

AEROSOL INFECTION OF MICE WITH MYCOBACTERIA USING A NOSE-ONLY EXPOSURE DEVICE

Liana Tsenova,* M.D., André Moreira,* M.D.,Ph.D.,
Esmeralda Party,** M.S., RBP, Victoria H. Freedman,* Ph.D., and Gilla Kaplan,* Ph.D.

*Laboratory of Cellular Physiology and Immunology

**Laboratory Safety & Environmental Health

The Rockefeller University, New York, New York

ABSTRACT

The effectiveness of an aerosol producing system (In-Tox Products, Albuquerque, NM) commonly used for toxicological studies of chemicals and radioisotopes was evaluated for experimental tuberculosis infection of mice. Previously, experimental tuberculosis of rodents has been accomplished by aerosol infection devices which exposed the entire animal to the aerosol within a chamber. We utilized the In-Tox system to infect animals via the respiratory route with *Mycobacterium bovis* strain Bacille Calmette-Guerin (BCG) as a model for experimental pulmonary tuberculosis. The advantage of the device tested is that exposure to the infectious aerosol is limited to the nose of the animal. The present instrument was found to yield good implantation of mycobacteria into the lungs. The infectious load was reproducible and, with the addition of some filtration, the infectious agent was well contained within the device. Our results showed that this device is easy to operate, produces an effective experimental aerosol infection and is safe for the investigator.

INTRODUCTION

Despite the decline of tuberculosis in the developed world throughout the 20th century, the disease remains an enormous global health problem. While the incidence of tuberculosis in the United States is approximately 9 cases per 100,000 per year, in Asia and Africa the rates approach 110-165 cases per 100,000 per year (Leonard 1990). Tuberculosis is now the world's foremost cause of death from a single infectious agent (Snider 1994). HIV infection, which renders individuals significantly more susceptible to mycobacterial infection, has caused a marked increase in the incidence of tuberculosis.

In order to study the course of tuberculosis infection and the host response it is important to develop experimental animal models of pulmonary tuberculosis. It has been shown that aerosol infection of rodents results in progressive granulomatous disease and persistence of mycobacterial replication (North 1995) indicating the usefulness of this model. Aerosol systems have been previously used to induce experimental respiratory infection in animals with *Bordetella pertussis* (Sato 1980; Oda 1983; Shahn 1990), influenza virus (Schulman 1963; Sullivan 1976; Johansson 1991), and *Mycobacterium tuberculosis* (Wiegeshaus 1970; North

1995). In all these systems, the animal is placed within a chamber and is exposed to aerosolized bacteria. Following exposure to the virulent organisms the animal must be irradiated with ultraviolet light for 30 minutes to disinfect the animal's skin and hair. Snout only or nose only aerosol delivery systems have been previously used to deliver substances such as radioactive materials (Raabe 1973) or pesticides (Newhouse 1978; Ferguson 1982). It has been reported that *Legionella pneumophila* was induced in guinea pigs using an inhalation system that limited delivery of the aerosol to the snout (Davis 1982). However, to date, nose only delivery systems have not been used to induce experimental tuberculosis.

We report here the use of a modified device for aerosol infection of mice (In-Tox Products, Albuquerque, NM), which differs from other previously described devices (Wells 1940, Lurie 1950, Middlebrook 1952). The system tested here is a negative pressure system that exposes only the nose of the animal to the infectious aerosol ensuring a pulmonary infection and reducing the potential spread of infectious organisms.

A protocol for the use of the system was developed and tested by infecting mice via the respiratory route with avirulent *Mycobacterium bovis* strain Bacille Calmette-Guerin (BCG). Once a satisfactory protocol was established, *Mycobacterium tuberculosis* strain Erdman, an infectious virulent strain, was used as a model for experimental pulmonary tuberculosis.

