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# Sulfuric Acid-Induced Changes in the Physiology and Structure of the Tracheobronchial Airways

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Sulfuric acid aerosols occur in the ambient particulate mode due to atmospheric conversion from sulfur dioxide ($SO_2$). This paper describes the response of the rabbit tracheobronchial tree to daily exposures to sulfuric acid ($H_2SO_4$) aerosol, relating physiological and morphological parameters. Rabbits were exposed to filtered air (sham control) or to submicrometer-sized $H_2SO_4$ at 250 $\mu g/m^3$ $H_2SO_4$, for 1 hr/day, 5 days/week, with sacrifices after 4, 8, and 12 months of acid (or sham) exposure; some rabbits were allowed a 3-month recovery after all exposures ended. $H_2SO_4$ produced a slowing of tracheobronchial mucociliary clearance during the first weeks of exposure; this change became significantly greater with continued exposures and did not improve after exposures ended. Airway hyperresponsiveness was evident by 4 months of acid exposure; the condition worsened by 8 months of exposure and appeared to stabilize after this time. Standard pulmonary mechanics parameters showed no significant trends with repeated acid exposure, except for a decline in dynamic lung compliance in animals exposed to acid for 12 months. Lung tissue samples obtained from exposed animals showed a shift toward a greater frequency of smaller airways compared to control, an increase in epithelial secretory cell density in smaller airways, and a shift from neutral to acidic glycoproteins in the secretory cells. The effect on airway diameter resolved after the exposures ceased, but the secretory cell response did not return to normal within the recovery period. No evidence of inflammatory cell infiltration was found due to $H_2SO_4$ exposure. Thus, significant alterations in the physiology of the tracheobronchial tree have been demonstrated due to repeated 1-hr exposures to a concentration of $H_2SO_4$ that is one-fourth the current 8-hr threshold limit value for exposure in the work environment. The cumulative dose inhaled by the rabbits is similar to current peak daily doses from ambient exposure in North America. The results obtained in the rabbit model provide insight into early changes in the tracheobronchial tree due to repeated irritant exposure and may be involved in the pathogenesis of chronic airway disease.

## Introduction

The relationship between repeated air pollutant exposures and the response of the respiratory tract is a complex but important aspect of toxicologic assessment involved in the proper setting of environmental exposure standards. The reason for this is that any organ system of the body has a limit as to the amount and duration of insult that can be withstood before its defenses are overwhelmed and pathology results. Recent studies performed in this laboratory have examined temporal changes in the tracheobronchial tree from repeated exposures to the common atmospheric irritant, sulfuric acid mist (*1–3*). Sulfuric acid ($H_2SO_4$) is often a significant component of the ambient accumulation mode aerosol in urban and rural areas (*4*); thus, large and very diverse populations are exposed to this pollutant. In addition, it has been suggested that exposure to $H_2SO_4$ may be a contributor to the pathogenesis of chronic bronchitis (*5*).

This paper describes and integrates the effects upon certain aspects of tracheobronchial function and structure in rabbits exposed to $H_2SO_4$ mist for 1 hr/days, 5 days/week for up to 1 year. The role of acid exposure in altering mucociliary clearance and respiratory mechanics in the development of airway hyperresponsiveness and in changing the morphology and morphometry of tracheobronchial airways was explored. Except for respiratory mechanics, individual results have been previously reported (*2,3*).

## Materials and Methods

### Experimental Animal

Male, SPF (*Pasteurella multocida*) New Zealand White rabbits (~3 kg) were housed in stainless-steel

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cages at 20°C and 50% relative humidity (RH), with food (Purina Rabbit Chow-HF) and water provided *ad libitum*. The animals were quarantined for a 2-week period prior to the start of the study. They were examined daily for any indications of infection, and clinical screening was performed monthly to monitor population health.

## Generation and Characterization of Sulfuric Acid Mist

The techniques for generation and monitoring of $H_2SO_4$ have been described (*1,3*). Submicrometer mass median diameter (0.3 μm) $H_2SO_4$ aerosols were produced by nebulizing a dilute solution of the acid with a Laskin nebulizer. The size distribution of the aerosol was monitored with a cyclone sampler; mass concentration analysis for sulfate was performed by flame photometry. The aerosol was equilibrated to 25°C, 80% RH in a mixing chamber and was delivered to the rabbits via a manifold system. The mean concentration of $H_2SO_4$ for all exposures was 254 μg/m$^3$ (± 24, SD).

## Measurement of Tracheobronchial Mucociliary Clearance

The technique for measuring mucociliary clearance from the tracheobronchial tree has been described previously (*6*). Clearance was measured after exposing rabbits to a radioactively tagged ($^{99m}$Tc) insoluble tracer aerosol of ferric oxide ($Fe_2O_3$) microspheres. Each rabbit was passively restrained within a sling placed between a pair of opposed, collimated, and electrically coupled 2-inch diameter NaI (Tl-activated) scintillation detectors aligned on a common axis on either side of the rabbit's thorax. Clearance of tracer particles was observed as a progressive decrease in the radioactive counts within the thoracic field obtained in serial measurements due to the proximal mucociliary transport of deposited particles out of the respiratory tract.

Clearance was quantitated by computer integration of the area under the tracheobronchial clearance curve, resulting in a parameter designated as mean residence time, MRT (*1*). The MRT is a reflection of clearance rate, in that an acid exposure-related decrease indicates an acceleration of clearance, whereas an increase in MRT indicates a retardation, as long as the initial regional deposition of the tracer particles was not altered by acid exposure.

