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Immunotoxicity of sulfuric acid aerosol: effects on pulmonary macrophage effector and functional activities critical for maintaining host resistance against infectious diseases ☆

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Abstract

Despite the widespread occurrence of acidic sulfur oxides in the ambient environment and their potential risks to human health, effects associated with pulmonary immune defenses have been poorly studied. The current in vivo study was designed to provide some insight into this relatively unexplored area by investigating the impact of inhaled sulfuric acid on immune defense mechanisms critical for maintaining pulmonary resistance against infectious diseases. Results of this study demonstrate that repeated inhalation of sulfuric acid reduces the uptake and intracellular killing of pathogenic bacteria by exposed pulmonary macrophages, and depresses the activity/production of important biological modifiers critical for maintaining pulmonary immunocompetence. These findings have important implications for human health, and may contribute to a better understanding of the possible mechanism(s) underlying the epidemiological evidence which suggests an association between total sulfates in the ambient air and increased incidence of acute bronchitis and lower respiratory illness in school-age children.

Keywords: Macrophage; Sulfuric acid; Immunosuppression; Pulmonary defense

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1. Introduction

There is increasing concern about the human health effects associated with the inhalation of ambient acid aerosols, which are produced directly from fossil fuel combustion and indirectly from the catalytic or photochemical oxidation of sulfur dioxide (Amdur et al., 1986). Studies have demonstrated that sulfuric acid (H_2SO_4): is a potent irritant to both the upper airways and deep lung (Amdur et al., 1986; Folinsbee, 1989), alters airway responsiveness (Gearhart and Schlesinger, 1986; Raizenne et al., 1989), decreases pulmonary function (particularly in young asthmatics) (Koenig et al., 1989; Raizenne et al., 1989), causes cell damage leading to alveolitis, bronchiolar epithelial desquamation, and edema (Cavender et al., 1977), and produces a persistent hypertrophy and hyperplasia of epithelial secretory cells in the tracheobronchial tree (Gearhart and Schlesinger, 1989). In addition, occupational exposure to H_2SO_4 appears to contribute to the development of upper respiratory tract cancer (Soskolne et al., 1984; Beaumont et al., 1987; Soskoline et al., 1992).

The pulmonary immune system is one of many potential targets of inhaled pollutants. Numerous antigens are present in the air and the possibility exists that inhaled pollutants may modulate non-specific and/or specific immunological reactions in the lung and, thereby, produce altered responses to subsequently inhaled infectious agents. In some cases, inhaled pollutants produce a decrease in immune function, which may lead to a partial or complete compromise of host defenses. Alternatively, inhaled contaminants may enhance immune activity, resulting in an augmentation of the antigenic response and, ultimately, to hypersensitivity.

While the data is somewhat limited, H_2SO_4 exposure, either alone or in combination with other air pollutants, has been shown in experimental animal studies to interfere with non-immunological (Naumann and Schlesinger, 1986; Schlesinger and Gearhart, 1986; Holma, 1989), as well as immunological defense mechanisms in the lung (Fairchild et al., 1975; Gardner et al., 1977; Pinto et al., 1979; Fenters et al., 1979; Zelikoff and Schlesinger, 1992). Although the results are inconclusive, epidemiological studies support these toxicological findings. Studies in human subjects suggest a correlation between total ambient sulfate concentration (often a surrogate for acid sulfates) and increased incidence of cough, bronchitis, and lower respiratory tract illness in school-age children (Ware et al., 1986; Speizer, 1989).

Pulmonary macrophages (PM), the main cellular defense against pathogenic organisms depositing in the lungs, play a key role in specific immunological defense mechanisms. As such, they are involved in the effector phase of immunity as inflammatory, microbicidal, and tumoricidal cells, as well as secretory and regulatory cells that can either enhance or suppress various immune reactions (Adams and Marino, 1984). As secretory cells, macrophages produce and release a number of diverse products which can participate in both non-specific and specific lung defenses. These factors include cytokines (i.e., interleukins and tumor necrosis factor) and reactive oxygen intermediates (ROI). Alterations of any of these membrane-related macrophage activities could potentially offset the balance necessary for immunoregulation in the lung and, thus, produce a cascade of detrimental secondary events, including compromised host resistance or local tissue damage.

While relatively little is known concerning the effects of acid aerosols on overall humoral- and cell-mediated immunity (Pinto et al., 1979; Frampton et al., 1992), effects on PM function have been investigated in greater detail (Naumann and Schlesinger, 1986; Schlesinger, 1987; Schlesinger et al., 1990, 1992; Frampton et al., 1992; Zelikoff and Schlesinger, 1992). For example, a single inhalation exposure of rabbits to environmentally-relevant (and higher) concentrations of H_2SO_4 depressed the biological activity of lipopolysaccharide-stimulated tumor necrosis factor-α (TNFα) and reduced the ability of PM to produce superoxide anion radical (O_2^-) in response to opsonized zymosan (Zelikoff and Schlesinger, 1992). Inhalation exposure to H_2SO_4 also altered PM activity in human volunteers. Macrophages recovered by bronchoalveolar lavage (BAL) from human subjects exposed to H_2SO_4 at 1 mg/m^3 for 2 h, demonstrated increased antibody dependent cytotoxicity (Frampton et al., 1992).