PROCEDURES

FACILITY

The work was carried out at an animal biosafety level 3 (ABSL3) facility equipped with hoods in which animal cages were placed. Following the CDC recommendations for handling cultures with *M. tuberculosis* (CDC/NIH 1993), a Class II, Type A/B3 biological safety cabinet, Model SG 400 (Baker Co., Sanford, Maine) was used as a Type A cabinet in the ABSL3 facility. Ventilation studies of the animal room using a theatrical fog machine (Party 1996) showed that the fog was exhausted through the animal hoods. These hoods had face velocities of about 40 feet per minute (fpm).

AEROSOL SYSTEM

The aerosol producing system consisted of a compressed air nebulizer (Lovelace Nebulizer Model No. 01-100, Style L009, In-Tox Products, Albuquerque, NM), a nose-only exposure chamber, a control panel, a vacuum pump and vacuum surge tank with controls, and a clean compressed air surge tank with controls. In-line 0.2 μm hydrophobic membrane filters (Gelman, Acro 50) were added to the connecting tubes to protect the control box from microbial contamination (FIG 1). The acrylic nebulizer, operated by compressed air, generated a high concentration of aerosol droplets.

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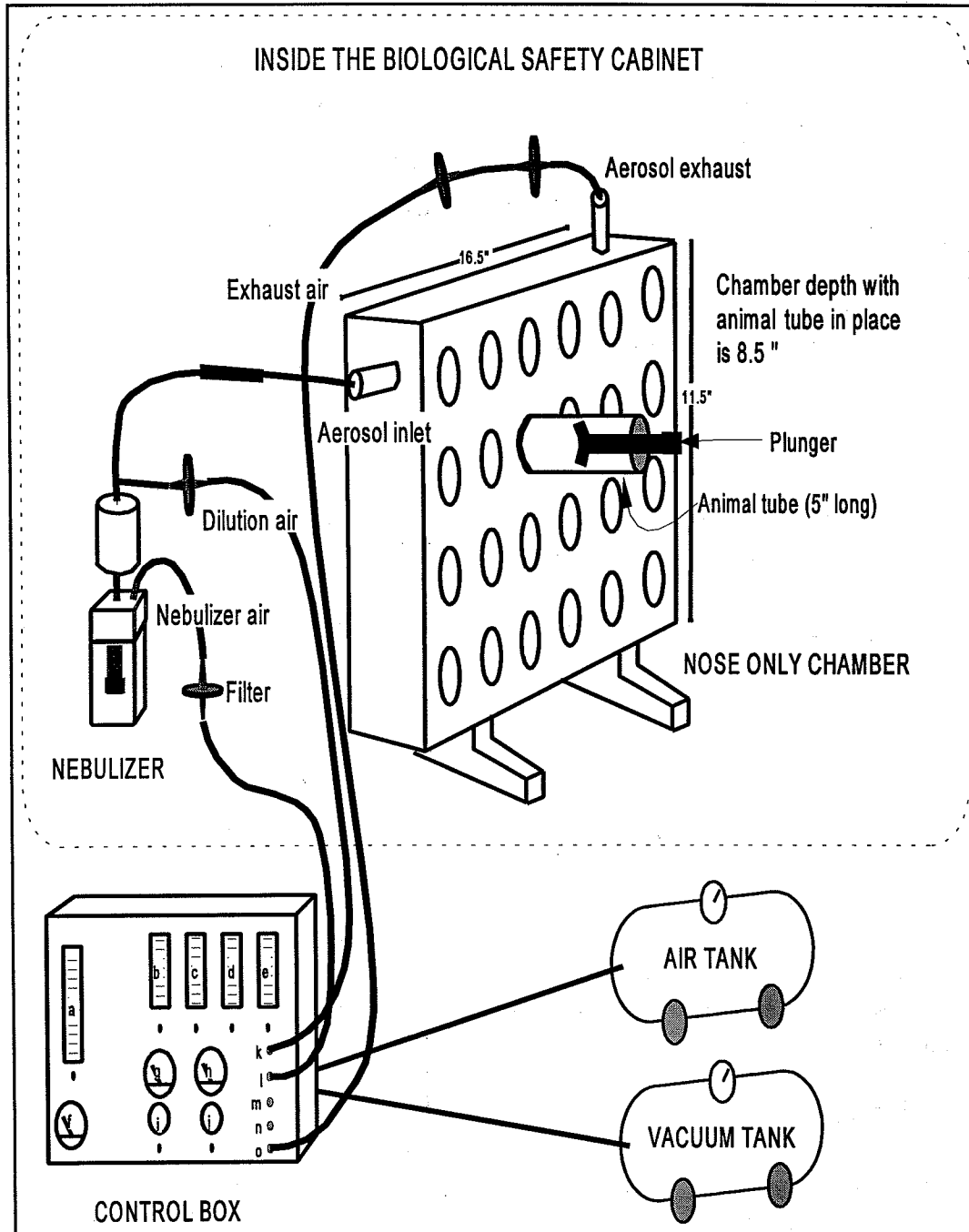


