Developmental Exposure to Lead Causes Persistent Immunotoxicity in Fischer 344 Rats

T. E. Miller, K. A. Golemboski, R. S. Ha, T. Bunn, F. S. Sanders, and R. R. Dietert

Department of Microbiology and Immunology, College of Veterinary Medicine, and Institute for Comparative and Environmental Toxicology, Cornell University, Ithaca, New York 14853-6401

Received August 8, 1997; accepted January 9, 1998


Lead has been shown to exert toxic effects during early development. In these in vivo and ex vivo experiments, the effect of lead on the immune system of the developing embryo was assessed. Nine-week-old female Fischer 344 rats were exposed to lead acetate (0, 100, 250, and 500 ppm lead) in their drinking water during breeding and pregnancy (exposure was discontinued at parturition). Offspring received no additional lead treatment after birth. Immune function was assessed in female offspring at 13 weeks of age. Dams in lead-exposed groups were not different from controls with respect to the immune endpoints used in these experiments; however, in the offspring, lead modulated important immune parameters at modest exposure levels. Macrophage cytokine and effector function properties (tumor necrosis factor-α and nitric oxide production) were elevated in the 250 ppm group, while cell-mediated immune function was depressed, as shown by a decrease in delayed-type hypersensitivity reactions in the 250 ppm group. Interferon-γ levels were decreased in the 500 ppm treatment group. Serum levels of IgE were increased in rats exposed to 100 ppm lead. These results indicate that exposure of mothers to moderate levels of lead produces chronic immune modulation in their F344 rat offspring exposed in utero. Since the mothers were not susceptible to chronic immune alterations, a developmental bias to the immunotoxic effects of lead is indicated. The differences observed are consistent with the possibility that lead may bias T helper subset development and/or function, resulting in alterations in the balance among type 1 and type 2 immune responses.

Levels of lead in blood as low as 10 to 15 μg/dl in infants can result in cognitive and behavioral deficits (Bellinger et al., 1991; Dietrich et al., 1991). All children appear to be at risk due to the prevalence of lead and the narrow margin of safety associated with lead’s low effect level (Rosen, 1995).

Lead also has been shown to modulate various functions of the immune system (Dean and Murray, 1991); exposure may either suppress or enhance immune responses, dependent upon the treatment and the specific parameter in question (Zelikoff et al., 1994). Little information is available concerning the immunological consequences of fetal exposure to lead. Rats exposed to lead in utero and for 4 subsequent weeks demonstrated changes in some humoral and cell-mediated immune parameters (Luster et al., 1978; Faith et al., 1979); however, these studies examined neither the differences among in utero and perinatal exposure nor the persistence of these effects into adulthood. In addition, the relative susceptibility of various age groups for immunotoxic responses has not been extensively examined.

Comparisons of the relative sensitivity and predictability of immune assessment techniques have shown that as few as two or three specific immune parameters can be informative for prediction of immunotoxic effects in rodents (Luster et al., 1992). The present study was designed to assess a variety of immune endpoints in rats exposed to lead during pregnancy or in utero, with the goal of describing possible persistent immunotoxic effects (defined for the purposes of this study as those effects which are present in rats which were exposed either in utero and have reached adulthood or during pregnancy and no longer harbor a significant body burden of lead) and the relative sensitivity of rats in these two groups.

METHODS AND MATERIALS

Animals. Seven- to eight-week-old Fischer 344 (F344) rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Animals were housed three per polycarbonate cage for a 2-week acclimation period (two per cage during mating). The AIN-93G purified rodent diet (Dyets, Inc., Bethlehem, PA) was fed ad libitum to all rats. Weekly feed and water intake and body weights were documented during pregnancy and lactation. A 12-h light/dark cycle was maintained during the entire experiment. Temperature and humidity were maintained between 68 and 75°F and 40–60%, respectively. Protocols...
were approved by the Cornell University Institutional Animal Care and Use Committee and complied with NIH guidelines.

