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IMMUNOTOXICITY OF PARTICULATE LEAD: IN VITRO EXPOSURE ALTERS PULMONARY MACROPHAGE TUMOR NECROSIS FACTOR PRODUCTION AND ACTIVITY

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Rabbit pulmonary macrophages were exposed in vitro to particulate lead oxide (PbO) for periods of up to 72 h and then assayed for the activity of tumor necrosis factor-alpha (TNFα) released after stimulation with lipopolysaccharide (LPS). The levels of TNFα obtained from PbO-treated cells were decreased in a dose-dependent manner as compared with metal-free control cells for each time point examined. Cells treated simultaneously with both LPS and PbO yielded less monokine than did cells receiving LPS alone. In addition, incubation of cell-free TNFα with PbO resulted in a diminution of cytotoxicity directed against TNFα-sensitive tumor target cells. Macrophage burdens of PbO particles increased with both the length of incubation and concentration of PbO used; increases in cellular lead burdens were paralleled by reductions in cell viability. Thus, under in vitro conditions, PbO affects the levels of the immunoregulatory monokine TNFα and also disrupts its cytotoxic properties after release from activated macrophages.

Lead (Pb) compounds are known to be toxic to both experimental animals and humans. The toxicity of lead is a major concern because of its prevalence in the environment and its potential threat to human health (U.S. EPA, 1986). Lead is a well-known immunotoxicant and has been shown to affect several immune functions, resulting in enhanced host susceptibility to bacterial and viral infections (Hemphill et al., 1971; Gainer, 1974; Blakley & Archer, 1981; Koller, 1990) and increased growth and metastases of implanted tumors (Kobayashi & Okamoto, 1974; Kerkvliet & Baecher-Steppan, 1982). While the aforementioned studies demonstrated that lead exposure resulted in a broad systemic immunotoxicity, other studies indicated that specific organ defense mechanisms, that is, pulmonary, were also adversely affected (Bouley et al., 1978; Zelikoff et al., 1993).

The pulmonary alveolar macrophage (PAM) represents the first line of defense against inhaled particles and microorganisms in the lungs (Cohn, 1968; Hocking & Golde, 1979). Exposure of PAM to lead in vitro reduces their viability (Kaminski et al., 1977) and depresses phagocytic activity; the degree of inhibition is directly proportional to the concentration of lead used

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(Jian et al., 1985; Zhou et al., 1985). Lead-induced damage to PAM mitochondria, nuclear membrane, microtubules and other cytoskeletal components, and both smooth and rough endoplasmic reticuli has also been observed (DeVries et al., 1983; Governa et al., 1987). The aforementioned results suggest that lead exposure may impact on cellular energy metabolism and secretory functions. While studies have shown a relationship between lead-altered energy metabolism and decreased phagocytic activity (Mustafa et al., 1971), less is known about the effects on PAM monokine production (Kowolenko et al., 1988). Disturbances in the production or release and/or activity of macrophage-derived cytokines [i.e., interleukin-1α (IL-1α) or TNFα] could play a critical role in the overall mechanism by which lead disrupts host immunocompetence.

TNFα is a nonglycosylated protein that is released from macrophages following activation with stimulating agents such as γ-interferon or bacterial endotoxin, or by the ingestion of particles (Le & Vilcek, 1987). TNFα causes necrosis of certain transplantable animal tumors in vivo and is cytotoxic to several animal and human tumor cell lines in vitro (Carwell et al., 1975). It has also been shown to have a role in vivo in inflammation, resistance to infection, and in the pathology of endotoxemia (Nathan, 1987). Lead exposure has been shown to decrease host TNFα production in vivo while simultaneously giving rise to an adaptive enhanced host sensitivity to these lowered levels of monokine (Dentener et al., 1989). Direct effects from lead on released TNFα are also highly probable as a result of interactions between lead ions and the two cysteine residues involved in a crucial transitional intraprotein disulfide bond necessary for optimizing cytotoxic activity (Jones et al., 1989; Narachi et al., 1989).

Our earlier studies in rabbits demonstrated that inhalation of lead oxide (PbO) produced a time-dependent alteration in the production of several PAM products including superoxide anion radical, hydrogen peroxide, and TNFα (Zelikoff et al., 1993). In the current study, PAM were exposed to particulate PbO in vitro in order to better understand the possible mechanisms by which effects on TNFα observed in vivo may have occurred. The direct effect from PbO on TNFα cytotoxic activity was measured to determine if extracellular PbO (either as particles or as solubilized Pb^{2+} ions) could affect TNFα after its release from activated PAM.

MATERIALS AND METHODS

Chemicals

The PbO used in all assays was obtained from Aldrich Chemical Company (Milwaukee, Wis.). The particles were sized by differential filtration and examination by scanning electron microscopy. Only PbO particles ≤ 2 μm were used; PbO particles of this size are found in the environment and workplace settings as a result of combustion and industrial use, respectively,

and are readily respirable and penetrate deep into the distal branches of the bronchial tree. Particles were sonicated, washed repeatedly with sterile saline, and vortexed prior to incubation with PAM to prevent particle agglomeration.

All tissue culture reagents were from Gibco (Gibco Co., Grand Island, N.Y.). Tissue culture plates and chamber slides were from Nunclon Products (Naperville, Ill.); microtitre plates for the TNFα cytotoxicity assays were from Falcon Industries (Lincoln Park, N.J.).

Rabbits

Male, specific-pathogen-free (*Pasturella multocida*) New Zealand White rabbits (14 wk old and weighing 2.5–3.0 kg) obtained from HARE Laboratories (Hewitt, N.J.) were used as the source of pulmonary macrophages. Upon arrival, animals were quarantined for 2 wk and were checked daily for any outward signs of infection. Animals were housed in individual stainless steel cages in a temperature- and light-controlled room and were fed Purina rabbit chow-HF (Ralston Purina, St. Louis, Mo.) and water ad libitum.