In terms of regional deposition, the bulk of clearance of material initially depositing in the tracheobronchial tree is completed within 24 hr after the tracer aerosol exposure, and residual lung activity remaining at this time is assumed to represent particles deposited within the nonciliated alveolar region (*7,8*). Thus, the percentage of total deposition of tracer particles that occurred in the tracheobronchial tree is taken as the percentage of the initial lung burden cleared by 24 hr after tracer aerosol exposure, i.e., $C_{24}$.

## Measurement of Respiratory Mechanics

Pulmonary resistance ($R_L$), dynamic compliance ($C_{dyn}$), respiratory rate (f), and tidal volume ($V_t$) were assessed with a whole body plethysmograph, as described previously (*3*). Tidal volume was determined by measuring plethysmographic pressure changes with a pressure transducer. Intrathoracic pressure was assessed with a 5-cm esophageal balloon attached to another pressure transducer via polyethylene tubing containing side holes in its closed end. Volume and pressure signals from the transducers were amplified using a carrier demodulator and were sent to a Charles River Data System MF-211 computer for real-time analysis of pulmonary mechanics values. Compliance was calculated by dividing full inspiratory and expiratory tidal volume, i.e., points of zero flow, by the corresponding differential esophageal pressure. Pulmonary resistance was calculated by dividing peak flow (~50% full tidal volume) into the corresponding differential pressure required to generate this flow.

## Measurement of Airway Responsiveness

The measurement of airway responsiveness by assessing changing in $R_L$ due to bronchoprovocation challenge with intravenously (*iv*) administered acetylcholine (ACh) has been previously described (*3*). Baseline mechanics values were obtained for each rabbit prior to determination of individual dose-response relationships for $R_L$ by delivery of ACh in sequentially increasing dose rates.

A positive response to ACh challenge was regarded as a change in excess of twice the normal intrasubject coefficient of variation (CV) of $R_L$ (*9*). The greatest CV in baseline measurements observed for any animal was 25%. Thus, the dosing regime was continued until the mean postprovocation $R_L$ for each rabbit increased to at least 150% of the mean preprovocation baseline value. In all cases, at least five doses of ACh were employed to produce the dose-response relationship, which was, in effect, a cumulative dose-response, since $R_L$ was not allowed to return to baseline level between successive acetylcholine administrations.

## Quantitative Histological Analysis

At designated times, animals were sacrificed, the lungs removed and inflated with fixative, embedded in plastic, sectioned at 3 μm, and stained with hematoxylin and eosin (H & E) or alcian blue (pH = 2.5)/Periodic acid Schiff (AB/PAS), as described previously (*2*).

Morphometric and morphologic responses to acid exposure were assessed by determining changes in airway diameter, epithelial secretory cell density, and secretory cell glycoprotein histochemistry (*2*). All air-

ways were examined in each section assessed using light microscopy. Each section was chosen at random from the serial sections prepared from each tissue block and were coded to avoid any bias during reading.

Airway diameters were determined by measurement from basement membrane to basement membrane. In oblique sections, the smallest diameter was determined by measuring perpendicular to the longest diameter of the airway (*10*). Diameters of respiratory bronchioles were not assessed; only the total number of such airways were counted. In addition, the total number of all airways in each section examined was determined and normalized to the area of the tissue section.

Epithelial secretory cell density (ESCD) was quantitated as the total number of PAS and/or AB staining cells per millimeter of epithelial length (*11*). To determine any shift in the pH of intracellular mucus, the number of AB-positive epithelial secretory cells was assessed as a fraction of all secretory cells. For this analysis, all AB-positive cells were counted whether or not they also stained with PAS.

## Experimental Design

Initially, 40 rabbits were randomly assigned into 2 groups of 20 rabbits each, with 1 group destined to receive acid exposures while the other served as a sham control. Each group of 20 was then randomly subdivided into 4 cohorts of 5 rabbits each, representing those animals scheduled to be sacrificed for tracheobronchial airway morphometric analyses after 4, 8, or 12 months of exposure, or after a recovery period of 3 months beyond the end of the 12-month exposure period. Prior to sham or acid exposures, each rabbit underwent a series of 10 control clearance tests to provide baseline values for MRT and $C_{24}$. Rabbits were then exposed to either the $H_2SO_4$ or the sham atmosphere for 1 hr/day, 5 days/week. All clearance tests were performed 18 to 20 hr after the preceding day's acid or sham exposure, so as to separate the immediate (acute) effect of the current exposure from the effect due to chronic exposure. Mucociliary function was measured once a week in all groups for the first 5 weeks of acid or sham exposure; thereafter, clearance was measured in each group every second to third week. Baseline respiratory mechanics values were determined before any acid or sham exposures began. Measurements were then made every third to fourth week during exposures. Bronchoprovocation challenge testing was performed at the end of 4, 8, or 12 months of acid or sham exposure. Three days after the end of each of these exposure series or at 15 months (recovery group), animals were sacrificed for histopathological analysis.

## Statistical Analysis

Each rabbit served as its own control for assessment of mucociliary clearance change due to exposure. A mean baseline value of MRT for each rabbit was obtained from the clearance tests performed prior to initiation of the acid or sham exposures. Overall exposure-induced changes in clearance for each cohort were determined by obtaining the mean MRT for all clearance tests for each constituent rabbit during the acid or sham exposure period and then using the paired *t*-test to determine the significance of any difference between these and respective baseline values.

To assess temporal changes in the clearance patterns of the acid or sham-exposed animals, linear regression analysis of $\overline{\%\Delta MRT}$ versus time of the clearance run (relative to initiation of the acid or sham exposures) was performed separately for each exposure cohort, i.e., the 4, 8, 12, and 15 month animals. The $\overline{\%\Delta MRT}$ is the mean percentage change from baseline of the entire exposure cohort for each clearance test performed. Regression analysis was also applied to the complete data set of $\overline{\%\Delta MRT}$ values for the acid- and sham-exposed animals.