**Breeding and lead exposure.** Following acclimation, 12 females were randomly assigned to each of four treatment groups (0, 100, 250, and 500 ppm lead, as lead acetate, in drinking water) and housed with males for 10 days. Control rats received sodium acetate equivalent in acetate concentration to that of the 500 ppm lead treatment group. Acetic acid (0.00125%) was added to the water to aid in dissolution. The treated water was provided ad libitum following acclimation, through mating, and until parturition. At parturition, lead treatment ceased and all rats were switched to normal drinking water. Tapwater was tested for lead contamination and shown to be below laboratory detection limits (<0.001 mg/L). Female offspring were weaned at 21 days, separated by litter, and housed 3 to 4 per cage. At initiation of analyses, dams were 7–8 weeks postpartum, and female offspring were old (a total of 11–14 dams and 12–17 offspring were tested per treatment group); male offspring were not tested in this series of experiments.

**Reagents.** Monoclonal antibodies for flow cytometry were obtained from Harlan Bioproducts for Science, Inc. (Madison, WI); FITC-, peroxidase-, and alkaline phosphastase-conjugated antibodies were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Sigma Chemical Company (St. Louis, MO) supplied Escherichia coli lipopolysaccharide (LPS), concanavalin A (Con A), Histopaque (1.083 g/ml), α-phenylmercuriaine dihydrochloride (OPD), lactate dehydrogenase (LDH) substrate mixture, bovine serum albumin (BSA), recombinant rat interleukin-2 (rRat IL-2), p-nitrophenyl phosphate, and Tween 20. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (La Jolla, CA). Monoclonal anti-rat IgE heavy chain and Rat IgE-κ were purchased from Serotec USA (Washington, DC). CTL-2 target cells were obtained from American Type Culture Collection (Rockville, MD). Rat TNF-α (Con A), Histopaque (1.083 g/ml), ophenylenediamine dihydrochloride (OPD), lactate dehydrogenase (LDH) substrate mixture, bovine serum albumin (BSA), recombinant rat interleukin-2 (rRat IL-2), p-nitrophenyl phosphate, and Tween 20. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (La Jolla, CA). Monoclonal anti-rat IgE heavy chain and Rat IgE-κ were purchased from Serotec USA (Washington, DC). CTL-2 target cells were obtained from American Type Culture Collection (Rockville, MD). Rat TNF-α and IFN-γ cytoscreen immunoassay kits were purchased from Biosource International (Camarillo, CA).

**Blood collection and lead analysis.** Peripheral blood was collected from the neck vein or by cardiac puncture of dams and offspring at various times during and after lead treatment for blood lead determination. Heparin was used as an anticoagulant in all samples. Tibia samples were collected after euthanasia, at the conclusion of experiments. Blood and tibia lead levels were determined by atomic absorption using the platform furnace technique (Prusz-kowski et al., 1985). Blood samples from rats were frozen at −70°C until analyzed for IFN-γ by ELISA. Lead levels were determined by diethanolamine buffer for 1 h (Negrao-Correa et al., 1985).

**Spleen cell preparation.** Spleens were removed on day 15 after KLH administration; single-cell suspensions were prepared using 400 μm sterile nylon mesh. Erythrocytes were lysed with a buffer containing 0.15 M NaCl, 1.0 mM KHCO₃, and 0.1 mM EDTA (pH 7.2). Splenocytes were washed twice with HBSS and plated in 24-well tissue-culture-treated plates (3 × 10⁶ cells/well). For some experiments, 6 × 10⁵ cells were incubated at 37°C and 5% CO₂ for 2 h to isolate adherent cells. When adherent cell monolayers were stimulated to produce metabolites, duplicate unstimulated wells were analyzed for protein content to ensure similar adherent cell numbers, using the bicinchoninic acid (BCA) binding method (Pierce Biochemical, Rockford, IL).

**Tumor necrosis factor-α (TNF-α).** Adherent spleen cells in RPMI 1640 medium containing 2% FBS were stimulated with 10 ng/ml LPS during a 16-h incubation. Supernatants were frozen at −70°C until TNF-α was analyzed by ELISA.

