considered to be significant and all values are presented as means $\pm$ SD.

RESULTS

A significant ($p < .0045$) treatment main effect was evident for relative weights of kidney of cotton rats (Figure 1). Relative kidney weight of cotton rats from reference sites (overall mean = 7.99 ± 1.29) was slightly greater than contaminated sites (7.65 ± 1.30).

There were no treatment main effects for the severity or prevalence of histologic lesions in either liver or kidney tissues between cotton rats from reference and contaminated sites, and the appearance of organ sections of cotton rats from both sites was indistinguishable. Both severity and prevalence of histologic lesions in liver and kidneys differed seasonally ($p < .001$), where severity and prevalence were 49-78% lower in winter than summer on both reference and contaminated sites (Table 2). A significant study unit by season interaction was evident in severity of histologic lesions in liver ($p < .008$) and kidneys ($p < .0011$) of cotton rats. Least square means analysis revealed that severity of histologic lesions in liver and kidneys was significantly lower in winter compared to summer in 7 of the 13 study units.

A form of chronic nephropathy, characterized by chronic interstitial inflammation, interstitial fibrosis, tubular mineralization, and tubular dilatation with proteinaceous casts, was observed in cotton rats from all study sites (Figure 2). Acid-fast intranuclear inclusions (Fite's Ferraco method) were found in renal epithelial cells of two cotton rats from the contaminated site on unit 11; these two cotton rats had lead concentrations in bone of 316 and 128 mg/kg, respectively (Figure 3). No other cotton rats from ether contaminated sites or reference sites had renal acid-fast inclusions and lead concentrations in bone ranged from 57 to 169 mg/kg and 0 to 9 mg/kg, respectively.

Cysticercus infection was commonly found in the liver of cotton rats from both contaminated sites and reference sites. Discrete multifocal granulomatous hepatitis with occasional necrotic foci with neutrophilic infiltrates was observed in cotton rats from both sites (Figures 4 and 5).

Strongyloides sp. was found in the intestinal mucosa of cotton rats from all study sites (Figure 6). There were seasonal and treatment-related differences in the prevalence of *Strongyloides* sp. in histologic sections of small intestine of cotton rats. Prevalence of *Strongyloides* sp. in cotton rats from contaminated sites (73/312; 23%) was lower than reference sites (116/310; 37%; $p < .001$). There were 42% and 53% decreases in prevalence of *Strongyloides* sp. from summer to winter in cotton rats from contaminated sites and reference sites, respectively. One cotton rat from a contaminated site had intranuclear inclusion bodies in intestinal epithelium that may have been due to adenovirus infection (Figure 7).

Multifocal mineralization in the heart was seen in one cotton rat from a reference site and three cotton rats from contaminated sites. Randomly distributed infiltration of

lymphocytes and macrophages was seen in the myocardium of two cotton rats from reference sites and one from a contaminated site. Myocardial fibrosis was observed in one cotton rat from a reference site.

Lung sections showed alveolar histiocytosis composed of distended alveoli filled with foamy macrophages in two cotton rats from reference sites. One cotton rat from a contaminated site had pneumoconiosis characterized by focal granulomatous inflammation with macrophages containing dark brown granular pigments. Another cotton rat from a contaminated site had eosinophilic granulomatous arteritis with locally extended interstitial inflammation due to parasitic organisms.

DISCUSSION

It has been suggested that histopathological examination can be an extremely sensitive approach to evaluating immunotoxicity of various agents in laboratory rodents (Crevel et al., 1997; Doi et al., 1996; Gopinath, 1996; Schuurman et al., 1994). We hypothesized that such techniques would also be sensitive in wild rodents inhabiting industrial waste sites contaminated with petrochemicals, as a variety of chemical mixtures of organic hydrocarbons and heavy metals exist in such soils, many of which are known immunotoxicants. Surprisingly, our study failed to support this hypothesis and we concluded that histopathological parameters of cotton rats are of limited value for detecting exposure to low levels of environmental contaminants found on petrochemical waste sites. Resistance of cotton rats to many pesticides and toxins, which is possibly related to enhanced capacity to perform detoxication reactions, might be partly

responsible for the insensitivity of these indices (Rattner et al., 1993). It is also possible that there are low enough levels of contaminants, and histological lesions would not be found in any species.