The current study continues and expands our previous investigations which examined the effects of a single inhalation exposure of rabbits to H_2SO_4 on two PM-derived biological mediators. This study is designed to further investigate the relationship between H_2SO_4 aerosol and the pathogenesis of respiratory disease by describing the immunomodulating effects of repeated acid inhalation on pulmonary macrophage microbicidal activity, and on the production/activity of a number of biologically-active mediators critical for maintaining host resistance against infectious diseases. The macrophage parameters selected for study included phagocytosis and intracellular killing of viable bacteria, formation of ROI, F_c and C_{3b} receptor expression, and cytokine production/activity.

2. Methods

2.1. Animals and experimental design

Male (14–16 weeks; 2.5–3.5 kg) specific pathogen-free (*Pasteurella multocida*) New Zealand white rabbits (Hazelton Research Products, Denver, PA) were used for this study. Upon arrival, animals were quarantined for 2 weeks on a 12-h light/dark cycle. All animals were housed individually in stainless steel cages and provided food (Purina Rabbit Chow-HF) and water ad libitum.

Groups of five rabbits each were exposed by inhalation (nose only) to H_2SO_4 at either 500, 750 or 1000 μg/m^3 for 4 days (2 h/day). Matched controls for each acid concentration consisted of five rabbits exposed to filtered clean air. Another group of five rabbits was exposed to ammonium bisulfate (NH_4HSO_4) at 1 mg/m^3. Rabbits were then sacrificed either immediately (t_o), or 24 (t_{24}) hours after the final exposure and the pulmonary macrophage (PM) collected by bronchopulmonary lavage.

3. Materials

All tissue culture medium and serum were obtained from GIBCO (Grand Island, NY) unless otherwise indicated. Cell culture reagents and glassware were analysed (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 h) prior to use.

L-M cells (American Type Cell Culture, Rockville, MD), a subclone of a continuous transformed cell line (L-929) derived from C_3H/H_2N mice, were the target cells used to evaluate TNFα-induced cytotoxicity. Cells were maintained in 75-cm^2 tissue culture flasks by serial passage in medium 199 (M-199), supplemented with 5% bactopeptone (Difco, Detroit, MI) and 1% L-glutamine (GIBCO).

Staphylococcus aureus (Type 1, Cowan) cultures were maintained in log phase growth by serial inoculation into nutrient broth (NB), with overnight incubation at 37°C. Prior to the actual phagocytosis/killing experiments, bacterial numbers were assessed based on the extinction coefficient for the *S. aureus* cultures estimated from a spectrophotometric absorbance (540 nm).

Rabbit serum-opsonized zymosan (SOZ), used to stimulate the production of ROI, was prepared by a modification of the method described by Zelikoff et al. (1991).

3.1. Generation and characterization of sulfate aerosols

Generation and characterization of aerosols were carried out as described previously by our laboratory (Zelikoff and Schlesinger, 1992; Schlesinger et al., 1992). Briefly, submicrometer (mass median diameter = 0.3 μm; σg = 1.6) aerosols were produced by nebulization of aqueous solutions of H_2SO_4 or NH_4HSO_4. All aerosols were equilibrated at 25°C, 60% relative humidity (RH), in a mixing chamber and delivered to individual nose-only exposure ports via a manifold system.

The mass concentration of H_2SO_4 and NH_4HSO_4 was assessed by flame photometric analysis for sulfur (Meloy, Model SA-260) during each exposure and were as follows: 1002 ± 3, 749 ± 2, 502 ± 2 for H_2SO_4 and 999 ± 4 for NH_4 for NH_4HSO_4. Each value represents the mean ± S.D. of values as determined at ten points during continuous monitoring during each exposure.

3.2. Bronchopulmonary lavage and pulmonary macrophage collection

Rabbits were euthanized by injection of sodium pentobarbital (50 mg/kg body weight) into the marginal ear vein. Animals were then exsanguinated by severing the abdominal aorta, and a bilateral pneumothorax was induced by an incision in the diaphragm. Bronchopulmonary lavage was performed as described previously (Zelikoff and Schlesinger, 1992). Aliquots of the acellular lavage fluid (obtained following centrifugation) were used to evaluate lactate dehydrogenase (LDH) activity and total protein content. Cell numbers and viability were determined by hemocytometer counting and trypan blue exclusion, respectively. Briefly, lungs were lavaged six times with a calcium-magnesium-free phosphate-buffered saline solution (PBS (pH 7.2)) at a volume equal to 70% of total lung capacity; approximately 80% of the total instilled lavage fluid was recovered. Aliquots of the counted cell suspension were taken for differential counts to assess the percentage of PM and other immune cell types in the recovered cell population and for identification of macrophages by nonspecific esterase staining. The remaining cells were respun at 400 × *g* for 10 min and resuspended in the appropriate medium for carrying out the individual in vitro bioassays.

3.3. Measurements of total protein and LDH 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 using a 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. LDH activity was measured colorimetrically (460 nm) from freshly-collected lavage samples using commercially-available kits (Sigma), and the activity expressed as Berger-Broida units per milliliter (B-B units/ml).

