Arch. Environ. Contam. Toxicol. 39, 251–256 (2000)
DOI: 10.1007/s002440010102

ARCHIVES OF
Environmental
Contamination
and Toxicology

A Role for Oxidative Stress in Suppressing Serum Immunoglobulin Levels in Lead-Exposed Fisher 344 Rats

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Received: 9 January 2000/Accepted: 21 March 2000

Abstract. Evidence implicating oxidative stress in toxicity during lead intoxication *in vivo* has opened new avenues for investigation of the mechanisms of lead-induced immunosuppression. The current study explores the possibility that lead-induced oxidative stress contributes to the immunosuppression observed during lead poisoning. Fisher 344 rats were exposed to 2,000 ppm lead acetate in their drinking water for 5 weeks. One week following removal of lead from the drinking water, significant reductions in serum levels of IgA, IgM, and IgG were detected. Significant increases in oxidative damage, based on malondialdehyde (MDA) content, were observed in peripheral blood mononuclear cells (PMCs) collected during the same experiments. In addition, MDA content increased in livers from lead-exposed rats. Following 5 weeks of lead exposure, administration of either 5.5 mmol/kg *N*-acetylcysteine (NAC) or 1 mmol/kg *meso*-2,3-dimercaptosuccinic acid (DMSA) in the drinking water for 1 week significantly reversed the inhibitory effects of lead on serum immunoglobulin (Ig) levels. Also, all parameters indicative of oxidative stress returned to control levels. These results suggest that oxidative stress contributes to suppressed serum Ig levels during lead intoxication *in vivo*, and that intervention with either a thiol antioxidant (NAC) or a metal chelator (DMSA) will alleviate this lead-induced suppression by correcting the prooxidant/antioxidant imbalance caused by lead exposure.

The immunosuppressive effects of lead intoxication were first reported in the 1970s, when it was noted that lead-exposed mice had a reduced immune response following challenges with viral and bacterial pathogens (Hemphil *et al*. 1971; Gainer 1974; Koller 1977). Further studies of lead-induced immunosuppression demonstrated significant decreases in lymphocyte activation and proliferation (Luster *et al*. 1978; Faith *et al*. 1979; Neilan *et al*. 1980) as well as decreases in natural killer (NK) cytotoxicity (Neilan *et al*. 1983; Talcott *et al*. 1985) and macrophage motility and migration (Blakley and Archer 1981; Kiremidjian-Schumacher *et al*. 1981). Despite evidence that lead damages immune function, a mechanism for lead-induced immunosuppression has not been completely elucidated. A recent report suggests modified cAMP regulation as a potential cause of the effects of lead on the immune system (Heo *et al*. 1998). Although the details of activation of adenylate cyclase by lead have not been determined, an important role for this action was demonstrated.

Recent animal studies suggest that lead can cause oxidative stress and lipid peroxidation in the liver, kidney, and brain (Lawton and Donaldson 1991; Sandhir *et al*. 1994; Sandhir and Gill 1995; Ercal *et al*. 1996a). *In vitro* studies also suggest that lead causes oxidative stress in red blood cells (Ribarov *et al*. 1981; Ribarov and Bochev 1982; Quinlan *et al*. 1988). This finding is supported by studies that show oxidative damage in red blood cells (RBCs) from lead-exposed workers and Fisher 344 rats (Ito *et al*. 1985; Monterio *et al*. 1985; Sugawara *et al*. 1991; Gurer *et al*. 1998). Furthermore, cellular thiol antioxidant capacity, based on glutathione content measurements, was compromised in tissues from lead-intoxicated animals (Gurer *et al*. 1998; Ercal *et al*. 1996a). In addition, lead may cause oxidative stress by inhibiting δ-aminolevulinic acid dehydratase (ALAD), leading to the buildup and autooxidation of δ-aminolevulinic acid to form H_2O_2 (Monteiro *et al*. 1986; Gurer *et al*. 1998). In the same study, the activity of catalase (an H_2O_2 metabolizing enzyme) increased in RBCs from lead-exposed rats (Gurer *et al*. 1998). Since data suggest that lead exposure results in oxidative stress, and oxidative stress may profoundly affect the immune function (Buttke and Sandstrom 1995; Bajer-Bitterlich *et al*. 1997; Cayota *et al*. 1996), it is

Dr. Nuran Ercal was supported a grant from the by University of Missouri—Rolla Research Board. Dr. Paula M. Lutz was supported by R01 ES06065-OIAI from the National Institutes of Health. Dr. D. R. Spitz was supported by RO1HL51469 from the National Institutes of Health.