To analyze for any changes in regional deposition during and after the acid or sham exposures, analysis of variance was performed on the parameter $C_{24}$ for all groups. The determination of significant changes in respiratory function parameters was made by using each animal as its own control and using the paired *t*-test to compare results between acid or sham exposure. Temporal changes were assessed by linear regression.

The response to bronchoprovocation challenge for each individual rabbit was quantitated by dividing the mean value of each respiratory mechanical function determined from the series of breaths obtained after each challenge by the mean prechallenge baseline value (i.e., at 0 dose) for that same function and multiplying by 100 to obtain a percentage of this baseline for each dose of acetylcholine. All subsequent statistical analyses were performed using these values for individual rabbits within each exposure group.

Two parameters based upon the change in $R_L$ with increasing dose of ACh, namely sensitivity and reactivity, were used to assess the state of the airways after exposure to $H_2SO_4$ (*12*). Sensitivity is defined as the dose of acetylcholine at which the criterion of a significant response, i.e., an increase in $R_L$ to 150% baseline, is reached. This was expressed in terms of an effective dose, the $ED_{150}R_L$. On the other hand, reactivity is the actual slope of the dose-response relationship for $R_L$, and provides information on the response of the airways at challenge doses above the minimally effective level, i.e., $> ED_{150}R_L$.

To obtain values for sensitivity, plots of $R_L$ (as a percentage of the baseline value) versus dose of ACh were constructed for each individual animal. From these, values for $ED_{150}R_L$ were determined for each rabbit by interpolation. Comparisons of $ED_{150}R_L$ between rabbits in each cohort were then performed by analysis of variance, followed by the Newman-Keuls tests. Reactivity for each cohort was determined by linear regression analysis using individual values of percentage of baseline $R_L$ for rabbits in each group versus

$\log_{10}$[ACh]; comparison of slopes between cohorts was then performed with analysis of covariance, followed by the Newman-Keuls test.

Intrapulmonary airway diameters for each exposure cohort were initially quantitated as a frequency distribution, and the one-sided Kolmogorov-Smirnov test for continuous data was used to determine whether the distribution for each acid exposure group differed significantly from that of their respective sham control group. Although this type of analysis is a powerful test to assess an overall shift in the distribution of airway sizes, it does not provide any indication of temporal differences for specific sizes. Thus, intrapulmonary conducting airways were also grouped into categories encompassing specific diameter ranges (< 250 μm, and then at 125 μm intervals, i.e., 251–375 μm, etc.) to examine acid-induced changes within airway size classes in the individual exposure cohorts. To assess any temporal shift in airway size due to acid exposure, regression analysis was performed on percentages of airways within each classification across acid or sham exposure cohorts sacrificed at either 4, 8, or 12 months. Finally, the density of airways, i.e., the total number per unit section surface area, was assessed in acid and control groups by the *t*-test.

To determine the significance of differences in secretory cell density and staining pattern, conducting airways were separated into size classifications similar to those described, except that all intrapulmonary airways > 500 μm were included in a single grouping. The respiratory bronchioles, main bronchi, and trachea were classified separately. Differences in cell density and staining pattern for each airway size class between acid exposure and control groups were then assessed using the *t*-test. Statistical significance for all tests was accepted at $p < 0.05$, unless noted otherwise.

# Results

## Mucociliary Clearance

Statistically significant differences were found in the mean MRT between baseline tests and those tests performed during acid exposure for the 4, 8, and 12 month cohorts (Table 1). Although the recovery cohort showed the greatest degree of slowing in clearance, statistical significance was not achieved due to the small number of remaining animals (final $n = 3$).

Mucociliary clearance during sham exposures, expressed as the group mean percentage change from baseline MRT (i.e., $\overline{\%\Delta MRT}$), is shown in the top panel of Figure 1. At no time was the group mean outside of the 95% confidence interval of $\overline{\%\Delta MRT}=0$. Although a regression of $\overline{\%\Delta MRT}$ with time for all sham controls showed a slight decrease in slope, i.e., a trend toward speeding in clearance, this change was not statistically significant.

The pattern of clearance response for all animals exposed to acid for 12 months is shown in the bottom panel of Figure 1. These animals, as well as those in the 4- and 8-month sacrifice groups, showed a slowing of mucociliary clearance within the first weeks of exposure, and this appeared to become progressively worse as the number of exposures increased. Regression analysis of $\overline{\%\Delta MRT}$ with time for the individual exposure cohorts indicated no significant relationship for the 4-month animals, but a significant slope was found for the 8-, 12-, and 15-month cohorts (Table 1). An estimation of the point in time at which clearance retardation became progressive with repeated acid exposure was obtained by an iterative regression analysis, beginning with data from all acid-exposed animals and sequentially removing from the analysis individual daily $\overline{\%\Delta MRT}$ values until the overall regression coefficient lost statistical significance. It was determined that progressive slowing of clearance began at approximately week 19 of acid exposure.

