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Effects Of Atmospheric Ammonia On The Pathogenesis Of Murine Respiratory Mycoplasmosis In Rats.

Trenton Robert Schoeb
University of Alabama at Birmingham

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EFFECTS OF ATMOSPHERIC AMMONIA ON THE PATHOGENESIS OF MURINE RESPIRATORY MYCOPLASMOSIS IN RATS

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EFFECTS OF ATMOSPHERIC AMMONIA ON THE PATHOGENESIS OF MURINE RESPIRATORY MYCOPLASMOSIS IN RATS

by

TRENTON ROBERT SCHOEB

A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title Effects of Atmospheric Ammonia on the Pathogenesis of Murine Respiratory Mycoplasmosis in Rats

Ammonia released from soiled cage bedding has been demonstrated to enhance the severity of murine respiratory mycoplasmosis (MRM) in rats. To test the hypothesis that this enhancement is due to interference with pulmonary bacterial clearance, known pathogen-free Fischer rats were exposed to 0 or 100 ppm ammonia for 1 week, then inoculated intratracheally with either radiolabelled Staphylococcus aureus, Staphylococcus epidermidis, or Mycoplasma pulmonis. Clearance rates were calculated from ratios of colony-forming units (CFU) to radioactivity in homogenized lungs of rats killed immediately or 4 to 6 hours postinoculation (PI). There were no statistically significant differences between ammonia-exposed and control rats. Clearance of S. aureus was variable, but there was little variation in clearance of S. epidermidis, which averaged 90% in 6 hours. No clearance of M. pulmonis was detected. Similar results were obtained with unlabelled M. pulmonis at PI intervals up to 48 hours. Further evidence against the hypothesis that ammonia alters pulmonary clearance is provided by data showing that all detectable ammonia was removed from inspired air containing less than 500 ppm ammonia by the time it reached the trachea.

The effect of 100 ppm ammonia on population dynamics of M. pulmonis in the respiratory tracts of rats was examined by quantitative culture

of nasal passages, larynxes, tracheas, and lungs at intervals of up to 28 days after intranasal inoculation of high (4 to 5 x 10^6 CFU) and low (1 to 4 x 10^4 CFU) doses of M. pulmonis. Populations of M. pulmonis increased more rapidly and to much higher numbers in rats exposed to ammonia. In both ammonia-exposed and control rats M. pulmonis populations increased first in the nasal passages, followed sequentially by larynx, trachea and lungs. Serum IgM and IgG anti-M. pulmonis antibody concentrations, measured by an enzyme-linked immunosorbent assay, generally paralleled population increases of M. pulmonis but were not highly correlated with quantitative culture data for any one site.

These findings indicate that ammonia exerts a local effect in the nasal passages which causes a rapid increase in numbers of M. pulmonis, followed by the appearance of larger numbers in the larynx and tracheobronchial tree. Ammonia probably increases the growth of M. pulmonis in the nasal passages through effects on the host, rather than by enhancement of M. pulmonis growth because 1 mM ammonium ion inhibited the growth of M. pulmonis in vitro. Analysis of nasal washings for nucleic acids by ethidium bromide fluorescence enhancement did not show differences between ammonia-exposed and control rats. However, further studies are needed to define the effects of ammonia on the availability of essential mycoplasmal nutrients in nasal secretions and other aspects of respiratory physiology.

Abstract Approved by: Committee Chairman J. Russell Lindsey

Program Director

Date _____ Dean of Graduate School

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To my wife, Sandy, and my son, Zack, I owe a special gratitude for their many sacrifices, especially for the many hours devoted to this study which we might otherwise have spent together.

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BALT . . . . . bronchus-associated lymphoid tissue

CFU. . . . . .colony-forming units

CPM. . . . . radioactivity counts per minute

CRD. . . . . chronic respiratory disease

C3 . . . . . .third component of complement system

ELISA. . . . . enzyme-linked immunosorbent assay

Fc . . . . . .crystallizable fragment of immunoglobulin molecule

HMPA . . . . . hexamethylphosphoramide

^{125}I . . . . . .iodine-125 radioactive isotope

IgA. . . . . .immunoglobulin A

IgG. . . . . .immunoglobulin G

IgM. . . . . .immunoglobulin M

LSC. . . . . liquid scintillation counting

mCi. . . . . .millicurie

mM . . . . . .millimolar

MRM. . . . . .murine respiratory mycoplasmosis

^{32}P. . . . . phosphorus-32 radioactive isotope

PBS. . . . . phosphate-buffered saline

PI . . . . . .post inoculation

PPLO . . . . . .pleuropneumonia-like organisms

ppm. . . . . parts per million (volume/volume)