Our observations are in agreement with those of Elangbam et al. (1989) who reported that morphologic evaluations, including relative organ weights and histopathologic examination of major parenchymal organs, did not reveal any remarkable lesions associated with exposure to environmental contaminants in cotton rats. In our study, mild interstitial nephritis, cardiac fibrosis and mineralization, intestinal strongyloidiasis, nematodiasis, and cestodiasis, and hepatic cysticercus, necrosis and granulomatous inflammation were commonly found in cotton rats from both contaminated and reference sites, similar to descriptions in other studies (Boggs et al., 1991; Elangbam et al., 1991, 1990, 1989; Faith et al., 1997; Montgomery and Seely, 1990; Rattner et al., 1993). Identification of nematodes and cestodes recovered from the small intestine of cotton rats has been reported in other studies. Boggs et al. (1991) identified *Longistriata adunca* nematodes and *Raillietina* sp. cestodes from cotton rats in Oklahoma. *Strongyloides* sp. was previously found in the intestinal mucosa of cotton rats collected from central Oklahoma (Elangbam et al., 1990). Prevalence of *Strongyloides* sp. infection observed in our study was 37% (116/310) and 23% (73/312) in reference sites and contaminated sites respectively, which was lower than the 78% (31/40) reported by Elangbam et al (1990). Elangbam et al. (1989) reported 50% of cotton rats from each toxic waste disposal site and control site had *Strongyloides* sp. within the intestinal crypts. Two previous studies did not report the seasonal and treatment-related differences

in the prevalence of *Strongyloides* sp. infection in cotton rats, which was significant in our study.

Significantly increased relative liver and kidney weights have been reported in white footed mice (*Peromyscus leucopus*) inhabiting an area contaminated with polychlorinated biphenyls and metals (Batty et al., 1990). Laboratory mice exposed to soils of the Love Canal chemical dump site had increased relative liver and kidney weights (Silkworth et al., 1984). In comparison to these studies, cotton rats in this study had relative kidney weights that were lower on contaminated sites than reference sites. Such a discrepancy in results between our study and others may be due to differences in type and concentration of contaminants in the soil among study sites.

There was a significant seasonal effect in severity and prevalence of histologic lesions in liver and kidneys of cotton rats, where severity and prevalence were lower in winter than summer on both reference and contaminated sites, but the mechanism is not fully understood. The only histological lesion directly associated with exposure to environmental contaminants was the renal intranuclear inclusions, which were acid-fast by Fite's Ferraco method, in only two cotton rats with bone lead concentrations of 316 and 128 mg/kg, respectively. McMurry et al. (1995) reported lead-induced toxicity in cotton rats receiving 1,000 ppm lead acetate for 7 weeks in a laboratory setting, including alterations in male reproductive organs, lymphoid tissues, and hematological parameters. They also observed that kidneys of cotton rats receiving 1,000 ppm lead acetate for 7 weeks had altered renal proximal tubular epithelium with occasional acid-fast staining renal intranuclear inclusions. Deer mice (*Peromyscus maniculatus*) with a mean lead concentration in bone of 80 mg/kg had intranuclear inclusions within renal tubular

epithelial cells (Kisseberth et al., 1984). White-footed mice (*Peromyscus leucopus*) and shorttail shrews (*Blarina brevicauda*) at a trap and skeet range were found to have mean levels of lead in bone of 245 and 437 mg/kg, respectively, with renal intranuclear inclusions (Stansley and Roscoe, 1996). The discrepancy in results of the presence of intranuclear inclusions and bone lead concentration between our study and others is possibly attributed to varying in concentration of lead through the sites, individual sensitivity to exposure to lead, and species and individual variability in lead metabolism.

Recent field studies have reported that environmental contamination induced by petrochemical wastes induced immune alterations, hepatic cytochrome P-450 induction, bone metal accumulation, and fluorosis in resident cotton rats (Elangbam et al., 1989, 1991; McBee et al., 1987; McBee and Bickham, 1988; McMurry et al., 1993, 1999; Paranjpe et al., 1994; Propst et al., 1999; Rafferty, 1998; Schroder et al., 1999). We conclude that histopathological parameters of cotton rats in petrochemical waste sites were not sensitive enough to detect subtle toxicity induced by low-dose long-term exposure to hazardous wastes of petroleum industry. Seasonal influence on the prevalence and severity of histological lesions in liver and kidneys of cotton rats were evident in this study, and contributing factors to the seasonal influence should be determined in future studies. There is a need for well-defined sensitive assay system for evaluating the effects and risks of environmental contamination on assemblages of organisms in terrestrial environment. Morphological and histological parameters could support the result of other assays for complete evaluation of impact of environmental contaminants on terrestrial ecosystem.

