

Comparative Toxicity of Ambient Air Pollutants: Some Aspects Related to Lung Defense

by Richard B. Schlesinger*

Clearance mechanisms are an integral part of pulmonary defense, serving to rid the lungs of inhaled particles that deposit upon airway surfaces. This is accomplished by mucociliary transport in conducting airways and to a large extent by alveolar macrophages in the respiratory region. This paper compares the effects of acute exposure to sulfuric acid (H_2SO_4), nitrogen dioxide (NO_2), or ozone (O_3) on mucociliary clearance in rabbits and on phagocytic activity of macrophages recovered by bronchopulmonary lavage from animals exposed *in vivo*. The possible toxicologic mechanisms underlying dysfunction of clearance mediated by these irritants is discussed in terms of response to a pure acid (H_2SO_4), a pure oxidant (O_3), and a material (NO_2) that is a direct oxidant but which may produce secondary oxidants and acids upon dissolution in lung fluids.

Introduction

The internal surfaces of the respiratory tract provide an extensive interface directly exposed to the external environment, as 10,000 to 20,000 L of air are inhaled each day. This air often contains a variety of gases and suspended particles resulting from anthropogenic activities that have the potential to produce injury upon contact with airway tissue. Although the lungs maintain a number of defenses that protect them from the adverse effects of these pollutants, many inhaled chemicals may alter the effectiveness of these defenses making the lungs more susceptible to disease (1).

One of the major functions of lung defense is the physical removal of inhaled particles that contact and deposit upon airway surfaces, a process known as clearance. By affecting residence time in the lungs, the rate and extent with which particles are removed frequently plays a role in determining the ultimate risk from exposure for the particles themselves or for other inhaled material. Both gaseous and particulate pollutants may alter clearance efficiency by affecting various aspects of the system, which differs in different parts of the lungs. In the tracheobronchial tree, clearance occurs via the movement of a mucous layer by the coordinated beating of respiratory cilia. On the other hand, the major mechanism of clearance from

the gas-exchange region of the lungs is via specialized cells, the alveolar macrophages.

This paper discusses the comparative effects of three important ambient air pollutants upon two aspects of lung defense related to clearance function, specifically, mucociliary transport, as assessed by physiological measurements of the clearance of tracer particles from the tracheobronchial tree, and a critical macrophage function, phagocytosis, as assessed by examination of cells recovered by lavage following pollutant exposure.

The specific pollutants assessed are ozone (O_3), nitrogen dioxide (NO_2), and sulfuric acid (H_2SO_4). The first two exist as gases; the last pollutant exists in the particulate phase, generally as a submicrometer aerosol. These three are of interest not only because of the extent of their occurrence but also because they presumably exert toxic action by different mechanisms. The effects of H_2SO_4 are likely due to the deposition of hydrogen ion (H^+) within the fluid lining of the lungs (2,3). Both O_3 and NO_2 are absorbed by this lining, as well as by the underlying epithelial cells, but the subsequent reaction pathways of these gases are not completely known. The toxic effects of O_3 are generally ascribed to direct oxidation of critical molecules, e.g., lipids and proteins, in the lungs (4). NO_2 should react with these same constituents, and its toxicity may be due in whole or in part to direct oxidation. On the other hand, unlike O_3 , NO_2 may produce nitric and nitrous acids when it is absorbed into lung fluids, with resultant toxicity due to this secondary production of acids (5). Furthermore, nitrite (NO_2^-), also produced by reaction in water, may result in oxidative damage (6-8).

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Methods

For the most part, methodological details are provided elsewhere and are only broadly described herein. All exposures used male New Zealand white rabbits (2.5–2.7 kg). Submicrometer [$0.3 \mu\text{m}$ mass median aerodynamic diameter (MMAD)] H_2SO_4 aerosols were generated via nebulization from dilute aqueous (0.01–0.1 N) solutions, and mass concentration was determined by turbidometric or calorimetric analyses (9). Delivery air was maintained at 24°C , 75% relative humidity (RH); exposures were via oral tube (10).

