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Inhalation of Particulate Lead Oxide Disrupts Pulmonary Macrophage-Mediated Functions Important for Host Defense and Tumor Surveillance in the Lung

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Lead, an immunomodulator and potential human carcinogen, is a major airborne pollutant in industrial environments which poses a serious threat to human health. Despite the widespread occurrence of respirable lead particles in the air, and the potential human health risks, effects associated with inhalation of particulate lead on the lung have been poorly studied. This study was performed to determine whether inhalation of particulate lead oxide (PbO), at a concentration below the currently acceptable air lead standard for occupational exposure, disrupts macrophage (Mø) functions important for maintaining pulmonary immunocompetence. These functions include phagocytosis, production of reactive oxygen intermediates, and the biological activity of tumor necrosis factor-α (TNF-α). Rabbits exposed to PbO at 30 $\mu g/m^3$ for 4 days (3 hr/day) were sacrificed and their lungs lavaged immediately, 24 hr, and 72 hr after the final exposure. Lactate dehydrogenase (a marker of lung cell damage) and lysozyme activity (a marker of lysosome permeability), measured in the lavage fluid, were significantly increased 24 and 72 hr after exposure. PbO produced no neutrophil infiltration nor effects on Mø viability or total numbers. Effects on Mø functions were as follows. Phagocytic uptake of latex particles was reduced with increasing post-exposure time reaching a maximum inhibition at 72 hr. Inhalation of PbO enhanced hydrogen peroxide (H_2O_2) and superoxide anion radical ($^{\cdot}O_2^-$) production in a time-dependent manner; effects on H_2O_2 began at 24 hr and were persistent up to 72 hr. Effects on TNF-α release/activity appeared earliest and were persistent up to 72 hr. Immediately and 24 hr after exposure, lipopolysaccharide-stimulated activity of TNF-α was depressed by 62 and 50%, respectively; after 72 hr, TNF-α release was significantly enhanced compared to control levels. Results demonstrate that the lung is a sensitive target for the toxic effects of inhaled lead. This study provides the first evidence that inhalation of particulate lead, at an occupationally relevant concentration, and in the absence of elevated blood lead levels, alters pulmonary Mø functions critical for lung defense against inhaled antigens. Our findings may have important implications for human health and should be considered when evaluating the health risks associated with inhaled lead. © 1993 Academic Press, Inc.

INTRODUCTION

Lead is a regulated air pollutant, and although no longer used as a gasoline additive, it is still widely distributed in the ambient urban environment (U.S. EPA Air Quality Criteria for Lead, 1986). The highest and most prolonged exposures to respirable lead, however, are in occupational settings (U.S. EPA Air Quality Criteria for Lead, 1986; Froines *et al.*, 1990). In the workplace, and localities surrounding high-temperature combustion sources, submicrometer lead particles, most often in the form of lead oxide (PbO), are emitted into the air and pose a substantial threat to human health (Cullen *et al.*, 1983; U.S. EPA Air Quality Criteria for Lead, 1986; Froines *et al.*, 1990).

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The toxicity of lead on hematopoesis, renal function, and the peripheral and central nervous systems are well documented (Reviewed in Vallee and Ulmer, 1972). Lead compounds are genotoxic (Nishioka, 1975; DiPaolo *et al.*, 1978; Zelikoff *et al.*, 1988) and are animal and potential human carcinogens (Kobayashi and Okamoto, 1974; Cooper, 1978; IARC, 1980; Sakai *et al.*, 1990). Exposure to lead has also been shown to disrupt systemic immunity of experimental animals (Reviewed in Koller, 1990) and humans (Jaremin, 1983; Coscia *et al.*, 1987; Cohen *et al.*, 1989). For example, *in vivo* exposure of rodents to soluble lead compounds have been shown to decrease host resistance against a variety of antigenic challenges and to compromise specific aspects of the humoral and cell-mediated immune responses (Hemphill *et al.*, 1971; Koller, 1973; Kobayashi and Okamoto, 1974; Cook *et al.*, 1975; Blakely and Archer, 1981; Kerkvliet and Baecher-Steppan, 1981; Hilliam and Ozkan, 1986; Kowolenko *et al.*, 1988; Malveya *et al.*, 1989; Sakai *et al.*, 1990). *In vitro* exposure to lead enhances interactions between B- and T-lymphocyte populations, inhibits delayed-type hypersensitivity, reduces the release of interleukin-2, and interferes with specific macrophage (Mø) functions important for maintaining host immunocompetence (Müller *et al.*, 1977; Castranova *et al.*, 1980; Exon *et al.*, 1985; Maüel *et al.*, 1989; McCabe and Lawrence, 1990, 1991). In addition, epidemiological studies suggest that occupational exposure of particulate lead impairs host immunity in exposed workers (Kimber *et al.*, 1986; Coscia *et al.*, 1987; Alomran and Shleamoon, 1988; Cohen *et al.*, 1989).