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logical to hypothesize that oxidative stress plays a significant role in lead-induced immunosuppression.

If oxidative stress contributes to lead-induced immunosuppression, a partial remedy for the immune dysfunction associated with environmental lead exposure may be to supplement peripheral lymphocyte antioxidant capacity. This could be accomplished by either reducing blood and tissue lead levels via chelation or by bolstering cellular antioxidant defenses through exogenous supplementation. We have investigated the feasibility of using chelators or thiol antioxidants to counteract lead-induced oxidative stress in mice, rats, and Chinese hamster ovary (CHO) cells (Ercal *et al*. 1996a, 1996b; Gurer *et al*. 1998). Intervention with the chelator *meso*-2,3-dimercaptosuccinic acid (DMSA), the preferred treatment for children with blood lead levels exceeding 25 μg/dl, reduced oxidative stress *in vivo*, presumably by facilitating excretion of lead. Treatment with *N*-acetylcysteine (NAC), a well-known synthetic thiol antioxidant that supplies cysteine for glutathione synthesis, also mitigated lead-induced oxidative stress in both CHO cells and animal tissues. The mechanism by which NAC suppressed lead-induced oxidative stress was not attributable entirely to chelation, but included the bolstering of cellular thiol antioxidant capacity (Ercal *et al*. 1996a, 1996b; Gurer *et al*. 1998).

The current study was initiated to determine the involvement of oxidative stress in reduced immune function in lead-intoxicated Fisher 344 rats. Serum levels of IgM, IgG, and IgA were assessed as indicators of the functional state of B cells. Measures of lipid peroxidation (malondialdehyde content) were utilized to assess oxidative damage in PMCs and liver homogenates. Catalase activity in PMCs and liver homogenates was determined as an indicator of cellular H_2O_2 metabolic capability. The effects of therapeutic intervention with NAC or DMSA on blood and tissue lead levels; serum concentrations of IgG, IgA, and IgM; and selective parameters indicative of oxidative stress were also determined. Results support the hypothesis that oxidative stress contributes to lead-induced suppresion of serum Ig levels and pharmacological intervention with either a metal chelator or thiol antioxidant reverses the suppressive effects of lead exposure *in vivo*.

Materials and Methods

Animals

All experiments were performed with adult male Fisher 344 rats, weighing 100–150 grams, housed in stainless steel cages in a temperature-controlled (25°C) room equipped to maintain a 12-h light:dark cycle. The rats were allowed standard rat chow (Purina) and water *ad libitum* throughout the experiment. Eighteen animals were randomized into four groups. Group I (n = 4), the control, was given only standard rat chow and water. Group II (n = 5) received 2,000 ppm lead acetate (Sigma Chemical, St. Louis, MO) in the drinking water for 5 weeks. During the 6th week, this group received plain water only. Group III (n = 5) received 2,000 ppm lead acetate in the drinking water during weeks 1–5 and NAC (5.5 mmol/kg; Sigma Chemical) was dissolved in the drinking water during week 6. Group IV (n = 4) received 2,000 ppm lead acetate in the drinking water during weeks 1–5 and DMSA (1 mmol/kg), dissolved with an equimolar concentration of HCO_3^- to bring the solution to pH 7.0, was added to the drinking water during week 6. Water consumption was carefully monitored in all of the experiments. Water consumption was found to be 18 ± 2 ml/rat throughout the experiment.

At the end of week 6, the animals were anesthetized with metofane and blood samples were collected with lead-free needles via intracardiac puncture. Three milliliters of each blood sample were aliquoted for lymphocyte isolation (see below). The remainder of each blood sample was maintained in EDTA and used for blood lead determinations. The animals were sacrificed and tissue samples were rinsed in ice-cold saline. Extraparenchymal tissue was trimmed and then the samples were maintained at −70°C until analysis.