Exposures to O_3 or NO_2 were performed in 1.6 m^3 stainless-steel dynamic exposure chambers, maintained at 23°C , 60% RH (11,12). O_3 was produced by passing oxygen through an ultraviolet O_3 generator; NO_2 was generated from an NO_2 cylinder (0.5% in N_2). Concentrations were varied by diluting the gas stream with filtered room air. The chamber concentration of O_3 was continuously monitored with an ultraviolet photometer, while that for NO_2 was monitored with an NO_x chemiluminescent analyzer. Table 1 provides details of the exposure atmospheres, exposure durations, and numbers of animals used for the various tests.

Comparative Effects on Tracheobronchial Mucociliary Clearance

Tracheobronchial mucociliary clearance was assessed from external measurements of the retention of radioactively tagged tracer aerosols [$^{99\text{m}}\text{Tc}$ -labeled $4.5 \mu\text{m}$ (MMAD) ferric oxide microspheres], which were inhaled after pollutant exposure. Serial retention measurements were performed during the first 24-hr postexposure (13,14).

The results of each clearance test were quantitatively described in terms of a parameter termed mean residence time (MRT). This represents the mean time, over the measurement period, that tracer particles that initially deposited in the tracheobronchial tree reside there. MRT is derived by computer integration of the clearance curve, i.e., the retention curve corrected for residual activity remaining at 24-hr post-tracer exposure.

Alterations in mucociliary clearance due to pollutant inhalation were assessed as follows: Each rabbit serves as its own control, and a mean control value for MRT is obtained for each animal based on a series of five clearance tests performed prior to any pollutant exposure. The mean MRT for the clearance tests conducted after pollutant exposure is obtained for each rabbit, and a value of $\% \Delta\text{MRT}$, i.e., the percentage change in MRT from mean preexposure control, is determined. A group mean percentage change, i.e., $\% \Delta\text{MRT}$, is then obtained for the entire cohort for each specific exposure condition.

The statistical significance of $\% \Delta\text{MRT}$ was initially assessed using the Kruskal-Wallis test. The significance of $\% \Delta\text{MRT}$ at any specific exposure level was then compared to that for sham control exposures using a nonparametric multiple-comparison test. The level of significance chosen was $p = 0.05$.

Figure 1 shows $\% \Delta\text{MRT}$ after 2-hr exposures to H_2SO_4 , NO_2 , and O_3 ; the data for H_2SO_4 and O_3 have been previously reported (3,15). Exposure to H_2SO_4 resulted in a significant retardation of clearance at the highest level used, i.e., 1 mg/m^3 . Exposure to NO_2 at concentrations ranging from 0.58 mg/m^3 (0.3 ppm) to 18.61 mg/m^3 (10 ppm) produced no significant alteration in mucociliary clearance from the bronchial tree. On the other hand, exposures to O_3 at 0.20 to 1.22 mg/m^3 (0.1–1 ppm) produced significant retardation of bronchial clearance only at the highest level.

The results of the 2-hr exposures to H_2SO_4 should not

Table 1. Summary of exposure conditions.

| Pollutant | Inhalation technique | No. of test animals | Exposure duration, hr | Mean concentration, ^a mg/m^3 (ppm) | End point ^b | Reference |
|-------------------------|----------------------|---------------------|-----------------------|---|------------------------|-----------|
| H_2SO_4 | Oral tube | 10 | 1 | 0.98 — | M | (19) |
| | Oral tube | 5 | 2 | 0.2 — | C | (3) |
| | Oral tube | 5 | 2 | 0.3 — | C | (3) |
| | Oral tube | 5 | 2 | 0.4 — | C | (3) |
| | Oral tube | 5 | 2 | 0.5 — | C | (3) |
| | Oral tube | 5 | 2 | 1.0 — | C | (3) |
| NO_2 | Chamber | 5 | 2 | 2.14 (1.14) | M | (21) |
| | Chamber | 5 | 2 | 18.84 (10.01) | M | (21) |
| | Chamber | 5 | 2 | 0.58 (0.31) | C | |
| | Chamber | 5 | 2 | 1.94 (1.03) | C | |
| | Chamber | 5 | 2 | 5.83 (3.10) | C | |
| | Chamber | 5 | 2 | 18.61 (9.90) | C | |
| O_3 | Chamber | 5 | 2 | 0.22 (0.11) | M | (17) |
| | Chamber | 5 | 2 | 2.39 (1.22) | M | (17) |
| | Chamber | 5 | 2 | 0.20 (0.1) | C | (15) |
| | Chamber | 5 | 2 | 0.5 (0.25) | C | (15) |
| | Chamber | 5 | 2 | 1.22 (0.62) | C | (15) |

^aCoefficients of variation were $\leq 10\%$.