**Nitric oxide (NO).** Adherent spleen cells in RPMI 1640 medium with 2% FBS were stimulated with 0, 1, 10, or 100 ng/ml LPS during a 24-h incubation. NO production was evaluated by measuring the accumulation of the more stable end product, nitrite, by the Griess reaction (Green et al., 1982).

**Superoxide (SO).** Superoxide anion was measured by a modification of the procedure described by Golombiak et al. (1990). Briefly, adherent spleen cells were exposed to 0 or 10 ng/ml LPS during a 24-h incubation. Mononuclear cells were then incubated with 2 μg/ml PMA and 80 μM ferricytochrome C, with or without 133 U/ml superoxide dismutase. After 1 h of incubation at 37°C and 5% CO₂, absorbances were read at 550 nm with a slitwidth of 1 nm on a microplate reader (Biotek EL312).

**Interferon-γ (IFN-γ).** Unseparated splenocytes were incubated in RPMI 1640 medium with 5% FBS and 0 or 5 μg/ml Con A for 72 h. Supernatants were frozen at −70°C until analyzed for IFN-γ by ELISA.

**Interleukin-2.** Unseparated splenocytes were incubated for 24 h with 5 μg/ml Con A, and the supernatants frozen for later testing of IL-2 activity. Each cell-free supernatant was serially diluted in a 96-well flat-bottom microtiter plate. The CTL-2 target cells (5 × 10⁵ cells in 100 μl) were added to the diluted supernatants and incubated for 24 h in 5% CO₂ at 37°C (Gillis et al., 1978). Proliferation was measured using MTT reduction (Mosmann, 1983). Standard curves were created with known units of rRat IL-2.

**Delayed-type hypersensitivity (DTH).** Injections of KLH were conducted as described by Exon et al. (1990). Briefly, 200 μl of 5 mg/ml KLH in sterile, deionized water was injected into the caudal tail fold on days 1 and 8. Control rats received 200 μl sterile water. On day 14, 100 μl of 20 mg/ml heat-aggregated (80°C for 1 h) KLH in saline was injected into the right footpad and 100 μl of sterile saline was injected into the left footpad. DTH reactions were measured 24 h after the heat-aggregated KLH injections using spring-loaded calipers (Dyer, Model 304). Results were reported as the percent difference between the right footpad (KLH) and the left footpad (sterile saline).
Euthanasia. No differences were noted for offspring or dams at various time points and dividing by the treatment mean and the associated control mean (Minitab Statistical Software, State College, PA). A p value of <0.05 was used to determine statistical significance.

**RESULTS**

**Blood lead levels (BLL).** During pregnancy and lactation, dam BLL in the 250 and 500 ppm treatment groups differed significantly from controls for the same time period (p < 0.01) (Table 1). Samples collected from the 100 ppm group during pregnancy and lactation did not differ from controls. No significant differences in BLL were noted among groups (data not shown).

**Tibia lead accumulation.** Offspring (13–14 weeks old) and dams (8–9 weeks postpartum) showed dose-dependent increases in bone lead accumulation across groups, as seen in Table 2. In both dams and offspring, bone lead levels in the 250 and 500 ppm groups were significantly (p < 0.05) higher than controls. Accumulation in dams was much greater than that in offspring.

**Growth rates.** Growth rates were determined by weighing offspring and dams at various time points and dividing by the number of days for the growth period. Offspring weights were started on the day of weaning and completed at the time of euthanization. No differences were noted for offspring or dams at various time points and dividing by the treatment mean and the associated control mean (Minitab Statistical Software, State College, PA). A p value of <0.05 was used to determine statistical significance.

**Statistical analyses.** One-way analysis of variance (ANOVA) tested for differences among the four treatment groups in this study. Post ANOVA (multiple comparison) tests determined which groups were statistically different when the overall ANOVA showed differences among the groups. Two different multiple comparison tests were employed. Fisher’s LSD provided a confidence interval for the difference between each treatment mean and the control mean (Minitab Statistical Software, State College, PA). A p value of <0.05 was used to determine statistical significance.

