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Intracage Ammonia Promotes Growth of *Mycoplasma pulmonis* in the Respiratory Tract of Rats

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Ammonia (NH_3) from soiled cage bedding is known to enhance the progression and severity of murine respiratory mycoplasmosis in rats. To test the hypothesis that NH_3 directly or indirectly enhances the growth of *Mycoplasma pulmonis* in vivo, pathogen-free F344 rats were inoculated intranasally with 1×10^4 to 4×10^4 or 4×10^6 to 5×10^6 colony-forming units of *M. pulmonis* and exposed to ≤ 1.5 or 76 μg of NH_3 per liter (≤ 2 or 100 ppm, respectively). Nasal passages, larynges, tracheas, and lungs from rats killed at intervals up to 28 days after inoculation were quantitatively cultured. Growth of *M. pulmonis* was much greater in NH_3-exposed rats than in controls, particularly in those inoculated with the lower dose. Increases in *M. pulmonis* populations were more rapid in proximal airways than in distal airways. Serum immunoglobulin G and M antibody responses to *M. pulmonis* as measured by an enzyme-linked immunosorbent assay were greater in NH_3-exposed rats. In other experiments, the nasal passages absorbed virtually all NH_3 when the rats were exposed to less than 380 μg of NH_3 per liter (500 ppm), indicating that NH_3 induced increases in the numbers of organisms in the distal respiratory tract, probably by a secondary, rather than a direct, effect. Also, NH_3 exposure did not inhibit pulmonary antibacterial activity as measured by clearance of radiolabeled *Staphylococcus epidermidis*. The growth of *M. pulmonis* in vitro was inhibited by 1 mM NH_4^+ added to the medium as NH_4OH but not by NH_4^+ concentrations of 0.5, 0.1, or 0.01 mM, suggesting that NH_3 increases growth indirectly through effects on the host.

Although murine respiratory mycoplasmosis (MRM) in rats has been recognized since the early 1900s (8), *Mycoplasma pulmonis* was not established as the causative organism until the early 1970s (10, 12, 13, 21). Earlier studies on the etiology of MRM were hindered because rats free of *M. pulmonis* and other pathogens were not available and because it was not recognized that environmental and other factors influence the expression of MRM (3, 14). Among such factors, the ammonia (NH_3) released from soiled cage bedding is of primary importance (2).

To obtain information on possible mechanisms by which environmental NH_3 affects the expression of MRM, we studied the effects of exposing pathogen-free F344 rats to 76 μg of NH_3 per liter (100 ppm) on the growth of *M. pulmonis* in the respiratory tract and on pulmonary bacterial clearance (7, 17). We also examined the absorption of NH_3 in the respiratory tract and the effect of NH_4^+ on the growth of *M. pulmonis* in liquid medium.

MATERIALS AND METHODS

Rats. F344 rats, 6- to 8-weeks old, from a gnotobiotic breeding colony were used in all experiments. Rearing and maintenance were as previously described (2, 13). Results of monitoring procedures (2, 13) consistently indicated that the colony was free of bacterial and viral pathogens.

NH_3 exposure. Gaseous NH_3 from a tank of liquid anhydrous NH_3 (PB & S Chemical Co., Henderson, Ky.) was introduced through a specially constructed regulator-filter apparatus into the air supply of a plastic film isolator equipped with two fans, two inlet filters, and two exhaust filters to increase the air flow (2). NH_3 concentration in the isolator was maintained at 76 μg/liter. This concentration is commonly found with rat cages (J. R. Lindsey, unpublished) and is well above the lowest concentration, 19 μg/liter (25 ppm), shown by Broderson et al. to exacerbate MRM (2). Control rats were housed in an identical isolator without NH_3. The intestinal floras of the rats contained no urease-producing organisms, but to reduce exposure to any naturally produced NH_3, the rats were housed in stainless steel wire mesh cages (19 by 41 by 25 cm) suspended 13 cm over paper bedding which was changed daily. NH_3 concentrations in both isolators were monitored daily with a Drager Multi Gas Detector (Dragerwerk, Lubeck, Federal Republic of Germany) or continuously with a Honeywell model UVH-10C3 NH_3 detector (Honeywell, Ft. Washington, Pa.). The NH_3 concentration in the control isolator was usually not measurable and never exceeded 1.5 μg/liter (2 ppm).