FIG 1 Nose only aerosol exposure system. Control box components: a) chamber flow rotameter; b) filter flow rotameter; c) impact flow rotameter; d) nebulizer air rotameter; e) dilution air rotameter; f) pressure chamber (magnehelic gauge); g) filter pressure (magnehelic gauge); h) impactor pressure (magnehelic gauge); i) nebulizer air connection; j) air pressure gauge; k) dilution air connection; l) nebulizer air connection; m) impactor connection (not in use); n) filter connection (not in use); o) chamber exhaust connection.

The nose-only exposure chamber can be customized to accommodate animals of different sizes. The chamber used here for mice was a 24-port single-sided chamber made of anodized aluminum with polycarbonate tubes. All inlets and outlets were made of brass outfitted with o-rings to provide tight seals. A polyvinylchloride (PVC) plunger was also used (FIGS 1 and 2) to ensure that the animals were maintained in the appropriate position. The chamber was designed to operate at a slightly negative pressure, drawing the aerosol past the animal's nose. The aerosol was brought into the chamber by top entry and delivered in front of the animals through a small orifice in the nose cone. The aerosol was drawn past the animal's nose and then pulled into the exhaust tube through a series of holes around the nose cone. The chamber has a rectangular distribution, top to bottom and left to right. It was not necessary to have all 24 animals in place for an experiment. If fewer animals were needed the unused ports were plugged with aerosol inlet caps and animal tube port plugs.

The control box allowed the adjustment of the nebulizer, dilution and chamber air flow. A tube connecting the chamber to a magnehelic gauge on the control panel enabled monitoring of chamber conditions during exposure. The impactor pressure and the vacuum and air pressure could also be monitored (FIG 1).

The nebulizer and nose-only chamber, which are both relatively small and compact, were placed inside a biological safety cabinet. The control panel was placed under the biological safety cabinet, on a low platform on wheels. The air and vacuum tanks and pump were placed away from the biological safety cabinet. Sound-proofing, if feasible, is recommended to reduce the noise caused by the pump.