The immune system is crucial for protection of the host against infectious agents and developing neoplasms. Macrophages, along with other immune and nonimmune cells and a variety of soluble factors, play a central role in mediating these responses in the deep lung. Enhancement or diminution of any essential Mø function could, potentially, offset the balance necessary for immunoregulation in the lung and, thus, produce a cascade of detrimental secondary events, including compromised host resistance, tissue damage, or possibly cancer.

Previous immunotoxicological studies have clearly demonstrated the ability of lead to alter host defense by a number of different mechanisms. Most, if not all, of these experimental studies have examined the immunomodulating potential of lead using water-soluble compounds. Since the toxic effects of metal compounds are dependent upon their solubility in water (Costa and Mollenhauser, 1980; Zelikoff *et al.*, 1988) results from these studies may not accurately predict human health risks associated with exposure to inhaled particulate lead. Since almost all respirable lead exists in the particulate form (U.S. EPA Air Quality Criteria for Lead, 1986) and inhalation is the primary route of occupational exposure, more studies using inhalation exposure and particulate lead compounds, which more accurately reflect human exposure, are needed. Thus, our study was performed to evaluate whether inhalation of particulate PbO, at a concentration below the currently acceptable air lead standard for occupational exposure (U.S. EPA Air Quality Criteria for Lead, 1986; Froines *et al.*, 1990), disrupts pulmonary immunocompetence by altering Mø activity important for maintaining host resistance against infectious agents and disease.

MATERIALS AND METHODS

Animals and Experimental Design

Fourteen to sixteen-week-old (2.5 to 3.5 kg) male specific pathogen-free (*Pas-*

teurella multocida) New Zealand white rabbits (Millbrook farms, Amherst, Mass) were used for this study. Upon arrival, animals were quarantined for 2 weeks on a 12-hr light/dark cycle. All animals were housed individually in stainless steel cages and given food (Purina Rabbit Chow-HF) and water *ad libitum*.

Groups of 12 rabbits each were exposed by inhalation (nose only) for 3 hr/day (for 4 days) to PbO at 30 $\mu g/m^3$. Matched controls consisted of animals exposed to filtered air. Cohorts of 4 animals were then sacrificed either immediately (t_0), 24 hr (t_{24}), or 72 hr (t_{72}) after the final exposure, and bronchopulmonary lavage was performed as described below. Recovered Mø were used to assess PbO-induced effects on phagocytosis, production of reactive oxygen intermediates (ROI), and on biological activity of the regulatory cytokine TNF-α. Groups of animals were also exposed to latex particles of the same mass median diameter as PbO; these served as the inert particle control to assess any generalized particle-induced response. Inhalation of the latex particles produced no significant effects on any of the end points evaluated.

Materials

Cell culture reagents and glassware were analyzed (prior to experimentation) for endotoxin contamination by the *Limulus* amebocyte lysate assay (Wittaker M.A. Bioproducts, Walkerville, MD) and found to be negative (<0.5 ng). Cell culture reagents and lavage fluid were also screened routinely for bacterial and fungal contamination using standard microbiological growth medium. All glassware was autoclaved and heat sterilized (170°C, 4 hr) prior to use.

L-M cells (American Type Cell Culture Collection, ATCC; Rockville, MD), the target cells used to evaluate TNF-induced cytotoxicity, are a subclone of a continuous transformed cell line (L-929) derived from C_3/H_2N mice. L-M cells were maintained in 75-cm^2 tissue culture flasks by serial passage in medium 199 (M-199; GIBCO, Grand Island, NY), supplemented with 5% Bactopeptone (Difco, Detroit, MI) and 1% L-glutamine (GIBCO).

Rabbit serum opsonized zymosan (SOZ) was prepared by a modification of the method described by Scott and Klesius (1981). Following a second centrifugation at 500 *g* for 5 min, the recovered pellet was resuspended in 2 ml of sterile saline to yield a final SOZ concentration of 50 mg/ml.

Exposure System and Generation of PbO

The exposure and generation system used in this study were designed and built by this laboratory specifically for the generation of metal particles between 0.5 and 3.0 μm. Lead monoxide [PbO, ultrapure grade (99.998%); Aldrich Chemical, Milwaukee, WI] was generated as a monodispersed suspension (in 18-MΩ-distilled water) at 10 PSI using an acorn II nebulizer (Marquest Med. Prod., Englewood, CA; Model No. 122014). The nebulized aerosol was diluted with temperature-controlled, humidified zero air to assure an accurate concentration. To assess exposure concentration, the lead aerosol was collected on 47-mm glass fiber filters (placed close to the rabbit site of inhalation) using a mass-flow controlled pump. The filters, along with laboratory blanks, were extracted in concentrated HNO_3, diluted to volume in 5% HNO_3, and analyzed for lead, against a certified calibration curve, by flame atomic absorption spectrophotometry. The particle size distribution was determined by measuring the diameter of 200 aerosolized particles using scanning electron microscopy. Table 1 shows the target and

TABLE 1
CHARACTERIZATION OF LEAD OXIDE (PbO)

Compound	Target concentration (μg Pb/m^3)	Actual concentration (μg Pb/m^3)	Mass median aerodynamic diameter (μm)	Geometric standard deviation
PbO	30	31 (24–38)[a]	2.0	1.1

[a] The mean and value ranges of the concentrations observed for 4 exposure days.

actual concentrations for the PbO aerosol; the size of the particles was expressed as the mass median aerodynamic diameter (MMAD). Lead oxide particles being inhaled had an aerodynamic particle size of ~2 μm which should have allowed for penetration into the deep lung.