***M. pulmonis* cultures.** Mycoplasma broth was prepared in liter quantities by adding 22.5 g of Frey mycoplasma broth base (GIBCO Diagnostics, Madison, Wis.) and 2.0 ml of 1% phenol red (Fisher Scientific Co., Fairlawn, N.J.) to 660 ml of deionized water. The pH was adjusted to 7.8 to 8.0 with concentrated NaOH solution, and the mixture was autoclaved for 15 min and allowed to cool. A 200-ml portion of sterile, gamma globulin-free, heat-inactivated horse serum (GIBCO Laboratories, Grand Island, N.Y.), 100 ml of sterile 25% yeast extract solution (GIBCO Laboratories), 10^6 U of penicillin G potassium (Pfizer Inc., New York, N.Y.) in 5 ml of sterile distilled water, 25 ml of filter-sterilized 2% thallous acetate (Sargent-Welch, Skokie, Ill.), and 10 ml of filter-sterilized 50% dextrose (J. T. Baker Chemical Co., Phillipsburg, N.J.) were then added. Solid medium was made from the same ingredients by adding 10 g of Noble agar (Difco Laboratories, Detroit, Mich.) before autoclaving.

M. pulmonis (stock culture 5782 C4, isolated from a rat with natural MRM) was grown by inoculating 10 ml of broth and then increasing the volume daily with fresh broth to 100, 250, 500, and 1,000 ml. The organisms were separated from the broth by centrifugation at 8,000 × *g* for 30 min and suspended in phosphate-buffered saline (PBS).

Experimental infections. In six experiments, 295 rats were injected intramuscularly with 0.10 ml of Innovar-Vet (Pitman-Moore, Inc., Washington Crossing, N.J.) diluted 1:10 in saline and then inoculated intranasally with 1×10^4 to 4×10^4 or 4×10^6 to 5×10^6 colony-forming units (CFU) of *M. pulmonis* in 50 μl of PBS. Half of the inoculated rats were exposed to NH_3. Exposure to NH_3 was begun either 1 week before or simultaneously with inoculation. Five rats each from the control and NH_3-exposed groups were killed at intervals up to 28 days post-inoculation. Serum from each rat was collected for determination of immunoglobulin G (IgG) and IgM antibodies against *M. pulmonis* by an enzyme-linked immunosorbent assay (9).

The fur of each rat was wetted with absolute ethanol, and the lungs, trachea, and larynx were aseptically excised. Ths skin was removed from the head, and the nasal passages were collected by transecting the head through the anterior edge of the orbits with a small circular saw mounted on a dental drill. The teeth were removed, and the bone and mucous membrane of the nasal passages were fragmented with small bone cutters. The lungs, larynx, and trachea were each homogenized in PBS in individually autoclaved ground-glass homogenizers (Duall no. 23; Kontes Biomedical Products, Vineland, N.J.) driven at 800 rpm by an electric motor (Master Servodyne; Cole-Parmer Instrument Co., Chicago, Ill.). Homogenates and nasal tissues were sonicated (model W220F; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) at full power for 30 s in ice water and quantitatively cultured by serial dilution and plating. Although homogenates of tissues are reported to inhibit mycoplasmal growth (11, 15), this effect is reduced or eliminated by dilution (15, 20). In these experiments, the organism grew well except in the first dilution tubes and in plated undiluted homogenate.

Measurement of pulmonary bacterial clearance. Pulmonary bacterial clearance was measured by a method similar to that described previously (7, 17). *Staphylococcus epidermidis* (ATCC 12228) was labeled with ^{32}P (as hydrochloride-, carrier-free phosphoric acid; New England Nuclear Corp., Boston, Mass.) in a medium containing the following (per 500 ml of distilled water): ammonium sulfate, 1.0 g; hydrated magnesium sulfate, 0.10 g; ammonium citrate, 0.5 g; Casamino Acids (Difco), 2.5 g; lyophilized yeast extract dialysate (BBL Microbiology Systems, Cockeysville, Md.), 1 10-ml vial; glucose, 2.5 g; Tris buffer, 3.0 g; and monopotassium phosphate, 0.07 g. This medium was sterilized by filtration. The organisms, in 50 ml of the medium containing 1 mCi of ^{32}P, were grown overnight in a shaker bath and then separated from unincorporated label by centrifugation at 1,500 × g for 30 min followed by washing and centrifugation twice in PBS. They were resuspended in PBS, and the suspensions were frozen at −70°C in 5-ml aliquots.