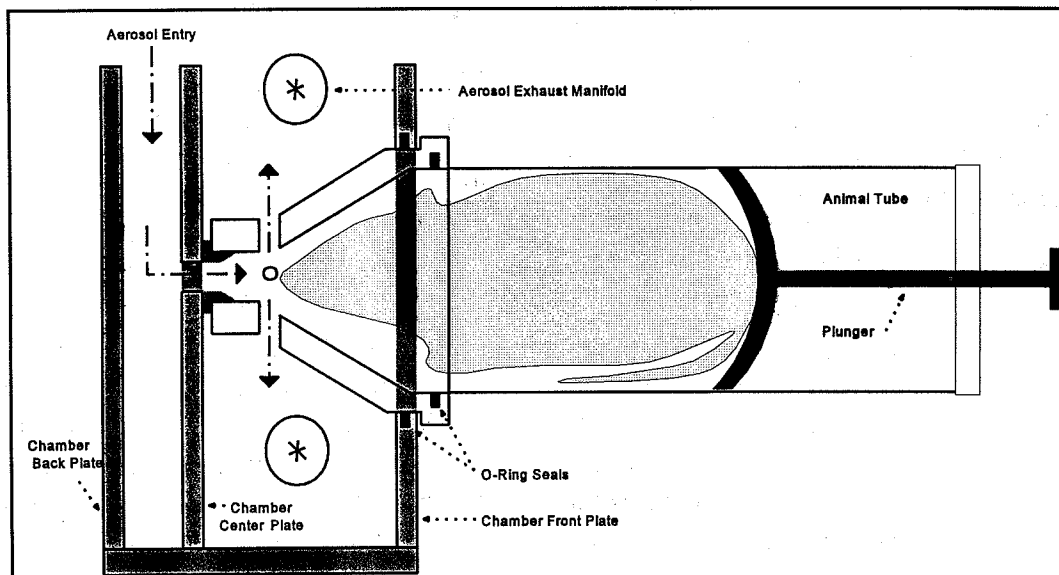


FIG 2 Cross section of aerosol chamber and air flow pattern.

WORKING PROTOCOL

A working protocol for the infection of mice via the respiratory route was designed and evaluated using *M. bovis* strain BCG. Personnel protective equipment was utilized for all work with the aerosol infection system. Tyvek™ coveralls and two pairs of latex gloves were always used. For face and respiratory protection either a positive air pressure respirator (PAPR) (Racal Breathe-Easy 12) or a N95 disposable respirator and safety glasses were used.

1. Preparation Nebulizer Suspension

M. bovis strain BCG, stored at -70 C was thawed, bath sonicated for 10 s to produce a single cell suspension, and diluted with Proskauer-Beck medium (Difco Laboratories, Detroit, MI) containing 0.01% Tween 80. The bacterial suspension contained $0.5-1.5 \times 10^7$ colony forming units (CFU) per mL. Mycobacteria are rod shaped organisms 4-5 microns x 1 micron in size. The nebulizer was adjusted according to the manufacturer's specifications to generate a droplet size of 5-10 microns. In this way, mycobacteria remained as single infectious particles and did not clump. Droplet size was kept constant so that there was 1-5 mycobacteria per droplet. According to the manufacturer's specification, the nebulizer was thus providing 50-80 μL /min of very fine mist.

2. System Settings

System settings were used according the manufacturer's specific guidelines to achieve desired aerosol droplet size and uniform exposure of mice. A 20-25 g mouse requires 25 mL/min airflow. For the 24 port chamber, therefore, 0.6 L/min are needed. As specified by the manufacturer, impactor air flow requires 1 L/min and filter flow requires 5 L/min. Since there are 2 filters, filter flow required for this system is 10 L/min. Thus, a minimum flow of 11.6 L/min is required. Nebulizer output is set to 1.6 L/min, so the total chamber flow should minimally be 13.2 L/min. The aerosol system was operated at a total chamber flow rate of 15-18 L/min, a slightly higher air flow than needed. Negative pressure in the system was maintained during the entire course of the experiment.

3. Placement of Animals

Each mouse was carefully inserted into an animal tube with the nose pointing to the aerosol outlet. The animal tubes are specifically designed to contain one mouse per tube. The tube are 86 mm long and have an inner diameter of 30 mm. Using the plunger in the tube the mouse was gently immobilized in the correct position. This step required experimentation to determine adequate immobilization. If the mouse was too closely confined in the holder tube it would die during the course of the experiment. A mouse that was too loose within the holder tube could turn around several times during the exposure, receiving a smaller infective dose of mycobacteria and potentially exposing the hair to bacterial contamination. Different strains of mice exhibited different behavior within the tube, necessitating careful adjustment of the plunger for immobilization.

4. Exposure to Aerosol

Exposure was carried out within the biological safety cabinet. The holder tubes with animals in place were inserted into the chamber while fresh air was circulating in the system. The nebulizer was turned on for 5 min to allow the bacterial aerosol to equilibrate in the chamber. The mice were then exposed to the aerosol for an additional 20 min. Next, dilution air was run for 5 min after exposure to flush the system. At the end of this 30 minute exposure period, the air system was turned off, holder tubes were removed from the chamber and the animals were placed back into their cages.