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Table 1. Scoring criteria for assessing severity of histologic lesions in liver and kidney tissue of cotton rats collected from petrochemical-contaminated sites and reference sites in Oklahoma

Lesion score	Organ	Characteristics
0 (Normal)	Liver	No lesion
1 (Mild)		Few minute granulomatous inflammation
2 (Moderate)		Discrete multifocal granulomatous inflammation and occasional necrotic foci with neutrophilic infiltrates
3 (Severe)		Severe multifocal granulomatous inflammation and locally extensive necrotic foci with neutrophils
0 (Normal)	Kidney	No lesion
1 (Mild)		Minimal lymphoplasmacytic infiltrates in cortex
2 (Moderate)		Mild to moderate multifocal interstitial fibrosis and Lymphoplasmacytic infiltrates
3 (Severe)		Moderate to severe multifocal interstitial fibrosis and Lymphoplasmacytic infiltrates with frequent dilated tubules containing protein casts

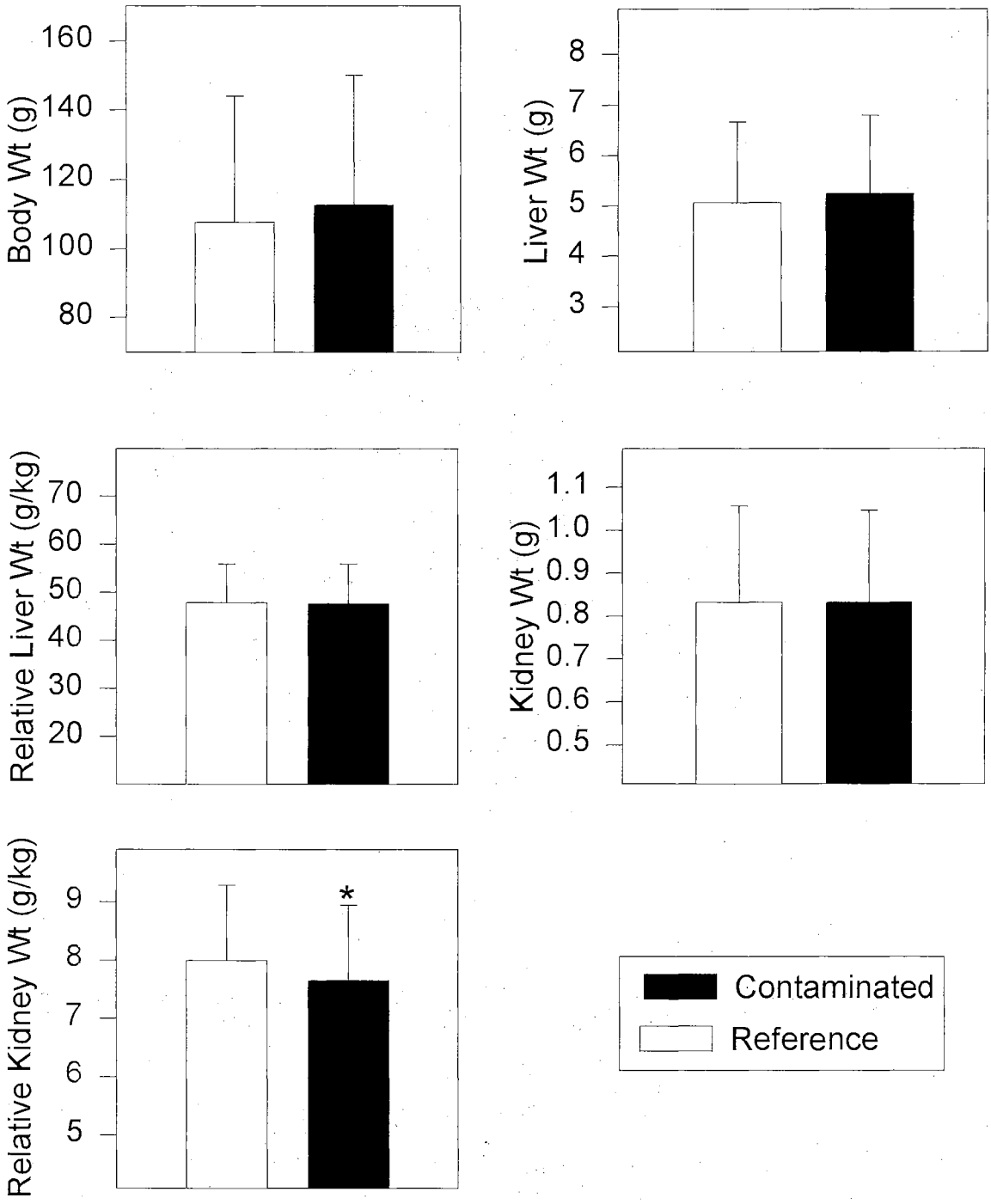
Table 2. Seasonal comparison of the prevalence (animals with lesions/total number examined) and severity (mean ± SD) of lesions in cotton rats with histologic lesions in liver and kidneys from contaminated sites and their matched reference sites in Oklahoma