^bAbbreviations: C, mucociliary clearance; M, bronchopulmonary lavage for macrophages.

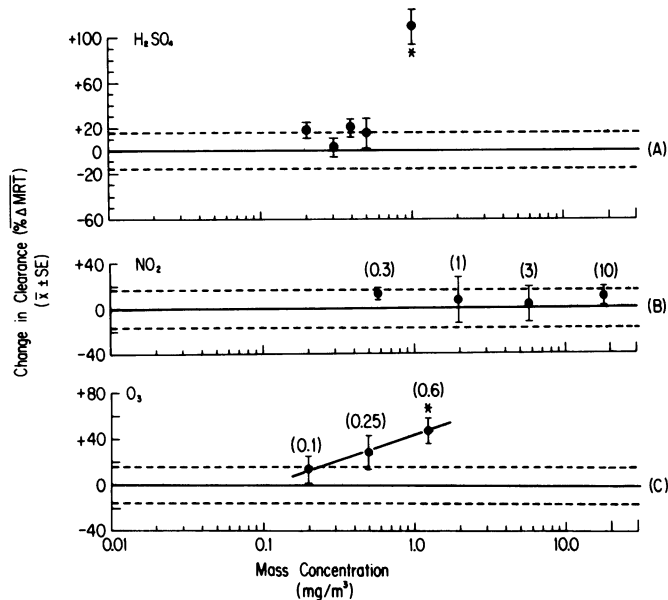


FIGURE 1. Change in mean residence time of tracer particles after exposures to H₂SO₄ (A), NO₂ (B), or O₃ (C). A negative change represents an acceleration in bronchial mucociliary clearance; a positive change represents a slowing. Values are mean \pm SE. The horizontal dotted lines represent 95% confidence limits for % Δ MRT = 0. Numbers in parentheses for O₃ and NO₂ represent concentration in ppm. Asterisk (*) denotes significant change from preexposure control at an individual concentration ($p < 0.05$). Data for H₂SO₄ from Schlesinger (3); data for O₃ modified from Schlesinger and Driscoll (15).

be interpreted as meaning that levels $< 1 \text{ mg/m}^3$ produce no effect. It was previously demonstrated with 1-hr exposures that clearance may be stimulated by low levels beyond a threshold concentration, but a maximum acceleration is eventually reached, and exposures at increasing concentrations result in a lessening acceleration, a cross-over through baseline, and then a slowing of clearance (16). It is possible that the lowest concentration used with the 2-hr exposures was not low enough to result in acceleration.

Although only the highest level of O₃ produced a significant change in clearance, the data strongly suggested a concentration-response relationship. Accordingly, regression analysis was performed for % Δ MRT versus $\log_{10} [\text{O}_3]$ (15). A significant relationship was found ($p < 0.01$, $r^2 = 0.98$), indicating a dependence of change in clearance with O₃ concentration. The threshold level of O₃ required to significantly alter clearance after a 2-hr exposure is between 0.25 and 0.6 ppm.

It is evident from Figure 1 that H₂SO₄ appears to be more potent than O₃ in altering mucociliary clearance; for example, a 2-hr exposure to 1 mg/m^3 H₂SO₄ produced a greater change than did a 2-hr exposure to the same mass concentration of O₃. On the other hand, exposure to NO₂ at up to 18.61 mg/m^3 did not alter mucociliary clearance. Thus, neither the production of secondary hydrolysis products from NO₂ nor direct oxidation effects were sufficient to change this particular end point. Therefore, it appears that O₃ impacts to a greater extent along the con-

ducting airways than does NO₂ in terms of altering mucociliary clearance, but the greatest impact in this regard is due to H₂SO₄.