3.4. Uptake and intracellular killing of Staphylococcus aureus

The uptake and killing of a viable bacterial culture (*S. aureus*) by harvested PM was performed using a modified technique of Cohen et al. (1989). Briefly, an overnight culture of *S. aureus* was combined with Eagles minimum essential medium with Earle's salts (EMEM-E) (containing 10% heat-inactivated autologous rabbit serum) and the mixture incubated for 20 min (at 37°C). An equal volume of the bacterial suspension (1×10^7 cells) was then combined with a sample of freshly suspended PM (5×10^6), and the mixture reincubated for an additional 20 min. After incubation, the bacterial/PM suspension was centrifuged ($750 \times g$ for 5 min at 4°C) to pellet only the PM, and the supernatant containing free *S. aureus* removed. The cell pellets were washed twice with serum-free EMEM-E, and resuspended in rabbit serum-containing medium.

To assess bacterial phagocytosis, aliquots of the macrophage suspension were transferred to individual tubes containing EMEM-E, and held on ice to halt intracellular killing. The PM were lysed by three successive freeze-thaw cycles in dry ice:acetone and the final solution serially-diluted and plated (in triplicate) onto nutrient agar plates for overnight incubation. Cell-free control tubes were similarly subjected to repeated freeze-thaw to determine effects on bacterial viability. Phagocytic uptake was expressed as the number of bacteria recovered from the PM after 20 min compared to the total number of bacteria found in the PM-free controls. While phagocytic uptake was being assessed, the remaining parental cell/bacteria suspension was incubated for an additional 45 min. The change in bacterial numbers over 45 min, as a percentage of the initial (t_0) bacterial burdens, was used as the index of intracellular killing.

3.5. Modulation of receptors

To assess the distribution of F_c receptors, PM were resuspended at 2×10^6 cells/ml in EMEM-E. An aliquot was transferred to each of four centrifuge tubes and the cells spun at $1000 \times g$ for 5 min at 4°C. The supernatants were removed and 0.25 ml of a 1:2.5 dilution of commercially-available fluorescein isothiocyanate (FITC)-labelled rabbit anti-goat IgG antibody was added to two of the pellets. The third tube received sorter buffer (0.2% bovine serum albumin, 0.1% NaN_3 in

Dulbeco's PBS) and served as the negative control. A second control (FITC-labelled $F(ab)'_2$-rabbit IgG fragment) was added to the fourth pellet to assess non-specific immunoglobulin binding. The contents of each tube were mixed and the samples incubated with constant inversion for 30 min at 4°C. After incubation, the cells were repelleted and then cleared of unbound antibody by repeated washes with sorter buffer. The final pellet was fixed in 2% paraformaldehyde and placed in the dark until measured by FACS analysis (EPIC Model 751, Coulter Electronics, Hialea, FL).

Assessment of EAC rosette formation, the assay used to quantitate C_{3b} receptors, was performed using a modification of the method described by Cohen et al. (1986) for mouse macrophages. Briefly, sheep red blood cells (10% SRBC, Cappell Labs, Malvern, PA) were washed twice in 0.1 M PBS and resuspended in PBS to yield a 5% solution. Heat-inactivated (56°C, 30 min) rabbit anti-sheep red blood cell serum (Cappell Laboratories) was diluted with PBS to yield a 1:40 final concentration of the antiserum. Sheep red blood cells (E) were sensitized with an equal volume of rabbit anti-SRBC serum (A) for 30 min in a 37°C water bath. The EA complex was then sensitized with an equal volume of a 1:10 dilution (in PBS) of autologous rabbit complement (C) for 30 min at 37°C. After centrifugation and two washes in cold veronal buffered saline (VBS), the EAC complex was resuspended in cold Hank's balanced salt solution (HBSS) at eight times the initial volume of SRBC. One hundred thousand PM were then incubated with the EAC complex (at 37°C for 5 min). Background populations (E and EA) were prepared in parallel using either SRBC or SRBC-antibody solutions, respectively. After centrifugation (200 × *g* for 5 min), the cells were incubated on ice for 2 h and an aliquot of cells stained with 0.5 ml crystal violet solution (0.025% (w/v)). Two hundred PM were examined, and the percentage of positive rosettes recorded. Only stained cells with three or more bound RBC were counted as EAC rosettes; true EAC rosette frequencies were calculated after adjusting for background levels of E and EA rosette formation.

3.6. Production of reactive oxygen intermediates (ROI)

Hydrogen peroxide (H_2O_2) production by resting and zymosan-stimulated rabbit PM was assessed using a microassay system as previously described (Zelikoff et al., 1993) and based upon the horseradish peroxidase (HRPO)-dependent oxidation of phenol red (PR) by H_2O_2. The quantity of H_2O_2 produced was determined spectrophotometrically (at 600 nm) using an automated microtiter platereader (Anthos, Lab Tek Instruments, Durham, NC). Superoxide anion radical (O_2^-) production was also assessed using a microassay method (Zelikoff et al., 1993) and based upon the reduction of ferricytochrome *c*. All results were expressed as nmol ROI/3 × 10^5 cells. Values were corrected for cell attachment (which was based upon the number of detached cells and upon protein determination).

3.7. Cytokine production/activity by rabbit macrophages

Tumor necrosis factor-α (TNFα). Recovered PM were plated at 2 × 10^6 cells/35-mm dish in unsupplemented EMEM-E. Following a 2 h incubation at 37°C (5% CO_2), PM were washed, nonadherent cells counted, and fresh medium, with and without 1 μg/ml lipopolysaccharide (LPS; phenol-extracted *Eschericia coli*, serotype 011:B; Sigma) was added. In all cases, the percentage of attached cells (after washing) was ≥98%; there appeared to be no difference in attachment/adherence between cells from acid- and air-exposed rabbits. After 24 h, EMEM-E 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 PM-conditioned medium (MCM) used for evaluation of TNFα-induced cytotoxicity and for interleukin-1α (IL-1α) measurements.