Blood and Tissue Lead Determinations

Blood lead levels were determined by atomic absorption spectrophotometry (Varian SpectrAA) at the CDC-certified Analytical Laboratory of the Springfield-Greene County Department of Public Health, Springfield, MO. Liver lead levels were also measured using atomic absorption spectrophotometry at the Environmental Trace Substances Laboratory (ETSL) of the Center for Environmental Sciences and Technology (CEST), UM-Rolla, in Rolla, MO.

Peripheral Blood Mononuclear Cell Isolation Technique

Blood samples were diluted with 1:1 (v/v) Hank's buffer and layered on top of 3.0 ml of Fico-Lite in 15-ml centrifuge tubes (Fisker *et al.* 1990; Boyum *et al*. 1991). The samples were then centrifuged at 340 *g* for 30 min in a swinging-bucket rotor. The lymphocyte buffy coat was removed with a transfer pipet and placed in sterile 15-ml centrifuge tubes. The PMCs were diluted to a total volume of 11 ml in 0.83% NH_4Cl and incubated for 5 min to remove contaminated red blood cells. The PMCs were then pelleted and the pellet resuspended in another 10 ml of 0.83% NH_4Cl. After a 5-min incubation, the cells were pelleted (150 *g* for 20 min) and the pellet was resuspended in 1.0 ml of HPLC-grade water. Samples were immediately homogenized with Tissue-Tearor and analyzed for MDA and catalase levels.

MDA Assay by HPLC

Lipid peroxidation, as estimated by the formation of MDA, was determined for liver samples and isolated PMCs using a previously described HPLC technique (Draper *et al*. 1993). Briefly, the tissue samples were minced and homogenized in buffers containing 1 ml 10% trichloroacetic acid (TCA) and 0.25 ml of 500 ppm butylatedhydroxytoluene (BHT) in methanol, with the addition of 0.30 ml of 1 mM diethylenetriaminepentaacetic acid (DETAPAC) to prevent artifactual autooxidation. The samples were heated in a boiling water bath for 30 min, then cooled to room temperature and centrifuged to pellet tissue. The supernatant was reacted with a saturated aqueous thiobarbituric acid (TBA) solution and placed in a boiling water bath for an additional 30 min. The concentration of the thiobarbituric acid–malondialdehyde (TBA-MDA) complex in the samples was determined by HPLC with fluorescence detection, using the calibration curve obtained from a 1,1,3,3,-tetraethoxypropane standard solution. The retention time of the standard was utilized to identify the TBA-MDA adduct in the experimental samples.

Catalase Activity Assays

Catalase activity was determined spectrophotometrically on tissue and cell homogenates by the Beers and Sizer (1952) method and expressed

in units/mg protein, as described by Aebi (1984). This method measures the exponential disappearance of hydrogen peroxide (H_2O_2; 10 mM) at 240 nm in the presence of tissue and cellular homogenates. The equation used to fit the exponential decay of H_2O_2 was $A_{60\ s} = A_{initial}e^{-kt}$, where k = the rate constant, which is dependent on the catalase activity. This assay easily detects 0.01 units/mg protein in homogenates.

IgG, IgM, and IgA Determinations

IgG, IgM, and IgA were determined on serum from rats that were not immunized using a radial immunodiffusion (RID) assay (Mancini *et al*. 1965; Fahey and McKelvey 1965) from Binding Site (Birmingham, England).

Protein Determination

The Bradford method was used to determine the protein content of the tissue and lymphocyte samples (Bradford 1976).

Statistical Analysis

Tabulated values are means ± SD. InStat© by GraphPad Software (San Diego, CA), using the Student-Newman-Keuls multiple comparisons test, provided statistical significance of the data from experimental and control groups, with p values <0.05 considered significant.

Results

Animal Weights

Weights were recorded at the beginning of the experiment, at the end of week 5 (before treatment with NAC or DMSA), and prior to sacrifice. Food and water consumption were monitored throughout the experiment, but no meaningful differences between groups were noted. However, significant differences between ending mean-body weights were observed (Table 1). Animals treated with lead (Group II) or DMSA + lead (Group IV) weighed significantly less than control rats (Group I) and rats treated with NAC + lead (Group III).