The variability of MRT in the acid exposure cohorts was significantly greater than that in the sham control cohorts throughout all exposure periods, and this variability was increased during the recovery period. The control animals also showed some increase in variability during the recovery period compared to that seen during the actual sham exposures, but this was not statistically significant. No significant change

**Table 1. Mucociliary clearance.**[a]

| Group, month | Treatment | Average ΔMRT, min[b] | Level of significance ($p$) | |
|---|---|---|---|---|
| | | | Paired *t*-test[c] | Linear regression[d] |
| 4 | Sham | − 8 | 0.10 | 0.67 |
| 4 | Acid | + 36 | 0.02 | 0.65 |
| 8 | Sham | − 6 | 0.14 | 0.59 |
| 8 | Acid | + 31 | 0.03 | 0.02 |
| 12 | Sham | − 13 | —[e] | 0.55 |
| 12 | Acid | + 43 | 0.01 | 0.001 |
| 15 | Sham recovery | − 14 | 0.14 | 0.45 |
| 15 | Acid recovery | + 55 | —[e] | 0.02 |

[a] Modified from Gearhart and Schlesinger (2).
[b] Difference between mean baseline and exposure MRT values.
[c] For comparison of baseline versus exposure means.
[d] For assessing relation between %ΔMRT versus time.
[e] Due to the small $n$, it was not possible to test for significance in these groups.

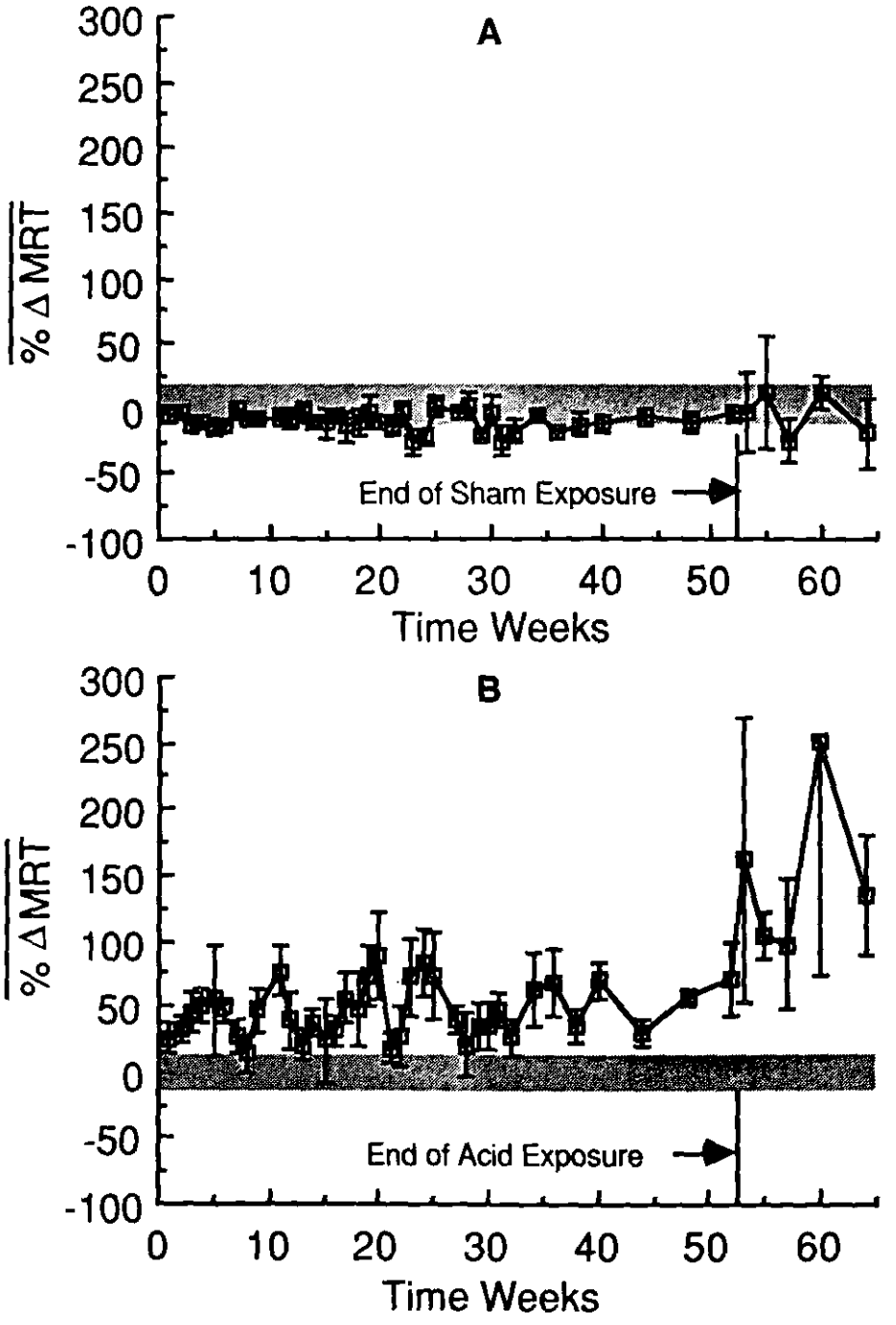


FIGURE 1. Effects of acid or sham exposure on mucociliary clearance. Each point represents the group mean (±SE) change from preexposure control values. The shaded band is the 95% confidence interval for $\overline{\%\Delta MRT} = 0$. (*A*) Change in MRT for sham control animals. (*B*) Change in MRT for all animals exposed to $H_2SO_4$ aerosol for 12 months. From Gearhart and Schlesinger (*2*).

in regional deposition of tracer particles occurred during the course of the acid or sham exposures.

## Respiratory Mechanics

The only statistically significant change in respiratory mechanics was in $C_{dyn}$. Figure 2 shows dynamic compliance values obtained during baseline measurements prior to any exposures and values obtained after 8 or 12 months of exposure. Both of the acid exposure groups exhibited a decrease in $C_{dyn}$, but only in the 12-month group was this change significant. However, linear regression of $C_{dyn}$ from acid-exposed animals showed a significant slope, suggesting that this effect on pulmonary mechanics worsened with time. This is in contrast to the regression of $C_{dyn}$ versus time for the sham-exposed animals, which showed a nonsignificant increase in slope.