Radiolabeled *S. epidermidis* in PBS was inoculated via the trachea into the lungs with the aid of an otoscope and a tuberculin syringe. A 20-guage needle (3.8 cm) tipped with 3 cm of small-diameter polyethylene tubing was attached to the syringe, which allowed deposition of the inoculum at the bifurcation of the major bronchi. Examination of sections of lungs from rats thus inoculated showed that the distribution of bacteria in the alveoli was reasonably uniform and that no acute inflammatory changes resulted.

Half of each group of rats was killed immediately post-inoculation, and half was killed at 6 h post-inoculation. The lungs were aseptically removed and then homogenized as described above. Homogenization was done in sterile distilled water to help break up the lung cells. Homogenates were brought to a uniform volume and sonicated for 2 min in ice water. The number of CFU per milliliter of homogenates was determined by serial dilution and plating.

Radioactivity in the lung homogenates was determined by liquid scintillation counting. To 1.0 ml of homogenate in a scintillation vial, 3 ml of tissue solubilizer (Protosol; New England Nuclear Corp.) was added, and the mixture was incubated overnight at 37°C. Five drops of 30% hydrogen peroxide were then added as a bleach. After 4 to 6 h, 15 ml of liquid scintillation cocktail (Aquasol-2; New England Nuclear Corp.) was added. To reduce chemiluminescence and further reduce color quenching, the pH was adjusted to 6.0 to 7.0 with hydrochloric acid. Samples were counted in an LS-150 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

The percentage of bacteria cleared from the lungs of each rat killed 6 h post-inoculation was calculated from the CFU/radioactivity (counts per minute [cpm]) ratios in lung homogenates by the formula: percent clearance = 100[1−(individual ratio of CFU per milliliter to cpm per milliliter at 6 h/mean ratio of CFU per milliliter to cpm per milliliter at 0 h)].

The rate of spontaneous loss of label in the inoculum was determined by taking samples for CFU and cpm determinations at the beginning of each experiment and again at the end after centrifugation and resuspension.

Statistical analysis. CFU counts and CFU/cpm ratios were transformed to common logarithms and analyzed by two-way analysis of variance and the least-significant-difference test (18). A computer analysis of the data from all quantitative culture experiments by a general linear model procedure was used to evaluate