Bronchopulmonary Lavage and Macrophage (Mø) Collection

Rabbits were anesthesized by injection of sodium pentobarbital (50 mg/kg body wt) in the marginal ear vein. Animals were then exsanguinated by severing the abdominal aorta, and a bilateral pneumothorax was induced by an incision made in the diaphragm. Bronchopulmonary lavage was performed as described previously (Schlesinger, 1987). Aliquots of the acellular lavage fluid were used to evaluate the levels of lactate dehydrogenase (LDH), lysozyme, and total protein content (Sigma). The recovered cell pellets were resuspended in Eagle's minimum essential medium with Earle's salts [EMEM(E); GIBCO] and pooled (within a given exposure condition). Cell numbers and viability were determined by hemocytometer counting and trypan blue exclusion, respectively. Aliquots of the counted cell suspension were taken for differential counts to assess the percentage of Mø and other immune cell types in the recovered cell population and for identification of Mø by nonspecific esterase staining (Sigma). The remaining cells were respun at 400 g for 10 min and resuspended in the appropriate solution for carrying out the individual *in vitro* bioassays.

Measurements of Total Protein, LDH, and Lysozyme in the Lavage Fluid

Total protein was determined from lavage samples using a modification of the standard Bio-Rad protein assay originally described by Bradford (1976) and performed exactly as outlined in the commercially available kit (Bio-Rad Laboratories, Richmond, CA). The amount of protein in the lavage fluid was calculated from a standard curve and the results expressed as micrograms of protein per milliliter of lavage fluid. Lysozyme activity was measured using a turbimetric assay originally described by Parry *et al.* (1965). A unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance of 0.001/min. LDH activity was measured colorimetrically from freshly collected lavage samples using commercially available kits (Sigma). Absorbance was determined using a spectrophotometer at 460 nm and the amount of LDH activity calculated from a standard curve. The results were expressed as Berger-Broida units per milliliter (B-B units/ml).

Phagocytosis

Macrophage phagocytosis of opsonized polystyrene latex microspheres (<3 μm; Duke Sci, Palo Alto, CA) was evaluated using a previously described sus-

pension assay (Schlesinger, 1987). The prepared smears were stained with Diff Quik (Sigma) and 300 cells/slide examined microscopically to determine the phagocytic index (PI), i.e., the numbers of Mø containing at least one particle. Values were corrected for cell viability.

Production of Tumor Necrosis Factor-α (TNF-α)

Collection. Macrophages recovered by bronchopulmonary lavage from PbO and air-exposed rabbits were plated at 2×10^6 cells/35-mm dish in 1 ml of unsupplemented EMEM(E). Following a 2-hr incubation at 37°C (5% CO_2), Mø were washed, nonadherent cells were counted, and fresh medium, with and without 1 μg/ml lipopolysaccharide (LPS; phenol-extracted *Escherichia coli,* serotype 011:B; Sigma), was added. In all cases, the percentage of attached Mø (after washing) was ≥97%; there appeared to be no differences in attachment/adherance between Mø from PbO- and air-exposed rabbits. After 24 hr, the medium was collected, filtered through a 0.22-μm filter, and stored at 4°C until used (no longer than 1 week later). This represented the Mø-conditioned medium (MCM) used for evaluation of TNF-induced cytotoxicity.

TNF-induced cytotoxicity assay. TNF-induced cytotoxicity toward sensitive tumorigenic target cells (L-M) was assessed as described previously by our laboratory (Zelikoff *et al.*, 1991). Results were expressed as the final dilution producing 50% cytotoxicity. Specificity of the TNF-induced response was assured by abrogation of cytotoxicity with polyclonal anti-TNF antibodies (Genzyme, Cambridge, MA).

Production of ROI

Hydrogen peroxide (H_2O_2). Lead oxide-mediated effects on H_2O_2 production were assessed for resting and zymosan-stimulated Mø using a modification of the microassay system originally described by Pick (1986) and based on the horseradish peroxidase (HRPO)-dependent oxidation of phenol red (PR) by H_2O_2. Briefly, Mø (3×10^5) were added to each well of a 96-well microtiter dish in unsupplemented EMEM (E) and allowed to attach for 90 min at 37°C (5% CO_2). Following incubation, the medium was removed and the number of detached cells counted from random wells. In several cases, protein determinations (Bio-Rad Laboratories) were performed to validate cell numbers. PR-HRPO mixture added to 3 wells with cells and 3 wells without cells served as the reference blanks and the Δ absorbance was read from the blanking wells. One-hundred microliters of a 200 μg/ml PR solution, containing 100 μg/ml HRPO, with and without SOZ (0.5 mg/ml), was added. After 60 min, the reaction was halted by the addition of 1 *N* NaOH. The quantity of H_2O_2 produced was determined spectrophotometrically at 600 nm using an automated microtiter plate reader. Results were expressed as nmoles $H_2O_2/3 \times 10^5$ Mø. Values were corrected for cell attachment.

