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Carbon Monoxide Exposure from Aircraft Fueling Vehicels

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Effect of Ferrous Sulfate Aerosols and Nitrogen Dioxide on Murine Pulmonary Defense

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ABSTRACT. A murine infectivity model was used to test the effect of exposure to atmospheres containing 290 ± 50 $\mu g/m^3$ of respirable sized ferrous sulfate ($FeSO_4$) particles (0.4 μm mass median aerodynamic diameter) and 1.0 ppm nitrogen dioxide (NO_2) prior to infection with aerosols of *Staphylococcus aureus* or group C streptococci. Exposure to these combined pollutants for 24 or 48 hr did not impair pulmonary inactivation of *S. aureus*. Exposure to $FeSO_4$ or NO_2 for 48 hr, or to both pollutants for 24 or 48 hr, resulted in significant decreases in inactivation of inhaled group C streptococci. Mortality studies following pollutant exposure demonstrated earlier, but not an increased number of deaths. These studies demonstrate the importance of the test organism in assessing air quality standards with the infectivity model and enhanced toxicity with prolongation of exposure to relatively low levels of submicron-size particles of $FeSO_4$ and NO_2.

MUCH CONTROVERSY exists concerning the risk of exposure to high ambient levels of submicron-size sulfate (SO_4^{2-}) particles. Some epidemiologic studies indicate that these exposures increase the frequency and severity of respiratory infection.[1-3] In contrast, other studies have failed to demonstrate increases in respiratory infection following exposure to these pollutants.[4,5] Because of the complexity of these studies, definitive epidemiologic evidence concerning the toxicity of ambient concentrations of SO_4^{2-} is unlikely in the near future.

Although extreme caution is necessary when extrapolating data from experiments conducted in animals to instances of human exposure, such investigations do allow qualitative inferences regarding toxicity. In particular, rodent models of infection permit direct testing of the relationship of pollutant exposure to pulmonary susceptibility to infection.[6,7] Studies with these models have confirmed the ability of ozone,[8-10] nitrogen dioxide (NO_2),[11-13] sulfuric acid,[14] and combinations of these pollutants[15,16] to enhance susceptibility to infection at concentrations approximating those associated with physiologic abnormalities in humans.

Recent studies with this rodent model have shown that brief, 3-hr exposures to high concentrations (1200 $\mu g/m^3$) of submicron-size [mass median diameter (MMD) = 0.63 μm] zinc sulfate particles increase susceptibility to aerosol challenge with virulent group C streptococci.[17] More prolonged 17-hr exposures to atmospheres containing near ambient concentrations of 100-150 $\mu g/m^3$ of 2.1-2.5 μm sized iron sulfate particles did not alter pulmonary susceptibility to infection.[18] Present studies extended these observations by determining the effect of 24-48 hr exposures to atmospheres containing 200-400 $\mu g/m^3$ of submicron-size SO_4^{2-} particles [mass median aerodynamic diameter (MMAD) = 0.4 μm] on the ability of the murine lung to withstand bacterial infection. Because SO_4^{2-} particles often coexist with NO_2 in pol-

luted atmospheres, additional studies were undertaken to test the effect of exposure to both pollutants on murine susceptibility to bacterial infection. This report documents the results of these investigations.

MATERIALS AND METHODS

Animals. Male, Swiss Webster chronic respiratory disease-free albino mice weighing 21 to 30 g were used in these experiments. The animals were housed in filter-topped metal cages and fed food and water ad libitum. Nutriments were present during the exposure periods, and the animals were weighed prior to and following exposure to the pollutants.

Pollutant exposure. The facilities used to test the effect of exposure to fixed concentrations of air pollutants are described elsewhere.[12,19] Briefly, animals were placed in type 304 open mesh stainless steel cages within the inhalation chambers and then exposed to atmospheres containing NO_2, $FeSO_4$, NO_2 and $FeSO_4$, or filtered air for 24 to 48 hr prior to, or 4 hr after, infection with *Staphylococcus aureus* or group C streptococci. No pollutant-induced effect was observed on the stainless steel cages; the amount of NO_2 recovered by the sampling methods used correlated very closely with the calculated NO_2 concentrations delivered, indicating a negligible interaction between NO_2 and the stainless steel cage material. Exposure chambers were maintained at a temperature of 24°C with a relative humidity of 40%. The desired concentration of NO_2 was attained within the chamber by metering the flow of NO_2 from cylinders containing 0.21% NO_2 in nitrogen mixed with the filtered chamber air. All outside air drawn into the inhalation chamber passes through high efficiency particulate and activated charcoal filters. Attainment of the desired NO_2 concentration within the chamber was validated by continuously measuring and recording chamber levels with a chemiluminescent oxides of nitrogen analyzer (Thermoelectron Corporation). Samples of chamber air were obtained at random intervals for further verification of the concentration using the method of Saltzman.[20]