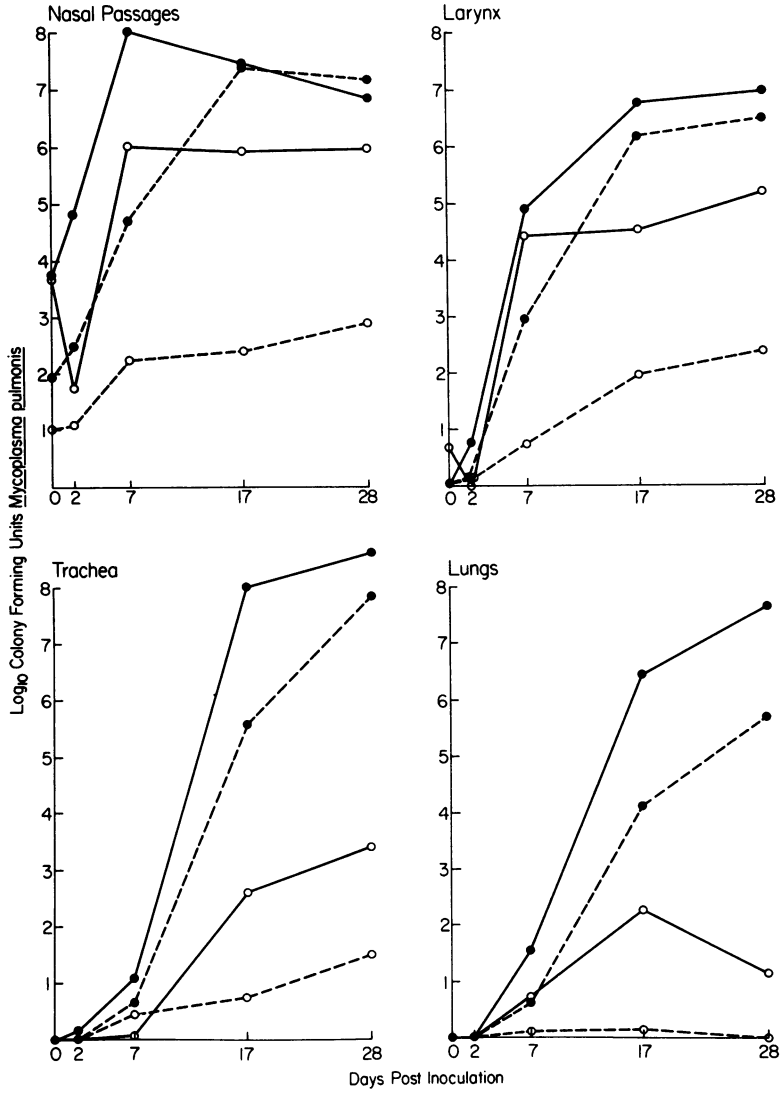

FIG. 1. Growth of *M. pulmonis* in respiratory tracts of rats after intranasal inoculation. Graphs show combined data from six experiments in which 295 rats were given 1×10^4 to 4×10^4 (---) or 4×10^6 to 5×10^6 (—) CFU of *M. pulmonis* and exposed to NH_3 at 76 μg/liter (●) or ≤1.5 μg/liter (○).

effects of dose and preexposure to ammonia. Differences were considered significant when $P \leq 0.05$.

RESULTS

Effects of NH_3 on respiratory tract populations of *M. pulmonis*. Within an individual dose, quantitative culture results were similar, although the times at which peak populations of *M. pulmonis* occurred varied somewhat from experiment to experiment. The effect of NH_3 preexposure could not be distinguished from variations among experiments, so the results of all high-dose inoculations were combined, as were those of low-dose inoculations (Fig. 1). Each point in Fig. 1 represents the results for at least 15 rats, except the point showing data for the 10 high-dose control rats at day 0 and the two points each showing data for 5 NH_3-exposed rats at day 0.

In both NH_3-exposed and control rats, *M. pulmonis* CFU increased most rapidly in the nasal passages and subsequently in the larynges, tracheas, and lungs. In all experiments, *M. pulmonis* CFU increased more in the organs of rats exposed to NH_3. The combined data (Fig. 1) show a difference in the response of *M. pulmonis* populations to NH_3 between the rats