Tumor necrosis factor-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).

Interleukin-1α. A standard comitogenesis assay using phytohemagglutinin-stimulated C_3H mouse thymocytes, as described previously by our laboratory (Cohen et al., 1993), was employed to assess IL-1α release/activity by the acid- and air-exposed rabbit PM. Briefly, freshly harvested C_3H mouse thymocytes were pelleted and resuspended in complete RPMI-1640 (containing 10% fetal bovine serum) supplemented with 25 μM 2-mercaptoethanol. Cells were then counted and the final concentration adjusted to 2×10^7 cells/ml in complete RPMI-1640.

The IL-1α samples were serially-diluted until a final titer of 1:64 was reached. Thymocytes (50 μl:1 $\times$ 10^6 cells/well) and phytohemagglutinin mitogen (PHA, *Phaseolus vulgaris*, 25 μl, 1 μg/ml) were added to each well, the contents mixed, and the plates incubated at 37°C (5% CO_2). After 66 h, 1 μC [^{3}H]thymidine (20 μl) was added to each well, and the plates incubated for an additional 6 h. Following incubation, the cellular DNA contents from each well was recovered on glass fiber filters using a cell harvester (Cambridge Instruments Inc., Cambridge, MA). Control wells received thymocytes or thymocytes plus PHA to monitor spontaneous proliferation, or complete RPMI-1640 medium with and without radiolabel, to monitor background counts. Incorporated label was counted using a Beckman LS9800-spectrophotometer (Beckman Instruments Inc., Irvine, CA). Results were expressed as counts/min rather than IL-1α units/ml because of the lack of purified rabbit IL-1α commercially available to generate a standard curve.

3.8. Statistical analysis

Analysis of results from all six control groups (one each per dose and exposure time) indicated no significant differences between macrophage functional/biochemical/effector 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 analyses used the air-exposed controls associated with each dose and post-exposure time group.

Differences in the-functional/biochemical activities between macrophages collected from control, NH_4HSO_4,- and H_2SO_4-exposed rabbits were assessed using two-way (treatment, time) ANOVA followed, when appropriate, by the Dunnett test. Statistical significance was accepted at $P < 0.05$.

4. Results

4.1. *Effects of NH_4HSO_4 on macrophage activity*

Inhalation of NH_4HSO_4 had no effects (compared to air-exposed controls) on macrophage number and viability nor on any macrophage functional/biochemical/effector activity examined.

4.2. *Characterization of lavaged cells and effects of H_2SO_4 on total protein and LDH activity in the lavage fluid*

The total number, viability, and purity of pulmonary lavage preparations, as well as H_2SO_4-induced effects on LDH activity and on total protein content (general markers of lung cell damage) in the lavage fluid, are shown in Table 1. The only immune cell type other than PM observed in the recovered cell population were polymorphonuclear leukocytes (PMN). None of the H_2SO_4 exposures produced infiltration of PMN into the lavage fluid (a hallmark of inflammation), nor affected the total number or viability of the recovered cells. Macrophage viability (as measured by trypan blue) was consistently $\geq 96\%$. In addition, exposure of rabbits to 1 mg acid/m^3 significantly increased LDH activity and total protein levels by 40 and 23%, respectively, as assessed 24 h after the final exposure; no effects were observed at the two lower acid concentrations (750 and 500 $\mu g/m^3$).

4.3. *Effects of H_2SO_4 on bacterial uptake and intracellular killing*

While no effects were observed immediately following exposure, uptake and intracellular killing of rabbit serum opsonized *Staphylococcus aureus* by PM was significantly reduced by H_2SO_4 24 h after the final exposure (Fig. 1). Inhalation of acid at 1000 and 750 $\mu g/m^3$ significantly reduced intracellular killing of opsonized bacteria by PM by 37 and 60% (presented as percent of control), respectively, and once a critical exposure level of acid (750 $\mu g/m^3$) was reached no further effects were observed. Bacterial uptake was significantly depressed (compared to the air control) (49%) 24 h following exposure only to the highest acid concentration (1 mg/m^3). Actual values for killing, calculated as the change in bacterial numbers after 45 min relative to initial bacterial burdens, showed that macrophages collected after 24 h from air-exposed rabbits decreased the number of ingested intracellular bacteria by 54%, while PM recovered from rabbits exposed to 1000 and 750 $\mu g/m^3$ reduced intracellular bacterial numbers by only 34 and 21%, respectively (data not shown). Phagocytic uptake, calculated as the number of bacteria recovered after 20 min from PM relative to the total number of bacteria observed in PM-free controls, was 47% in the control macrophages, but only 24% in macrophages collected 24 h following exposure to 1 mg H_2SO_4/m^3 (data not shown).