Submicron-size aerosols were generated with a Babington type nebulizer (Solo Sphere, McGaw Respiratory Therapy) using dilute solutions of $FeSO_4$ (0.6 g/L $FeSO_4 \cdot 7H_2O$). The nebulizer was immersed in an ice-water bath during operation to diminish evaporation from the solution. The aerosol was diluted with clean dry air to decrease the relative humidity. A ^{85}Kr discharger reduced the aerosol electrostatic charge to Boltzmann equilibrium.[19] The Chamber's SO_4^{2-} concentration was determined by capturing the iron sulfate droplets from known volumes of air passed through a 0.4 μm Nuclepore filter, washing with 1.0 N HCl, and measuring the amount of iron in the wash fluid by a spectrophotometric assay using ferrozine,[21] and by atomic absorption spectrophotometry using an iron lamp.[22] Sulfur concentrations were calculated by assuming a one-to-one ratio of iron and sulfur on the Nuclepore filters. This relationship has been confirmed previously in a similar system by particle-induced X-ray emission spectra.[18]

The aerodynamic size distribution of particles within the exposure chamber was determined by drawing the contaminated air through a cascade impactor and measuring the amount of iron on the individual glass stages and on the after filter. The values from each sample set of data were graphed in a log-normal distribution to determine the MMAD and geometric standard deviation (σg). Continuous measurements of SO_4^{2-} particles were made throughout the exposure period with an optical particle counter (Royco 245). A thermometer and hygrometer recorded temperature and relative humidity.

Infection schedules. The methods used for determining bacterial clearance or killing rates within murine lungs have been published elsewhere.[23,24] In the first series of experiments, mice were exposed to atmospheres of NO_2 and SO_4^{2-} for 24 hr prior to, or 4 hr after, infection with finely dispersed aerosol of *S. aureus*. After infection equal numbers of control and treated mice were sacrificed with ether. The left lung was removed aseptically and the number of viable bacteria was determined by pour plate techniques. The right lung was perfused with buffered formalin and examined for pathologic changes. After 4 hr, equal numbers of remaining untreated and treated mice were sacrificed. The numbers of viable bacteria and the pathologic status of lungs were again determined using the same techniques.

Rates of bacterial clearance or killing were calculated by comparing the mean bacterial count for each group of mice at each time period according to the following formula: % bacterial clearance = $(N_o - N_t/N_o) \times 100$, where N_o and N_t were mean bacterial counts for mice sacrificed at 0 or t hours. These data were analyzed by the Theorem of Wilks for significance of difference.[25]

In the second series of experiments mice were exposed for 24 and 48 hr to atmospheres containing SO_4^{2-} particles, NO_2, or combinations of these pollutants, and then infected with aerosols of a virulent group C streptococcus. Rates of bacterial clearance were compared in mice exposed to polluted atmospheres and in controls at 0, 8, 24, and 48 hr after aerosol infection. The proliferation of group C streptococci within the lungs of normal mice invalidates analysis by the Theorem of Wilks. Differences between control and pollutant-exposed animals were analyzed by ranking the numbers of bacteria located in the lung with the Mann-Whitney U test.[26] In addition to these comparisons, the frequency of streptococcal proliferation, defined as the presence of greater numbers of intrapulmonary streptococci than the mean value in 0 hr animals, was compared for treated and control mice at each post-infection time period. These results were tested for significance by an adjusted chi-square (χ^2) analysis.[27] Finally, mortality rates were determined during a 6-day period following streptococcal infection in mice previously exposed to polluted or control atmospheres, and were also analyzed by the adjusted χ^2 test. Bacterial clearance and mortality rates were determined in two ore more experiments for each pollutant exposure.