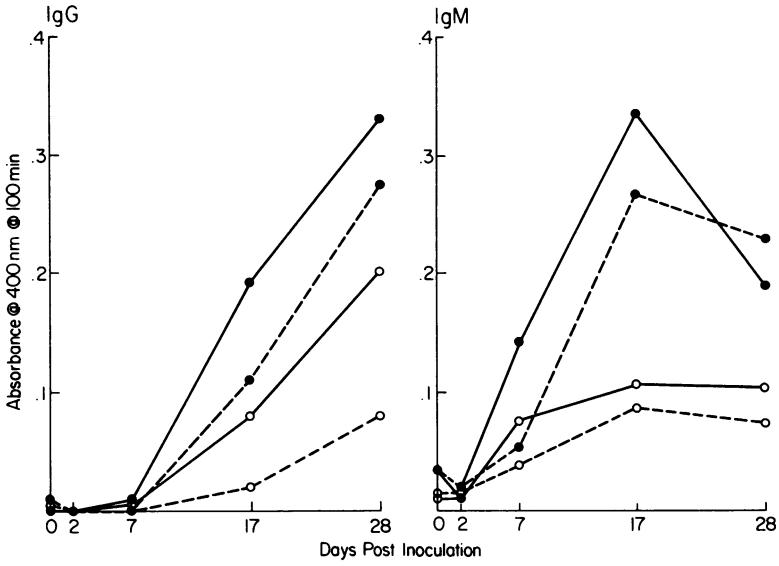

FIG. 2. Enzyme-linked immunosorbent assay of serum IgG and IgM antibodies against *M. pulmonis*. Graphs show combined data from six experiments in which 295 rats were inoculated intranasally with 1×10^4 to 4×10^4 (---) or 4×10^6 to 5×10^6 (—) CFU of *M. pulmonis* and exposed to NH_3 at 76 μg/liter (●) or ≤1.5 μg/liter (○).

inoculated with the high dose and those inoculated with the low dose, particularly in the nasal passages and larynges. In these sites, the maximum numbers of *M. pulmonis* were similar in the NH_3-exposed rats regardless of inoculation dose, although these maximum numbers were attained somewhat later in rats inoculated with the low dose. In contrast, control rats given the larger inoculation dose had many more CFU in their respiratory tracts than did the control rats given the lower dose. In the tracheas and lungs this difference was less obvious, although it was statistically significant at all sites.

IgG and IgM enzyme-linked immunosorbent assay values generally paralleled *M. pulmonis* populations but were not highly correlated with numbers at any one site (Fig. 2).

Absorption of NH_3 from the respiratory tract. It seemed likely that the larger nasal populations of *M. pulmonis* resulted in a more rapid spread of the organism to the distal parts of the respiratory tracts in NH_3-exposed rats as compared with controls. NH_3 probably would not have a direct but delayed effect in the trachea and lungs because it is reported to be absorbed in the nasal passages (4, 5). However, studies of NH_3 absorption in the respiratory tracts of rats have not been reported. To determine whether NH_3 could be detected distal to the nasal passages, 11 rats, 3 of which has been exposed to 76 μg of NH_3 per liter for the preceding week, were anesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, Ill.) diluted 1:10 in sterile PBS and injected intraperitoneally at 40 mg/kg. The rats were exposed to NH_3 via a face mask constructed from the barrel of a 50-ml plastic syringe. The large open end was covered by a thin, centrally perforated rubber diaphragm which sealed the mask to the muzzle of the rat. Air was supplied through plastic tubing from the ammonia exposure isolator, and the mask was vented to provide a constant supply of fresh air with a known ammonia concentration. Tracheas were surgically exposed, cannulated, and connected to the Drager gas detector by a short length of plastic tubing. NH_3 was not detected in the tracheal air of any of the rats during exposure to less than 380 μg of NH_3 per liter (500 ppm). At 380 μg/liter, 8 to 15 μg/liter (10 to 20 ppm) was detected in the air reaching the tracheas of some rats.

Pulmonary clearance of *S. epidermidis*. To assess the effect of NH_3 on nonspecific resistance of rat lungs to bacterial pathogens, we measured the rates of pulmonary bacterial clearance (7, 17) in NH_3-exposed and control rats. In three experiments with a total of 76 rats, half of the rats were exposed to 76 μg of NH_3 per liter for 1 week. Rats were inoculated with 10^6, 10^7, or 10^8 CFU of radiolabeled *S. epidermidis*. Half of the control rats and half of the exposed rats were killed immediately thereafter, and the rest were killed 6 h later. There was no statistical difference in the rate of pulmonary clearance between exposed and control rats or among experiments. In the combined results, both NH_3-exposed and control rats cleared 91 ± 6% of the bacteria in 6 h.

Effects of NH_4^+ on in vitro growth of *M. pulmonis*. To address the question of whether the increased populations of *M. pulmonis* in NH_3-exposed rats resulted directly from enhancement of growth of the organism or indirectly from effects on the host, experiments were done to determine the effect of NH_4^+ on the growth of *M. pulmonis* in liquid medium. Media containing 1, 0.5, 0.1, and 0.01 mM NH_4^+ (as NH_4OH) and control medium were inoculated with *M. pulmonis*. The pH values of all media were adjusted to 7.8 to 8.0. Determinations of CFU were done immediately after inoculation and 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, 60, 66, 72, and 84 h later. The results of replicate experiments were combined for statistical analysis. NH_4^+ at 1 mM inhibited the growth of *M. pulmonis*. Lower concentrations had no effect.