Isolation and Collection of PAM

Pulmonary alveolar macrophages were retrieved by repeated broncho-pulmonary lavage of unexposed rabbits. Briefly, rabbits were sacrificed with an overdose injection of Nembutal (50 mg/kg; Abbott Laboratories, North Chicago, Ill.) into the marginal ear vein. Immediately after death, a single midline incision was made to expose the abdominal aorta and the vessel was severed to allow blood to exit the abdominal cavity and not accumulate in the thoracic cavity. The trachea was exposed and a 30-mm tube was inserted between adjacent rings and anchored by suture.

An appropriate volume (approximating 70% by body weight) of Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS) was instilled into the lungs and the thoracic cavity was gently massaged for 15–30 sec. The lavage fluid was slowly withdrawn to prevent tearing of lung tissues and the fluid was placed in polypropylene tubes held on ice. The lavage procedure was repeated 6 times and the recovered fluid was centrifuged at 400 × g for 15 min at 4°C. The cell pellets were resuspended in Eagle's minimal essential medium containing Earle's salts [EMEM(E)] and the cell numbers were determined by counting in a hemacytometer; initial viabilities were determined by trypan blue exclusion. The PAM were resuspended at 10^6/ml in EMEM(E) containing antibiotics (penicillin, streptomycin, and gentamycin at 100 U/ml, 100 μg/ml, and 50 μg/ml, respectively), placed in 35-mm sterile culture dishes, and incubated for 90 min at 37°C to permit cell adherence before use in the in vitro bioassays.

PAM Viability

Following a 90-min incubation, the dishes were randomly separated into experimental and untreated control groups. The medium was removed from

each dish and replaced with 1 ml medium containing PbO (10, 30, or 100 μg) or sterile saline. These concentrations, although somewhat above the acceptance limit for occupational exposures (50 $\mu g/m^3$), were selected to investigate the mechanisms underlying the effects observed in our previous PbO inhalation studies. All plates were then incubated for 3, 24, 48, or 72 h, and PAM viabilities were than measured by trypan blue exclusion following gentle trypsinization.

Phagocytic Uptake of PbO Particles

Five hundred thousand PAM were plated onto double-welled chamber slides and allowed to attach for 90 min at 37°C. The medium was removed and the cells were gently washed with fresh EMEM(E) to remove nonadherent cells. Medium containing varying concentrations (10, 30, or 100 μg PbO/ml) of PbO was then added to the cells. To monitor normal phagocytic processes, PbO-free control PAM were incubated with sonicated fetal bovine serum (FBS) opsonized latex microspheres (~3 μm, Duke Scientific, Palo Alto, Calif.) in EMEM(E) (100 : 1 particle to cell ratio). All slides were then incubated at 37°C for either 3 h or for 24-h intervals up to 72 h. Slides were removed at the designated times, air dried, and fixed in 2.5% cold glutaraldehyde; the latex-treated slide were subsequently placed in xylene overnight to dislodge any noningested particles (Gardner et al., 1973). Slides were then stained with Diff-Quick solution (Fisher Scientific, Springfield, N.J.) to visualize internalized particles. Internalized PbO particles were differentiated from extracellular membrane-associated PbO by the presence of lead particles within intracellular vacuoles and by varying the plane of focus during microscopic examination. The PAM were examined using oil immersion (100x; minimum 100 cells counted per field, total of 3 fields) and the phagocytic activity was expressed as the phagocytic index. Phagocytic index was determined as the ratio of the total numbers of phagocytically active cells (PAM were considered active if cells contained at least one PbO or latex particle) to the total numbers of cells counted.

Determination of Rabbit PAM-Derived TNFα

Collection One-milliliter aliquots of PAM were plated at 10^6 cells/35-mm dish and incubated 90 min as described earlier. The adherent cells were rinsed with fresh EMEM(E) and then treated with 1 ml EMEM(E) containing PbO (30 μg) or with medium only. After 3, 24, 48, or 72 h of incubation, the PAM were washed again and 1 ml of EMEM containing 1 μg/ml *Escherichia coli* endotoxin (lipopolysaccharide, LPS, type 055:B5, Sigma Corporation, St. Louis, Mo.) was added to stimulate TNFα production; spontaneous TNFα production was determined in the absence of LPS. After an additional 24 h, the cell-free supernatant was removed, filtered through 0.22-μm Acrodisc filters (Gelman Scientific, Ann Arbor, Mich.), and stored at 4°C (for no longer than 7 d). This macrophage-conditioned medium (MCM) was used to assess TNFα-induced cytotoxicity against tumorigenic target cells, an indirect measurement of TNFα production by activated PAM. In separate experiments,

PbO and LPS were incubated simultaneously with the cells to evaluate possible effects due to extracellular PbO–LPS interactions or due to effects of PbO on cell–LPS interactions.

To account for possible differences in TNFα production due to PbO-induced variations in cell survival or adherence, freshly harvested PAM were plated and treated with PbO as already described. After 3, 24, 48, or 72 h of exposure, PAM were harvested by mild trypsinization, pooled, and reseeded into 35-mm dishes at 10^6 PAM/dish. After a 2-h reattachment period, EMEM(E)-containing LPS solution was added to each dish; unstimulated control cells received EMEM(E) without LPS. After a 24-h incubation, MCM were collected and assayed as described next.

TNFα-Induced Cytotoxicity Assay Murine fibroblastic cells (L-M, a clone of L929, American Type Cell Culture, Rockville, Md.) were maintained in culture in Medium 199 (M199) containing 5% Bactopeptone and 1 m*M* L-glutamine. Cells were passaged at 90% confluency twice weekly, and only cells at low passage (<p15) were used for the cytotoxicity assays. Wells of a 96-well flat bottom plate were each loaded with 5×10^4 L-M cells and the plates were incubated overnight at 37°C. The medium was then removed and replaced with 100 μl of complete M199 containing 1 μg/ml actinomycin D. Into the first well of each row, 50 μl of M199 containing 2 μg/ml actinomycin D and 50 μl of test MCM was added. Twofold serial dilutions were made by transferring 0.1-ml aliquots until a final MCM titer of 1 : 1024 was achieved. Plates were incubated for 24 h at 37°C; cells were then fixed and stained in a 0.5% crystal violet/ethanol solution, the plates were air dried, and the absorbance was read (550 nm) on an automated plate reader (Anthos Labtech Instruments, Durham, N.C.). Results were expressed as the inverse of the MCM dilution producing 50% toxicity toward the L-M target cells (Zelikoff et al., 1991).