4.4. *Effects of H_2SO_4 on H_2O_2 and O_2^- production*

Resting, unstimulated rabbit PM produced 1 nmol $H_2O_2/3 \times 10^5$ cells in 60 min, which increased ~2-fold (1.0 vs. 1.9 nmol) following in vitro stimulation with SOZ. Exposure to H_2SO_4 had no effect on either spontaneous or zymosan stimulated production of H_2O_2 by rabbit PM (data not shown). However, exposure to H_2SO_4

Table 1
Effects of H_2SO_4 on recoverable cell populations and lavage fluid

Treatment group	Time after exposure (h)	Concentration of H_2SO_4 ($\mu g/m^3$)	Total number viable PM/kg BW[a] ($\times 10^6$)	PM viability (%)[a]	% PMN[a,b]	LDH (B-B units/ml)[a]	Protein (μg/ml)[a]
Air Control[c]	—	—	8.19 (±0.5)	95 (±0.4)	2.5 (±0.2)	39 (±4)	18 (±0.1)
H_2SO_4	0	1000	8.10 (±0.7)	96 (±1.0)	2.6 (±0.4)	40 (±5)	16 (±0.2)
	24		7.20 (±0.9)	97 (±0.8)	2.4 (±0.6)	61 (±5)*	23 (±1.0)
H_2SO_4	0	750	6.66 (±1.6)	96 (±1.0)	2.1 (±0.5)	37 (±3)	15 (±0.2)
	24		6.49 (±0.6)	98 (±0.8)	2.0 (±0.1)	35 (±6)	16 (±0.3)
H_2SO_4	0	500	7.89 (±0.6)	98 (±0.4)	2.9 (±0.4)	42 (±2)	19 (±0.2)
	24		7.26 (±0.6)	99 (±0.1)	1.8 (±0.3)	34 (±7)	20 (±0.2)

*$P < 0.05$
[a]Mean ± S.E.
[b]Percentage of polymorphonuclear leukocytes in recovered cell population.
[c]Results for air-exposed sham control animals at two sacrifice times and three doses were pooled.

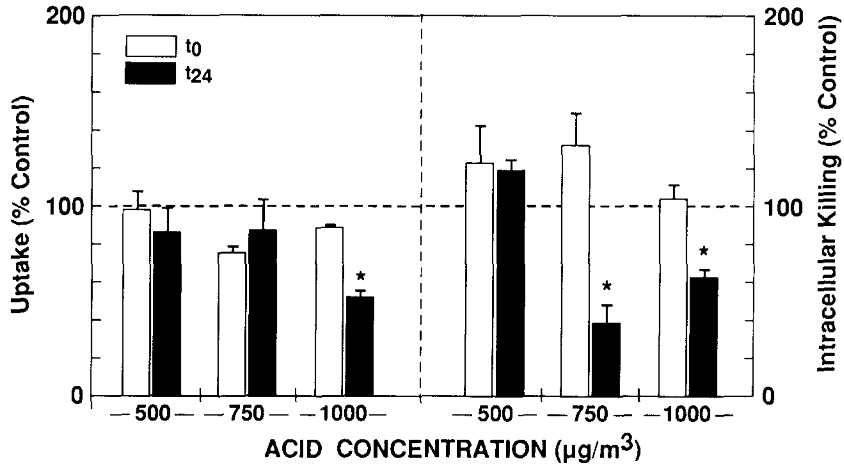

Fig. 1. Effects of inhaled H_2SO_4 on bacterial uptake and intracellular killing of *Staphylococcus aureus* by rabbit pulmonary macrophages. Values represent the mean (± S.E.) for five rabbits in each of the exposed groups and 30 rabbits for the pooled air control group. Inhalation of H_2SO_4 at 1 mg/m^3 significantly ($P < 0.05$) reduced bacterial uptake 24 h post-exposure compared to the air-exposed control. Phagocytic uptake, calculated as the number of bacteria phagocytosed after 20 min compared to the total number of bacteria measured in PM-free controls, is presented as a percent of control. H_2SO_4-exposure at 1000 and 750 μg/m^3 significantly ($P < 0.05$) reduced intracellular killing of *S. aureus* 24 h following the final acid exposure. Intracellular killing, calculated as the percent change in the number of intracellular microorganisms after 45 min relative to initial cell burdens, is also presented as percent of control.

significantly altered the production of O_2^- (Fig. 2). Spontaneous O_2^- production was reduced by 97% immediately following acid exposure at 750 μg/m^3, while H_2SO_4 at both 1000 and 750 μg/m^3 depressed spontaneous production by approximately 78% 24 h after the final acid exposure (3.1 vs. 0.07 nmol O_2^-). In contrast to the depression observed at these higher acid concentrations, exposure to H_2SO_4 at 500 μg/m^3 increased spontaneous production of O_2^- ~3-fold (0.3 vs. 0.78 nmol/90 min) 24 h after acid exposure. On the other hand, no significant effects from any of the acid exposures were observed on zymosan-stimulated O_2^- production (pooled SOZ air control = 1.9 ± 0.2 nmol/3 × 10^5 cells/90 min).

4.5. *Effects of H_2SO_4 on macrophage membrane receptors*

Although the distribution of membrane-bound F_c receptors (reported as molecules of equivalent soluble fluorochrome units (MESF × 10^4)) on the macrophage surface was slightly reduced following exposure to H_2SO_4 at 1 mg/m^3 (pooled air control = 25 MESF vs. 21 MESF), the results obtained for F_c and C_{3b} receptors failed to reach statistical significance ($P < 0.06$).