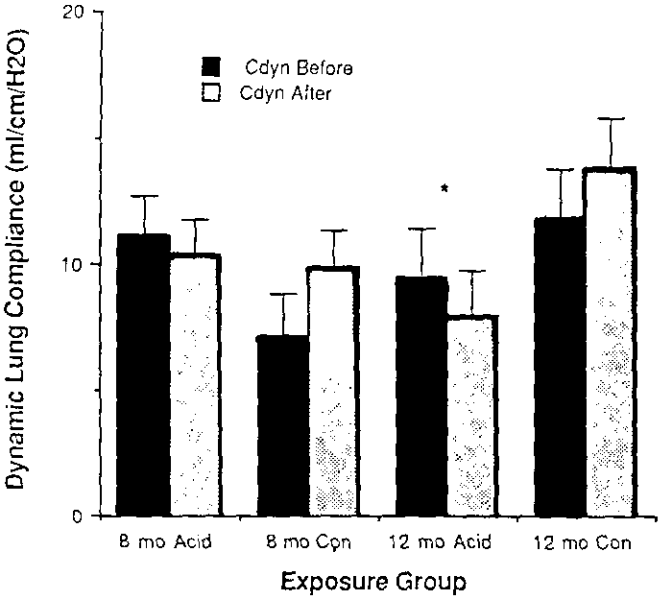


FIGURE 2. Change in dynamic lung compliance (mean ± SD) for the 8-month and 12-month acid and sham cohorts. $C_{dyn}$ before represents mean of measurements obtained prior to start of acid or sham exposures. $C_{dyn}$ after represents mean of measurements obtained at the end of 8 or 12 months of exposure. (*) Indicates those acid groups in which the difference before and after is significantly different from that in sham control ($p < 0.05$).

## Airway Responsivity

No temporal effect was found in the responsiveness of animals in any of the sham-exposed groups over the course of the study, as determined by two-way analysis of variance. As a result, all control cohorts were pooled for comparison to results obtained in the animals exposed to $H_2SO_4$.

The bronchial sensitivity for the sham-control and acid-exposed animals, as obtained via interpolation from individual rabbits in each group, is presented as group means and ranges of $ED_{150}R_L$ in Table 2. The mean $ED_{150}R_L$ values of the individual acid exposure groups were not significantly different, but the values for all acid groups differed significantly from that of the sham controls. This indicates that $H_2SO_4$ mist produced an increase in bronchial sensitivity by 4 months of repeated exposures, and this condition continued into the longer exposure periods.

**Table 2. Bronchial sensitivity.**[a]

| Group | $ED_{150}R_L$, μg/kg/min Mean (± SE) | Range |
|---|---|---|
| Control | 18.7 (± 3.7)[b] | 3.6–39.8[d] |
| Acid, 4 months | 2.0 (± 0.3)[c] | 1.3– 2.5 |
| Acid, 8 months | 1.5 (± 0.4)[c] | 1.1– 2.6 |
| Acid, 12 months | 1.2 (± 0.2)[c] | 1.0– 1.5 |

[a] From Gearhart and Schlesinger (*3*).
[b,c] Values with the same superscript letter are not significantly different from each other.
[d] Range of values for individual sham control groups: 4 months, 7.6–24; 8 months, 3.6–39.8; 12 months, 7.5–39.8

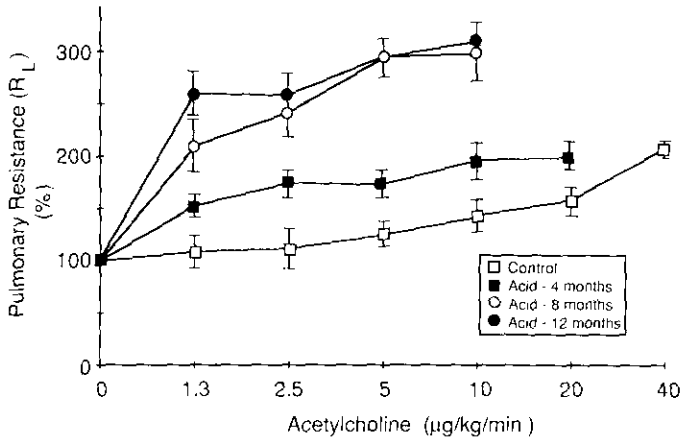


FIGURE 3. Effect of serial bronchoprovocation challenge on pulmonary resistance ($R_L$). The abscissa is expressed in terms of doubling doses of acetylcholine. Data are expressed as the group mean (± SE) percentage of baseline $R_L$ at each dose. From Gearhart and Schlesinger (*3*).

Figure 3 depicts the change in $R_L$ with increasing dose rate of ACh for each exposure cohort. Using individual values for rabbits in each group, linear regression analysis showed a significant relationship between change in $R_L$ and the $\log_{10}$ [ACh] in all cases. The slopes of these dose-response lines is a measure of bronchial reactivity (Table 3). Reactivity for the 8-month acid exposure cohort was found to be significantly greater than that for the control animals and greater than those exposed to acid for only 4 months. This higher reactivity was also present after 12 months of acid exposure. Although reactivity in the 4-month acid exposure group was not significantly different from control at $p < 0.05$, it was significant at $p < 0.075$, indicating that the 4-month group may have been beginning to show a response. Thus, repeated exposures to $H_2SO_4$ resulted in a temporal increase in bronchial reactivity over the course of 8 months of exposure; beyond this time, and with continued exposures, reactivity remained constant at this elevated level.

