Use of Experimental Airborne Infections for Monitoring Altered Host Defenses

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The success or failure of the respiratory system to defend itself against airborne infectious agents largely depends upon the efficiency of the pulmonary defenses to maintain sterility and to dispose of unwanted substances. Both specific and nonspecific host defenses cooperate in the removal and inactivation of such agents. Several studies have shown that these defenses are vulnerable to a wide range of environmental agents and that there is a good relationship between exposure to pollutant and the impaired resistance to pulmonary disease. There are numerous immunological, biochemical and physiological techniques that are routinely used to identify and to characterize individual impairments of these defenses. Based on these effects, various hypotheses are proposed as to what health consequences could be expected from these effects. The ultimate test is whether the host, with its compromised defense mechanisms, is still capable of sustaining the total injury and continuing to defend itself against opportunistic pathogens. This paper describes the use of an experimental airborne infectious disease model capable of predicting subtle changes in host defenses at concentrations below which there are any other overt toxicological effects. Such sensitivity is possible because the model measures not just a single “health” parameter, but instead is capable of reflecting the total responses caused by the test chemical.

Introduction

The lung, being a prime target organ to environmental insults, has been shown to be vulnerable to a wide range of noxious substances, some of which can cause serious dysfunction of normal respiratory (gaseous exchange) and/or nonrespiratory (host defenses, metabolism) functions of the lung. Such adverse effects would be expected to have numerous ramifications on the host and increase the unwanted risk of disease.

Under normal conditions the lung has the capability to maintain pulmonary sterility and disposal of unwanted substances by constantly removing or inactivating any such agents that are deposited in the respiratory tract. This is usually successfully accomplished through the cooperative efforts of both specific and nonspecific host defenses. A major challenge for the pulmonary toxicologist is to identify and to describe individually each and every subtle impairment of these pulmonary defenses caused by the inhaled chemicals. Such a comprehensive assessment, although relevant, would be costly, time consuming, difficult for routine toxicological testing and may not individually be sensitive enough to provide the necessary data base for establishing safety of the test compound.

The purpose of this discussion is to describe a much simpler approach for monitoring such subtle changes using a whole animal model and to illustrate the validity and the use of such a biological screening system for standard toxicological testing. The success of this test system depends upon detecting the ability of the host to cope with the total injury induced by the inhaled substance. With this testing procedure, animals with normal pulmonary defenses are quite capable of defending the lung against such assaults, but significant alterations in any of the several pulmonary defenses would be
expected to prolong microbial viability and to enhance the establishment of the potentially hazardous microorganisms, thereby increasing the risk of pulmonary disease.

The Microorganisms

Although a wide range of infectious agents, both bacterial and viral, has been successfully employed by various investigators, we primarily use a non-human pathogen (*Streptococcus pyogenes*, group C) that was originally isolated from a spontaneously infected pharynx of a guinea pig in our laboratory. These bacteria are maintained either lyophilized or on brain-heart infusion agar and are periodically passed in mice to maintain their virulence. Prior to the day of the test, the organism is grown for 24 hr in Todd-Hewitt broth, and the concentration of organisms adjusted spectrophotometrically so that approximately $1 \times 10^9$ organisms per exposure are aerosolized. Quantitative studies of viable agents have illustrated that such organisms may undergo considerable destruction during the aerosolization processes (1). As would be expected, the degree of lability varies with the microorganism. Factors responsible for this destruction may include shearing force, impaction, agitation, dehydration, and possibly some oxidative effects occurring during aerosolization.

If the infectious model system is to be an effective screen for determining the adequacy of the host's pulmonary defenses, the infectious agent must fulfill certain criteria. It must remain alive and infectious in aerosol; be deposited and capable of multiplying in susceptible tissue; have minimal influence on the mortality rate when small variations in dose or virulence occur; have low natural mortality in the normal host; have a method available for quantifying the bacteria dose; elicit reproducible quantitative data; and be nonpathogenic for humans for safety reasons.

Experimental Airborne Infection

The exposure methodology for this test system is shown schematically in Figure 1. Test animals are

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**Figure 1.** Schematic drawing of infectivity model system. Animals are exposed to either clean air or test chemical. After this initial exposure the two groups are combined and exposed to an infectious microorganism. The data is expressed as percent mortality between the test groups.
randomly selected to be exposed to either clean air or to the test substance. The operational procedures for this exposure, including the generation of exposure atmospheres, sampling and characterization methodology, and experimental design for delivering the airborne test materials, will be dictated by the objectives of the particular toxicologic investigation. The duration of the exposure and the mode of exposure, i.e., whole body, head only, nose or mouth only or lung only, can all be employed in this model system.