4.6. *Effects of H_2SO_4 on cytokine production/activity*

Rabbit PM are capable of producing TNFα and IL-1α spontaneously, although

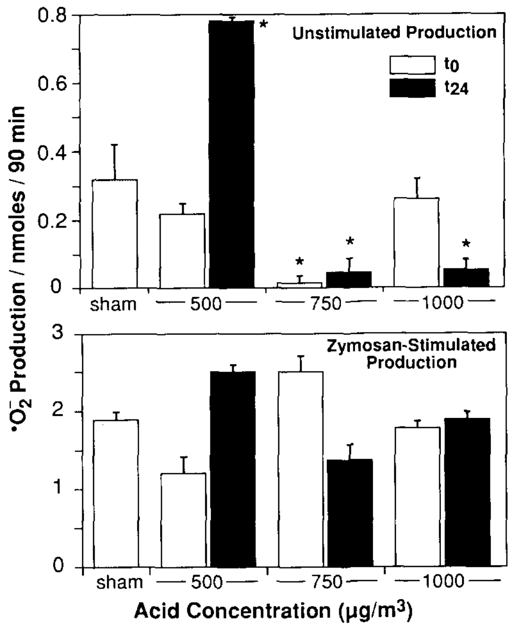

Fig. 2. Effects of inhaled H_2SO_4 on unstimulated and zymosan-stimulated O_2^- (nmol/90 min) production by rabbit pulmonary macrophages. Values represent the mean (± S.E.) for five rabbits in each of the exposed groups and 30 rabbits for the pooled air control group. While no effects on zymosan-stimulated production of O_2^- was observed, inhalation of H_2SO_4 at 1000 and 750 $\mu g/m^3$ significantly ($P < 0.05$) reduced spontaneous production of O_2^- 24 h following the final acid exposure; O_2^- production was also significantly ($P < 0.05$) reduced immediately following acid exposure at 750 $\mu g/m^3$. In contrast, inhalation of 500 $\mu g/m^3$ H_2SO_4 significantly ($P < 0.05$) enhanced O_2^- production 24 h post-exposure.

levels were quite low (3 units/ml and 2.5×10^2 counts/min, respectively); inhalation of H_2SO_4 had no effect on the spontaneous production of either cytokine. In vitro exposure to LPS at 1 $\mu g/ml$ proved to be a potent stimulant for TNFα and IL-1α activity by rabbit PM, increasing activity/production in cells from air exposed animals ~3000 and 10 000-fold, respectively, above constitutive levels. Exposure to acid at 1 mg/m^3 significantly reduced the biological activity of TNFα, as well as IL-1α production, compared to control levels (Fig. 3A,B). While exposure to 1 mg/m^3 H_2SO_4 depressed TNF and IL-1α activity/production at both post-exposure time points (t_0 and t_{24}), effects were greater after 24 h. Tumor necrosis factor-α was reduced by 43 and 79% immediately and 24 h following exposui to 1 mg/m^3 acid, respectively; IL-1α production was reduced by 49 and 79% at the same post-exposure time points. Exposure to acid at 750 $\mu g/m^3$ reduced TNF activity 74%, but only 24 h post-exposure; inhalation of 750 $\mu g/m^3$ acid had no significant effects on IL-1α production at either time point. At the lowest inhaled acid concentration (500 $\mu g/m^3$), both IL-1α and TNFα were somewhat elevated compared to control levels, but the differences were not statistically significant ($P > 0.05$).

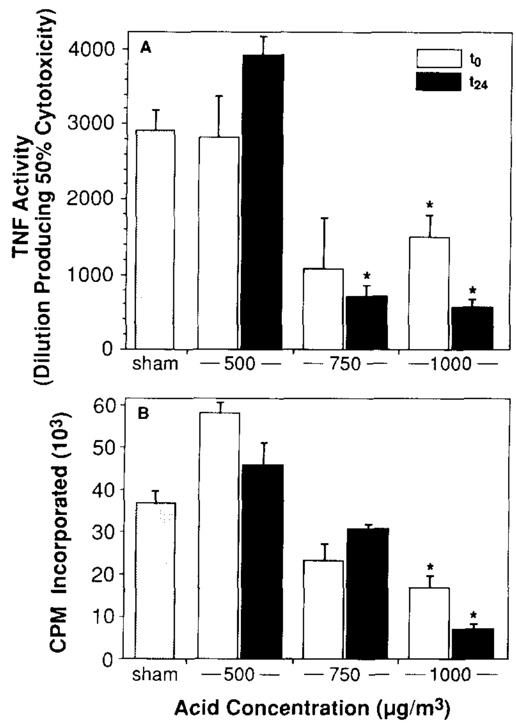

Fig. 3. Effects of inhaled H_2SO_4 on LPS-stimulated TNFα cytotoxic activity (A) and IL-1α production (B) by rabbit pulmonary macrophages. Results for TNF activity are expressed as the inverse of the dilution producing 50% cytotoxicity towards L-M tumor target cells. Results for IL-1α are presented as incorporated counts/min ($\times 10^3$). Sulfuric acid at 1 mg/m^3 significantly ($P < 0.05$) reduced TNF and IL-1α activity/production both immediately and 24 h post-exposure. Tumor necrosis factor activity was also significantly ($P < 0.05$) depressed 24 h after exposure to 750 μg H_2SO_4/m^3. Activity tended ($P < 0.06$) to be depressed immediately after exposure to 750 μg H_2SO_4/m^3, but the results were not statistically significant. Values represent the mean ($\pm$ S.E.) of five rabbits in each of the acid-exposed groups and 30 rabbits for the pooled air control group.