		Liver		Kidney	
Treatment	Lesion	Summer	Winter	Summer	Winter
Reference	Severity	0.55 ± 0.77	0.20 ± 0.42[a]	0.50 ± 0.75	0.14 ± 0.44[a]
	Prevalence	64/156 (41%)	28/155 (18%)[a]	61/155 (39%)	16/155 (10%)[a]
Contaminated	Severity	0.53 ± 0.69	0.24 ± 0.49[a]	0.45 ± 0.68	0.10 ± 0.34[a]
	Prevalence	66/155 (43%)	35/157 (22%)[a]	56/155 (36%)	14/157 (9%)[a]

[a] Significantly different at $p < .05$ compared to summer.

Figure Legend

FIGURE 1. Comparison of body weight and organ weight (absolute and relative) of cotton rats collected from contaminated sites and their matched reference sites in Oklahoma. Values represent mean ± SD; * = significant difference between reference and contaminated study sites ($p < .05$).


Body Wt (g)
160
140
120
100
80
Liver Wt (g)
8
7
6
5
4
3
Relative Liver Wt (g/kg)
70
60
50
40
30
20
Kidney Wt (g)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
Relative Kidney Wt (g/kg)
9
8
7
6
5
*
Contaminated
Reference

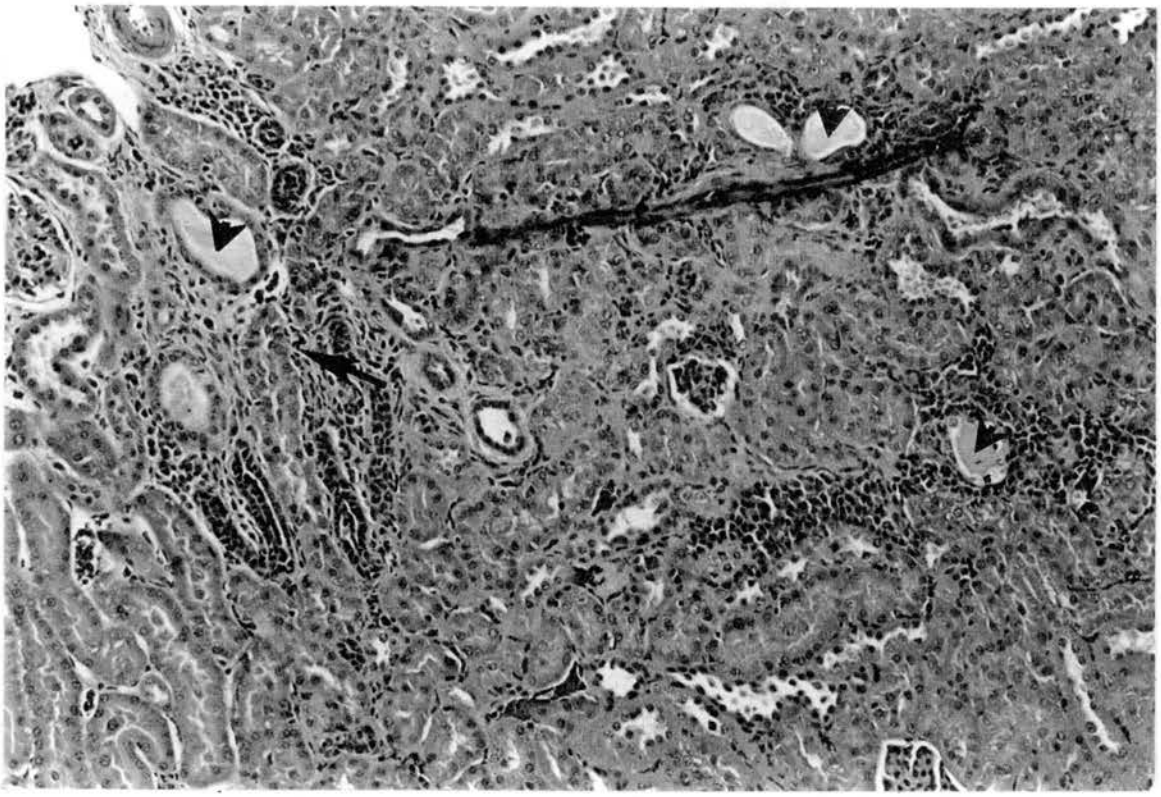


FIGURE 2. Renal cortex from a cotton rat. Chronic interstitial inflammation, interstitial fibrosis (arrow), and tubular dilatation with proteinaceous casts (arrowhead) are present. H&E. ×140.

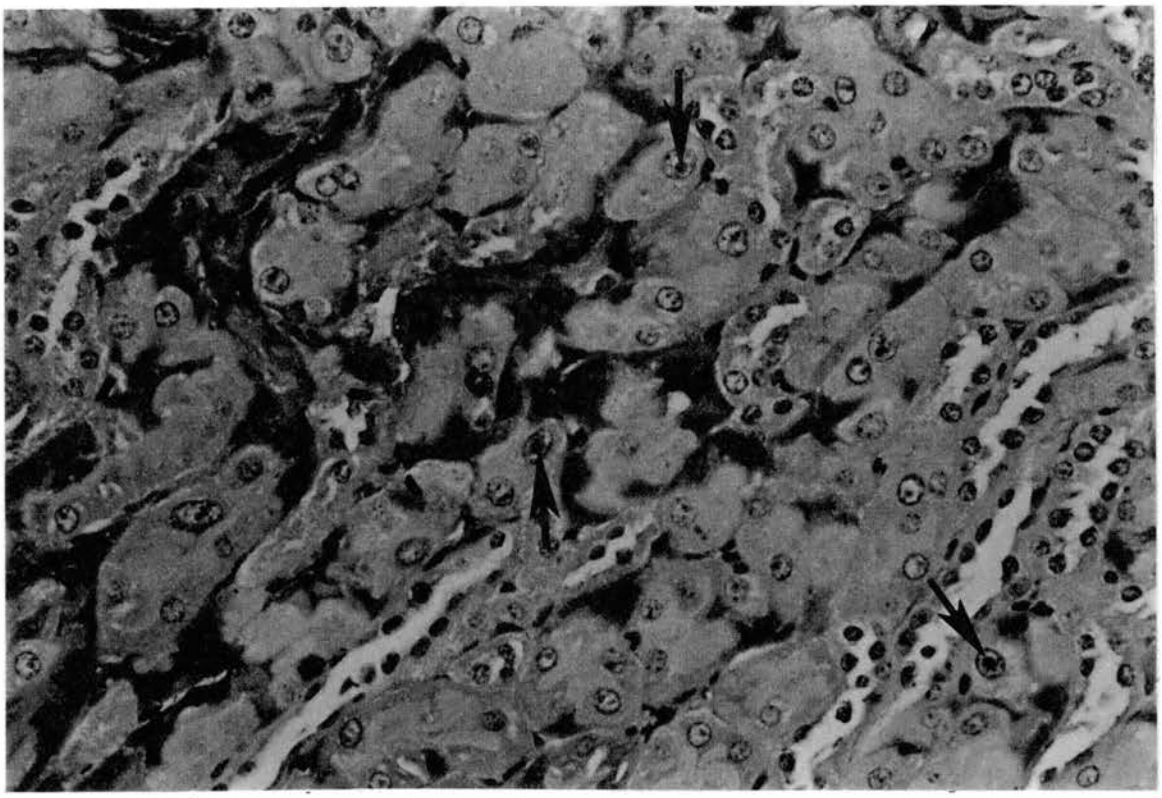


FIGURE 3. Fite's Ferraco staining of renal cortex from a cotton rat. Numerous acid-fast intranuclear inclusions (arrow) are present in renal epithelial cells. ×520.