The methods developed for operation of the infectious exposure chamber and the exposure of animals should be designed to insure the safety of the scientific personnel. It should be remembered that many microorganisms are potentially pathogenic and that standard microbiological safety techniques must be employed in these operating procedures.

At various times after the exposure to the test chemicals, both the pollutant exposed and control animals are combined and placed in another chamber for a 15 min exposure to an aerosol of infectious organisms. A flow diagram of the infectivity chamber is given in Figure 2.

**Atomization of Infectious Organisms**

The production of an evenly dispersed cloud of infectious agents under controllable conditions is fundamental for experimental studies of airborne infection. Considerable research has been conducted concerning the methodology for atomization of the infectious organisms (2-4). Atomizers are devices used to produce a continuous finely divided particle or droplet from a liquid suspension or solution. Although a number of different types of generators are available to disperse liquids to form aerosols (spinning disc atomizers, ultrasonic nebulizer, and vibrating orifice generators), we have found the DeVilbiss No. 40 compressed air nebulizer (5) to be highly efficient (Fig. 3).

These generators atomize a liquid by moving a blast of air across a narrow nozzle into which liquid has been drawn by the Bernoulli effect. Large droplets impinge on the upper wall of the exit duct while the smaller droplets remain aerosolized. The size range of the primary droplets is from 1 to 7 μm with a MMD of 1.3. The outputs are in the order of 1–30 μl of fluid/liter of air. A 5-ml portion of the fluid containing the infectious organisms is atomized over a 15-min period. Air continues to pass through the "dried" nebulizer for an additional 5 min (air wash) before the test animals are removed from the cham-

Figure 2. Diagram of the chamber exposure system for inhalation exposure to an infectious microorganism (Rochester type).

Figure 3. DeVilbiss No. 40 compressed air nebulizer for generation of microbial aerosol.
ber. In order to maximize the reduction in variability in total dose deposited in the respiratory tract, the animals are randomized in stainless steel mesh racks which individually partition animals. This prevents huddling and aids in insuring that each animal receives as nearly as possible, the same number of viable organisms.

The measurement of the concentration of microorganisms within the chamber fails to adequately define the actual viable dose delivered to the target organ. Therefore it is important to measure pulmonary deposition of the viable agents. This can be accomplished if, immediately following this aerosol exposure, two to four animals from each test group are sacrificed and the lung and trachea removed and completely homogenized in sterile broth. Aliquots of the homogenized sample are cultured in triplicate on blood agar plates and total number of colony-forming units (cfu) of viable organisms determined by standard microbiological techniques. The same techniques can be used at other time intervals during the experiment to provide comparison data on in vivo clearance (i.e., bacteriocidal activity) mechanism of the various treatment group.

For maximum sensitivity, it is important to maintain a low mortality rate in the control group. In this way, a slight shift in the functioning of the host defenses can be measured in the terms of enhanced mortality. However, day to day variation in the number of organisms initially deposited in the lung can be routinely expected. For this reason it is necessary to know the relationship between number of viable organisms deposited and the resulting mortality. This can be accomplished by examining the cumulative data from all of the control mice. Figure 4 illustrates, that with our organism, one can expect that, within the range of 200–4000 cfu/lung, there is no apparent correlation between number of bacteria deposited in the lung and resulting increase in percent mortality. However, at higher levels of bacterial deposition one can expect that the observed mortality will increase further (6,7).

After receiving the bacterial challenge, the test animals are separated into their appropriate treatment groups and maintained in clean air for 15 days, during which time the mortality rates are determined. With streptococci, the typical expected mortality rate in the control group ranges from 10–30%. This mortality reflects the natural susceptibility of the host and the virulence of the test microorganisms.

### Selection of Test Animals

To maintain the health of the individual test animal, many of the host's available defenses must be able to act rapidly and efficiently if they are to limit the extent of the induced laboratory infection. In the resistant animal, the host defenses are functionally capable of quickly reducing the activities of the organism in the tissue. The unhampered lung, with all of its bactericidal capability can, within hours, restore the sterility of its environment (8-10).