Superoxide anion radical ($^{\cdot}O_2^-$). Production of $^{\cdot}O_2^-$ was assessed using the microassay method described previously by our laboratory and based on the reduction of ferricytochrome c (Zelikoff and Schlesinger, 1992). Results were expressed as nmoles $^{\cdot}O_2^-/3 \times 10^5$ Mø. Values were adjusted for cell attachment (which was based upon the number of detached cells and by protein determinations).

Determination of Lung Lead Burden and Blood Lead Levels

Lung lead burden was determined from whole (unlavaged) lungs removed from PbO- and air-exposed rabbits. Excised lungs were weighed (wet weight), separated into right and left halves, reweighed, and frozen (at −20°C) until digested with HNO_3. Following tissue digestion, the samples were diluted to volume in 5% ultrapure HNO_3. The lead concentration was determined against a certified calibration curve by flame atomic absorption spectrophotometry. Lead levels in whole blood were measured at the New York City Department of Health (Bureau of Laboratories) using the standard method of MIBK-APDC chelation extraction.

Statistical Analysis

Analysis of results from all control animals (at each time point) indicated no significant difference among Mø functional, biochemical, or secretory activities, nor in cell number, viability, or differential counts (analysis of variance, $P < 0.05$). Thus, control data were pooled for graphical presentation; however, statistical analysis used the air-exposed controls associated with each PbO exposure group.

Differences in blood lead levels, lung burdens, and the functional and biochemical activities between Mø collected from control and PbO-exposed rabbits were assessed using two-way (treatment, time) ANOVA, followed, when appropriate, by the Dunnett test (Zar, 1986). Statistical significance was accepted at $P < 0.05$.

RESULTS

Total Protein, LDH, and Lysozyme Activity in the Lavage Fluid

Table 2 shows the effects of inhaled PbO on total protein content, LDH activity (general markers of lung cell damage), and lysozyme levels (a marker lysosomal enzyme) in the lavage fluid. While exposure to PbO at 30 $\mu g/m^3$ for 4 days produced no change in total protein levels, LDH and lysozyme activity were significantly enhanced (~160%) 24 and 72 hr after the final exposure.

Characterization of Lavaged Cells

The total number, viability, and purity of rabbit pulmonary lavage preparations are shown in Table 3. The only immune cell type other than Mø observed in the recovered cell population was polymorphonuclear leukocytes (PMN). Inhalation

TABLE 2
EFFECTS OF INHALED PbO ON LDH, TOTAL PROTEIN, AND LYSOZYME IN THE LAVAGE FLUID

Exposure group	Time postexposure (hr)	LDH activity (B-B units/ml)[a]	Total protein (μg)[a]	Lysozyme (units/min)[a]
Air control[b]	—	31 (±3)	17 (±0.7)	8.6 (±0.5)
PbO	0	24 (±0.6)	14 (±3)	10.3 (±0.7)
PbO	24	50 (±5)*	13 (±1)	13.5 (±2)*
PbO	72	48 (±5)*	18 (±1)	13.4 (±0.9)*

[a] Mean ± SE for 5 animals for the PbO-exposed groups and 12 rabbits for the pooled control group.
[b] Controls performed at each time point (t_0, t_{24}, and t_{72}) not significantly different; therefore, values pooled.
* $P < 0.05$

TABLE 3
CHARACTERIZATION OF LAVAGED CELLS

Exposure group	Time postexposure (hr)	Total no. viable Mϕ/kg body wt ($\times 10^6$)[a]	Cell viability (%)[a]	% PMN[a,b]
Air control[c]	—	7.34 (±1)	98 (±0.2)	1.7 (±0.2)
PbO	0	7.06 (±1)	97 (±0.6)	1.0 (±0.001)
PbO	24	7.40 (±0.8)	96 (±1)	1.8 (±0.5)
PbO	72	7.04 (±0.6)	97 (±0.6)	1.3 (±0.6)

[a] Mean ± SE for 5 animals for the PbO-exposed groups and 12 rabbits for the pooled control group.
[b] PMN, percentage of polymorphonuclear leukocytes in recovered cell population.
[c] Controls performed at each time point (t_0, t_{24}, and t_{72}) not significantly different; therefore, values pooled.

of PbO produced no infiltration of neutrophils (a hallmark of inflammation) and had no effect on the total number of Mø recovered. No significant differences in cell viability, as measured by trypan blue exclusion, were observed between the lead- and air-exposed groups; for all populations, viability was ≥96%.

Phagocytosis

The effects of inhalation exposure on the phagocytic uptake of opsonized polystyrene latex particles by rabbit pulmonary Mø are shown in Table 4. Phagocytic activity as expressed as the phagocytic index, a measure of the overall phagocytic competence of the lavaged cell population, decreased with increasing exposure time; 72 hr after the final exposure, phagocytic activity was significantly depressed to 74% of the control.