5. Disinfection of System

After removing the animal tubes, the chamber openings were plugged and a solution of 70% ethanol was run through the nebulizer for 10-15 min. The tubes and nebulizer were disinfected by dipping for 3 hr in a bath containing a solution of IODEX AR/18 (Quip Laboratories, Inc., Wilmington, DE) following manufacturer's instructions. The chamber side ports were opened, the internal chamber pipes were cleaned with the aid of very fine bottle brushes, and the chamber was rinsed with the IODEX disinfecting solution followed by 10% chlorine bleach (Clorox) internally and externally. The entire system was then rinsed extensively with water and allowed to air dry. After drying the system had to be carefully reassembled to prevent leaks.

RESULTS

AEROSOL INFECTION OF MICE

Separate experiments were performed to establish the working protocol and to evaluate the reliability of the system. To vary the initial infecting dose, bacterial suspensions were diluted to a concentration of 10^5 - 1.5×10^7 CFU/mL. The aerosol exposure time of each experiment was kept constant at 20 min. Three hours after the experiment, mice were sacrificed to evaluate the effectiveness of the aerosol infection. Lungs (without the large airways) were homogenized in phosphate buffered saline (PBS) containing 0.05% Tween 80 and plated on 7H10 agar (Difco Laboratories, Detroit, MI). Colonies were counted after 15 days incubation at 37 C.

All procedures including sacrifice of animals, dissection and homogenization of tissues, and plating were carried out within the biological safety cabinet. Plates were incubated in an incubator within the ABSL3 facility.

Table 1 shows the results of 9 such experiments carried out with aerosolized *M. bovis* BCG. For each experiment littermates (of the same sex, age and size) of either B6D2F1 or Balb/c mice (Charles River Laboratories, NJ), 8-10 wk of age were used. There was no significant difference in the number of CFU among the mice placed in different sections of the chamber. Mice from the same experiment had consistent numbers of mycobacteria implanted in the lungs.

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TABLE 1
Aerosol Infection of Mice

Experiment #	Mouse Strain	Number of Mice	Bacterial Suspension BCG(CFU/mL)	Mean CFU/Mouse (both lungs)
1	B6D2F1	7	1.5×10^7	260±140
2	B6D2F1	5	1.5×10^7	120±80
3	Balb/c	3	1.5×10^7	340±90
4	Balb/c	6	1.5×10^7	110±40
5	B6D2F1	6	1.5×10^7	300±80
6	B6D2F1	7	1.5×10^7	640±160
7	Balb/c	8	7×10^6	320±80
8	B6D2F1	6	7×10^6	370±150
9	B6D2F1	5	7×10^6	240±130

To determine the extent and uniformity of the pulmonary pathology induced by infection with the aerosol device, animals were sacrificed 40 or 50 days following the infection and lungs were fixed in 10% neutral buffered formalin (Fisher). Histological examination of the lungs revealed scattered granulomas composed of numerous T cells and macrophages containing intracellular acid fast bacilli (FIG 3).