![Figure 4](image-url) **Figure 4.** Correlation between the number of viable organisms deposited in the lungs of control mice and the resulting percent mortality. (●), (○), and (□) represent the data from experiments which used 100, 40 and 30 control mice, respectively (7). The correlation coefficient (0.002) shows no relationship between these variables.

![Figure 5](image-url) **Figure 5.** Difference in mortality in mice, hamsters and squirrel monkeys exposed to various concentrations of NO₂ for 2 hr and subsequently challenged with an infectious agent (15).
The ability to resist infection with any given organism is variable not only between species but also within species. Although we use primarily 4–6-week-old female CD-1, COBS mice, a variety of other animal species have been employed with the infectivity model system including the squirrel monkey, rat, hamster, guinea pig, and mouse (11-14). The choice of the species may vary with the nature of the information sought. A degree of caution is warranted when one tries to use this model system for comparing the effects of a given chemical on different species of animals. Figure 5 shows the response of mice, hamsters and squirrel monkeys to exposure to NO₂ and subsequently challenged with Klebsiella pneumoniae aerosol (15). These data would suggest that either the squirrel monkeys are more resistant to NO₂ than mice, since it took significantly more NO₂ to produce the observed effect, or they are less susceptible to that particular infectious agent. Such information is essential in regulatory settings. However, when one compares the mortality rate within the control animals, it indicates that the animal's natural resistance to the bacterial pneumoniae was quite different for the different species. The respective mortality rates in the control mice, hamsters and monkeys were 41%, 11% and 0%. This illustrates the importance of the selection of the proper organism for testing as well as indicating that one needs to be careful in interpreting the data.

Difference in natural resistance to the airborne infection has also been seen within different strains of mice (15). Table 1 shows the mortality in four different strains of mice after exposure to NO₂ and challenged with Klebsiella pneumoniae aerosol.

### Table 1. Mortality in different mouse strains after exposure to 5 ppm NO₂ and challenge with Klebsiella aerosol.

| Strain       | Air          |  | NO₂          |  | Change, % |
|--------------|--------------|---|--------------|---|-----------|
|              | D/T          | % | D/T          | % |           |
| BDF (black)  | 31/120       | 25.8<sup>b</sup> | 40/120       | 33.3 | 7.5<sup>b</sup> |
| C57BL (black)| 24/70        | 34.3<sup>c</sup> | 36/70        | 51.4 | 17.1<sup>c</sup> |
| Swiss (albino)| 164/390     | 42.1<sup>1</sup> | 262/390      | 67.2 | 25.1<sup>d</sup> |
| Balb (albino)| 48/100       | 48.0<sup>e</sup> | 72/100       | 72.0 | 24.0<sup>d</sup> |

<sup>a</sup>Means in a column with the same superscript are not significantly different (p ≤ 0.05).

<sup>b</sup>Significantly different from controls (p ≤ 0.05).

### Statistical Considerations

Since there is variability between replications of the individual experiments due to both number and the virulence of the viable microorganisms aerosolized and subsequently deposited within the lung, it is important that the data be examined using sound statistical analysis for reducing the experimental error. This can be achieved by expressing the data for each replication as the difference in mortality between the chemically exposed group and the air controls or by probit analysis using a correction for natural mortality. Table 2 provides an example, using our system, of the recommended number of replications necessary to detect percent mortality differences based on the number of mice per group. The clearance of the microorganisms from the lung can be analyzed by an analysis of variance which accounts for the assay error of the microbiological technique.

### Effects of Airborne Metals and Particulates

Evidence exists which indicates that in vitro exposure to a number of trace metals can significantly alter the basic functioning of several host defense systems (16-20). In many cases, these insults can be correlated to significant increases in susceptibility to infection. Inhalation of metals such as Ni and Cd affects not only the phagocytic and enzymatic activity of the alveolar macrophages, but these metals also affect the primary humoral immune system (21-24).

Figure 6 shows the dose-response relationship of various trace metals when tested in the infectivity system. It is important to notice that neither Fe₂O₃ nor carbon dust produced any significant effects. This indicates that the model system can distinguish between particulates which are inert and
with the coal fly ash. In these studies, Aranyi et al. (25) were successful in correlating the mortality effects with some adverse effects on alveolar macrophages that could account for this increase in susceptibility.