DISCUSSION

Our findings clearly show that NH_3 has striking effects on the population dynamics of *M. pulmonis* in the respiratory tracts of rats. Inasmuch as the development of lesions is probably related to the numbers of organisms in affected tissues, our results (Fig. 1) are consistent with those of Broderson et al. (2). In that study, intranasal inoculation with *M. pulmonis* induced rhinitis in most rats, but rats exposed to as little as 19 μg of NH_3 per liter had a greater incidence of pulmonary lesions and more severe lesions in all parts of the respiratory tract. We did not detect NH_3 in the tracheas of rats exposed to less than 380 μg of NH_3 per liter, in agreement with previous studies (4, 5). Therefore, we believe that the increased numbers of *M. pulmonis* in the lungs and the consequent exacerbation of lung lesions in MRM are secondary to events in the nasal passages rather than a direct effect of NH_3 in the lung itself. Consistent with this interpretation is the fact that increases in mycoplasmal populations in the distal respiratory tract did not occur until after large numbers appeared in the proximal structures (Fig. 1).

Because NH_3 did not penetrate to the distal airways in our study, we did not extensively examine the effects of NH_3 on pulmonary defense mechanisms. However, one measure of pulmonary antibacterial activity, intrapulmonary killing of radiolabeled staphylococci (7, 17), was unaffected by NH_3 exposure. NH_3-exposed rats had systemic antibody responses to *M. pulmonis* as great as or greater than those of control rats (Fig. 2). Inasmuch as the critical components of local and systemic immunological responses to mycoplasmal infections are not well understood, the significance of this finding is unclear, but it indicates that at least some responses to *M. pulmonis* were not affected by exposure to 76 μg of NH_3 per liter.

In our study of the effect of NH_4^+ on the growth of *M. pulmonis* in vitro, the selection of the range of concentrations used was based on an estimate of the concentration of NH_4^+ that might be attained in nasal secretions. Calculations based on the average respiratory rate and tidal volume for rats (1) indicated that a rat breathing 76 μg of NH_3 per liter in air would inhale about 800 μmol of NH_3 in 24 h. If all NH_3 was absorbed in the nasal passages and none was locally metabolized, 100 ml of nasal secretion in 24 h would be required for the concentration of NH_4^+ to be as low as 1 mM. No figures are available on the rate of secretion in rat nasal passages, but it does not seem likely that it would exceed 100 ml/24 h. The inhibition of growth at 1 mM was unexpected but not surprising in view of the sensitivity of mycoplasmas to many substances (6, 11, 15, 19, 20). Although it is possible that under different in vitro conditions NH_4^+ might have been stimulatory, this seems unlikely inasmuch as there is no known metabolic pathway by which NH_4^+ would be expected to increase *M. pulmonis* growth (16; M. F. Barile, personal communication). Therefore, rather than directly enhancing the growth of *M. pulmonis*, NH_3 probably influences the expression of MRM through effects on the respiratory tract, but the nature of these effects remains to be elucidated.

Our results provide quantitative information regarding the effects of NH_3 exposure and inoculation dose on experimental *M. pulmonis* infection in pathogen-free rats. They also demonstrate a basis for the exacerbation by NH_3 of lesions of MRM (2) and suggest possible avenues of future investigation into the mechanisms underlying these effects.