5. Discussion

Despite the widespread occurrence of sulfur oxides in the ambient and occupational environment (Amdur, 1986) and their potential risks to human health, except for well-documented irritant effects and acid-induced changes in lung clearance function (Schlesinger et al., 1986), other potential health effects, particularly, those associated with pulmonary immunocompetence, have not been well-characterized. In this study, exposure to H_2SO_4 suppressed macrophage-mediated immunity in the lung. Repeated acid exposures of rabbits reduced, in a time dependent, but dose-independent manner, the in vitro uptake and killing of pathogenic bacteria by recovered macrophages, as well as their ability to release cytotoxic mediators. The absence of any dose-dependency in this study is similar to that observed in our earlier in vivo investigations of acid-induced immunotoxicity in rabbit PM (Zelikoff and Schlesinger, 1992). Once a critical exposure level of acid is reached no further effects are observed. The underlying mechanism(s) responsible for this response remains to be determined.

Repeated inhalation of H_2SO_4 had no effect on neutrophil infiltration, nor produced any change in PM viability nor total numbers of lavageable cells. These findings are similar to those observed by Zelikoff and Schlesinger (1992) following a single inhalation exposure of rabbits to acid concentrations up to 500 $\mu g/m^3$, and in human subjects 18 h following a single 2 h exposure to H_2SO_4 at 1 mg/m^3 (Frampton et al., 1992). Conversely, Naumann and Schlesinger (1986) reported a marked increase in the number of neutrophils in rabbits exposed acutely to H_2SO_4 at 1 mg/m^3; neutrophil numbers began to increase at 4 h post-exposure and remained elevated for up to 24 h. Reasons for discrepancies between these studies have not been elucidated. It is possible, however, that in the current study neutrophil numbers increased transiently sometime after the initial measurement (t_0) and declined by 24 h.

The analysis of biochemical markers is a sensitive method for the evaluation of pulmonary damage and may provide 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, and LDH activity in the lavage fluid increased 24 h following exposure to 1 mg/m^3 H_2SO_4. Since no effects on LDH activity or protein levels were observed immediately after the final acid exposure, it appears that effects of inhaled acid on these endpoints may be delayed in their onset. In support of this hypothesis, Warren and Last (1987) demonstrated no change in total lavageable protein in rats exposed to acid at 1.0 mg/m^3 for 7 days and examined immediately after exposure. Furthermore, total protein levels in the BAL of human volunteers were also slightly increased (although not significantly) a number of hours (18 h) after a single 2 h H_2SO_4 exposure. The results of the aforementioned studies suggest that the observed change in lavageable protein is an adaptive response of the lungs that occurs once acid exposure is terminated, but not during the actual course of exposure.

Uptake and intracellular killing of infectious pathogenic organisms are critical effector functions carried out by alveolar macrophages in order to maintain sterility of the respiratory tract (Peterson et al., 1977; Nibbering et al., 1989). In the current study, repeated inhalation of H_2SO_4 depressed both the uptake and intracellular killing of ingested *S. aureus* in vitro by rabbit PM. These results support those of Schlesinger (1987), who showed that repeated inhalation exposure to H_2SO_4 (at 500 $\mu g/m^3$) reduced the phagocytic uptake of opsonized latex particles by rabbit PM. In vitro exposure to H_2SO_4 also reduced the phagocytic uptake and capacity of PM from this same species (Schlesinger et al., 1992). Similar effects on phagocytosis have also been observed in other animal species. Chen et al. (1992) demonstrated a decrease in macrophage phagocytic activity 24 h following a 4-day inhalation exposure of guinea pigs to ultrafine (0.03 μm diameter) H_2SO_4 at 300 $\mu g/m^3$. These investigators concluded that acid aerosols could produce prolonged alterations in phagocytosis. Since a decrease in intracellular pH (pH_i) has been shown to reduce surface ruffling, a marker of macrophage activation and phagocytic activity (Warheit et al., 1984), it is possible that inhalation of H_2SO_4 may have acted to reduce PM phagocytosis by reducing pH_i and, thus, interfere with maintenance of the activated state.

The first stage in the intracellular killing of most bacteria is attachment of the organism to the phagocyte surface (Peterson et al., 1977; Nibbering et al., 1989). This is an important interaction which may determine whether subsequent uptake occurs and whether killing mechanisms are triggered. Since binding of infectious agents to the phagocyte can be mediated by opsonin receptors (i.e., complement (C_{3b}) and antibody $F_{c\gamma}$) on the surface of the macrophage (Chandler et al., 1986), H_2SO_4-induced changes in F_c and C_{3b} receptor expression were examined to help explain the observed decrease in bacterial uptake by rabbit PM 24 h after the final acid exposure. Interestingly, no changes in either F_c or C_{3b} (with respect to control) were observed under the conditions used in this study. The lack of measurable changes in these parameters are not necessarily due to a lack of effect on these endpoints. While the results demonstrate that inhalation of H_2SO_4 had no effects on the distribution of F_c and/or C_{3b} macrophage receptors, reduction in the numbers of 'accessible' receptors per cell, or in receptor avidity/reactivity (which was not directly measured in this study), may still account, in part, for the decreases in bacterial uptake. Alternatively, since macrophages also recognize and clear bacteria via nonimmune receptor mechanisms on the macrophage surface (i.e., lectins), it is possible that alterations in carbohydrate receptors may have accounted for the observed decreases in bacterial uptake (Chandler et al., 1986).