INTRODUCTION

Murine respiratory mycoplasmosis (MRM) is one of the most important diseases of laboratory rats, because affected rats are unsuitable as research subjects (53, 65, 86, 129, 131, 184, 193, 216, 219, 291). Many investigations in respiratory disease, gerontology, nutrition, behavior, and toxicology have been rendered difficult or impossible to interpret by manifestations of MRM (193). The disease is ubiquitous among conventionally housed rats (195).

Although MRM has been recognized since the early 1900's (107), only recently has Mycoplasma pulmonis been established as the cause (146, 176, 179, 193, 294). Results of earlier investigations into the cause of rodent respiratory disease were not conclusive, probably because it was not recognized that many factors can influence the expression of natural and experimental MRM. Among such factors, the atmospheric ammonia produced by bacteria in soiled cage bedding is of primary importance (32, 196).

Elucidation of the role of ammonia in the pathogenesis of MRM could be of broad general significance, not only because of the importance of MRM, but also because of the potential for adverse effects on other species. Other laboratory animals besides rats are frequently exposed to ammonia pollution (80, 205, 253), as are swine (54, 55, 70, 71, 262) and poultry (7, 8, 35, 42, 43, 175, 296). There also is considerable potential for human exposure. Ammonia is

widely used in industry and agriculture (4, 252), and there are significant amounts in tobacco smoke (34, 226).

It is possible to formulate a rather wide variety of hypotheses regarding the mechanisms by which ammonia could influence MRM because no aspect of the problem is well understood. Mechanisms by which mycoplasmas in general produce disease are quite unclear, as are the host responses to, and defenses against, mycoplasmal infections. Furthermore, although the major mechanisms of respiratory tract defense against infectious disease appear to have been identified, the relative contribution of each has not yet been elucidated. Similarly, little is known about effects of chronic exposure to low concentrations of ammonia. The current knowledge regarding these subjects is summarized in the sections which follow.

LITERATURE REVIEW

Murine Respiratory Mycoplasmosis

General features. MRM is an insidious, chronic disease (40, 195). It usually is clinically inapparent unless far advanced, when weight loss, rough hair coat, nasal and ocular discharges, and dyspnea may be evident. Abnormal respiratory sounds often can be detected in the absence of visible signs. Head tilt resulting from labyrinthitis is seen occasionally. Morbidity is usually low, but epizootics occur in which nearly all rats have clinical manifestations. Sporadic deaths result in a cumulative mortality which is particularly vexing to long term studies such as those in gerontology, oncology and toxicology.

There is little relation between the severity of clinical signs and the extent of lesions. Gross lesions may not accurately reflect the degree of microscopic changes. Lungs of normal macroscopic appearance, for example, can contain severe histologic changes. Lesions are quite inconsistent, even among closely associated rats of the same age, strain, and environment.

Gross changes are most striking in the lungs, and include atelectasis, exudative consolidation, bronchiectasis, and abscesses, all of variable distribution. Entire lobes can be affected, sometimes being converted to masses of abscesses. Atelectatic and consolidated lobes are dark red or gray and contain multiple, discrete, often elevated yellow foci which impart a cobblestone appearance to the pleural

surface. These foci represent dilated bronchi and bronchioles filled with mucopurulent exudate. Similar exudate usually is present in the nasal passages, trachea, and tympanic bullae.

In all parts of the respiratory tract, microscopic lesions are characterized by chronic suppurative inflammation accompanied by epithelial hyperplasia and, to a lesser extent, squamoid metaplasia. Extensive submucosal accumulation of lymphocytes also is characteristic. In the lung, proliferation of bronchus-associated lymphoid tissue (BALT) is an early and often profound change, characterized by increases in T lymphocytes, IgA-bearing B lymphocytes, and null cells (65). This is associated with mucin and neutrophilic exudate in airway lumina. Bronchiectasis and bronchiolectasis are typical of the advanced disease and can be of sufficient severity to result in abscess formation, seen grossly as yellow confluent nodules in the lung. The parenchyma also can be affected by suppurative inflammation, but airway lesions predominate. Chronic active rhinitis, otitis media, laryngotracheobronchitis, bronchiectasis, and bronchopneumonia are thus the major morphologic features of the disease but frequently not all are present. Rhinitis and otitis media seem to be the most common. Other changes are loss of cilia and increased goblet cells in the nasal mucosa, fibrosis of the lining membrane in the tympanic bullae, and inflammation of the laryngeal glands (40, 195).