Comparative Effects on Macrophage Function: Phagocytosis

Alveolar macrophages are the first "line of defense" against particles that deposit in the alveolated airways of the lungs. If the functional integrity of these cells is impaired, subsequent dysfunction of lung defense, e.g., clearance, may result. The internalization and inactivation of deposited particles via phagocytosis is an essential component of macrophage function and is, perhaps, the most important function for the adequate performance of these cells in particle clearance. Pollutant-induced changes in phagocytosis may alter the efficiency of lung defenses; this was examined in cells obtained from rabbits exposed *in vivo*.

To obtain cells, rabbits were sacrificed immediately (within 1 hr), 1 day, or 7 days after pollutant exposure by injection (IV) of sodium pentobarbital. With techniques described (17), the lungs were lavaged *in situ* for recovery of cells, which were characterized by type, viability, and *in vitro* phagocytic function. Only phagocytic function will be discussed here.

Phagocytosis by alveolar macrophages was quantitated by either of two techniques: An attached cell assay was used after H₂SO₄ exposures (18); a cell suspension assay was used after NO₂ or O₃ exposures (17). Both techniques assessed the phagocytosis of 3- μm diameter polystyrene latex microspheres. Cells were incubated with the latex for 1 hr, after which time they were fixed, and unphagocytized latex was removed by xylene treatment. Two hundred cells per slide were screened to determine the phagocytic index (PI), i.e., the number of cells containing at least one completely internalized latex particle. This parameter provided a measure of the number of viable cells engaged in phagocytosis at each sacrifice time. Values for phagocytic index were compared between pollutant-exposed and sham-control animals with Dunnett's test. The level of significance chosen was $p = 0.05$.

Figure 2 shows the changes in PI due to pollutant exposure; results for H₂SO₄ and O₃ have been reported previously (17,19) (No change in viability was observed due to exposure under any condition compared to control.) There are obvious differences between H₂SO₄, NO₂, and O₃ in their effects upon phagocytosis. No change was found due to H₂SO₄. Ozone inhalation resulted in reduced PI at both concentrations, but this reduction was prolonged after the high level exposure. A depression in phagocytosis may be the result of O₃ reacting directly with the cells or from products produced in the lung fluid, either resulting, for example, in inhibition of metabolic chains within mitochondria, disruption of membrane receptors, or generalized membrane damage (4,20).

Nitrogen dioxide similarly resulted in a depression of phagocytosis at the lowest exposure level; however, enhanced phagocytosis was observed after exposure to the