H_2O_2 and $^{\cdot}O_2^-$ Production

Resting, unstimulated rabbit pulmonary Mø produced 0.79 nmole H_2O_2/3 × 10^5 Mø in 60 min, which increased ~twofold (0.79 vs 1.5 nmole) following *in vitro* stimulation with SOZ (Fig. 1). Repeated inhalation of PbO at 30 μg/m^2 significantly increased spontaneous production of H_2O_2, compared to control levels, 24 hr after the final exposure and remained elevated, though not significantly, at 72 hr. Production by zymosan-stimulated Mø was significantly enhanced 24 and 72 hr after exposure.

Figure 2 shows spontaneous and SOZ-stimulated production of $^{\cdot}O_2^-$ by Mø

TABLE 4
EFFECTS OF INHALED PbO ON PHAGOCYTIC ACTIVITY

Exposure group	Time postexposure	Phagocytic index (%)[a,b]
Air control[c]	—	70 (±3)
PbO	0	67 (±1)
PbO	24	63 (±2)
PbO	72	52 (±3)*

[a] % PI = (No. of Mϕ engulfing at least 1 particle)/(total no. of Mϕ counted) × 100.
[b] Mean ± SE for 4 animals for the PbO-exposed groups and 10 rabbits for the pooled control group.
[c] Controls performed at each time point (t_0, t_{24}, and t_{72}) not significantly different; therefore, values pooled.
* $P < 0.05$

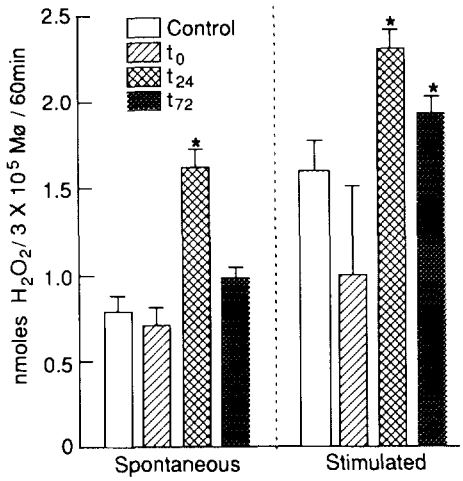

FIG. 1. Spontaneous and stimulated (zymosan, 0.5 mg/ml) production of H_2O_2 (nmoles) by control and PbO-exposed rabbit pulmonary macrophages collected by lavage immediately (t_0), 24 hr (t_{24}), 72 hr (t_{72}) after the final exposure. Values represent the mean ± SE for 4 rabbits in the PbO-exposed groups and 12 rabbits for the pooled control group. Spontaneous and zymosan-stimulated production of H_2O_2 was significantly enhanced ($P < 0.05$) compared to control 24 hr after the final exposure; stimulated production was still significantly ($P < 0.05$) enhanced at t_{72}.

recovered from air- and PbO-exposed rabbits after 60 min incubation. *In vitro* stimulation with zymosan increased Mø-mediated production of $^{\cdot}O_2^-$ ~threefold (0.36 vs 1.08 nmole). Spontaneous production of $^{\cdot}O_2^-$ was unaffected by PbO exposure; stimulated production began to increase 24 hr after the final exposure and reached statistically significant levels (1.08 vs 1.83 nmoles) by 72 hr.

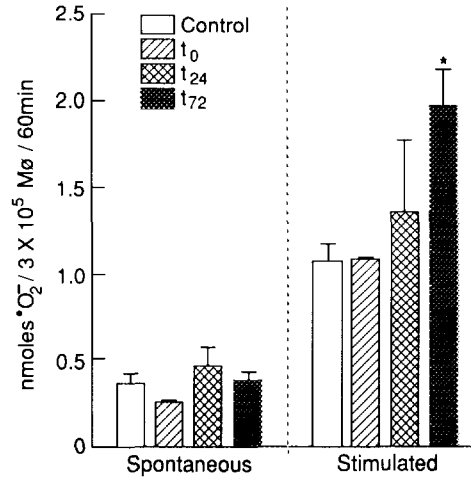

FIG. 2. Spontaneous and stimulated (zymosan, 0.5 mg/ml; cytochalasin B, 2.5 μg/ml) production of $^{\cdot}O_2^-$ (nmoles) by control and PbO-exposed rabbit pulmonary macrophages collected by lavage immediately (t_0), 24 hr (t_{24}), and 72 hr (t_{72}) after the final exposure. Values represent the mean ± SE for 4 rabbits in the PbO-exposed groups and 10 rabbits for the pooled control group. While spontaneous production of $^{\cdot}O_2^-$ was unaffected by exposure to PbO, zymosan-stimulated production began to increase at t_{24} and was significantly ($P < 0.05$) elevated at 72 hr.