**Effects of Gaseous Pollutants**

In the past, a number of investigators had reported that during chronic inhalation studies there was a significant increase in spontaneous pneumonia in the animals being exposed to gaseous test substances (26,27). These responses were substantiated under controlled laboratory experimentation where individual gases such as O₃ and NO₂ as well as complex mixtures such as irradiated auto exhaust, H₂SO₄ and O₃; O₃ and NO₂; O₃; SO₂ and hydrocarbons; were tested using the infectivity systems (28-32).

The effects of various exposure regimens have also been investigated. For example, using the those that are potentially toxic. In this study, the toxicity ranking for these metals was Cd > Ni > Mg. Ehrlich (15) also used this model system for comparing the toxicological effects of a number of sulfate compounds. From his studies, he estimated that concentrations of the compound (3 hr exposure) which produced a 20% excess in mortality over controls were: 0.4 mg/m³ CdSO₄, 1.0 mg/m³ CuSO₄, 2.5 mg/m³ ZnSO₄, 2.6 mg/m³ Al₂(SO₄)₃ and 3.8 mg/m³ Zn(NH₄)₂(SO₄)₂. Concentrations as high as 8 mg/m³ of NH₄NO₃, KNO₃, NH₄HSO₄, (NH₄)₂SO₄, and Na₂SO₄ did not result in any significant changes in mortality.

In addition to testing of pure compounds, this system has also been used to evaluate "real world" industrial particulates collected from copper smelter and from a fluidized-bed coal fired power plant (25). Mice were exposed for 5, 10, or 20 daily 3-hr (5 days per week) exposures prior to receiving the streptococcal infection. Figure 7 illustrates the excess mortality observed in the copper sample as compared

**Figure 6.** Increase in susceptibility to pulmonary infections following a 2 hr exposure to various airborne particulates.

**Figure 7.** Effects of multiple daily 3 hr exposure to aerosols of copper smelter dust or coal fly ash No. 2 on susceptibility to respiratory streptococcus infection (25).

**Figure 8.** Time necessary to elicit a 20% mortality in mice versus concentration of NO₂. The regression shows that concentration-time relationship for mortality can be represented by a straight line on a log-log scale.

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Table 3. Percent excess mortalities in mice exposed for 3 hr to pollutants and challenged with streptococcus over corresponding infected controls.

| NO₂ concentration, mg/m³ (ppm) | O₃ concentration, mg/m³ (ppm) | 0 | 0.1 (0.05) | 0.2 (0.1) | 0.98 (0.5) |
|---------------------------------|---------------------------------|---|------------|------------|------------|
| 0                               | 0                               | 5.4| 7.2        | 28.6*      |
| 2.82 (1.5)                      | -1.7                            | 4.6| 4.2        | 23.9*      |
| 3.76 (1.5)                      | 14.3*                           | 22.0*| --         | 56.2*      |
| 6.58 (1.5)                      | 25.2*                           | -- | 38.5*      | 68.7*      |
| 9.40 (1.5)                      | 35.7*                           | -- | --         | 65.3*      |

*Significant change in mortality over controls (p < 0.05).

enhancement of mortality as the biological endpoint for measuring the toxic effects of NO₂, studies have been conducted which varied the exposure time from a few minutes to several months (33). Different concentrations ranging from 0.94 mg/m³ to 526 mg/m³ (0.5–28 ppm), different exposure modes (i.e., continuous vs. intermittent) and various concentrations × time effects were evaluated. Figure 8 is a concentration-time (C × T) curve using the endpoint of a 20% mortality enhancement with this model system. The regression obtained shows that the C × T relationship for mortality can also be represented by a straight line on a log-log scale when the exposures were continuous.

The infectivity model system has also been employed to evaluate the effects on the dose-response relationship when two gases were combined (30). In this study the animals were exposed for 3 hr to NO₂ alone, O₃ alone, NO₂ and O₃ or clean air. The data in Table 3 clearly indicate that these two gases produce an additive effect since the differences in mortality rates were, in most cases, equivalent to the sum of those resulting from each of the individual pollutants.

Simplifying the Model System

In order to be able to increase the utilization of this model system and to make it more applicable for general screening of a large volume of chemicals, it would be advantageous to simplify the exposure system. Since it has been generally recommended that the test agent be administered by the route that most closely simulates the major route by which human exposure occurs, it is important for toxicological studies of airborne chemicals that the primary target organ continues to be the lung. However, since the use of sophisticated aerosol exposure techniques are extremely costly and are not readily available to most laboratories, it would be useful if an alternative method of exposure were available. The intratracheal injection (IT) method is a convenient, acceptable and easy-to-use approach for testing hazardous substances. This approach has several advantages over inhalation in that relatively small amounts of the test substances are required; delivered dose can be accurately measured; large size particles can be tested; skin contamination is avoided; fewer safety and engineering problems are encountered; large quantities can be introduced into the respiratory system provided the substances are not too toxic; and much less expense is involved than required to establish an inhalation facility for generating and monitoring the test aerosol.