Extracellular Lead Oxide–TNFα Interactions

To assess any direct effect from PbO on TNFα, MCM from untreated PAM (10^6 cells/dish) stimulated with LPS was harvested and aliquoted (900 μl) into Eppendorf tubes for incubations with 30 μg/ml PbO. Into triplicate tubes for each time point, 100-μl aliquots of PbO solution were added and the tubes were incubated for 3, 24, and 72 h at room temperature with gentle rocking. Two tubes served as untreated controls and received 100 μl of medium only. These two control tubes were immediately placed at 4°C for later determination of the initial TNFα titer. Additional MCM samples were prepared with medium only and were incubated along with the PbO-treated samples in order to account for any alterations in TNFα activity due to time-related protein degradation. Following incubation, samples were filtered to remove PbO particles and stored at 4°C until used for the cytotoxicity assays.

PbO Dissolution in Rabbit PAM

To assess intracellular processing of the ingested PbO particles by the PAM, 10^6 cells were added to 35-mm dishes and incubated for 2 h at 37°C

to allow for cell attachment. The medium was then removed and replaced with 1 ml of EMEM(E) containing 30 μg PbO. The dishes were incubated for 3-, 24-, 48-, or 72-h intervals before the physical state (particulate vs. solubilized form) of ingested PbO was determined. At each of the designated time points, random dishes were removed and the cells rinsed twice with EMEM(E). One milliliter of 0.25% trypsin was added to each dish and the plates were incubated for an additional 30 min. The PAM were harvested by gentle scraping and were placed in plastic culture tubes for lysis with 1 ml of 1% Triton X-100 followed by sonication for 30 min. All cell-associated solutions were then passed through a 0.22-μm Millipore (type GS) filter. The levels of solubilized PbO in the filtrates and of the intact insoluble PbO on the filters were analyzed by atomic absorption spectrophotometry using protocols previously described by our laboratory (Zelikoff et al., 1993). At least three samples per time point were examined.

To measure spontaneous extracellular dissolution of the particles, PbO at 30 μg/ml was incubated in EMEM(E) for the same lengths of time as in the cellular assays and then processed. To assess total lead levels in each set of samples, random plates were harvested at each time point and directly analyzed without any filtrations steps.

Statistical Analyses

All data were analyzed using the Student-Newman-Keuls test. Differences within and between treatment groups were designated as statistically significant at $p < .05$, and highly significant at $p < .01$.

RESULTS

In vitro treatment of freshly harvested rabbit pulmonary macrophages (PAM) with particulate PbO resulted in both a time- and dose-dependent decrease in viability (Fig. 1). The presence of PbO rapidly reduced cell survival; survival was reduced by 50% 38 h after exposure to 100 μg/ml PbO as compared with an estimated period of 106 h in untreated controls.

In the absence of PbO particles, PAM displayed a time-dependent decrease in the uptake of latex particles. Conversely, at concentrations of PbO less than 100 μg/ml, the phagocytic index appeared to remain relatively constant over time (Fig. 2). At 100 μg/ml PbO, the phagocytic index increased at 48 and 72 h. The apparent increase in the numbers of PAM containing PbO particles is in agreement with the observation that PAM treated with particulate PbO at 30 μg/ml (Table 1) continually solubilize the metal but only at the incubation times less than 48 h. Dissolution beyond this time period reaches a plateau, with about 60% of the original particulate PbO being solubilized by the PAM. Spontaneous dissolution of the particles in cell-free EMEM(E) was slow and reached a maximum of approximately 32% of the total recovered PbO after 72 h. The lack of particle dissolution, in conjunction with decreases in PAM viability, would then allow for the observa-

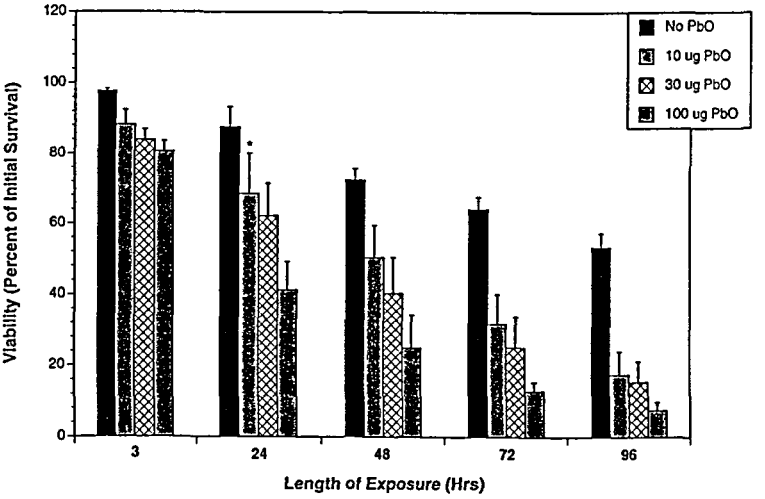

FIGURE 1. Effect of PbO on PAM viability. Survival was expressed as a function of incubation time with PbO at the concentrations indicated. PAM (10^6/dish) were exposed to PbO at the doses indicated for 3, 24, 48, 72, or 96 h. Viability was measured by trypan blue exclusion and the results were expressed as the percentage of the initial cell viability. All values given are the mean (± SE) of six dishes per treatment per time point. Within each time point, viabilities are significantly different ($p < .01$) between PbO-treated PAM and those of the metal-free controls (except where indicated by the single asterisk, where $p < .05$).