**Table 3. Bronchial reactivity.**[a]

| Group | Reactivity mean ± SE[b] |
|---|---|
| Control | 19.5 (± 11.6)[c] ($r^2$ = 0.69) |
| Acid, 4 months | 47.2 (± 4.8)[c] ($r^2$ = 0.98) |
| Acid, 8 months | 101.3 (± 15.4)[d] ($r^2$ = 0.94) |
| Acid, 12 months | 96.1 (± 10.8)[d] ($r^2$ = 0.91) |

[a] From Gearhart and Schlesinger (*3*).
[b] Each value represents the slope of the line generated for each group using individual values of change from baseline $R_L$ for rabbits within each group. The numbers in parentheses are the standard errors of this slope. The unit of reactivity is $\Delta R_L$/μg/kg/min, where $\Delta R_L$ is percentage of the baseline value. $r^2$, coefficient of determination.
[c,d] Values with the same superscript letter are not significantly different from each other.

## Airway Morphometry and Morphology

Frequency histograms of airway sizes for all exposure cohorts depict the effect of repeated acid exposure on airway diameters (Fig. 4). Animals exposed to $H_2SO_4$ and sacrificed at 4, 8, or 12 months were found to have a significantly greater percentage of smaller airways than did their respective controls; no significant difference from control was found for those acid-exposed animals sacrificed at the end of the 3-month recovery period. No significant difference was found in the total airway number (per unit section surface area) between control cohorts and any of the cohorts exposed to acid.

Regression analysis indicated that a progressive increase in the percentage of total airways ≤ 250 μm occurred in the acid-exposed animals from 4 months to 12 months ($p < 0.003$). This shift toward smaller airways was reflected in a temporal reduction in the relative proportions of the next two larger-sized airway categories.

A statistically significant increase in epithelial secretory cell density (ESCD) was found in certain airway size classes for all acid exposure cohorts compared to control (Fig. 5). Although only intrapulmonary airways in the 375 μm size class, i.e., those with diameters between 251 and 375 μm ($p < 0.003$), and the trachea ($p < 0.001$) were increased in the 4-month exposure cohort, there was a significant change in ESCD for all airway classes in the 8-month acid cohort. This difference was significant as well for most airways in the 12-month acid exposed animals. The acid recovery group showed significantly increased ESCD only in airways ≤ 250 μm or between 376 and 500 μm in diameter.

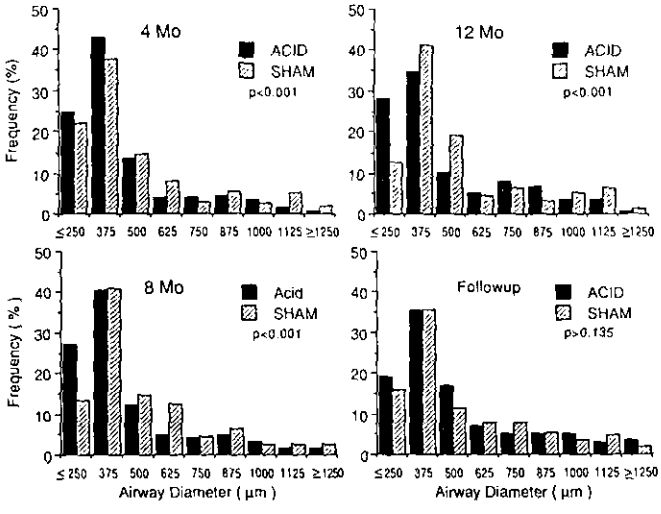


FIGURE 4. Frequency histogram of airway diameters. The diameters shown are the end of each range; complete ranges are as follows: < 250; 375 = 251–375; 500 = 376–500; 625 = 501–625; 750 = 626–750; 875 = 751–875; 1000 = 876–1000; 1125 = 1001–1125; 1250 = 1125–1250; > 1250 μm). Statistical differences between each acid-exposed and sham-exposed cohort was determined by the Kolmogorov-Smirnov test, and the $p$ values are shown. From Gearhart and Schlesinger (*2*).

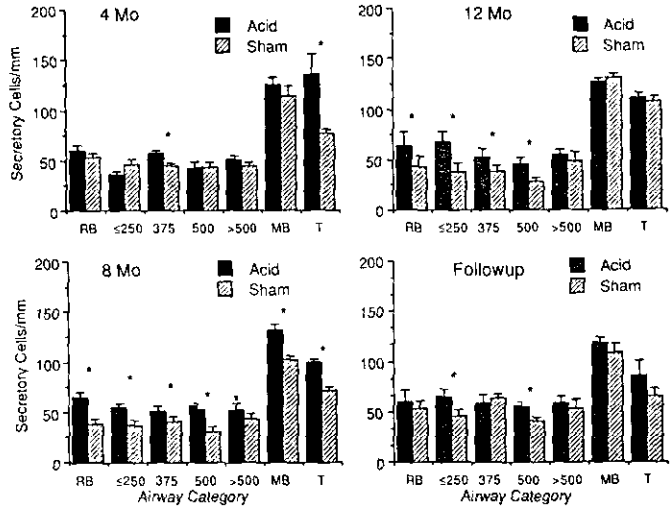


FIGURE 5. Epithelial secretory cell density (mean ± SE) in airways of various diameter ranges (in micrometers). The diameters shown are the end of each range; complete ranges are as follows: < 250; 375 = 251–375; 500 = 376–500; > 500 μm. RB, respiratory bronchioles; MB, main bronchus; T, trachea. (*) Indicates significant difference between acid and sham cohorts ($p < 0.05$). From Gearhart and Schlesinger (*2*).