Blood and Tissue Lead Levels

Rats treated with lead (Group II) had a mean blood lead (PbB) level of 45 μg/dl, which represents a significant elevation when compared to the control (Group I, Table 1). A PbB level of this magnitude in humans would require prompt intervention with chelation therapy (Center for Disease Control 1991). Animals given the chelator DMSA (Group IV) in the final week of the experiment had significantly lower PbB levels when compared to the lead-exposed (Group II) rats. Both DMSA and NAC gave significant reductions in PbB levels.

The results of liver lead determinations were consistent with the PbB findings (Table 1). Liver lead levels had significantly increased in all lead-treated groups. Rats treated with NAC + lead (Group III) or DMSA + lead (Group IV) had significantly lower liver lead levels than animals given lead (Group II) alone, although Groups III and IV did not differ significantly from one another. Overall these results indicate that lead was cleared more rapidly from animals treated with either DMSA or NAC than from lead-treated animals receiving no further intervention.

Table 1. Effect of lead exposure on body weight, blood, and liver lead levels in Fisher 344 rats. Blood and tissue lead levels were determined by atomic absorption

Group	Ending Body Weights (g)	Blood Lead Levels (μg/dl)	Liver Lead Levels (μg/g)
Control (I)	243 ± 10	3.0 ± 0.8	0.07 ± 0.06
Lead (II)	218 ± 7[a]	45.0 ± 6.9[a]	2.10 ± 0.25[a]
NAC + lead (III)	238 ± 8[b]	26.0 ± 4.5[a,b]	0.67 ± 0.05[a,b]
DMSA + lead (IV)	220 ± 9[a,c]	12.5 ± 1.7[a,b,c]	0.61 ± 0.10[a,b]

Results represent means ± SDs.
[a] $p < 0.05$ compared to the corresponding data collected for Group I.
[b] $p < 0.05$ compared to the corresponding data collected for Group II.
[c] $p < 0.05$ compared to the corresponding data collected for Group III.

MDA Levels and Catalase Activity

MDA levels in the lymphocytes and the liver samples significantly increased following lead exposure (Table 2). NAC and DMSA reduced MDA in both tissues to levels that were not significantly different from those of the control (Group I). After lead exposure, catalase levels in lymphocytes increased significantly, but no changes were observed in the liver samples. Treatment of the lymphocytes with either NAC or DMSA returned catalase levels to control levels.

Determination of IgM, IgG, and IgA by Radial Immunodiffusion

IgM, IgG, and IgA determinations appear in Table 3. Significant decreases in these levels were observed in serum samples collected from rats given lead (Group II). Animals treated with lead plus NAC (Group III) or DMSA (Group IV) had IgG, IgM and IgA levels that were not significantly different from those of the control (Group I). There was no significant difference between the treatment groups (Group III and Group IV) and the control group.

Discussion

Significant declines in IgM, IgG and IgA levels in lead-exposed rats confirm the ability of lead to affect B cell function *in vivo*. The hypothesis that this effect on B cells may be a manifestation of increased oxidative stress is supported by the finding that lead intoxication is accompanied by increases in lymphocyte and liver MDA levels (indicative of increased lipid peroxidation) as well as increases in protein oxidation as assessed by protein carbonyl content (data not shown). These results are consistent with previous reports of increased liver

Table 2. Effect of lead exposure on liver and lymphocyte MDA levels in Fisher 344 rats

Group	Lymphocyte MDA (nmol/100 mg protein)	Liver MDA (nmol/100 mg protein)
Control (I)	0.15 ± 0.01	12.5 ± 1.1
Lead (II)	0.95 ± 0.04[a]	27.8 ± 1.9[a]
NAC + lead (III)	0.16 ± 0.07[b]	15.2 ± 1.4[a,b]
DMSA + lead (IV)	0.13 ± 0.01[b]	15.4 ± 1.1[a,b]

Results represent means ± SDs.
[a] $p < 0.05$ compared to the corresponding data collected for Group I.
[b] $p < 0.05$ compared to the corresponding data collected for Group II.