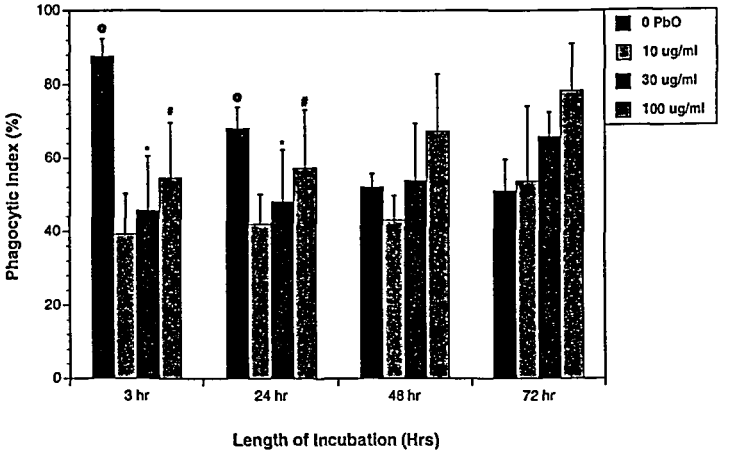

FIGURE 2. Phagocytic activity of PAM toward particulate PbO. Cells were exposed to varied concentrations of PbO or to FBS-opsonized latex microspheres for the times shown. At least 100 cells per field (3 fields per sample) were examined to determine the phagocytic index as defined in the Materials and Methods. Values significantly different within each lead treatment ($^{@}p$, .01; $^{*}p$, .05; and $^{\#}p < .05$) are indicated.

TABLE 1. Dissolution of PbO Particles in Rabbit Pulmonary Macrophages and in Culture Medium (EMEM[E]) as a Function of Incubation Time

	Lead oxide dissolution in rabbit pulmonary macrophages			Spontaneous dissolution of PbO particles in EMEM(E) medium		
Incubation time	Soluble PbO[a]	Insoluble PbO[a]	Insoluble PbO (% of total PbO)	Soluble PbO[a]	Insoluble PbO[a]	Insoluble PbO (% of total PbO)
3 h	0.59 ± 0.41	14.42 ± 2.90	96.07%	0.51 ± 0.40	11.94 ± 2.87	95.90%
24 h	2.40 ± 0.06	11.18 ± 4.63	82.20%	0.40 ± 0.04	8.17 ± 2.40	95.33%
48 h	10.52 ± 0.80	7.86 ± 0.22	42.76%	5.43 ± 1.90	15.22 ± 1.90	74.70%
72 h	10.08 ± 0.73	8.12 ± 2.91	44.37%	6.76 ± 1.71	14.73 ± 0.11	68.54%

[a]Cells were treated with ~30 μg PbO and then the cell samples were split to obtain replicate dishes. Values are the recoveries (in μg PbO) obtained from at least four plates per time period. Average total lead burden in the sample plates was 16.30 μg (±2.38).

tion of more PAM containing intact PbO particles with the longer periods of incubation.

The TNFα production by PAM appears to be dependent upon the length of PAM incubation prior to LPS stimulation; prolonged incubation of the cells prior to LPS stimulation resulted in decreased PAM TNFα production. As the unstimulated (no LPS added) formation of TNFα in PbO-treated and control cells was consistently below the limits of detection in the assay system employed (data not shown), it was concluded that PbO treatment itself did not enhance spontaneous LPS production by PAM. As shown in Figure 3, TNFα production by metal-free cells decreased 30% after only 3 h of culturing, and was reduced by 90% after 48 h of incubation prior to stimulation. PbO-treated cells showed a similar pattern in LPS responsiveness in relation to the increased lengths of incubation prior to LPS treatment. However, while there were no significant differences between the PbO-treated PAM and the metal-free control cells with respect to the magnitude of the reductions in TNFα activities (at each time point) as compared with initial TNFα titers, the TNFα yields from PbO-treated PAM were consistently lower, with maximal depression being reached by 48 h (5% of initial titer). By readjusting all recovered titer values on the basis of changes in PAM viability over the increasing incubation period with PbO, it was observed that the recoverable TNFα activities (with respect to both absolute and relative titers) were still lower for the PbO-treated PAM (except at the final 72-h time point) as compared with the metal-free control cultures (data not shown).

Since the possibility existed that the observed reduction in TNFα levels might have been a result of PbO-dependent changes in the total numbers of viable adherent cells, experiments were performed to address this question. Using plates containing equal numbers of reseeded viable cells, TNFα production in PbO-treated PAM was consistently lower than that from control

cells even at the earlier timepoints (3 and 24 h; Fig. 4A), with the magnitude of inhibition at the 3-h time point being greatly enhanced by reseeding. While a comparison of the levels of TNFα recovered from stimulated control PAM indicated little effect from the trypsinization/reseeding steps (Fig. 4B), PbO-related decreases in PAM adherence (DeVries et al., 1983) may have unexpectedly resulted in an enrichment of viable PbO-damaged PAM (during the mild trypsinization) for use in the reseeding experiments. In this case, the effects upon TNFα production would be amplified at the earliest time points, but would become less obvious over time as overt toxicity from the PbO eventually overwhelms the cultures.