Historical perspective. The large number of terms (40, 41) applied to syndromes resembling MRM is a result of numerous conflicting and inconclusive reports regarding the cause or causes of "chronic respiratory disease" (CRD) of rats. Today the term murine respiratory mycoplasmosis (MRM) is preferred (195), but many of the concepts and

doubts expressed in the early literature persist. A critical review of these papers should be helpful in understanding how this situation has come about.

The first known description of MRM appeared in the early 1900's (107). A few other reports followed (78, 201, 204), and in 1930 the first of many papers authored or co-authored by Dr. J.B. Nelson of Rockefeller University appeared (222). Nelson probably influenced the early concepts of rodent respiratory disease more than any other single individual.

Nelson and Gowen (222) reported isolation of several bacteria from the tympanic cavities of rats with otitis media. Several of these organisms produced otitis when injected directly through the tympanic membrane but would not do so after intranasal inoculation. In 1931 Nelson and Gowen (223) reported the establishment of a subline of rats from young breeders free of lesions of rhinitis, otitis, and pneumonia. The incidence of otitis in this subline was greatly reduced but rhinitis and pneumonia soon reappeared. They cited this as evidence that the causes of otitis and pneumonia were different. King (169) described the elimination of labyrinthitis by selective breeding in a manner similar to that of Nelson and Gowen (223) but his rats still had otitis media and pneumonia. Again the author interpreted this as suggesting the cause of labyrinthitis was different from that of the other lesions.

In 1940 Nelson (211) described an outbreak of respiratory disease among brown hooded rats. The disease could be transferred to rats from his previously described colony (223) by nasal instillation of nasal and tympanic exudates. The disease was maintained for many passages in this manner. In early passages deaths were common and pneumonia was

severe. Actinobacillus muris (now Streptobacillus moniliformis) and Bordetella bronchiseptica were frequently isolated. However, in later passages the incidence of pneumonia decreased and bacteria were rarely isolated, but the rats still had rhinitis and otitis. Nelson interpreted these findings to indicate that rhinitis and otitis were manifestations of a disease he called "infectious catarrh." He thought bacteria such as B. bronchiseptica were responsible for the acute pulmonary manifestations of a mixed infection. Yet, inoculation of cultures of B. bronchiseptica into his selectively bred rats would not produce the disease. These results are difficult to interpret because his rats already had a chronic enzootic rhinitis and pneumonia (223).

In 1937 (174) and 1940 (172) Klieneberger and Steabben described the isolation of an organism they called "L3" (now M. pulmonis) from rats with chronic pneumonia. However, this organism also did not reproduce the disease (172). Nelson described in 1940 (212) the finding of "coccobacilliform bodies" in exudates from rats with "infectious catarrh" (211). This organism was isolated in tissue culture but would not grow on "PPLO" medium. At the time, Nelson did not believe the organism was a mycoplasma but later (217) he attributed "infectious catarrh" to the previously described "PPLO" (172, 174), and still later (220) he stated that M. pulmonis was the cause of "a CRD."

Nelson reported in 1946 (213) that homogenates of the lungs of old rats from his colony with "endemic pneumonia" caused rhinitis, otitis and pneumonia in mice, his "mouse test." This syndrome closely resembled natural "infectious catarrh" of mice, but he was unable to demonstrate the "coccobacilliform bodies" of "infectious catarrh" or to isolate mycoplasmas from the mice (214). Nelson found that the

non-cultivable agent would pass through a Berkefeld V filter but not a Berkefeld N, indicating a size comparable to pox viruses (214). Although it was known at that time that these characteristics also could apply to a "PPLO" or mycoplasma, he thereafter usually referred to the agent as a virus (217-220). There is no evidence that any known virus of rodents alone can cause disease resembling MRM, but it is quite possible that Nelson's rat lung homogenates contained Sendai virus, and that this virus could have exacerbated existing mycoplasmal infections in rats and mice in his studies.