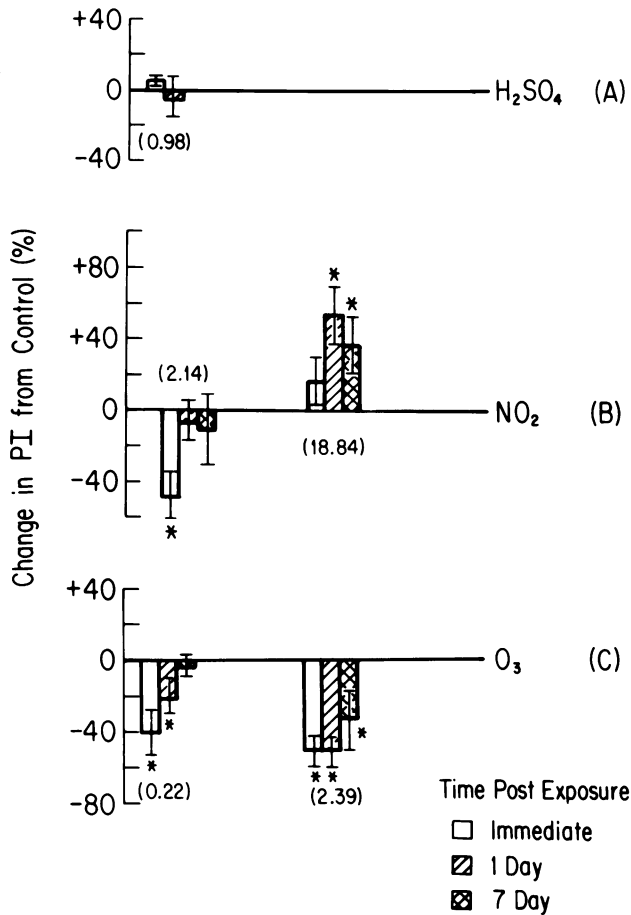


FIGURE 2. Change in PI from sham control after exposures to H₂SO₄ (A), NO₂ (B), or O₃ (C). A negative change represents a reduction in PI; a positive change indicates an increase. Numbers in parentheses represent exposure concentration in mg/m³. Asterisk (*) denotes significant change ($p < 0.05$). Data for H₂SO₄ derived from Naumann and Schlesinger (19); data for O₃ derived from Driscoll et al. (17).

higher concentration. The difference in effects at the two exposure levels of NO₂ could be due to different active reaction products. The precise mechanisms of NO₂ reaction in the lungs are still uncertain. However, because H⁺ and NO₂⁻ are two reaction products presumed to be formed, further evaluations in this laboratory were made *in vitro* to assess their effects on phagocytosis (21). As exposure to NO₂ both stimulated and depressed phagocytosis, it was hypothesized that the relative amounts of each chemical species formed may be a factor in producing this observed response.

To assess the effects of NO₂⁻, macrophages were obtained from naive rabbits by lung lavage as previously described. The cells were resuspended at a concentration of 2.8×10^6 viable macrophages/mL with MEM (Hanks Salts, MEM-H, pH = 7.4) containing 2 mM L-glutamine, 1% antibiotic-antimycotic mixture, and 50 μ g/mL gentamycin. Aliquots (0.5 mL) of this suspension were placed into 15-mL polypropylene centrifuge tubes and 2.0 mL of an appropriate stock solution of sodium nitrite [(NaNO₂) in MEM-H (pH = 7.4)] added to yield final NO₂⁻ concen-

trations of 0.5, 1.0, 2.0, 10.0, or 20.0 mM. The tubes were incubated at 37°C in closed culture for 2 hr in a shaking water bath. Following incubation, the volume was adjusted with fetal bovine serum (FBS) to yield a 10% FBS suspension of 5×10^5 macrophage/mL. Of this suspension, 1 mL portions were used to assess phagocytosis after a 1-hr incubation with latex particles as previously described. MEM-H (pH = 7.4) was used as a control.

To assess the effect of H⁺, collected macrophages were resuspended at 2.8×10^6 viable cells/mL of unbuffered MEM-H (no sodium bicarbonate, pH = 7.4) containing 2 mM L-glutamine, 1% antibiotic/antimycotic mixture, and 50 μ g/mL gentamycin. Additional volumes of this unbuffered MEM-H were adjusted with sterile 1.0 N HCl or 1.0 N NaOH to yield hydrogen ion concentrations of 1.6×10^{-6} M (pH = 5.8), 6.0×10^{-7} M (pH = 6.2), 2.0×10^{-7} M (pH = 6.8), or 1×10^{-8} M (pH = 8.0). Aliquots (0.5 mL) of the macrophage suspension were placed into 15-mL polypropylene centrifuge tubes, and 2.0 mL of pH-adjusted media was added. The control was 2 mL MEM-H (40×10^{-8} M, pH = 7.4) added to one tube. The tubes were incubated for 2 hr and processed as described for the phagocytosis assay.

Another series of tests examined the response to mixtures of NO₂⁻ and H⁺. Nitrite (as NaNO₂) stock solutions were prepared using unbuffered MEM-H, and they were adjusted to the desired pH using sterile 1.0 N HCl or 1.0 N NaOH. The combinations examined were 1.0 mM NO₂⁻ (at pH 6.2), 1.0 mM NO₂⁻ (at pH 6.8), 2.0 mM NO₂⁻ (at pH 6.2), or 2.0 mM NO₂⁻ (at pH 6.8). Unbuffered MEM-H (at pH 7.4) was used as a control. Macrophages were incubated in these mixtures for 2 hr prior to measuring phagocytic activity.

Statistical differences in PI were examined using a two-factor analysis of variance without replication. The Newman-Keuls test was used to assess differences between individual group means. The level of significance chosen was 0.05.