Studies were also performed to provide possible explanations for the observed effects from PbO on LPS stimulation of PAM TNFα production. To determine whether PbO produced any indirect effects on TNFα production via interference with PAM–LPS interactions, control cells were treated simultaneously with both PbO and LPS and then incubated for 24 h. The quantities of TNFα released following the simultaneous PbO–LPS treatment was 34–38% of that obtained with cells receiving LPS alone (Fig. 5). In comparison, TNFα production in PAM treated with PbO for 24 h prior to LPS stimu-

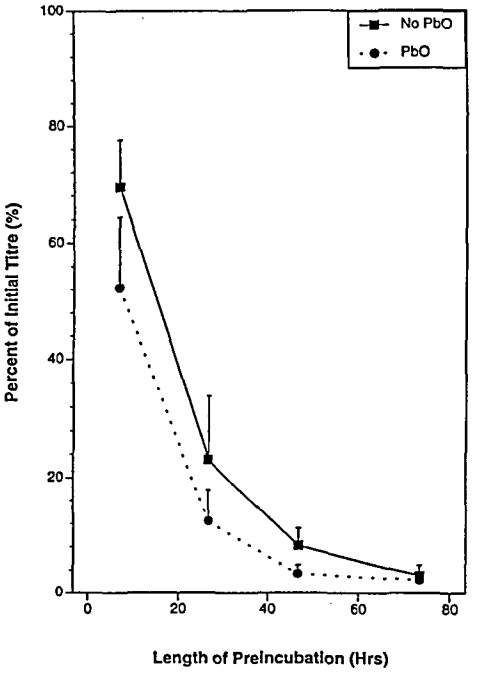

FIGURE 3. Effect of exposure duration on PAM TNFα production. All values are expressed as the percentage of the initial TNFα titers (initial titer ≈ 623 ± 58). All plates received either medium or 30 μg/ml PbO for the designated periods prior to addition of 1 μg/ml LPS as outlined in Materials and Methods. Data shown are the mean (± SE) of five separate experiments, with all samples from each time point run in triplicate.

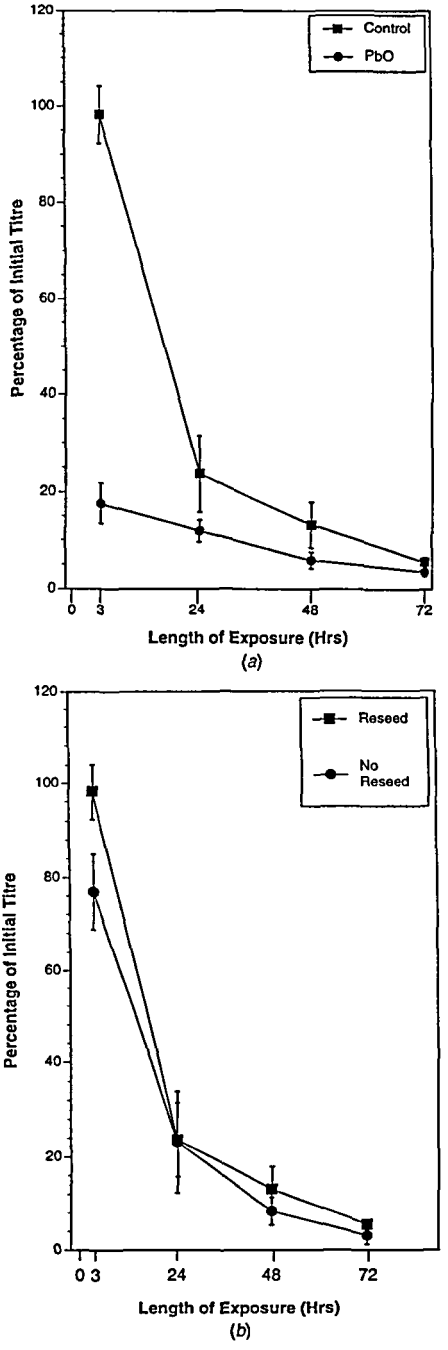

FIGURE 4. LPS-dependent TNFα release in reseeded controls and PbO-treated (30 μg/ml PbO) PAM. Cells were treated as described in Materials and Methods and either reseeded or left as is prior to 24-h treatment with 1 μg/ml LPS. All values are the mean titers (± SE) of six separate experiments with each TNFα sample analyzed in triplicate. (a) Reseeded PbO-treated and metal-free cells. (b) Effects from trypsinization and replating steps on PbO-free PAM control cells.

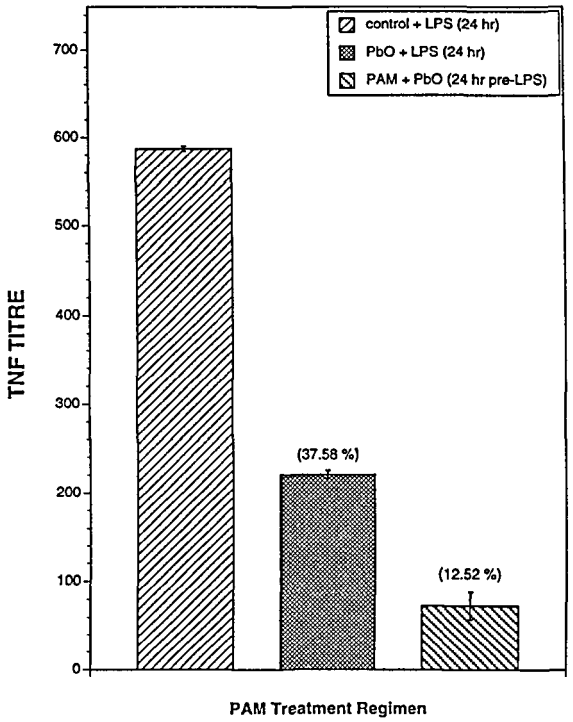

FIGURE 5. Effect of PbO on PAM–LPS interactions and subsequent TNFα production. PAM were treated with LPS alone (1 μg/ml), with LPS and 30 μg/ml PbO for 24 h, or for 24 h with PbO prior to stimulation with LPS. All values are the mean titers ± SE of three separate experiments with each sample analyzed in triplicate. Numbers in parentheses indicate the relative titer values as compared with PAM receiving LPS only.

lation was affected to a greater extent than in the cells receiving simultaneous LPS and PbO treatments (TNFα production ~12.5% of PbO-free control PAM).