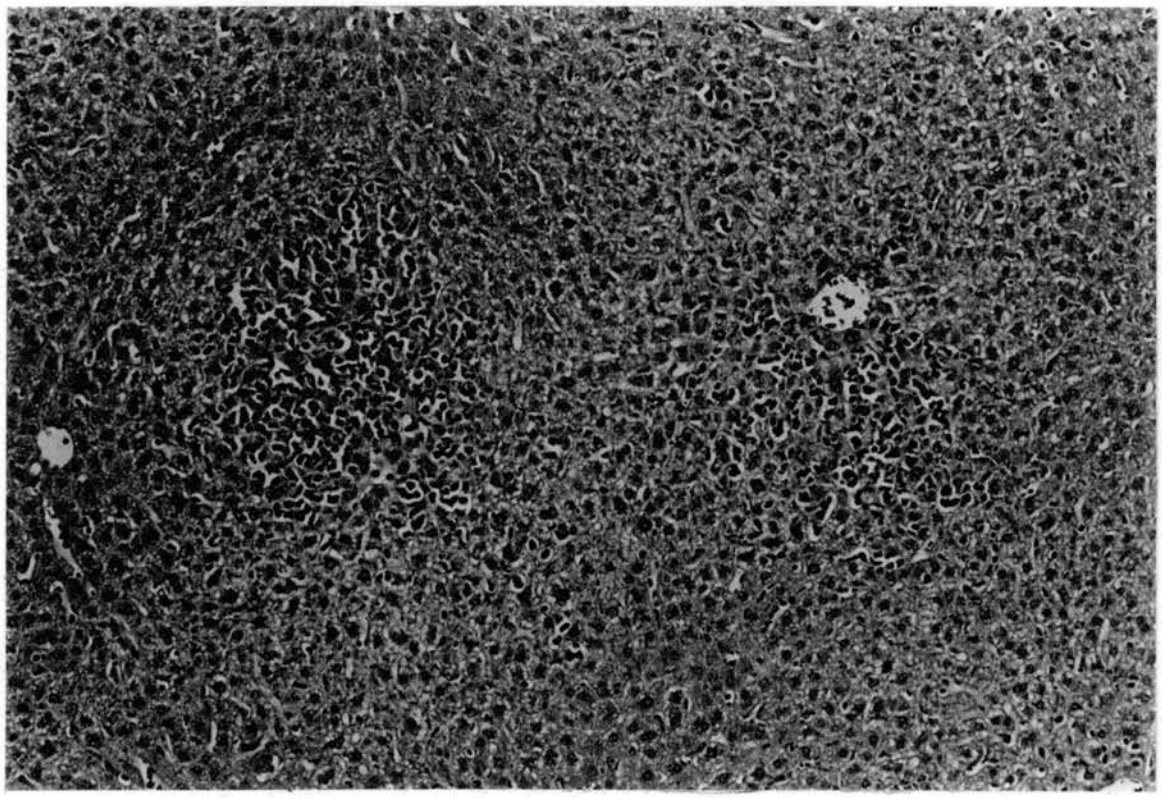


FIGURE 4. Histological section of liver from a cotton rat. Note discrete multifocal granulomatous inflammation. H&E. ×130.