RESULTS

In each experiment approximately 10^8 bacterial particles of respirable size (1 to 3 μm diameter) were present in each cubit foot of air. Mice inhaled 1 to 5×10^5 bacteria

Table 1.—Effect of Exposure to Ferrous Sulfate and Nitrogen Dioxide on the Number of Group C Steptococci Cultured from Murine Lungs following Aerosol Infection

Exp. Group	Exposure	Time following Infection: O hr		8 hr		24 hr		48 hr	
$FeSO_4$	290 $\mu g/m^3$* – 24 hr	40†	(22)‡	1.7	(8)	8.7§	(21)	39	(14)
Control		42	(22)	1.1	(8)	2.4	(21)	37	(14)
$FeSO_4$	310 $\mu g/m^3$ – 48 hr	23	(21)	0.3	(16)	7.9§	(22)	260§	(13)
Control		25	(22)	0.5	(16)	1.6	(22)	2.7	(14)
NO_2	1.0 ppm – 24 hr	43	(8)	1.9	(8)	7.8	(8)	9	(8)
Control		41	(8)	0.9	(8)	6.2	(8)	10	(8)
NO_2	1.0 ppm – 48 hr	41	(8)	2.0	(8)	8.8§	(8)	100§	(8)
Control		45	(8)	2.3	(8)	1.8	(8)	9.4	(7)
$FeSO_4$-NO_2	290 $\mu g/m^3$; 1.0 ppm – 24 hr	55	(28)	3.3	(22)	18§	(24)	94§	(14)
Control		70	(31)	1.9	(22)	2.2	(23)	6.6	(14)
$FeSO_4$-NO_2	295 $\mu g/m^3$; 1.0 ppm – 48 hr	61	(30)	3.8§	(14)	14§	(21)	57§	(16)
Control		86	(30)	1.3	(14)	2.2	(22)	4.7	(16)

*Mean pollutant concentration.
†Geometric mean of number of bacteria cultured × 10^3.
‡Number in parentheses is number of animals studied.
§$P < .05$ Mann Whitney U rank test.

during the 20-min exposure period.

Mice exposed to atmospheres containing NO_2 and SO_4^{2-} for 48 or 24 hr prior to, or 4 hr after, infection with *S. aureus* had similar rates of bacterial inactivation [48 hr, 77%; 24 hr, 81%; 4 hr, 76% ($P > .05$)] to those of controls [48 hr, 80%; 24 hr, 85%; 4 hr, 74% ($P > .05$)] for each comparison. Sulfate concentrations varied from 270 to 396 $\mu g/m^3$ (mean ($\bar{X}$) = 317 $\mu g/m^3$) with particle sizes of 0.4 μm MMAD and a corresponding σg of 2.8. The NO_2 concentrations varied from 0.89 to 0.99 ppm.

Table 1 shows the effect of exposure to 206-412 $\mu g/m^3$ ($\bar{X}$ = 292 $\mu g/m^3$) of approximately 0.4 μm MMAD SO_4^{2-} particles on the capacity of the murine lung to kill inhaled group C streptococci. The values for SO_4^{2-} concentration

Table 2.—Effect of Exposure to Ferrous Sulfate and Nitrogen Dioxide on the Fate of Subsequently Inspired Group C Streptococci

Exp. Group	Exposure	8 hr: Clear*	Pro‡	24 hr: Clear*	Pro‡	48 hr: Clear*	Pro‡
$FeSO_4$	290 $\mu g/m^3$† – 24 hr	8	0	17	4	10	4
Control		8	0	21	0	9	5
$FeSO_4$	310 $\mu g/m^3$ – 48 hr	16	0	18	4	4	9§
Control		16	0	22	0	14	0
NO_2	1.0 ppm – 24 hr	8	0	8	0	8	0
Control		8	0	7	1	7	1
NO_2	1.0 ppm – 48 hr	8	0	8	0	6	2
Control		8	0	8	0	7	0
$FeSO_4$-NO_2	290 $\mu g/m^3$; 1.0 ppm – 24 hr	22	0	19	5	5	9§
Control		22	0	23	0	13	1
$FeSO_4$-NO_2	295 $\mu g/m^3$; 1.0 ppm – 48 hr	14	0	19	2	13	3
Control		14	0	22	0	15	1

*Number of mice with streptococcal concentration below 0 hr.
†Mean pollutant concentration.
‡Number of mice with streptococcal concentration above 0 hr.
§$P < .01$ (adjusted chi-square test).

and particle size were obtained by the ferrozine assay. The ferrozine and atomic absorption assays agreed within 20% of each other. Because these data were analyzed by the Mann-Whitney *U* rank test, geometric mean values are listed in the Table. The concentrations of inspired steptococci were reduced by more than 90% in control and treated mice 8 hr after infection. Higher concentrations of streptococci were observed in almost all experimental groups 24 hr after infection. This increase was significantly greater in mice previously exposed to polluted atmospheres ($P < .05$). The concentrations continued to increase in the 24- to 48-hr post infection period with larger increases again occurring in the treated mice.