To ascertain if PbO in the external culture milieu could affect TNFα activity after its release from stimulated PAM, MCM-containing TNFα was incubated with 30 μg PbO for 24 h. Any changes in TNFα activity due to extracellular PbO–TNFα interactions could give rise to inaccurate interpretations of the measured PAM TNFα production. After 24 h of co-incubation, TNFα activity was reduced to 75% of the original titer (Fig. 6). After 72 h, activity was reduced to 40% of initial levels; this was 28% lower than the activity demonstrated by PbO-free control TNFα solutions that were incubated in parallel.

DISCUSSION

The results from this study indicate that PbO may contribute to the immunosuppression of an exposed host by affecting the production and activity of the immunoregulatory monokine TNFα. In addition, the data suggest

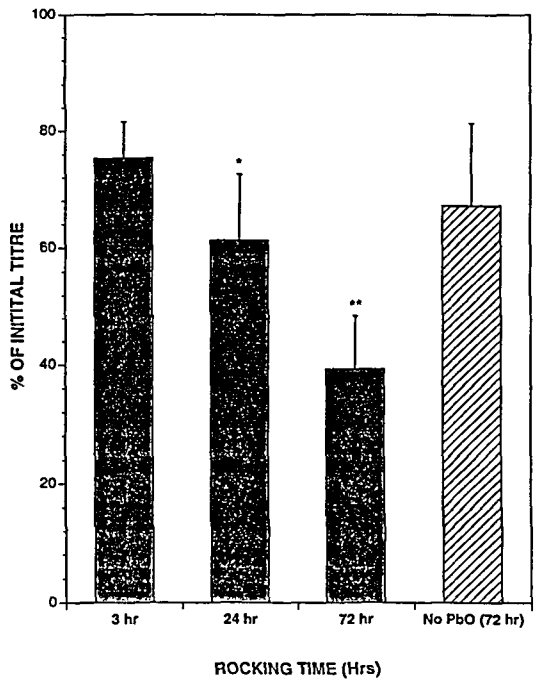

FIGURE 6. Effect of PbO on extracellular TNFα activity. The TNFα recovered from untreated PAM was treated with 30 μg/ml PbO and gently rocked at room temperature for the indicated periods. Control tubes received medium only. Duplicate control tubes immediately placed at 4°C were designated as the "initial titer" samples. Other PbO-free samples were maintained in parallel for 72 h. Each bar represents the mean titers (± SE) of at least two replicate experiments. Titers significantly (**$p < .01$ or *$p < .05$) lower than the "initial titer" values (initial titer ≈ 584 ± 126) are indicated.

that the presence of lead may alter PAM responsiveness to bacterial endotoxin (LPS) either at the initial stages of cell–LPS interaction or at subsequent stages following LPS stimulation.

In macrophages, LPS is internalized either as a free molecule or as an aggregate via endocytic pathways for eventual storage and slow detoxification in secondary lysosomes (Bona, 1973; Munford & Hall, 1986; Dijkstra et al., 1988). However, some extracellular LPS disaggregates prior to endocytosis and becomes associated with hydrophobic plasma membrane components via the LPS lipid A moiety. This binding triggers an increase in TNFα mRNA transcription and translation, resulting in the production of a 26-kD pre-TNFα molecule (Oppenheim et al., 1990). The pre-TNFα protein is excreted and anchored to the cell membrane for proteolytic processing to release the fully active 17-kD TNFα species (Kriegler et al., 1988; Scuderi, 1989; Vilcek & Lee, 1991).

Lead ions have been shown to alter both membrane lipid and membrane fluidity in viable cells (Amoruso et al., 1987; Apostoli et al., 1988). Thus, a possible explanation for the observed reduction in the amounts of TNFα

recovered from LPS-stimulated PbO-treated cells may be via interference with LPS–PAM interactions at the PAM cell surface, or due to secondary alterations in PAM endocytic/phagocytic pathways. A second possible explanation for the observed reductions in LPS-stimulated TNFα levels is that the PbO-induced alterations in PAM membrane fluidity might disturb pre-TNFα anchoring and therefore result in errors in posttranslational processing. By this mechanism, an 18-kD pseudo-TNFα possessing a similar tertiary structure but devoid of cytotoxic activity would result (Cseh & Beutler, 1989).

The data presented here indicate that treatment of PAM with PbO, either before or during LPS stimulation, results in the decreased formation of functional TNFα. Although we have postulated that decreased LPS uptake is one likely cause for the decrease in TNFα production, the apparent continuous uptake of the highest concentration of PbO particles (i.e., 100 μg/ml) over time suggests that nonspecific phagocytic activity was not greatly affected by PbO. It is possible that at the lower concentrations of PbO employed, the processes of PbO particle dissolution and phagocytic uptake might have been in equilibrium (regarding rates and magnitudes) such that the phagocytic indices appeared to remain unchanged. At the higher concentrations of PbO, in conjunction with a decreased number of viable PAM to actively solubilize ingested PbO particles, an increased number of particles is likely to be left intact within the phagosomes of the remaining viable PAM and so give rise to the appearance of increased phagocytic activity. Nevertheless, unlike the mechanisms for LPS binding and internalization, the ingestion of inert materials (such as PbO and latex particles) is believed to occur at varied points along the cell membrane and without the need for any specialized ingestion pits or receptor molecules (Vray et al., 1981). The possibility remains, therefore, that PbO particles might interact directly with, or induce secondary changes in, the 73-kD LPS receptor on the PAM surface to affect LPS stimulation primarily and TNFα production secondarily. Studies are ongoing to quantitate the magnitude of any changes in PAM binding of LPS after PbO exposure.