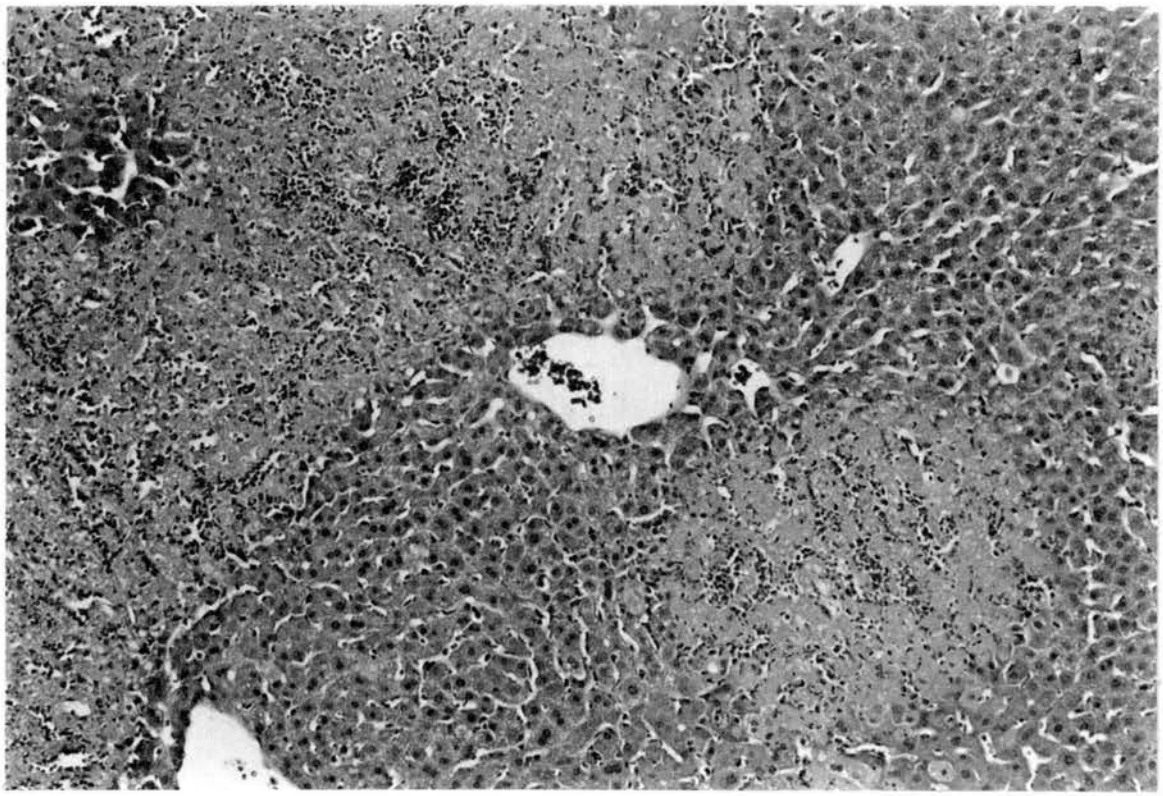


FIGURE 5. Histological section of liver from a cotton rat. Note multifocal, locally extensive areas of necrosis with neutrophilic infiltrates. H&E. ×130.