The possibility of combining IT instillation, as the route of exposure of the test substance, prior to the infectious challenge has been tested in our laboratory by Hatch and co-workers (34). This modification would increase the possible utilization of this infectious model system providing there was good correlation between the effects seen following the IT injection and those observed in inhalation studies of the same substances.

In these investigations, 23 compounds were initially tested. Each substance was injected IT in concentrations which were calculated to approximate the dose of the chemical which would be deposited in the lung as reported for inhalation studies. Comparison of the dose-effect curves of these two routes of administration indicates there was a general agreement between results of inhalation and IT injection studies.

Four of the chemicals tested that showed significant increased mortality (> 40%) by inhalation also showed similar mortality increases when the test substance was administered by IT instillation at levels calculated to be equivalent to those achieved during inhalation. Five of the chemicals caused small 20-30% enhancement by intratracheal, but not all produced an effect by inhalation. The remaining 14 compounds which showed no significant effect by inhalation also produced no effect when given by IT. Table 4 gives a representative sample of the data from this study illustrating the correlation between these two methods of administration (34). Although additional testing is required, the present data for
Table 4. Comparison of inhalation and intratracheal routes of exposure on enhancement of bacterial infection.*

| Excess mortality (p ≤ 0.05), % | Chemicals | Lung dosage, µg/mouse lung |
|-------------------------------|-----------|---------------------------|
|                               |           | Inhalation † | Intratracheal |
| > 40                          | CdCl₂     | 0.44         | 0.46          |
|                               | CdSO₄     | 1.12         | 1.30          |
|                               | CuSO₄     | 0.81         | 0.70          |
| 20–30                         | AlNH₄(SO₄)₂| 1.82         | 2.10          |
|                               | ZnSO₄     | 1.40         | 0.7           |
| No effect                     | Ca(NO₃)₂ | 2.29         | 2.10          |
|                               | Carbon black | 2.29 | 3.50 |
|                               | Fe₃O₄ | 1.03         | 3.50          |
|                               | Pb(NO₃)₂ | 3.30         | 5.61          |
|                               | NH₄NO₃   | 3.60         | 2.10          |

*From Hatch et al. (54).
†Estimation of quantities of chemical per lung following inhalation.
†No significant effect with inhalation.

Metals indicate that similar effects on susceptibility to lung bacterial infection can be determined whether the chemicals are deposited in the lung by inhalation or by intratracheal injection. Thus, IT injection appears to be a reliable method for establishing the likelihood of pulmonary toxicity of inhaled chemicals.

Summary

A reliable toxicological test should have the sensitivity to demonstrate a failure in some system to perform a normal function. In the infectivity models, the inability of the animals to defend themselves against opportunistic pathogens is the ultimate test of the host's defenses. Although the alveolar macrophages appear to be the major initial line of defense against these viable agents, there is evidence that local, specific immune responses also occur in the lung. Unfortunately, the actual mechanisms describing the interaction of defense cells and the local humoral and cell-mediated immunity in protecting the host against such infectious insults is not yet clearly defined. In evaluating the usefulness and reliability of this whole animal model for predicting altered host defenses, several things can be considered. First, there is a possibility that a similar effect may occur in man, providing the microorganism present is capable of exploiting any alterations in host defenses and that the concentration of the inhaled pollutant reaching these defenses is sufficient to cause an adverse effect. Secondly, the model can also be used to provide information on the pathogenesis of infectious disease, and aid in understanding how the lung protects itself against various environmental challenges. Finally, because of the unique sensitivity of this infectious model, it is capable of identifying subtle changes at exposure concentrations that do not produce any other overt toxicological effects such as morphological changes, edema or gross physiological alterations. This last item is an important consideration when one is developing and evaluating various tests for possible use in screening applicability. The ideal test should be one that produces few false positives or false negatives, is predictive of human effects and be capable of measuring not just a single host response, but instead capable of reflecting the total responses caused by the test chemical resulting in subtle alterations to many functional systems present in the whole organism.

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