The effects of NO₂⁻ on phagocytosis are shown in Figure 3. A 2-hr preincubation of macrophages with NO₂⁻ at 1.0 mM to 20.0 mM significantly increased phagocytosis when compared to the control with no NO₂⁻ present. The lowest concentration, 0.5 mM NO₂⁻, did not affect phagocytosis. A dose-response relationship was not observed between NO₂⁻ concentration and PI. Rather, the increase in phagocytosis plateaued at 10 mM, and this concentration produced a response similar to that observed after preincubation with 20.0 mM NO₂⁻. These results do not agree with those of Vassallo et al. (22), who reported impaired phagocytosis due to NO₂⁻. They are, however, more consistent with the increase in macrophage phagocytosis seen after NO₂ exposure. The discrepancy in the two studies may be due to the choice of test particles used, i.e., bacteria versus latex beads.

The phagocytic activity of macrophages at various media pH levels is presented in Figure 4. Lowering pH (i.e., increasing [H⁺]) to either 6.8, 6.2, or 5.8 did not significantly alter phagocytosis when compared to macrophages from a control suspension medium of pH 7.4. Some decrease in PI was observed at pH values of 6.2 and 5.8,

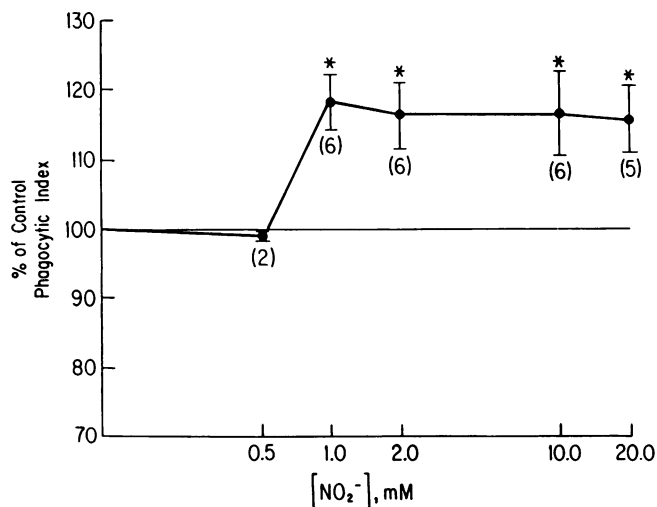


FIGURE 3. Effects of NO_2^- on macrophage phagocytosis. Each point represents the mean \pm SE (n = number of experiments). PI is expressed as percentage of control. Asterisk (*) denotes significant change from control. From Vollmuth (21).

suggesting a tendency toward reduced phagocytosis with decreasing pH. This is consistent with results of Tucker et al. (23), who found little difference in phagocytosis in the pH 6.0 to 7.4 range; however, phagocytosis was depressed at pH levels below approximately 5.5 to 6.0. One possible explanation offered was that pH might affect the absorption by the macrophage of phagocytosis-promoting serum proteins through the addition of H^+ to the protein surface, creating a net positive surface charge.

Also shown in Figure 4 is the phagocytic activity of macrophages preincubated for 2 hr at pH 6.2 and 6.8 in the presence of 1.0 mM or 2.0 mM NO_2^- . Whereas phagocytosis was shown to be significantly increased by the presence of either 1.0 or 2.0 mM NO_2^- (Fig. 3), this effect was mitigated by lowering the suspension pH to 6.8 and 6.2, in that a slight, but not statistically significant, depression in phagocytosis was observed in macrophages

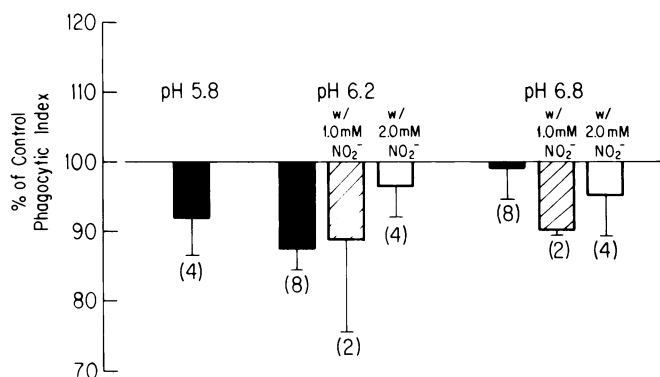


FIGURE 4. Effects of H^+ and NO_2^-/H^+ mixtures on macrophage phagocytosis. Bars represent the mean \pm SE (n = number of experiments). PI is expressed as percentage of control. From Vollmuth (21).