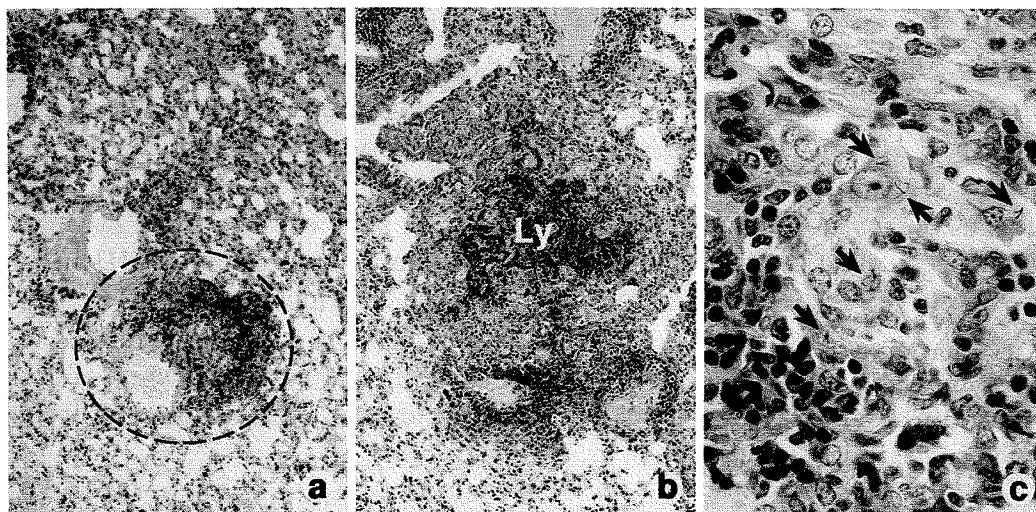


FIG 3 Histological sections of the lung of a mouse infected by aerosol with 300 cfu of *M. bovis* strain BCG. a) 40 days post infection; acid-fast stain of the sections revealed formation of a granuloma in the lung parenchyma (circle). The granuloma is surrounded by lung tissue which is partially infiltrated by inflammatory cells, occluding the air spaces (magnification x 10). b) 50 days post infection; acid-fast stain. Most of the lung parenchyma is occupied by a large granuloma with numerous lymphocytes (Ly) with dark staining nuclei and macrophages with lighter staining cytoplasm (magnification x 10). c) 50 days post infection; acid-fast stain. Acid fast bacilli are seen (arrows) within the cytoplasm of macrophages. A cuff of T lymphocytes with dark staining nuclei is seen at the bottom left of the micrograph (magnification x 60).

CONTAINMENT AND STERILITY TESTS

As provided by the manufacturer, the control box and the compressed air surge tank appeared vulnerable to microbiological contamination. Therefore inline filtration was added as described above. To function properly the animal exposure device must be leak-free. To verify that the instrument did not leak into the surrounding environment, the aerosol system was submitted to two tests: a pressure test and a microbiological test.

Pressure

Five empty animal tubes were placed in the upper row of the chamber. All other chamber openings were plugged using the aerosol inlet caps and port plugs. The chamber exhaust was connected to the vacuum source through a line with a needle valve. Vacuum was drawn into the system to -1.8 in. w.g. (-0.45 kPa) as indicated on the magnehelic gauge. The valve was closed and pressure was maintained for at least 20 min.

Since the system held a steady pressure, it was concluded that the system did not leak and the unit was properly sealed.

Microbiological Leak

All areas of the animal chamber were tested for microbial leaks using an aerosol prepared from a suspension of approximately 1.5×10^7 CFU/mL of *M. bovis* strain BCG. While the instrument was in operation 7H10 agar plates (regular size, 100 mm in diameter) were placed under potentially leaky connections so that any leaking aerosol could impinge onto the surface of the agar (FIG 4). An unexposed plate was used as a negative control and 0.1 mL of the bacterial suspension was plated as a positive control. Plates were incubated for 15 days at 37 C.

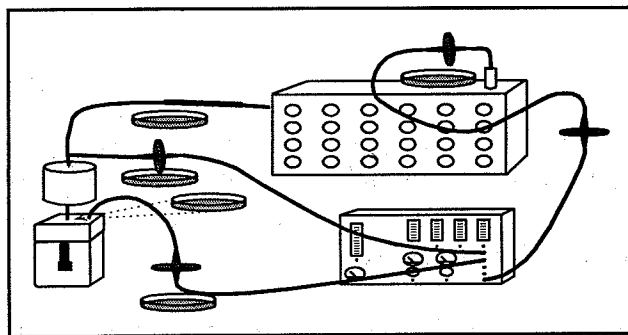


FIG 4 Microbiological leak test indicating agar plate positions.

The test plates and the negative control were all bacilli free. The positive control had confluent bacillary growth. It was therefore concluded that the system did not leak.