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LITERATURE CITED

1. **Bivin, W. S., W. P. Crawford, and N. R. Brewer.** 1979. Morphophysiology, p. 74–104. *In* H. J. Baker, J. R. Lindsey, and S. H. Weisbroth (ed.), The laboratory rat. Academic Press, Inc., New York.
2. **Broderson, J. R., J. R. Lindsey, and J. E. Crawford.** 1976. The role of environmental ammonia in respiratory mycoplasmosis of rats. Am. J. Pathol. **85**:115–130.
3. **Cassell, G. H., J. R. Lindsey, H. J. Baker, and J. K. Davis.** 1979. Mycoplasmal and rickettsial diseases, p. 243–269. *In* H. J. Baker, J. R. Lindsey, and S. H. Weisbroth (ed.), The laboratory rat. Academic Press, Inc., New York.
4. **Cralley, L. V.** 1942. The effect of irritant gases upon the rate of ciliary beating. J. Ind. Hyg. **24**:193–198.
5. **Dalhamn, T., and J. Sjoholm.** 1963. Studies of SO_2, NO_2,

and NH_3. Effect on ciliary activity in the rabbit trachea of single *in vitro* exposure and resorption in rabbit nasal cavity. Acta Physiol. Scand. **58**:287–291.

6. **Del Giudice, R. H., R. S. Gardella, and H. E. Hopps.** 1980. Cultivation of formerly uncultivable strains of *Mycoplasma hyorhinis*. Curr. Microbiol. **4**:75–80.
7. **Green, G. M., and E. H. Kass.** 1964. The role of the alveolar macrophage in the clearance of bacteria from the lung. J. Exp. Med. **119**:167–176.
8. **Hektoen, L.** 1915. Observations on pulmonary infections in rats. Trans. Chicago Pathol. Soc. **10**:105–109.
9. **Horowitz, S. A., and G. H. Cassell.** 1978. Detection of antibodies to *Mycoplasma pulmonis* by an enzyme-linked immunosorbent assay. Infect. Immun. **22**:161–170.
10. **Jersey, G. C., C. K. Whitehair, and G. R. Carter.** 1973. *Mycoplasma pulmonis* as the primary cause of chronic respiratory disease in rats. J. Am. Vet. Med. Assoc. **163**:599–604.
11. **Kaklamanis, E., K. Stavcopoulos, and L. Thomas.** 1971. The mycoplasmacidal action of homogenates of normal tissues, p. 27–35. *In* S. Madoff (ed.), Mycoplasmas and L forms of bacteria. Gordon and Breach, New York.
12. **Kohn, D. F., and B. E. Kirk.** 1969. Pathogenicity of *Mycoplasma pulmonis* in laboratory rats. Lab. Anim. Care **19**:321–330.
13. **Lindsey, J. R., H. J. Baker, R. G. Overcash, G. H. Cassell, and C. E. Hunt.** 1971. Murine chronic respiratory disease. Significance as a research complication and experimental production with *Mycoplasma pulmonis*. Am. J. Pathol. **64**:675–716.
14. **Lindsey, J. R., G. H. Cassell, and H. J. Baker.** 1978. Mycoplasmatales and rickettsiales, p. 1481–1550. *In* K. Benirschke, F. M. Garner, and T. C. Jones (ed.), Pathology of laboratory animals, vol. 2. Springer-Verlag, New York.
15. **Mardh, R. A., and D. Taylor-Robinson.** 1973. New approaches to the isolation of mycoplasmas. Med. Microbiol. Immunol. **158**:259–266.
16. **Razin, S.** 1978. The mycoplasmas. Microbiol. Rev. **42**: 414–470.
17. **Ruppert, D., G. J. Jakab, D. L. Sylvester, and G. M. Green.** 1976. Sources of variance in measurement of intrapulmonary killing of bacteria. J. Lab. Clin. Med. **81**:544–558.
18. **Snedecor, G. W., and W. G. Cochran.** 1967. Statistical methods, 6th ed. p. 294–380. Iowa State University Press, Ames, Ia.
19. **Tauraso, N. M.** 1967. Effect of diethylaminoethyl dextran on the growth of *Mycoplasma* in agar. J. Bacteriol. **93**:1559–1564.
20. **Tully, J. G., and R. Rask-Nielsen.** 1967. Mycoplasma in leukemic and nonleukemic mice. Ann. N.Y. Acad. Sci. **143**:345–352.
21. **Whittlestone P., R. M. Lemcke, and R. J. Olds.** 1972. Respiratory disease in a colony of rats. II. Isolation of *Mycoplasma pulmonis* from the natural disease, and the experimental disease induced with a cloned culture of this organism. J. Hyg. **70**:387–409.