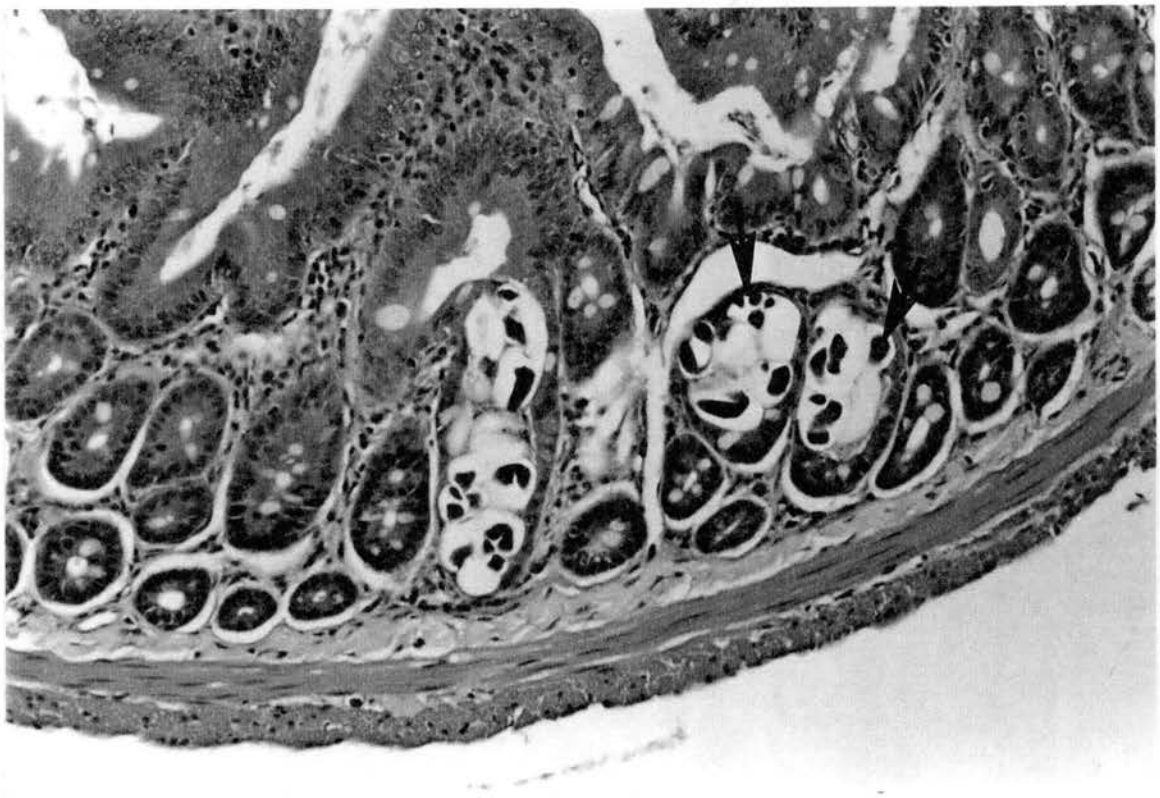


FIGURE 6. Small intestine from a cotton rat. Note the larvae of *Strongyloides* sp. (arrow) within the intestinal crypts. H&E. ×150.

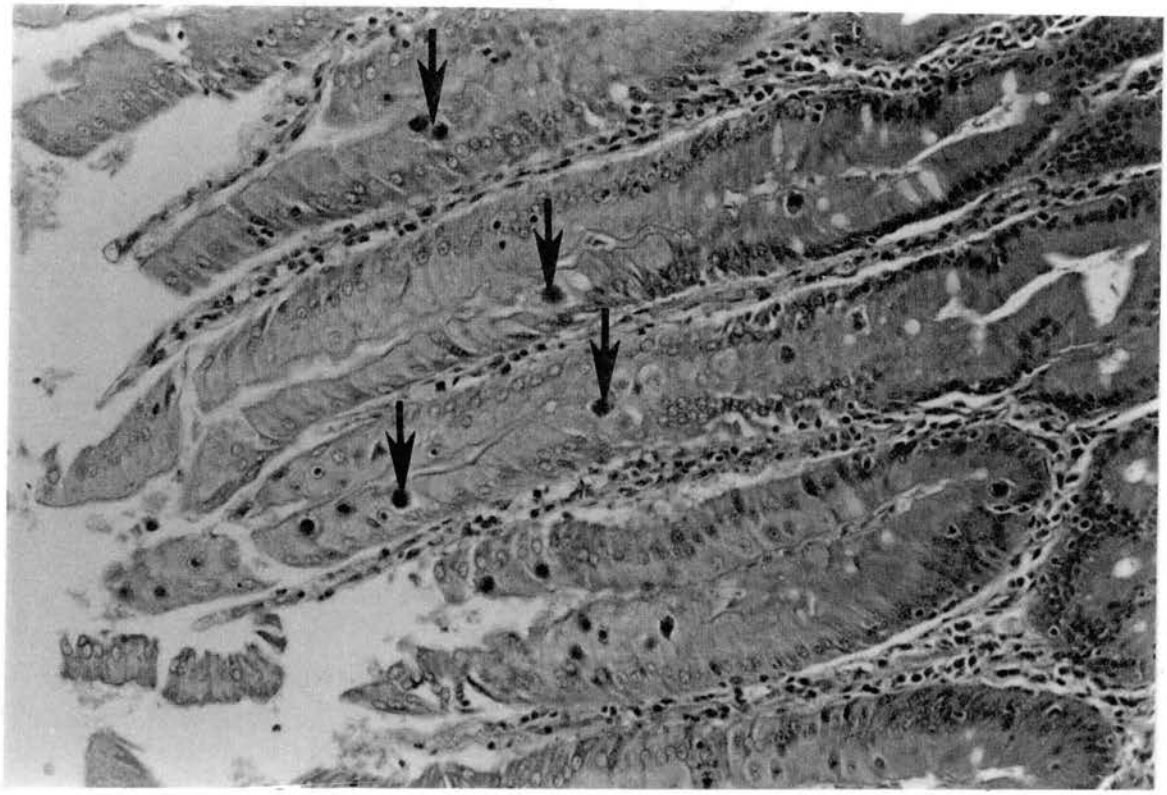


FIGURE 7. Small intestine from a cotton rat from a contaminated site. Numerous intranuclear inclusion bodies (arrow) are present in intestinal epithelium. H&E. ×150.

VITA

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