Subsequently, Nelson (215) showed that lung suspensions from old rats that caused pneumonia in 100% of intranasally inoculated mice did not cause pneumonia in young rats from the "infectious catarrh"-free colony (which had an enzootic chronic pneumonia). Suspensions of pneumonic lung from the inoculated mice also caused no increase in pneumonia in these rats. Nelson found that lungs of young uninoculated rats less than about 4 weeks old were not infective for mice, but that nasal washings were infective when the rats were as young as 5 days of age. He considered this suggestive of infection acquired by contact from infected parents. These findings are quite similar to those of a recent study of enzootic M. pulmonis infection in a conventional colony of Sprague-Dawley rats (62) and to Lemcke's (190) study of the isolation of "PPLO" from rats of various ages.

In 1951 Nelson (216) reported the establishment of a colony of rats free of lesions of the respiratory tract. This was done by selecting young rats from litters of parents free of lesions and with lungs negative to the "mouse test." The original stock were infected with "PPLO," S. moniliformis, and the rat pneumonia agent demonstrable by the "mouse

test." However, he later reported (217) that the "enzootic bronchiectasis" agent was again detectable in almost 100% of mature rats from this colony, even though they remained lesion-free.

Other investigators (129, 231) were unable to produce "CRD" in Nelson's rats with mycoplasmas from rats with natural "CRD." Pankevicius et al. (232) isolated mycoplasmas from only a few rats with natural "CRD." Newberne et al. (224) reported inconsistent isolation of bacteria from rats with "CRD." They did isolate an "agent" in tissue culture, but did not characterize it.

By the middle 1960's the view was expressed that "CRD" of rats was of complex etiology with a variety of infectious agents together or independently producing the "CRD" syndrome (30, 86, 87, 110, 130, 220). This view is commonly held today, but available evidence does not support it. Gay (84) showed that the "gray lung virus" of Andrewes and Glover (9) and Nelson's non-cultivable rat pneumonia agent had the morphology of mycoplasmas in respiratory tissues of mice. A subsequent study of the rat pneumonia agent in rats also showed that it was a mycoplasma, but the authors were unable to grow it on artificial media or in tissue culture (85).

Production of "CRD" lesions in the lungs of gnotobiotic rats inoculated with cloned cultures of M. pulmonis and fulfillment of Koch's postulates was first described by Kohn and Kirk (179) and Kohn (176, 177). The rats were serologically negative for Sendai virus, pneumonia virus of mice, and reovirus type 3 infections and no bacterial pathogens were isolated. However, only 7 of 45 rats had bronchiectasis in the first experiment, and 6 of 23 in the second. In a 1970 outbreak of respiratory disease among barrier-maintained gnotobiotic rats of 1

strain, mycoplasmas were consistently isolated from rats with lung lesions (186). Mycoplasmas also were isolated from non-affected rats of 2 other strains in the same facility. No viruses were isolated although some rats did have serum antibody titers to Sendai virus.

Lindsey et al. (193) described production of lung lesions, including bronchiectasis, in 12 of 23 gnotobiotic Fischer rats inoculated with a strain of M. pulmonis and killed 1 month later. The rats were intensively monitored to assure pathogen-free status. All 3 strains of M. pulmonis tested produced lesions of the proximal respiratory tract but 2 strains, including 1 obtained from Nelson, did not cause lung lesions. M. pulmonis was demonstrated by indirect immunofluorescence in lesions in all parts of the respiratory tract, by culture in tracheal lavages, and by electron microscopy in lung lesions. Bacteria were isolated from 35% of the rats, but in less than 10% of the rats were the organisms considered potential pathogens (Staphylococcus aureus, Pseudomonas sp.).

Whittlestone et al. (294) used a cloned culture of M. pulmonis to produce "CRD" lesions, including otitis interna and lung lesions, in rats serologically negative for Sendai virus infection and free of cultivable bacterial pathogens. Jersey et al. (146) again fulfilled Koch's postulates with M. pulmonis. They reported that M. pulmonis was the principal isolate from pneumonic lungs of germ-free rats exposed to rats with natural "CRD." Either cell-free filtrates from pneumonic rat lungs or broth cultures of M. pulmonis produced lesions of "CRD" in 2 to 6 weeks in germ-free or exgerm-free rats. M. pulmonis was isolated from lesions in these rats. In contrast, attempts to produce "CRD" in

rats with intranasal inoculation of bacteria isolated from natural cases have been unsuccessful (85, 211, 222).