A statistically significant shift in the staining characteristics of the secretory cells in specific airway size classes was found to be related to acid exposure (Fig. 6). This change was toward an increase in AB staining, indicating an increase in the acidic glycoprotein content of the intracellular mucus (*13*). The greatest magnitude of response in the 4-, 8-, and 12-month acid exposure cohorts was at the level of the respiratory bronchioles. The animals allowed a recovery period did not show significantly increased AB-positive cells in the small airway categories, although there was still

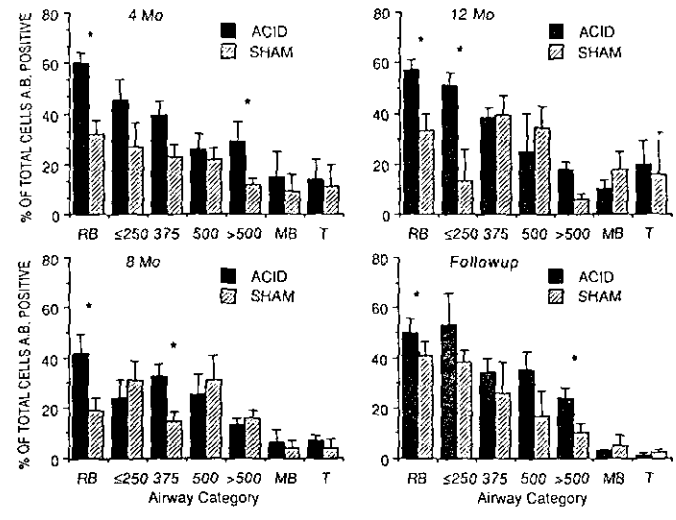


FIGURE 6. Percentage (mean ± SE) of total epithelial secretory cells staining with alcian blue (AB) in airways of various diameter ranges (in micrometers). Ranges as in Fig. 5. RB, respiratory bronchioles; MB, main bronchus; T, trachea. (*) Indicates significant difference between acid and sham cohorts ($p < 0.05$). From Gearhart and Schlesinger (*2*).

such a trend in all airway classes, and a significant change in those airways > 500 μm.

The 4-month acid exposure cohort showed a trend toward increased acidic glycoproteins in all airway size classes, but the respiratory bronchioles and the > 500 μm bronchi were the only categories with statistically enhanced AB staining. This pattern was also evident in the 8- and 12-month acid cohorts, but for increasingly smaller airways; the greatest difference was in the 375 μm diameter airways at 8 months; the 250 μm diameter airways showed the greatest change after 12 months of acid exposure.

Tissue sections stained with H & E showed no significant histopathologic effects due to acid exposure. For example, there was no bronchiolar inflammation or peribronchiolar lymphocytic infiltration, both of which are characteristics of chronic irritation of the respiratory tract (*14*). Thus, the changes in airway size distribution and secretory cells were early responses to acid exposure which occurred prior to overt chronic inflammation.

**Table 4. Effects of repeated $H_2SO_4$ exposure on tracheobronchial function and structure.**

| End point | 4 Months | 8 Months | 12 Months | Post exposure (3 Months) |
|---|---|---|---|---|
| Mucociliary clearance | ↓ | ↓ | ↓ | ↓↓ |
| Pulmonary resistance | —[a] | NC[b] | NC | — |
| Dynamic compliance | — | NC | ↓ | — |
| Airway sensitivity | ↑ | ↑↑ | ↑↑ | — |
| Airway reactivity | NC | ↑ | ↑ | — |
| Airway diameter | ↓ | ↓ | ↓↓ | NC |
| Secretory cell number | ↑ | ↑↑ | ↑↑ | ↑ |
| Secretory cell pH | ↓ | ↓ | ↓ | ↓ |

[a] —, not measured.
[b] NC, no change.

## Discussion

The objective of the research described in this paper was to examine the types and progression of functional and structural changes induced in the tracheobronchial tree by repeated inhalation of a common atmospheric irritant, namely $H_2SO_4$ aerosol. By assessing multiple parameters, it is possible to gain a broader understanding of the events involved in the early pathogenesis of airway disease, not just by relating function with structure, but by ordering the appearance of aberrant airway responses as irritant exposures proceed. Determination of the magnitude of each response helps in understanding its relationship to the overall disease process. The parameters assessed were clearance of particles from the tracheobronchial tree via the mucociliary escalator; respiratory mechanics and airway responsiveness; and tissue structure, as determined by histopathologic, morphologic, and morphometric assessments. Table 4 presents a summary of the results of acid inhalation.

Rabbits exposed to $H_2SO_4$ for 4 months showed a slowing of tracheobronchial mucociliary clearance, increases in airway responsiveness, increases in the relative number of small airways and in the number density of secretory cells, and a decrease in the pH of intercellular glycoproteins. The slowing in clearance occurred within the first week of acid inhalation, but as histological evidence for any epithelial cell changes directly responsible for this alteration was lacking due to the sacrifice schedule, it must be inferred from other studies of chronic irritant exposure that changes in both secretory cell number and glycoprotein pH may also occur early in the irritant response (*15*). For example, an increase in secretory cell number in $H_2SO_4$-exposed rabbits after only 1 month of exposure to a comparable concentration was found in another study conducted in this laboratory (*1*). Such cellular responses are believed to be the first indications of functional responses involved in the disease process (L. Reid, personal communication, 1987). Thus, a change in clearance is a general measure of the response of the airway epithelium to irritant challenge. Mucociliary clearance is a major lung defense mechanism, responsible for maintaining pulmonary homeostasis by removing inhaled organisms and foreign particles that deposit on epithelial surfaces. Clearance dysfunction has been associated with, and is perhaps a factor in, the pathogenesis of chronic obstructive lung disease, notably chronic bronchitis (*16,17*). The inability to effectively clear mucus from the small airways may also contribute to the exacerbation of asthma (*18,19*).