Secondary Containment

To ensure that in case of a failure of the integrity of the aerosol system, the infectious microorganisms would be contained, the animal chamber and nebulizer were placed inside the Class II Type A/B3 biological safety cabinet that was used as a Type A cabinet. The performance of the cabinet with the aerosol system in place was evaluated according to the NSF 49 (NSF 1992) specifications. The smoke test was carefully observed for signs of any disturbance in the smoke pattern, changes in the split between the front and back air flow and in particular air losses out of the cabinet.

Downflow and inflow measurements were within acceptable NSF 49 ranges. Dioctylphthalate (DOP) test indicated that there were no leaks in the cabinet filters. A titanium tetrachloride smoke tube was used to test the airflow patterns. The smoke test indicated that the nose-only chamber and nebulizer units placed inside the cabinet did not produce airflow disturbances that affected the performance of the cabinet. All the smoke was contained within the cabinet and the air flow patterns were normal.

Aerosol Distribution

To determine whether the aerosol was spread equally to all 24 tubes, we evaluated the seeding of aerosolized mycobacteria onto 7H10 agar small petri dishes (35 mm in diameter). Each dish was placed inside the conic part of the animal tubes against the aerosol opening and exposed to the aerosol containing 1.5×10^7 mycobacteria, for 20 min. The same tubes were used for either animal exposure or exposure of the petri dishes. After 15 days of incubation at 37 C, all petri dishes were shown to have similar numbers of mycobacterial colonies ($380 \text{ CFU} \pm 112/\text{dish}$). These results indicate that there is an equal distribution of aerosolized mycobacteria within the chamber.

External Contamination

Animal. Following completion of the aerosol infection, those mice which had been turning in the tube during the experiment were withdrawn, and the fur and tail were touched to 7H10 plates. Following incubation, no colonies were observed indicating that despite their movement the mice were not contaminated by the aerosol.

Investigator. Similarly, at the end of each experiment the gloved fingers of the investigators that handled the mice were touched to 7H10 plates. No colonies developed after the incubation.

CONCLUSIONS

Previously aerosol infection has been carried out using a device which exposed the hair and skin of the animal and potentially the investigator to bacterial contamination. We therefore modified and evaluated the InTox aerosol producing device for use as a respiratory infection system for experimental animals.

Since the system is intended for use with infectious, virulent microorganisms it is important to establish the effective containment of the organisms within the biological safety cabinet. Even with the aerosol system in place, the cabinet performed according to NSF Standard 49 (NSF 1992) specifications and all smoke was contained within the cabinet. Within the cabinet, the system maintained a steady air pressure without any air leaks. During operation with an aerosolized bacterial suspension, the animal chamber was tested for leaks of microorganisms. No bacilli were detected. Moreover, the fur and tail of the mice were uncontaminated following exposure to the aerosol obviating the need for UV irradiation of the experimental animals which is necessary in some other systems.

We then established the protocol for aerosol infection of mice using a nonpathogenic strain of mycobacteria, *M. bovis* BCG. The aerosol generated by the nebulizer contained single microorganisms or small numbers of bacilli. Following infection, mice from the same experiment had similar numbers of mycobacteria implanted in the lungs (Table 1). The number of mycobacteria implanted in the lungs was independent of either the mouse strain or the number of mice infected per experiment. The absolute number of CFU in both lungs of infected mice differed from experiment to experiment within the acceptable variation for microbiological systems. Histologic examination of the lungs of infected mice revealed pathology similar to that obtained using conventional aerosol infection systems (North 1995). After 50 days, granulomas containing intracellular bacilli were observed in the lungs of all infected mice confirming the potential usefulness of this system in studies of pulmonary tuberculosis (FIG 3).

The aerosol infection system described here proved easy to operate, eliminated the need for irradiation of the experimental animal and provided an adequate safety barrier for the investigator. Our results indicate that this device can be successfully used for respiratory infection of mice with aerosols containing mycobacteria as an experimental model for human pulmonary tuberculosis.

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