Because of the propensity of group C streptococci to proliferate in some, but not all mice, comparisons of streptococcal clearance and proliferation for the two experimental groups at each time period are shown in Table 2. Bacterial clearance was defined as a pulmonary bacterial concentration less than the mean bacterial concentration of mice sacrificed at 0 hr, while bacterial proliferation was defined as a concentration exceeding that of the mean 0 hr concentration. Streptococcal clearance occurred in all animals 8 hr after infection (Table 2). At 24 hr after infection, proliferation seldom occurred in control mice (1/104 mice). Although not statistically significant for individual comparisons ($P > .05$), proliferation was more common 24 hr after infection in mice previously exposed for 24 or 48 hr to atmospheres containing only SO_4^{2-} particles or in combination with 1.0 ppm of NO_2. This difference between treated and control mice was even more pronounced 48 hr after infection. At this post infection period, streptococcal proliferation occurred in 25 of 57 mice exposed to atmospheres containing SO_4^{2-} compared to 7 of 58 controls. The differences were significant at the $P < .01$ level for comparisons of the 48-hr SO_4^{2-} pre-exposure and the 24-hr NO_2-SO_4^{2-} pre-exposure and their appropriate controls.

Figure 1 depicts the mortality data for mice exposed for 24 or 48 hr to atmospheres containing NO_2 and $FeSO_4^{2-}$ alone and in combination. Although overall mortality was unchanged, mice exposed to 1.0 ppm NO_2 for 48 hr or 260-340 $\mu g/m^3$ SO_4^{2-} (MMAD = 0.4 μm) for 24 to 48 hr died sooner than did controls. These differences were most apparent in mice exposed to both pollutants with the mean time to death being 3.3 and 3.2 days for animals exposed to both SO_4^{2-} and NO_2 for 24 and 48 hr, as compared with 4 and 3.5 days, respectively, for comparable controls.

Histological examination of pulmonary tissues did not reveal structural abnormalities or pneumonia in dead animals or those dying of streptococcal infection. Cultures of blood, lung, liver, and spleen from dying or deceased animals contained large numbers of streptococci, confirming that death was associated with widespread septicemia, as well as pulmonary infection.[13]

DISCUSSION

These experiments demonstrate that exposure to 206-412 $\mu g/m^3$ of submicron-size SO_4^{2-} particles or 1.0 ppm of NO_2 for 48 hr prior to aerosol infection increases the propensity of virulent group C streptococci, but not minimally virulent *S. aureus*, to proliferate within murine lungs. Consequently, the streptococcal mortality curve is shifted with deaths occurring earlier than is normal during the 6-day post infection period.

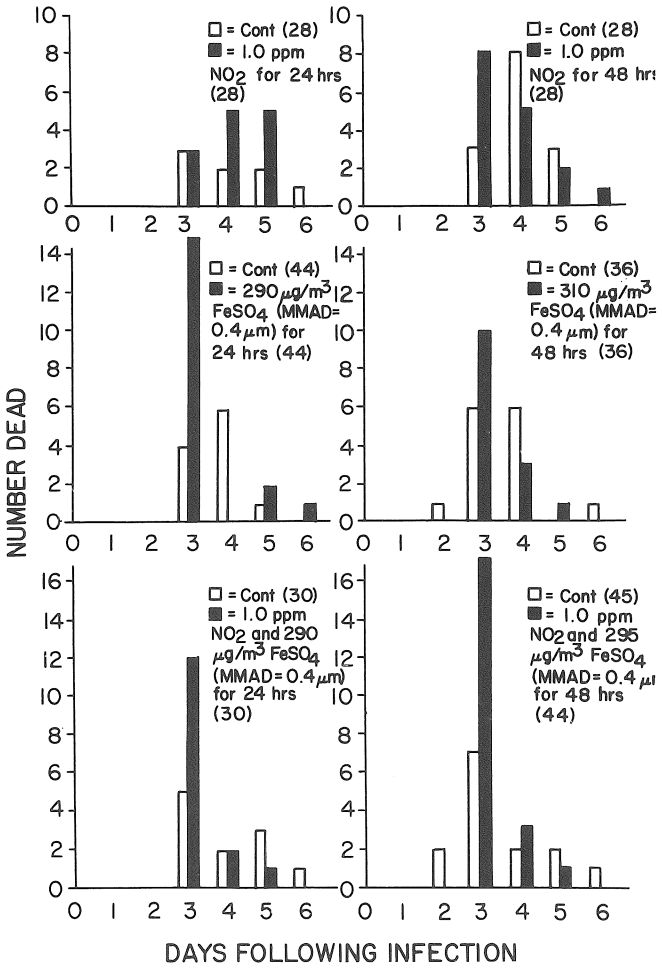

Fig. 1. Mortality following aerosol infection with group C streptococci in mice exposed for 24 or 48 hr to ferrous sulfate and nitrogen dioxide. Numbers in parentheses indicate number of mice treated.