**Total leukocyte counts.** Offspring TLCs were decreased significantly (p < 0.02) in the 250 and 500 ppm lead groups when compared with controls (Fig. 1). Dam TLCs in the lead-exposed groups were not different from those of controls.

**Leukocyte subpopulation distribution.** Flow cytometry data was assessed by analyzing both percentage of positive cells and mean fluorescence for each monoclonal antibody. No significant differences were noted in either category, in data from offspring or from dams. Control values for dams were as follows: CD4, 53.6%; CD8, 31.8%; NK, 3.6%; macrophage/monocyte, 1.6%; IA, 9.8%; SLg, 12.4%. Control values for offspring were: CD4, 48.6%; CD8, 38.0%; NK, 4.2%; macrophage/monocyte, 2.9%; IA, 17.3%; SLg, 30.2%.

**Natural killer cells.** No lead treatment related effects were noted with respect to NK cell mediated lysis; however, inter-experiment variability was significant for this bioassay (data not shown).

**Antigen-specific ELISA.** IgG and IgM antibody titers to the KLH antigen were compared by lead treatment group. Figure 2 shows IgG titers for offspring showing an upward trend in IgG production with increased lead dose. These results are not statistically significant. αKLH IgM titers were highly variable and did not differ significantly among groups (data not shown).

**FIG. 1.** Offspring total blood leukocyte counts were counted with a hemacytometer. Data were analyzed by ANOVA (p < 0.02). *Significant difference when compared with controls.
IgE levels. Serum IgE levels were increased (*p < 0.05) in samples from offspring of the 100 ppm rats compared with those from control rats (Fig. 3). IgE levels in samples from offspring of the 250 ppm group were not significantly different from control samples. Samples from offspring of the 500 ppm group were not evaluated for IgE levels, nor were those from dams.

Tumor necrosis factor-α. TNF-α production by 16-h adherent splenocyte cultures (with 0.01 μg/ml LPS) was elevated (*p < 0.05) in offspring of the 250 ppm treatment group compared with that from control offspring splenocytes (Fig. 4). Dams exhibited consistent TNF-α production among treatment groups (6.73 pg/μg protein for control rats vs 6.18, 5.90, and 6.54 in 100, 250 and 500 ppm lead groups, respectively).

Nitric oxide. Nitric oxide production by macrophages from pups increased with lead treatment up to 250 ppm (Fig. 5). Levels in the 250 ppm group were significantly (*p < 0.05) increased compared to controls; however, NO production returned to control levels in the 500 ppm group. Nitrite levels in splenocyte cultures from dams averaged 58.65 μM/mg protein in control animals; no differences were noted among the four lead treatment groups (data not shown). Protein levels in adherent monolayers used for both NO and SO production did not differ among treatment groups (data not shown).

Superoxide anion. No differences in SO anion production were noted among the treatment groups, in cells from offspring or from dams (data not shown).

Interferon-γ. IFN-γ measured in 72 h Con A-stimulated culture supernatants from offspring was significantly lower in the 500 ppm group when compared with supernatants from either controls or 100 ppm lead group (*p < 0.05) (Fig. 6). IFN-γ production from dam splenocyte cultures (mean 1074.6 pg/ml from controls) did not differ among the treatment groups (data not shown).

Interleukin-2. IL-2 production by Con A-stimulated splenocytes, as assayed by proliferation of CTLL-2 cells followed by MTT reduction, did not differ among treatment groups, either for dams (controls, 34.7 ± 6 U/ml, overall mean 43.0) or for offspring (controls, 7.1 ± 2 U/ml, overall mean 5.1).