Table 3. Effect of lead exposure on liver and lymphocyte catalase activity in Fisher 344 rats

Group	Lymphocyte Catalase Activity (kunits/mg protein)	Liver Catalase Activity (kunits/mg protein)
Control (I)	0.12 ± 0.01	0.76 ± 0.07
Lead (II)	0.82 ± 0.12[a]	0.71 ± 0.09
NAC + lead (III)	0.12 ± 0.08[b]	0.76 ± 0.03
DMSA + lead (IV)	0.10 ± 0.05[b]	0.69 ± 0.13

Results represent means ± SDs.
[a] $p < 0.05$ compared to the corresponding data collected for Group I.
[b] $p < 0.05$ compared to the corresponding data collected for Group II.

MDA levels following lead exposure (Rehman 1984; Sandhir and Gill 1995). Furthermore, treatment with a metal chelator or a thiol antioxidant following lead exposure reduced oxidative stress in PMCs and livers and normalized serum Ig levels, indicating a reversal in lead-induced effects on B cell function.

Decreases in serum Ig levels following lead exposure have been previously reported in both animal and human studies. Luster *et al*. (1978) described an almost 30% decrease in serum IgG in rats immunized with sheep red blood cells (SRBCs) or lipopolysaccharides (LPS), however, the serum IgM and IgA levels were not inhibited. Greater than 50% decrease in SRBCs-specific plaque forming cells (PFCs) was also noted, showing a direct effect on B cells. Lawrence (1981) corroborated these results, showing decreased PFC responses to SRBC in mice treated with high levels of lead (10 mM), although lower levels were found to enhance the response. A study by Ündeger *et al*. (1996) in nonimmunized lead workers and controls showed a 29% decrease in serum IgG and a 34% decrease in serum IgM. Ewers *et al*. (1982) reported nonimmunized lead workers with chronic lead exposure had a 19% decrease in serum IgM and a 28% decrease in salivary IgA when compared to reference subjects lacking such exposure. These data agree with our findings.

Some conflicting data have been reported. A study in rabbits (Gainer 1974) and one in adult lead-exposed workers (Kimber *et al*. 1986) observed no such decreases. It should be noted that comparisons among studies, both animal and human, are often difficult. In animal studies, technical aspects may vary widely, such as species or strain of animal chosen, specific lead salt used, duration of exposure, the high content of antioxidants in standard animal diets, and concentration of lead. In human studies, the latter two parameters are a concern when making comparisons between studies, as well as the reference or control population used. For example, a study of children with environmental lead exposure who were immunized with tetanus toxoid (Reigart and Graber 1976) showed no effect on either total Ig levels or on the antibody titer to tetanus toxoid. However, comparisons were made between "low lead" (mean 23 μg/dl) and "high lead" (41–51 μg/dl) groups. This "low lead" population had exposure well above the 10 μg/dl intervention level the CDC now uses for children and cannot be considered negative controls.

Total Ig levels are one measure of proper B cell development and function. Our data show decreased serum Ig levels in the presence of lead in animal model. Although we do not directly show impaired host resistance to pathogens in this animal model, the lead exposed workers in Ewers *et al*. (1982) did show an increased incidence of colds and influenza (associated with a 19% decrease in serum IgM). This would indicate that decreases of 20–30% in serum Ig isotypes, such as we have seen in this study, may indeed have clinical relevance.

The concentrations of NAC and DMSA used here greatly improve but do not completely reverse the suppression of serum Ig levels. Adjustment of dosage and length of treatment may completely abrogate lead's effects. Such studies are under way.