The available evidence, then, clearly indicates that "CRD" is a mycoplasmal disease and is therefore properly termed murine respiratory mycoplasmosis (MRM) (195). The lesions described in experimental MRM (146, 176, 179, 193, 294) are compatible with the descriptions and pictures representing "CRD" which have been published over the last 50 years (53, 78, 86, 87, 129-131, 172, 222, 223, 227).

There have been objections to M. pulmonis as the primary cause of MRM. Mycoplasmas were not isolated from many cases of the natural disease (12, 81, 87, 212, 213, 220, 232, 276). Many mycoplasmal isolates did not produce all manifestations of "CRD," particularly the bronchopulmonary lesions (12, 41, 85, 129, 172, 193, 272), and in some instances mycoplasmas were associated with bronchiectasis only after bronchial ligation (173, 277, 278).

For several reasons these objections are of doubtful validity. First, early workers were hampered by the lack of "CRD"-free rats (131, 216). Meaningful data were not obtained until gnotobiotic or pathogen-free rats became widely available. Second, many investigators made the error of concluding that an agent not demonstrable in culture was not present. Mycoplasmas that are pathogenic for rats and mice but which did not grow on artificial media or in tissue culture have been described and include the "enzootic bronchiectasis" agent (84, 85). Van Zwieten et al. (276) described mycoplasma-like structures in some rats heavily infected with a filamentous organism. Mycoplasmas were not isolated, but the enzyme-linked immunosorbent assay (ELISA) demonstrated antibodies to M. pulmonis.

At least a partial explanation for the frequent difficulty in isolating some mycoplasmas appeared in recent reports of inhibitory substances in tissue suspensions (156, 157) and medium ingredients (68, 69, 267). Therefore, the practice of applying tissue suspensions to plates of solid medium, by which some investigators (81, 232) have attempted to isolate mycoplasmas in natural MRM, is likely to be unsatisfactory. Further, some mycoplasmas are reported to become so host-adapted in chronic infections that they are no longer cultivable although they are demonstrable by immunofluorescence (202). It may be that in such cases cultural techniques simply need improvement (69). Finally, the early studies in which agents were supposedly demonstrated by nasal instillation of lung suspensions are suspect because subclinical MRM in mice can be acutely exacerbated by nasal instillation of a variety of materials, even sterile broth (73, 199, 264). Thus, it is impossible to say whether any agent at all was present in the lung suspensions.

The failure to induce lung lesions with isolates of M. pulmonis also probably reflects that only recently have investigators recognized that a number of complicating factors must be considered (32, 146, 176, 294). Isolates of M. pulmonis vary considerably in pathogenicity (121, 193, 294) and it is possible that some may lose virulence after repeated cultivation on artificial media (294). More importantly, several factors can profoundly influence the extent to which disease develops as a result of infection by even the most virulent strain of M. pulmonis. For example, administration of toxic compounds can greatly enhance the expression of MRM (230). Several reports have suggested, and Davis et al. (66, 67) have conclusively demonstrated, that strains of rats

vary in susceptibility (186, 224, 294). Broderson et al. (32) were the first to show that ammonia released from soiled bedding has a profound effect on the development of MRM, although many other investigators have suggested that environmental conditions could be important in this regard (75, 87, 110, 186, 224, 294). Farris (75) suggested that poor sanitary practices (dirty cages) exacerbated the problem of "CRD" and Lane-Petter et al. (186) noted an increased odor of ammonia during a respiratory disease outbreak. Whittlestone et al. (294) were unable to produce lung lesions in experimental MRM until they reduced the frequency of cage cleaning, resulting in accumulation of ammonia in the room.

Broderson et al. (32) showed that atmospheric ammonia in concentrations of 25 to 250 ppm increased the severity of lesions of the proximal airways and both the incidence and severity of bronchopulmonary lesions, including bronchiectasis. This effect was the same when the ammonia source was either pure anhydrous ammonia or soiled cage bedding. Up to 33% of rats in the high-ammonia groups had grossly evident bronchiectasis as compared to 8% or less of the control rats. While this frequency of bronchiectasis is a little less than sometimes reported in the natural disease, it must be noted that these rats were young, 2 to 4 months old, that the disease had been allowed to progress for only 4 to 6 weeks, and that they were of a strain (Fischer) which is relatively resistant to the development of these lesions (66, 67).