Delayed-type hypersensitivity. In the female offspring, the reaction to KLH in the 250 ppm lead-exposed group was significantly decreased when compared with controls (*p < 0.05) (Fig. 7). Dams did not differ significantly in DTH reactions among the four treatment groups (control dams had a 33.6% difference between footpads, while the 100, 250, and 500 ppm groups differed by 45.9, 49.4, and 41.6%, respectively).
The immunotoxic effects of lead have been examined in some detail (Kerkvliet and Baecher-Steppan, 1982; Exon et al., 1985; McCabe and Lawrence, 1991), but few studies have included developmental status as a variable (Luster et al., 1978; Faith et al., 1979). Therefore, the potential for immunotoxicity during development, as well as the relative risks to the mother and fetus, required further examination.

The present study demonstrated that in utero lead exposure in rats can modulate the immune system of the offspring and, additionally, provided a direct comparison of relative lead immunotoxicity risk to developing offspring vs pregnant dams. Since the offspring were examined as adults, the altered function in the progeny of lead-treated dams reflects persistent effects on the immune system. After a similar time interval, no differences were noted in maternal immune function, suggesting that any effects of the same exposure in adults were transient. In a study which examined acute effects of lead exposure using adult mice, animals with blood lead levels similar to maternal levels in this study (38 μg/dl) exhibited increases in Th2-dependent immune functions, including IgE and IL-4 production (Heo et al., 1996).

It is important to note that no alterations in offspring growth rate were observed. This is consistent with the observations of Hammond et al. (1993) that lead-induced growth alterations were usually transitory and suggests that chronic immune alterations can result from in utero lead exposures which do not affect overall growth.

Lead exposures were restricted to the period of in utero development, but lead remobilized from maternal bone is transported in rat milk (Palminger and Oskarsson, 1995) and could have contributed to some postpartum exposure during lactation. However, recent studies using the same F344 in utero exposure system detected insufficient lead concentrations in maternal milk to produce significant oral exposure to the nursing pup (Chen et al., unpublished observations). Therefore, the observed immune alterations in the present study can be attributed to embryonic exposure.

Blood lead concentrations in dams during pregnancy were relatively high, ranging from 39.4 ± 6.7 to 112 ± 19.9 μg/dl. These levels are higher than those reported to affect behavioral function in young children (15–25 μg/dl) (Goyer, 1993). Offspring blood and bone lead levels at the time of immune assessment were remarkably low, suggesting that significant lead storage need not occur in the embryo for the induction of persistent immune alterations. Based on these results, if there are key periods of early immune development which are particularly susceptible to persistent lead-induced alterations, it is possible that even short-term exposure of pregnant females, in rodents or in other species including humans, could compromise offspring immune function.

The specific immune alterations observed in the present study support the hypothesis presented by other investigators that lead is capable of altering the balance of T helper cell (Th1 vs Th2) activity (McCabe and Lawrence, 1991; Heo et al., 1996). Because Th1 vs Th2 activities promote different immune functions resulting in different host defense processes (Kuchroo et al., 1995; Surcel et al., 1994; Kawakami and Parker, 1992; Clerici et al., 1993), significant shifts in the balance of these functions could alter disease resistance of the host. In studies using adult mice in vivo and murine in vitro models, Lawrence and colleagues found lead chloride-induced elevation of IL-4 and IgE levels with a significant reduction of IFN-γ levels in exposed Balb/c mice (McCabe and Lawrence, 1991; Heo et al., 1996).

In the present study, antigen-specific cell mediated immune function (delayed type hypersensitivity) associated with a Th1 response was depressed in the 250 ppm treatment group. In contrast, IgE production, a Th2-dependent response (Mosmann and Coffman, 1989) was significantly elevated in the 100 ppm lead-exposed group (specific IgG production to KLH was unaltered). The findings suggest that embryonic exposure to lead decreased Th1 function while elevating some Th2-dependent activities. These results are consistent with several prior neonatal and adult exposure studies in which antibody response was either unaltered or elevated in contrast to lead-induced depression of cell-mediated immunity (Lawrence, 1981; Kim-
ber et al., 1986; McCabe and Lawrence, 1990; Redig et al., 1991; Horiguchi et al., 1992).