The mechanism responsible for lead-induced oxidative stress is currently unknown. In contrast to transition metals like iron and copper, lead is not redox active metal. However, lead has a high affinity for sulfhydryl groups (Lima-Hermes *et al*. 1991). Intracellular soluble thiols, such as glutathione (GSH), cysteine (Cys), and γ-glutamylcysteine, contain reduced sulfhydryl groups (-SH) that are critical to maintaining functional cellular proteins in a reduced state. Binding of lead to sulfhydryls could alter thiol oxidation/reduction reactions and disrupt cellular function via the loss of reduced thiols, such as GSH. In fact, prior studies have found a correlation between decreases in GSH and the inhibition of lymphocyte activation and proliferation (Fischman *et al*. 1981; Hamilos and Wedner 1985; Hamilos *et al*. 1989). Other studies have also shown that lead exposure decreases tissue levels of GSH (Ercal *et al*. 1996a; Gurer *et al*. 1998). Alternatively, lead may inhibit δ-aminolevulinic acid dehydratase (ALAD) activity, leading to a buildup of δ-aminolevulinic acid (Monterio *et al*. 1986; Gurer *et al*. 1998). The autooxidation of δ-aminolevulinic acid forms reactive oxygen species, such as hydrogen peroxide, which may contribute to lead-induced oxidative stress. Though we have no data linking these proposed mechanisms to lead-induced immunosuppression, we have shown that ALAD activity in whole blood from Fisher rats is significantly reduced during lead intoxication (Gurer *et al*. 1998). Furthermore, in the current study, we found evidence that PMCs from lead-treated animals were apparently experiencing oxidative stress as evidenced by the accumulation of by-products of oxidation reactions (MDA) and increases in capacity to scavenge H_2O_2 via catalase. This is indirect evidence that H_2O_2 may be one of the reactive oxygen species contributing to lead-induced oxidative stress. In support of this, previous studies have demonstrated the capacity of pro-oxidant substances (like H_2O_2) to suppress lymphocyte responses (Freed *et al*. 1987).

A novel finding in this study (with significant therapeutic

Table 4. Radial immunodiffusion (RID) measurement of serum IgG, IgA and IgM levels in lead exposed Fisher 344 rats

Group	IgM (mg/L)	IgG (mg/L)	IgA (mg/L)
Control (I)	706 ± 12	6,506 ± 227	84 ± 7
Lead (II)	583 ± 26[a]	4,776 ± 1,086[a]	67 ± 3[a]
NAC + lead (III)	668 ± 37[b]	5,644 ± 637[b]	82 ± 6[b]
DMSA + lead (IV)	660 ± 60[b]	5,944 ± 297[b]	91 ± 1[b]

Results represent means ± SDs.
[a] $p < 0.05$ compared to the corresponding data collected for Group I.
[b] $p < 0.05$ compared to the corresponding data collected for Group II.

implications) is that pharmacological intervention, which decreases indices of lead-induced oxidative stress in PMCs, also decreases evidence of lead-induced suppression on Ig levels. This further supports the hypothesis that there might be a causal relationship between lead-induced oxidative stress and depressed Ig levels. Two pharmacological approaches to inhibiting lead-induced oxidative stress in animals were used in this study—chelation and thiol supplementation. The first approach used DMSA, an orally administered chelating agent whose popularity as a treatment for childhood plumbism has dramatically increased in recent years (Mann and Travers 1991). The chelating action of DMSA increases excretion of lead from tissues and, therefore, decreases oxidative injury by limiting the amount of lead that contributes to oxidative stress. The second approach was to increase thiol antioxidant capacity by feeding NAC to lead-intoxicated animals. When given orally, NAC quickly deacylates in the small intestine to yield cysteine, which can then be transported across the cell membrane to supplement cysteine pools as well as support increased GSH synthesis (Moldeus and Cotgreave 1994). Reinforcement of the cell's antioxidant defenses could mitigate lead-induced oxidative stress. Treatment with NAC or DMSA significantly decreased lead levels in blood and the liver, although DMSA was more effective in reducing blood lead levels (Table 1). DMSA and NAC also mitigated all of the effects of lead on parameters indicative of oxidative stress and immunosuppression in both PMCs and liver samples (Tables 2, 3, and 4). These findings show that both chelation therapy and thiol antioxidant therapy may aid in reversing the suppression of Ig levels and oxidative stress caused by lead intoxication.

Though DMSA is currently in use as a therapeutic intervention for symptomatic lead poisoning (PbB $>$ 25 μg/dl), its use in subjects with lower PbB levels ($<$25 μg/dL) is discouraged because of the side effects (Mann and Travers 1991). Given that detrimental cognitive and neurological effects have been reported in children with PbB levels of 10–25 μg/dl (Mushak *et al*. 1989), a need clearly exists for a therapy for subjects with modestly elevated PbB levels. NAC's abrogation of lead-induced oxidative stress and reversal of suppression of Ig levels, combined with its high toxicity threshold, oral administration, low cost, and 40-year history of safe clinical use (Moldeus and Cotgreave 1994), show that NAC may be an alternative therapy for modest levels of lead intoxication (PbB $>$ 25 μg/dl).

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