TNF-Induced Cytotoxicity

The effects of PbO on LPS-stimulated TNF-induced cytotoxicity toward L-M cells are shown in Figure 3. Rabbit pulmonary Mø were capable of producing TNF spontaneously, although levels (dilution of MCM-containing TNF needed to produce 50% cytotoxicity) were quite low (3 units/ml). While inhalation of PbO had no effect on spontaneous release of TNF at any of the measured time points (5 vs 3 units/ml), LPS-stimulated activity was altered at all post-exposure time intervals. Immediately and 24 hr following the last exposure, TNF-induced cytotoxicity was depressed by 62 and 50%, respectively; at t_{72}, TNF activity was significantly enhanced (~twofold) compared to control levels.

Blood Lead Concentration and Total Lung Lead Burden

As shown in Table 5, there was found to be no significant change (compared to those levels measured prior to exposure) in the blood lead levels of exposed animals following inhalation of PbO. Immediately after inhalation, the amount of total lead measured in the unlavaged lungs was ~fivefold above control values (0.37 vs 2.1 μg). Lead concentration in the lung decreased with increasing post-exposure time; 72 hr after exposure, the total lung burden of lead was similar to that observed for the air-exposed control (0.37 vs 0.42 μg). Interestingly, 90% of the total lung burden, at t_0, was deposited in the right lung. In addition to the results shown, there were no apparent differences in the overall appearance or total weight of the lead-exposed lung compared to that observed for the control lung.

DISCUSSION

Despite the widespread occurrence of respirable lead particles in the air (in both occupational and ambient environments), and their potential risks to human health, effects associated with inhalation of particulate lead on the lung has, for the most part, been ignored. The present study was designed to shed light on this

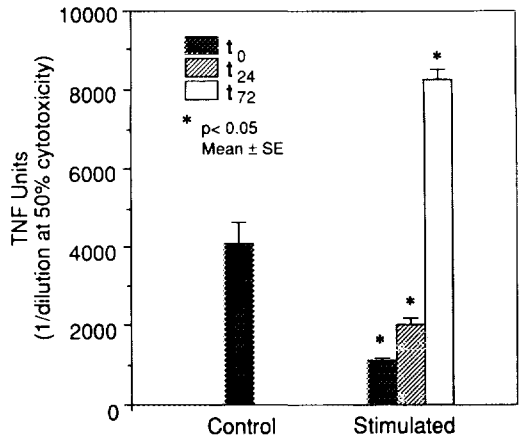

FIG. 3. Effects of inhaled PbO on LPS (1 μg/ml)-stimulated TNF release/activity by rabbit pulmonary macrophages collected immediately (t_0), 24 hr (t_{24}), and 72 hr (t_{72}) after the final exposure. Results are expressed as the dilution producing 50% cytotoxicity toward L-M cells. Values represent the mean TNF units ± SE for 4 rabbits in the PbO-exposed groups and 12 rabbits for the pooled control group. PbO significantly ($P < 0.05$) depressed TNF activity immediately and 24 hr after exposure; at t_{72}, TNF activity was significantly ($P < 0.05$) enhanced above control levels.

TABLE 5
BLOOD LEAD LEVELS AND TOTAL LUNG BURDEN IN RABBITS EXPOSED TO PbO

Exposure condition	Time post-final exposure (hr)	Blood lead levels (μg/dl)[a]	Concentration of lead in the lung (μg)[a]		Total lung burden of lead (μg)
			Left	Right	
Preexposure	—	1.2 (±0.1)	—	—	—
Air[b]	—	1.2 (±0.3)	0.15 (±0.06)	0.22 (±0.01)	0.37
PbO	0	2.0 (±1.0)	0.22 (±0.02)	1.90 (±0.05)*	2.12*
PbO	24	1.3 (±0.3)	0.19 (±0.06)	0.90 (±0.05)*	1.09*
PbO	72	2.0 (±0.1)	0.19 (±0.04)	0.23 (±0.07)	0.42

[a] Values represent means of four rabbits ± SE.
[b] Controls performed for each time point (t_0, t_{24}, and t_{72}) not significantly different; therefore, values pooled.
* $P < 0.05$

relatively unexplored area by investigating the impact of inhaled particulate lead on pulmonary immune defense mechanisms important for maintaining host resistance against infectious diseases and, possibly, cancer. In this study, inhalation of particulate lead, at a concentration below the acceptable limit set for occupational exposure, interfered with pulmonary Mø functions including phagocytosis, ROI production, and activity of the cytokine TNF-α, in a time-dependent manner.

Analysis of biochemical markers is a sensitive method for the evaluation of pulmonary damage and one which may help gain insight into mechanisms by which inhaled pollutants produce their effects. Biochemical markers in lavage fluid are often altered by pollutant exposures which fail to produce other changes (Conner *et al.*, 1989). Protein content, a measure of transudation of blood plasma proteins, was unchanged by repeated exposure to PbO while LDH and lysozyme activity (in the lavage fluid) increased 24 and 72 hr after the final exposure. These findings indicate that effects of inhaled PbO on these end-points are delayed in their onset and persist after exposures have ceased. In a study by Hirano *et al.* (1989), intratracheal instillation of another particulate divalent cation, cadmium oxide, also produced similar time-related effects on some of these same biochemical markers. It has been suggested in a previously reported *in vitro* study (DeVries *et al.*, 1983), that it is the solubilized lead ions released from Mø-ingested PbO particles that are responsible for the lead-induced cellular changes observed over time. Thus, intracellular dissolution of PbO and subsequent release of the free (possibly more toxic) lead ions may be responsible for the delayed effects observed in this study on LDH and lysozyme activity, as well as on the Mø activities.