Following microbe internalization, bacterial-containing phagosomes fuse with lysosomes to form phagolysosomes, and various killing mechanisms are activated. Macrophages possess a number of oxygen-dependent and independent antimicrobial mechanisms for pathogen destruction. Oxygen-dependent microbicidal activity is dependent upon the production of toxic mediators such as H_2O_2 and O_2^- released into the intracellular phagolysosome (Adams and Hamilton, 1984). Given their potent microbicidal activity, a reduction of either of these oxygen metabolites may compromise host resistance and, thus, increase the incidence of respiratory infections. In the current study, spontaneous production of O_2^- by rabbit PM was significantly depressed at all but the lowest acid concentration; exposure to acid at 500 $\mu g/m^3$ enhanced O_2^- production 24 h following the final exposure. Exposure to H_2SO_4 has been shown to significantly reduce pulmonary macrophage pH_i (Chen et al., 1992) which can influence membrane permeability, ion transport, intracellular calcium, and protein synthesis (Nuccitelli and Deamer, 1982). In this same study, changes in pH_i were shown to persist for up to 24 h following exposure to ultrafine (mass median diameter, 0.03 μm) acid aerosols. Since membrane permeability is reduced by a drop in pH_i, molecules necessary for the respiratory burst may have had limited access into the cell, thus reducing the ultimate generation of O_2^- as observed in the present study. The findings by Chen et al. (1992) concerning prolonged acid-induced reductions in pH_i support this hypothesis. The mechanism(s) by which H_2SO_4 may have acted to depress unstimulated O_2^- production by PM without affecting zymosan-stimulated production or producing a change in H_2O_2 formation is, however, somewhat confusing. The lack of effects observed on stimulated O_2^- production and on H_2O_2 production might lead to the conclusion that alterations in ROI production were not responsible for the decrease in microbicidal activity observed in this study.

Tumor necrosis factor-α and IL-1α, proinflammatory mediators produced and released in large quantities by activated macrophages (Adams and Marino, 1984), mediate a variety of biological responses associated with host defense and non-immunologically-related parameters (Vilcek and Lee, 1991). Cytokines in the lung provide the intercellular communication signals between the various inflammatory cells, such as PMN, PM, and epithelial cells, thereby regulating the degree of inflammation and the bactericidal capacity of the pulmonary defense system (Sharma et al., 1992). In the current study, inhalation of acid significantly depressed both LPS-stimulated TNFα and IL-1α activity/production. The effects on TNFα are similar to those observed following a single inhalation exposure of rabbits to acid concentrations ranging between 75 and 500 $\mu g/m^3$ (Zelikoff and Schlesinger, 1992). However, these results conflict with those observed by Chen et al. (1992) who demonstrated that acute/repeated in vivo exposures to 300 $\mu g/m^3$ of either fine (0.3 μm) or ultrafine (0.03 μm) H_2SO_4 increased endotoxin-stimulated TNFα activity by guinea pig PM. Discrepancies between the studies may be due to a number of factors, including interspecies differences.

The actual mechanisms by which H_2SO_4 may have acted to depress LPS-stimulated TNFα and IL-1α activity by PM is not known. However, since inhalation of H_2SO_4 has been shown to reduce pH_i, and this reduction is associated with alterations in intracellular homeostasis, cell function, protein helix formation, and de novo protein synthesis, acid-induced changes in pH_i may account, at least in part, for the observed changes in TNFα activity. In addition, since membrane permeability is also reduced by a drop in pH_i a reduction in the amount of TNFα and/or IL-1α released into the external mileiu could account for the decrease in cytokine activity.

One additional point which should not be overlooked in this study concerns the differences in effects between inhaled H_2SO_4 and NH_4HSO_4, the predominant acidic particles in the atmosphere. Although it might be expected that inhalation of acid sulfates of equal mass concentrations of H^+ would result in relatively similar biological responses, the results of this study, as well as other toxicological studies from this laboratory (Schlesinger et al., 1990), have shown inhaled H_2SO_4 to be disproportionately potent compared to NH_4HSO_4. Thus, it appears that when considering the health risks associated with inhaled acidic atmospheres the species of the acid must be considered.

In summary, our study has demonstrated the immunosuppressive effects of inhaled H_2SO_4. Repeated exposure to H_2SO_4 suppresses macrophage effector/functional and biochemical activities important for maintaining pulmonary immunocompetence. Although the acid concentrations used in this study are well above those found in the ambient environment (50–75 $\mu g/m^3$), the fact that inhalation of H_2SO_4

reduces the microbicidal activity of PM is significant, given the importance of this function for protection of the host against infectious respiratory diseases. Since people with abnormalities in the numbers or functions of their phagocytes usually have an increased susceptibility to respiratory infections (Hoidal and Repine, 1979) this further demonstrates the potential health risks associated with suppressed immunocompetence.

Furthermore, the results of this study may contribute to a better understanding of the possible mechanism(s) underlying the epidemiological evidence which suggests an association between total sulfates in the ambient air and increased incidence of acute bronchitis and lower respiratory tract illness in school age children (Ware et al., 1986; Speizer, 1989). These findings have important implications for human health, and demonstrate the need for further toxicological studies, at more environmentally relevant levels, examining the health risks associated with inhaled acid aerosols.

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