Modulations of various immune endpoints were observed at different lead exposure concentrations in these experiments. A number of parameters (TNF-α, NO, IgE, and DTH) were altered in one of the lower treatment groups (100 or 250 ppm) and then returned to control levels at a higher dose (250 or 500 ppm). Similar results were described in Heo et al. (1996) where IL-4 production in vitro was increased by exposure to 25 μM Pb but not by 50 μM Pb treatment. These data suggest the possibility that embryonic lymphocyte populations may differ in sensitivity to lead-induced modulation.

A potential neonatal bias to Th2 vs Th1 function has implications for subsequent host resistance to disease. For example, Th2 hyperresponsiveness can increase the susceptibility of the host to infectious challenges and could predispose the host to allergic atopic manifestations (Romagnani, 1995) and autoimmunity (Pelletier et al., 1994). Increased Th2-type responses with concomitant suppression of IL-2 production has been reported to cause severe immune dysfunction in NMRI mice (Faxvaag et al., 1995).

Shirakawa et al. (1997) have shown an inverse correlation between Th2-mediated atopy (asthma, rhinitis, eczema) and childhood conversion to positive tuberculin status. They suggest that the presence of a strong Th1-inducing stimulus, such as Mycobacterium tuberculosis exposure, could inhibit Th2 cytokines to an extent sufficient to decrease subsequent atopy. Applying the same hypothesis to the developing rat immune system, it is conceivable that a stimulus such as lead, which may increase Th2 cytokine levels could influence fetal immune development to persistently suppress Th1 responses.

In the present study, adult offspring exposed to lead in utero also exhibited altered macrophage function (nitric oxide production). While such changes could be associated with the shifts in T cell functional capacities and concomitant changes in cytokine production (e.g., IFN-γ production), other studies have reported direct lead-induced changes in macrophage function in vitro (Tian and Lawrence, 1996; Chen et al., 1997). However, the in utero exposure at 250 ppm resulted in enhanced TNF-α and NO production by rat splenocytes, in contrast with in vitro studies which suggest that lead can directly suppress NO production. The elevated TNF-α production seen in the present study is consistent with the observation of Guo et al. (1996) that exposure to lead chloride elevates TNF-α production by LPS-stimulated human peripheral blood monocytes. Since TNF-α is an autocrine factor for macrophage activation (Witsell and Schook, 1992), this could explain some of the effects of lead on NO production. TNF-α also plays a role in airway hypersensitivity and bronchoalveolar inflammatory cell accumulation (Renzetti et al., 1996). Based on these results, if lead induces increases in Th2 cytokine levels, these compounds could act in concert with lead-induced elevations in TNF-α to increase airway hypersensitivity.

Recent increases in both the prevalence and the severity of childhood asthma have prompted a search for potential environmental risk factors. Lead-induced Th1-Th2 immunomodulation (McCabe and Lawrence, 1991; Heo et al., 1996, 1997), the known role of other heavy metals in facilitating autoimmune reactions (Kiely et al., 1995), the relationship among elevated IgE levels and airway reactivity (Sears et al., 1991) and the present embryonic exposure results implicate lead exposure as a possible risk factor for the development of Th2-dependent chronic diseases. Exposure to lead concentrations which produce no (or only transient) maternal immunomodulation may increase the propensity for allergic disease and/or autoimmunity in the exposed fetus through persistent shifts in Th1 vs Th2 activity.

ACKNOWLEDGMENTS

The authors thank Supong Chen, Valerie Highfill, Christina Kim, and Kevin Fitch for their assistance with these experiments and Diane Colf for her administrative support. Additionally, the helpful discussions of Dr. Judy Zerlikoff are much appreciated. This publication was made possible by Grant ES05950 from the National Institute of Environmental Health Sciences, NIH, with funding provided by EPA.

REFERENCES

Faxvaag, A., Espevik, T., and Dalen, A. (1995). An immunosuppressive murine leukaemia virus induces a Th1 • Th2 switch and abrogates the IgM
DEVELOPMENTAL LEAD IMMUNOTOXICITY