Inhalation of PbO produced no neutrophil infiltration nor any change in cell viability or total numbers. Similar to the absence of lead-induced cytotoxicity toward Mø observed in this study, Kaminski *et al.* (1977) reported that intrapulmonary instillation with moderate doses of PbO for 15 to 40 days produced no change in the viability of rat pulmonary Mø although lead particulates were deposited in the alveoli and alveolar macrophages. PbO also proved relatively nontoxic (as measured by trypan blue exclusion) to mouse Mø following long-term *in vitro* exposures (Hilbertz *et al.*, 1986).

Our observed lack of a PbO-induced change in the number of recoverable Mø

is in contrast to an early study by Bingham *et al.* (1973), who reported that prolonged inhalation of particulate lead sesquioxide at 10 and 150 $\mu g/m^3$ dramatically reduced the total number of lavageable rat pulmonary Mø. Discrepancies between the studies may be due to differences in duration of lead exposure, animal model, or the specific type of lead particles used. Lead particulates differ in their solubilities and rate of dissolution within Mø, and these are important factors for predicting overall particulate toxicity. For example, particulate lead tetroxide has a faster rate of dissolution in rabbit alveolar Mø than lead monoxide, and the former is more cytotoxic as evaluated by ultrastructural and cytological alterations (DeVries *et al.*, 1983).

Clearance of inhaled antigens and particulate matter by phagocytosis is a critical function of pulmonary Mø. A toxicant-induced compromise of this activity can, thus, lead to lung damage and/or an increased susceptibility of the host to respiratory diseases caused by inhaled infectious agents. In our study, inhalation exposure to PbO reduced the phagocytic activity of rabbit pulmonary Mø in a time-dependent manner with maximum inhibition occurring 72 hr after the final exposure. This finding is similar to that reported previously by other investigators who demonstrated that exposure to moderate doses of soluble lead (both *in vitro* and *in vivo*) depressed the phagocytic uptake by murine Mø of latex particles, sheep red blood cells, and bacteria (Kerkvliet and Baecher-Steppan, 1981; Hilbertz *et al.*, 1986; Kowolenko *et al.*, 1988).

Mø surface features have previously been shown to correlate with phagocytic activity of the cells. Macrophages with ruffled membranes phagocytose a significantly greater number of metal beads *in vitro* than do smooth, unruffled cells (Warheit *et al.*, 1983). *In vitro* exposure to PbO particles (2–5 μm) has been shown to alter the appearance of rabbit pulmonary Mø by reducing the number of surface ruffles; ruffling decreased with increasing postexposure time (DeVries *et al.*, 1983). These results lend further support to our findings.

Inhaled PbO may act to depress Mø phagocytosis by direct effects on surface receptors important for binding and ultimate uptake of the opsonized latex particles. Jian *et al.* (1985) reported that *in vitro* exposure of rabbit pulmonary Mø to relatively low concentrations of soluble lead inhibits phagocytosis of opsonized sheep red blood cells and down-regulates F_c receptor expression. The investigators suggest that F_c receptors may be masked following lead exposure as a result of changes in Mø ultrastructure (i.e., loss of surface ruffling). Alternatively, since lead is known to compete metabolically with calcium (Costa *et al.*, 1984) and an optimal intracellular calcium $[(Ca^{2+})_i]$ level is required for F_c receptor-mediated phagocytosis to occur, it is possible that phagocytosis (in the present study) was altered via effects of lead on overall calcium homeostasis.

Mø-mediated cytotoxicity toward foreign antigens and developing neoplasms depends on target cell injury mediated by toxic factors, including H_2O_2 and $^{\cdot}O_2^-$ (Adams and Marino, 1981). While a depression of these metabolites may increase host susceptibility to infectious diseases, an overproduction can produce tissue and organ damage. In this study, spontaneous and stimulated production of H_2O_2 and $^{\cdot}O_2^-$ was enhanced in a time-dependent manner following lead exposure. This observed enhancement was rather surprising given the reported ability of soluble lead to depress oxidative metabolism and ROI production in rodent Mø (Kaminski *et al.*, 1977; Castranova *et al.*, 1980; Hilbertz *et al.*, 1986; Buchmuller-Rouiller *et al.*, 1989).