The discovery of the role of ammonia in MRM may offer some insight into a hitherto unexplained observation. Two papers report the elimination of "CRD" lesions in rats from the same original source by sulfamerazine in feed or water (81, 104). It is possible, although purely

speculative, that the antibiotic inhibited the urea-splitting flora of the rats enough to significantly reduce intracage ammonia. However, in one article it was stated that the effect of good husbandry practices could not be evaluated because it was not tested in the absence of sulfamerazine. Increased cleanliness might also have reduced ammonia production.

In 1971 Lindsey et al. (193) reviewed many investigations which had been complicated by MRM. Particularly instructive examples of such studies are reports attributing lesions of MRM which appeared in a toxicologic study of hexamethylphosphoramide (HMPA), and insect chemosterilant, to direct effects of the compound (167, 168). It was later suggested that HMPA might actually have exacerbated natural "CRD" (254). Overcash et al. (230) subsequently showed that HMPA caused a remarkable enhancement of lesions of MRM, with up to 60% mortality associated with extreme neutrophilic exudation. HMPA alone caused thinning and microulceration of the epithelium in the major bronchi. Additional studies complicated by MRM have been reported since 1971. Male rats affected by "chronic murine pneumonia" developed more lung neoplasms in response to administration of N-nitrosoheptamethyleneimine than did germ-free or pathogen-free rats (249). Morphologic studies of BALT in rats (77, 101, 102) are made suspect by a lack of adequate documentation of the absence of mycoplasmal infection. Rats with MRM were reported to have abnormalities of pulmonary clearance, mucociliary transport, and mucus production (132).

Several reports indicate that rats infected with M. pulmonis are particularly inappropriate for studies of respiratory immunology. Davis et al. (65) demonstrated qualitative and quantitative changes in lung

lymphocytes including increases in T cells, IgA-bearing B cells, and null cells as compared to uninfected rats. M. pulmonis is mitogenic for murine B and T lymphocytes (207, 209, 210), and therefore could alter responses of these cells. Several mycoplasmas, including M. pulmonis, are reported to induce or inhibit interferon production (49, 50). Since some mycoplasmal infections have been associated with immunosuppression (1), it is possible that similar effects could occur in rats with MRM under some circumstances.

Respiratory Defense Mechanisms

The defense mechanisms of the normal respiratory tract act in an integrated and interrelated fashion (91, 94, 164) but, for the sake of ease of discussion, can be considered as consisting of filtration, the mucociliary system, phagocytosis, and specific cellular and humoral immunity. There are major differences in the contributions of each of these systems between the upper and lower respiratory tract, that is, above and below the junction of terminal and respiratory bronchioles. Much information, which has been reviewed by several authors (47, 94, 164, 225), exists regarding these systems although the relative importance of each in defense against infectious agents is not entirely clear.

Filtration and mucociliary transport. These mechanisms constitute the major defense against inspired particulate matter. Most particles larger than about 3 μm are deposited on the mucus blanket of the upper respiratory tract (47, 93, 94, 97, 225). Particles less than 3 μm generally reach the lower respiratory tract, and many are deposited there (47, 94, 225). There is little deposition of particles smaller

than about 0.5 μm (94). The secretions of the proximal airways also absorb soluble noxious gases including ozone, ammonia, sulfur dioxide, and chlorine (97, 185, 225).

Physical removal of particles from the lung is complex, but mucociliary transport is by far the major, if not the only, means of removing particles deposited upon the ciliated airway surfaces (91, 94, 96, 97, 180, 188, 225). The rate of transport on ciliary surfaces is rapid, up to 10-20 mm/min in the trachea and main bronchi and up to 1 mm/min in the small airways, so that particulate clearance from the upper tract is usually complete within 24 hr (47, 93, 225). Both nasopharyngeal and tracheobronchial mucus is transported toward the oropharynx and usually is swallowed (47, 225). Particles deposited distal to the ciliated surface, in the lower tract, are removed much more slowly and may persist for months in the lung though not necessarily in the alveolar spaces (47, 93, 94, 225). These particles may be removed, with or without phagocytosis by alveolar macrophages, by movement from the alveoli to the ciliated surface and transport by the mucociliary system. The mechanism of transport from alveoli to bronchioles is poorly understood (93-95, 225).