preincubated in these mixtures. Therefore, the relative abundance of H^+ and NO_2^- ions seems to modulate the phagocytic response *in vitro*.

Conclusions

Based upon work using similar protocols in the rabbit model, this paper evaluated the role that H_2SO_4 , NO_2 , and O_3 may play in altering selected aspects of lung clearance function following acute exposures. It is evident that the effectiveness of any inhaled agent is dependent upon the end point examined and that observed responses may be due to actions via different mechanisms that can be exposure concentration dependent in some cases. It is generally accepted that both NO_2 and O_3 have comparable effects upon the lungs, but that the relative effective concentrations are different; O_3 is considered to be almost 10 times as potent as NO_2 . The results suggest that this proportionality may differ at different exposure levels and may also depend upon the specific end point assessed.

Differences in the relative potency of NO_2 , O_3 , and H_2SO_4 in altering mucociliary clearance may be explained by differences in their regional deposition and/or their reaction following deposition. Submicrometer H_2SO_4 aerosols should deposit maximally in the tracheobronchial region of the lungs (24). Dissolution of H_2SO_4 in the mucus then results in the release of H^+ . The ability of the mucus to subsequently bind this H^+ depends upon its buffering capacity and initial pH (25); the former can be overwhelmed with sufficiently high H^+ production. This may result in changes in mucus viscosity and/or ciliary beat, thereby altering clearance rate (2). In contrast, while NO_2 and O_3 can be absorbed along the entire tracheobronchial tree, the terminal bronchioles and respiratory region receive the maximum dose (26). Depending on the reactivity of NO_2 and O_3 in respiratory tract fluids and the absorption capacity of these fluids, which is a function of their thickness and physiochemical properties, variable percentages of NO_2 and O_3 are likely to be absorbed in the tracheobronchial region. Although the data base concerning the reactions of NO_2 and O_3 in these fluid layers is quite limited (27,28), it can be speculated, based upon the results of the mucociliary clearance assays presented here, that any H^+ produced by NO_2 reaction with water may be adequately buffered by the mucus, without a resultant change in viscosity. On the other hand, O_3 in addition to being more potent than NO_2 , is more readily removed in the conducting airways proximal to the terminal bronchioles than is NO_2 (26); this may provide some explanation for its ability to alter mucociliary clearance.

Although NO_2 was ineffective in the tracheobronchial region, it did alter macrophage function, indicating that doses penetrating down into the alveolar region were capable of inducing a response. In addition, although no statistically significant effects on mucociliary clearance followed 2-hr exposures of rabbits to O_3 at ≤ 0.25 ppm, exposures to 0.1 ppm did alter the phagocytic activity of macrophages recovered by lavage from exposed animals. Again, these regional response differences to O_3 and NO_2 may be due to the dose distribution pattern of the inhaled

gases, i.e., their major dose is delivered to the respiratory region, and/or to differences in the sensitivity of the cells lining the bronchial and respiratory airways. It is not known whether any H^+ formed upon NO_2 deposition in the respiratory region contributes to the observed macrophage response. This chemical species may be adequately buffered in the lower respiratory tract, and the oxidants formed (e.g., NO_2^-) may be solely responsible for altering macrophage function. On the other hand, interaction between NO_2^- and H^+ may occur. For example, a synergistic reaction between oxidants and H^+ has been suggested by Last et al. (29), who proposed that the lifetime of free radicals arising from the interaction of an oxidant with molecules within the lung is increased by local pH changes, thus increasing oxidant reactivity. Regardless of the uncertainty in the mechanism(s) by which NO_2 reacts in the lungs, it seems to produce responses characteristic of both an acid and an oxidant.

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