To elucidate which of the two postulated mechanisms (altered LPS uptake or posttranslational misprocessing of TNFα) is the more likely explanation for reduced TNFα levels observed in this study, LPS-stimulated PbO-treated PAM could be analyzed for TNFα mRNA levels. Our ongoing analyses of the PAM TNFα mRNA levels will help to clarify whether the LPS–PAM interaction (or LPS internalization and processing) is primarily affected during activation. Since lead itself is not known to significantly impair RNA synthesis in intact cells (Pounds et al., 1982; Frenkel & Middleton, 1987), any reductions in TNFα mRNA levels would most likely be due to defects in LPS-dependent stimulation events and not to effects of the metal on TNFα mRNA formation. Should the levels of TNFα mRNA remain unaffected in the PbO-treated cells, the stimulated PAM could then be evaluated to determine whether increased levels of functionally deficient TNFα molecules were being released. The results of these latter studies (currently in progress) would sug-

gest that posttranslational misprocessing is the likely mechanism for the PbO-derived defect in PAM TNFα production.

While results from this study demonstrate that PbO can impair production of functional TNFα via as yet undefined mechanisms, the results also indicate that PbO can act extracellularly to directly inhibit the functional catalytic (i.e., tumoricidal) activity of TNFα. Rabbit TNFα, like human and murine TNFα, contains two cysteine residues in each chain of the active trimer (Ito et al., 1986). The residues are often linked in an intraprotein disulfide bridge, which, although not essential to maintaining overall tertiary structure, is critical for maintaining optimal protein cytotoxic activity against tumor cells (Narachi et al., 1987; Jones et al., 1989). Based upon the well-documented affinity of lead for protein thiol groups (Passow, 1970; Moore et al., 1980; Al-Modhefer et al., 1991), it is possible that the increasing periods of PbO co-incubation with TNFα likely give rise to increased levels of Pb-TNFα complexes and fewer disulfide bridges. In addition to disturbing the crucial formation of a hydrophobic pit within the active TNFα molecule, the presence of lead among the pleated β sheets could further decrease catalytic activity by increasing overall regional hydrophilicity.

The possibility that the time-related decrease in activity of the native TNFα may have been due, in part, to its degradation over the course of the experiment was also considered. However, the nonoxidative nature (in aqueous systems) of this particular oxide of lead (R. K. Skogerboe, personal communication) would suggest that the observed decreases were more likely the result of the postulated Pb-TNFα complex formation rather than a physical removal of the native protein from the system. While enzyme-linked immunosorbent assay (ELISA) quantitation of the remaining TNFα could have provided supportive data for the foregoing hypothesis, the lack of a rabbit TNFα-specific antibody, along with the proposed changes in the TNFα antigenic epitope, would have rendered results from ELISA studies highly suspect.

The results of this study demonstrate that in vitro exposure to PbO can likely modify host PAM-derived TNFα production, possibly by affecting its induction by LPS (as shown in Fig. 5) and/or by inducing changes in the TNFα activity after its cellular export (as shown in Fig. 6). These disturbances in TNFα production and functionality after PbO exposure may explain, in part, many of the observations by ours and other laboratories regarding the in vivo immunosuppressed responses of lead-exposed hosts. Our continuing studies (i.e., quantitation of PAM TNFα mRNA and its posttranslational products, as well as analyses of PAM–LPS binding protein activity) will hopefully clarify more precisely the mechanisms by which lead alters PAM responsiveness to monokine inducers such as LPS, as well as the production of functional TNFα.

REFERENCES

Al-Modhefer, A. J., Bradbury, M. W., and Simons, T. J. 1991. Observations of the chemical nature of lead in human blood serum. *Clin. Sci.* 81:823–829.

Amoruso, M. A., Witz, G., and Goldstein, B. D. 1987. Alteration of erythrocyte membrane fluidity by heavy metal cations. *Toxicol. Ind. Health* 3:135–144.

Apostoli, P., Romeo, L., De Matteis, M. C., Menegazzi, M., Faggionato, G., and Vettore, L. 1988. Effects of lead on red blood cell membrane proteins. *Int. Arch. Occup. Environ. Health* 61:71–75.

Blakley, B. R., and Archer, D. L. 1981. The effect of lead acetate on the immune response in mice. *Toxicol. Appl. Pharmacol.* 61:18–26.

Bona, C. A. 1973. Fate of endotoxin in macrophages: Biological and ultrastructural aspects. *J. Infect. Dis.* 128:S74–S81.

Bouley, G., Dubreuil, A., Arsac, F., and Boudene, C. 1978. Effect of lead microparticles on the pulmonary defense mechanism of the mouse. *Environ. Sci.* 1:127–130.

Carwell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA* 72:3666–3670.

Cohn, Z. A. 1968. The structure and function of monocytes and macrophages. *Adv. Immunol.* 9:163–180.

Cseh, K., and Beutler, B. 1989. Alternative cleavage of the cachectin/tumor necrosis factor polypeptide results in a larger, inactive form of secreted protein. *J. Biol. Chem.* 264:16256–16260.

Dentener, M. A., Greve, J. W., Maessen, J. G., and Buurman, W. A. 1989. Role of tumor necrosis factor in the enhanced sensitivity of mice to endotoxin after exposure to lead. *Immunopharmacol. Immunotoxicol.* 11:321–334.

DeVries, C. R., Ingram, P., Walker, S. R., Linton, R. W., Gutknecht, W. F., and Shelburne, J. D. 1983. Acute toxicity of lead particulates on pulmonary alveolar macrophages—Ultrastructural and microanalytical studies. *Lab. Invest.* 48:35–44.

Dijkstra, J., Larrick, J. W., Ryan, J. L., and Szoka, F. C. 1988. Incorporation of LPS in liposomes diminishes its ability to induce tumoricidal activity and tumor necrosis factor secretion in murine macrophages. *J. Leukocyte Biol.* 43:436–444.

Frenkel, G. D., and Middleton, C. 1987. Effects of lead acetate on DNA and RNA synthesis by intact HeLa cells, isolated nuclei, and purified polymerases. *Biochem. Pharmacol.* 36:265–268.

Gainer, J. H. 1974. Lead aggravates viral disease and represses antiviral activity. *Environ. Health Perspect.* 7:113–119.

Gardner, D. E., Graham, J. A., Miller, F. J., Illing, I. W., and Coffin, D. L. 1973. Techniques for differentiating particles that are cell-associated or ingested by macrophages. *Appl. Microbiol.* 25:471–475.