Although our findings on ROI production may, at first, appear contradictory to those previously reported, it should be remembered that all of the former studies employed soluble compounds and exposure routes other than inhalation; most studies also employed relatively high lead concentrations. This is an important consideration since immunotoxic effects of metals have been shown to be dependent upon certain parameters, including metal concentration, solubility in water, exposure regimen, and route of metal exposure (reviewed in Treagan, 1975). In the only other study evaluating the effects of PbO on H_2O_2 production, *in vitro* exposure to moderate doses of PbO produced no effects on H_2O_2 production by rabbit pulmonary Mø (Labedzka *et al.*, 1989). The effects of lead on H_2O_2 formation are not that straightforward and appear to depend upon the length of cell contact with the ions. Hilbertz *et al.* (1986) reported that *in vitro* exposure of Mø to soluble lead chloride for up to 1 hr enhanced phorbol myristateacetate (PMA)-stimulated chemiluminescence, while longer exposures (i.e., 20 hr) to an equimolar concentration of lead substantially reduced lucigenin-induced chemiluminescence. Buchmuller-Rouiller *et al.* (1989) suggested that an increased respiratory-burst activity after relatively short exposures to lead might result from effects of the lead ions on the cell membrane; this interaction may promote activation of specific surface receptors involved in triggering the respiratory burst.

Our study provides the first evidence that particulate lead, under any exposure condition, can alter TNF activity. This finding is extremely important given the ability of this cytokine to mediate a variety of biological responses associated with host defense and non-immunologically related parameters (Kunkel *et al.*, 1989). In addition to its well-known tumoricidal activity, TNF disrupts hematopoietic precursor cells, produces fever by increasing prostaglandin synthesis, activates neutrophils, alters endothelial cells by increasing adhesive properties and procoagulant activity, destroys virally infected cells, and stimulates fibroblast proliferation (Ruff and Gifford, 1981; Kunkel *et al.*, 1989). Thus, alterations produced by inhaled PbO may have significant implications to the overall health and immune defense of the lungs. Lead-induced alterations in LPS-stimulated TNF activity appeared earlier than the other observed effects and persisted for up to 72 hr. The decrease in TNF activity observed immediately and 24 hr after PbO exposure may help explain a previous *in vitro* study by Maüel *et al.* (1989), who demonstrated a lead-induced diminution of tumor target-cell lysis by LPS-stimulated mouse Mø. The authors suggested that lead may be acting to depress Mø-mediated cytotoxicity by inhibiting Mø acquisition of the fully activated state. If the hypothesis is correct, this offers a possible mechanism by which TNF activity (in this study) may have been reduced. However, given the concurrent enhancement of other Mø activation markers (i.e., ROI production), this does not appear to be a likely explanation for our findings. In the same *in vitro* study, lead also reduced interferon-γ (IFN-γ) receptor turnover and degradation of bound IFN-γ by Mø. Since Mϕ are also capable of binding and internalizing TNF-α, inhaled PbO may have acted to reduce TNF activity by interfering with some aspect of TNF receptor metabolism.

Th PbO-induced enhancement of TNF observed after 72 hr in our study is difficult to explain. One could speculate that since pulmonary Mø represent a heterogenous population of cells which exhibit different morphological, functional, and biochemical properties (Murphy and Herskowitz, 1984), exposure to

lead, after 72 hr, has "selected out" those Mø subpopulations with a greater capacity to release TNF.

Historically, blood lead levels have been used as a primary indicator to predict the biological effects of lead exposure (U.S. EPA). In fact, lead exposure limits have been set based principally on blood levels. In our study, lead-induced effects on the lung (as measured by biochemical markers of lung cell damage) and on Mø functions occurred in the absence of concomitant increases (compared to controls) in blood lead levels. A failure to observe elevated blood lead levels at the time points examined suggests either that lead is confined to the lung and has not yet reached the circulation, or that it has already been cleared from the blood and is now localized in the next compartment (i.e., kidney or bone). This finding is extremely important when considering human health risks since it demonstrates that inhaled particulate lead can produce toxic effects locally in the lung in the absence of "predictive" elevated blood lead levels.

Lung lead burden was determined in this study in order to assess whether the amount of lead in the lung correlated with observed effects on Mø functions and lung cell damage. Lead concentration in the lung was significantly elevated (compared to the air-exposed control) immediately after exposure and then decreased with time reaching control levels by 72 hr. The complete absence of lead in the lungs by 72 hr was surprising given the observed effects on Mø functions at this same time point. Lead ions released from the particulates by intracellular dissolution within the Mø were most likely absorbed (Hilliam and Ozkan, 1986), while lead remaining in the alveoli as PbO was probably removed within Mø via the mucociliary escalator (Kaminski *et al.*, 1977). Differences in lead deposition between the right and left lung were most likely due to the increased size of the right lung and/or the larger bronchialdiameter.

CONCLUSIONS

An important route by which pathogenic and noxious agents enter a body is through the respiratory tract. If inhaled lead is able to alter immunoreactivity of the lung, it may not only increase the risk of respiratory disease but also enhance the occurrence of lung pathology. Our results demonstrate that the lung is an important target for the toxic effects of lead and that inhalation of airborne lead particles, at a concentration below the currently acceptable limit for occupational exposure, alters, in a time-dependent fashion, Mø-mediated functions critical for maintaining pulmonary immunocompetence. We feel that our findings may have important implications for human health and should be considered when evaluating the health risks associated with lead exposures.

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