These studies also indicate that exposure to submicron-size SO_4^{2-} particles and NO_2 may interfere with defense mechanisms other than the alveolar macrophage system. This conclusion is based on the following observations. The rate of killing of *Staphylococcus aureus*, a bacteria that is rapidly inactivated by alveolar macrophages, was unimpaired in mice exposed to polluted atmospheres.[24,28] Similarly, the initial killing of the group C streptococcus, a parameter that is also mediated by alveolar macrophages, was unaltered in control and exposed mice.[29] Unlike the minimally virulent staphylococcus, some group C streptococci survive this initial period of killing and proliferate 48 to 96 hr following infection.[9,18] The eventual eradication of these bacteria probably depends on secondary defense mechanisms, e.g., influxes of new phagocytes,[28-30] Although our studies do not identify a specific defect in these defense mechanisms, the finding that proliferation is common in mice exposed to pollutants 48 hr after infection, whereas such proliferation usually occurs 72 hr after

infection in untreated mice, suggests a pollutant-induced defect in these defenses.

The finding that exposure to submicron-size SO_4^{2-} particles and NO_2 permitted earlier proliferation of intrapulmonary streptococci provides an explanation for the premature deaths of the pollutant-exposed mice. In untreated mice, mortality from group C streptococcal infection correlates with elevated pulmonary bacterial counts and septicemia, with death usually occurring from 48 to 96 hr after aerosol infection.[9] Exposure to pollutants advanced this sequence of bacterial proliferation, septicemia, and death. Because overall mortality was unchanged by exposure to the pollutants, the earlier deaths probably occurred in mice which would have eventually succumbed to the bacterial infection.

The effect of exposure to atmospheres containing both submicron-size SO_4^{2-} particles and NO_2 was equivalent to the sum of that which would be expected from each individual pollutant acting alone. Exposure to both pollutants for 24 or 48 hr prior to infection caused an increase in earlier deaths, but not an overall increase in deaths. These increases are attributed to the SO_4^{2-} particles causing premature deaths in mice exposed for 24 hr prior to infection, and the NO_2 or SO_4^{2-} causing premature deaths in mice exposed for 48 hr prior to infection. Comparison of the mortality data shows that at both time periods the percentage increase in premature deaths due to the combined exposure is similar to the percentage increase noted for the individual pollutant at that time period.

These studies also confirm the importance of particle size in assessing the toxicity of air pollutants.[31,32] In an earlier study performed in this laboratory, 17-hr exposure to 100-150 $\mu g/m^3$ SO_4^{2-} (MMAD = 2.1-2.5) did not enhance the capacity of group C streptococci to proliferate within murine lungs.[18]

The following arguments indicate that the difference between the present results and previous ones probably reflects the use of submicron-size particles rather than slightly higher SO_4^{2-} concentrations (i.e., 300 $\mu g/m^3$) or longer exposure periods (24-48 hr). The amount of SO_4^{2-} deposited within murine lungs can be calculated by multiplying the exposure concentration X the volume of air inhaled X the percent of SO_4^{2-} aerosol assumed to be deposited for a specifically sized particle. Guyton's formula was used to determine the volume of air inhaled,[33] and the percent deposition was assumed to be 14 to 18% for a 0.4 μm particle.[34] Using these values, pulmonary burdens were 1.6 to 2.0 μg SO_4^{2-} for the 24-hr exposures. These amounts are 7 times higher than those calculated for the 17 hr exposures to micron-size SO_4^{2-} particles. An additional factor favoring the importance of the submicron size is the likelihood that these particles were deposited more deeply within the lungs, thus minimizing their removal by physical transport mechanisms.[34]

This study, together with that of Ehrlich et al.,[17] supports the need for an air quality standard for submicron-size sulfate particles. Ehrlich et al.'s[17] study demonstrated a threshold level for murine mortality of 1200 $\mu g/m^3$ for a 3-hr exposure to submicron-size particles. The present study shows that 24- or 48-hr exposures to 300 $\mu g/m^3$ of submicron-size SO_4^{2-} particles result in a reduced murine capacity to kill inspired, virulent bacteria and an earlier but unchanged mortality. Because the concentration of SO_4^{2-} was 10 times higher than the air quality standard of 25 $\mu g/m^3$ for a 24-hr period,[35] our results indicate that insofar as data obtained in this experimental model can be extrapolated to man, the present air quality standards are probably protective against respiratory infection.

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