There was no apparent change in respiratory mechanics within the first 4 months in the 8- and 12-month exposure cohorts. However, the responsivity of the airways of those animals exposed to acid for 4 months was increased over that of matched controls. How early prior to this time such a change occurred cannot be determined from this study. Nevertheless, the development of hyperresponsive airways is significant, as such a condition has been linked to both asthma and chronic bronchitis (*20–22*) and appears to develop in asymptomatic smokers with otherwise normal respiratory function (*23,24*).

The 8-month acid exposure cohort showed changes in the same parameters as the 4-month group, but the condition of the tracheobronchial tree evidently became worse with repeated acid exposures, since airway physiology was altered by 8 months. There was a significant increase in airway reactivity by 8 months compared to 4 months, so that between 4 and 8 months of acid exposure the responsiveness of the airway musculature became greater as clearance function worsened, and changes in airway morphology and morphometry progressed. The 12-month acid exposure group maintained the same level of airway respon-

siveness as the 8-month cohort, but there was a continued worsening of clearance function between 8 and 12 months of repeated acid insult and a further increase in the proportion of acidic glycoproteins.

It should be stressed that while not all airway categories exhibited an increase in secretory cell density in some of the acid-exposed cohorts, it is likely that changes in the smaller airways are the most biologically significant. These airways represent a large surface area and are the site of the earliest airway disease processes (*14,25*). Considering the small size of the $H_2SO_4$ aerosol to which the rabbits were exposed, it is not surprising that this is the apparent site of the maximal morphologic response.

Clearance alterations are the result of changes in mucus secretory capacity, alteration in the type of mucus produced, and disruption of normal morphology of cilia and ciliated cells (*26–28*). Thus, the slowing of tracer particles from the tracheobronchial tree observed in acid-exposed rabbits was likely a direct reflection of alterations in the cellular population of the epithelial surface and of the characteristics of its secretory products.

The animals allowed a 3-month recovery period after 1 year of acid exposure exhibited the worst clearance function, but showed some partial resolution in secretory cell density. Although only two airway size classifications had significant decreases in mucoglycoprotein pH, a trend toward decreased pH in all intrapulmonary airway categories was present in the recovery group; this may help explain the worsened clearance capacity of these animals.

The results of respiratory mechanics measurements suggest that changes in the smaller airways of the acid-exposed animals became significantly worse with continued exposure. This was evidenced by a decrease in $C_{dyn}$, which became worse by the end of 12 months of $H_2SO_4$ exposure. In the absence of parenchymal disease, changes in $C_{dyn}$ are reflective of alterations in small airways, whereas $R_L$ is more sensitive in detecting change in central airways (*9*). The decrease in $C_{dyn}$ in the acid-exposed animals seems even more pronounced if compared to both the 8-month and 12-month sham exposure groups, which exhibited a trend towards increased $C_{dyn}$. The progressive change in $C_{dyn}$ from 8 to 12 months of acid exposure was associated with a progressive shift toward a greater proportion of smaller-sized airways.

It is evident from the observations reported in this paper that rabbits repeatedly exposed to $H_2SO_4$ aerosol develop significant changes in tracheobronchial airway physiology and structure that are characteristic of chronic bronchitis and/or asthma. These findings have added significance when it is noted that recent studies of asymptomatic nonatopic cigarette smokers are finding some similar results (*29–31*); cigarette smoking is a known causal factor in chronic obstructive lung disease.

Although the $H_2SO_4$ concentration used is as much as 10 times that which normally occurs in the ambient atmosphere, it is only one-fourth the threshold limit value for 8 hours/day occupational exposures. However, Schlesinger (*32*), in this symposium, has shown that effects on clearance function in rabbits are due to some combination form of exposure concentration and exposure duration (i.e., $C \times T$), so that on a daily basis, actual total ambient exposures may approach those used herein.

Whether chronic exposures to current levels of ambient $H_2SO_4$ produce, in the general human population, those changes characteristic of lung disease that were measured in this study are not currently known. Perhaps a more important question is how are current $H_2SO_4$ levels affecting populations with existing airway disease (*33*). It is clear from examinations of human asthmatics that brief exposures to acidic aerosols very near the levels used in this study evoke responses from hyperresponsive airways (*34*). In light of this, it is interesting that in the healthy rabbits, fairly low levels of acid were able to induce hyperresponsivity. Some added factors related to the overall picture of developing lung disease similar to asthma that occurred in rabbits repeatedly exposed to $H_2SO_4$ aerosol are the slowing in mucus clearance rates and the alteration in the distribution of airway diameters (*35,36*).

In conclusion, recent work has resulted in an animal model of chronic airway irritation having some characteristics of both chronic bronchitis and asthma. There was a substantial slowing in the normal rate of mucociliary clearance from the tracheobronchial tree, increases in the number of epithelial secretory cells and the acidity of their glycoproteins, a shift toward a smaller size-distribution of airways, an alteration in the normal agonist response of the airway musculature, and a decline in normal airway mechanics of the smaller airways. It is most significant that these changes resulted from an $H_2SO_4$ exposure level well within current levels encountered by workers in various occupations and at a concentration within most commonly used safety factors employed for protection of public health.

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