Governa, M., Valentino, M., and Visona, I. 1987. *In vitro* impairment of human granulocyte functions by lead. *Arch. Toxicol.* 59:421–425.

Hemphill, F., Kaerberle, L. M., and Buck, W. B. 1971. Lead suppression of mouse resistance to *Salmonella typhimurium*. *Science* 172:1031–1033.

Hocking, W. G., and Golde, D. W. 1979. The pulmonary alveolar macrophage. *N. Engl. J. Med.* 301:580.

Ito, H., Shirai, T., Yamamoto, S., Akira, M., Kawahara, S., Todd, C. W., and Wallace, R. B. 1986. Molecular cloning of the gene encoding rabbit tumor necrosis factor. *DNA* 5:157–165.

Jian, Z., Ying-Han, X., and Hong-Fu, C. 1985. The effects of lead ion on immune function of rabbit alveolar macrophages: Quantitation of immune phagocytosis and rosette formation by ^{51}Cr *in vitro*. *Toxicol. Appl. Pharmacol.* 78:484–487.

Jones, E. Y., Stuart, D. I., and Walker, N. P. C. 1989. Structure of tumor necrosis factor. *Nature* 338:225–228.

Kaminski, E. J., Ficher, C. A., Kennedy, G. L., and Calandra, J. C. 1977. Response of pulmonary macrophages to lead. *Br. J. Exp. Pathol.* 58:9–12.

Kerkvliet, N. I., and Baecher-Steppan, L. 1982. Immunotoxicology studies on lead: Effects of exposure on tumor growth and cell-mediated tumor immunity after syngeneic or allogeneic stimulation. *Immunopharmacology* 4:213–224.

Kobayashi, N., and Okamoto, T. 1974. Effect of lead oxide on the induction of lung tumors in Syrian hamsters. *J. Natl. Cancer Inst.* 52:1605–1610.

Koller, L. D. 1990. The immunotoxic effects of lead in lead-exposed laboratory animals. *Ann. N.Y. Acad. Sci.* 587:160–167.

Kowolenko, M., Tracy, L., Mudzinski, S., and Lawrence, D. A. 1988. Effect of lead on macrophage function. *J. Leukocyte Biol.* 43:357–364.

Kriegler, M., Perez, C., DeFay, K., Albert, I., and Lu, S. D. 1988. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. *Cell* 53:45–53.

Le, J., and Vilcek, J. 1987. Tumor necrosis factor and interleukin-1: Cytokines with multiple overlapping biological activities. *Lab. Invest.* 56:234–248.

Moore, M. R., Meredith, P. A., and Goldberg, A. 1980. Lead and heme biosynthesis. In *Lead toxicity*, eds. R. L. Singhal and J. A. Thomas, pp. 80–110. *Baltimore: Urban and Schwarzenberg.*

Munford, R. S., and Hall, C. L. 1986. Detoxification of bacterial lipopolysaccharides (endotoxins) by a human neutrophil enzyme. *Science* 234:203–205.

Mustafa, M. G., Cross, C. E., Munn, R. J., and Hardie, J. A. 1971. Effects of divalent metal ions on alveolar macrophage adenosine triphosphatase activity. *J. Lab. Med.* 77:563–571.

Narachi, M. A., Davis, J. M., Hsu, Y. R., and Arakawa, T. 1987. Role of single disulfide in recombinant human tumor necrosis factor-a. *J. Biol. Chem.* 262:13107–13110.

Nathan, C. F. 1987. Secretory products of macrophages. *J. Clin. Invest.* 79:319–326.

Oppenheim, J. J., Dubois, C. M., Ruscetti, F. W., and Keller, J. R. 1990. Regulation of the production and receptors for interleukin-1 and tumor necrosis factor. *Prog. Clin. Biol. Res.* 349:183–194.

Passow, H. 1970. The red blood cell; Penetration, distribution, and toxic action of heavy metals. In *Effects of metals on cells, subcellular elements, and macromolecules,* eds. J. Maniloff, J. R. Coleman, and M. W. Miller, pp. 291–305. Springfield, Ill.: Charles C. Thomas.

Pounds, J. G., Morrison, D., Wright, R., Casciano, D. A., and Shaddock, J. G. 1982. Effect of lead on calcium-mediated cell function in the isolated rat hepatocyte. *Toxicol. Appl. Pharmacol.* 63:402–408.

Scuderi, P. 1989. Suppression of human leukocyte tumor necrosis factor secretion by the serine protease inhibitor *p*-toluenesulfonyl-L-arginine methyl ester (TAME). *J. Immunol.* 143:168–173.

U.S. Environmental Protection Agency. 1986. Air Criteria Quality for Lead, Environmental Criteria and Assessment Office. EPA-600/8-83/028dF (Vol. II of IV). June.

Vilcek, J., and Lee, T. H. 1991. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J. Biol. Chem.* 266:7313–7316.

Vray, B., Sint-Guillan, M., Reloup, R., and Hoebeke, J. 1981. Kinetic and morphologic studies of rat macrophage phagocytosis. *J. Reticuloendothel. Soc.* 29:307–319.

Zelikoff, J. T., Thomas, D. T., Yang, Z. J., and Schlesinger, R. B. 1991. Inhalation of lead oxide perturbs pulmonary macrophage functional and biochemical activities. *Toxicologist* 11:83.

Zelikoff, J. T., Parsons, E., and Schlesinger, R. B. 1993. Inhalation of particulate lead oxide disrupts pulmonary macrophage-mediated functions important for host defense and tumor surveillance in the lung. *Environ. Res.* 62:207–222.

Zhou, J., Xu, Y. H., and Chang, H. F. 1985. The effects of lead ion on immune function of rabbit alveolar macrophages. Quantitation of immune phagocytosis and rosette formation by chromium-51 *in vitro*. *Toxicol. Appl. Pharmacol.* 78:484–487.