The importance to the host of mucociliary transport is not completely clear. It generally is thought that decreased mucociliary activity is associated with increased susceptibility to infections but is still compatible with a fairly normal life (91, 225). Persons with immotile cilia syndrome have a tendency to develop chronic bronchitis, sinusitis and some degree of chronic obstructive lung disease (2).

The mucous secretion itself is produced by goblet cells, mucous glands, and other cells such as Clara cells. It contains about 95%

water and about 1% protein, carbohydrates, and lipids (224). Proteins in normal airway secretions include albumin, α_1 anti-trypsin, lysozyme, transferrin, interferon, IgG, IgA, free secretory component, and minimal amounts of complement components (47, 97, 225, 242). Alveolar fluids generally lack mucus and are rich in neutral lipids, proteins, and phospholipids, particularly phosphatidyl choline (97).

Alveolar macrophages and pulmonary bacterial clearance. Alveolar macrophages originate in the bone marrow and probably also in a proliferating pulmonary interstitial population (28, 88, 97, 111, 225). After a sojourn in the spaces of the alveoli and respiratory bronchioles, most alveolar macrophages reach ciliary airway surfaces and are transported to the pharynx and swallowed (28, 159). Large numbers of macrophages leave the lung in this way, 1 to 5 x 10^6/hr in small animals (97, 260). Much of the particulate matter reaching the respiratory spaces is ingested by these macrophages. Alveolar macrophages probably do not have an important phagocytic function during transit through the upper airways (97).

Very fine particles can enter the pulmonary interstitium and be phagocytized there, but significant clearance of particulates by macrophages re-entering pulmonary tissues and leaving the lung through lymphatics probably does not occur in normal lungs (28, 188). Macrophages that have phagocytized particles in the interstitium are thought to be able to enter the airways at the bronchioloalveolar junction (94, 95, 97).

Alveolar macrophages constitute the primary defense of the lower respiratory tract against bacteria (47, 90, 93, 96, 99, 159, 164, 225). Though the respiratory membrane is generally thought of as sterile,

recent studies indicate that viable bacteria may be commonly aspirated from the proximal airways (192). Thus alveolar macrophages are important in the routine policing of the respiratory surface as well as in defense against specific infectious diseases.

Most of the evidence regarding the role of lung macrophages has come from studies of pulmonary bacterial clearance. Laurenzi *et al.* (187) made the first quantitative study of pulmonary bacterial clearance in mice. They devised an apparatus for exposing mice to an aerosol of bacterial suspension of a droplet size sufficiently small to reach the alveoli, and showed that colony-forming units (CFU) of *S. aureus* deposited in the lungs in this manner decreased rapidly in roughly an exponential fashion such that clearance was 88% complete in 4 hr and 95% complete in 6 hr.

Green and Kass (99) used this technique to expose mice to aerosols of bacteria labelled with phosphorus-32 (^{32}P). Numbers of viable *S. aureus* and *Proteus mirabilis* in homogenized lungs decreased at a much greater rate than radioactivity, indicating that intrapulmonary killing of the bacteria was the major mechanism of pulmonary clearance rather than physical removal. Parallel histologic and immunofluoresence studies on additional mice showed that the staphylococci were rapidly phagocytized by alveolar macrophages. This was followed by the appearance of diffuse staphylococcal antigen in the alveolar macrophages indicating that the macrophages were responsible for the observed killing of the bacteria.

In similar experiments, Goldstein *et al.* (90) deposited more than 10^6 CFU of *S. aureus* in the lungs of rats. There were 77% less CFU in the left lungs of rats killed 5 hr after exposure than in those of

rats killed immediately. The right lungs were examined histologically. At 5 hr 92% of bacterial seen were within alveolar macrophages. Kim et al. (166) showed that ingestion of S. aureus by mouse alveolar macrophages preceded killing and that actual destruction followed inactivation by a considerable interval.

In sum, the available evidence indicates that in situ killing is the primary antibacterial defense of the respiratory membrane, and that it is accomplished primarily by alveolar macrophages. Recent work indicates that neutrophils also may participate in clearance of some gram negative bacteria (235, 273).

Different species of bacteria are cleared from mouse lungs at different rates. These rates are the net result of physical removal, in situ killing, and intrapulmonary growth (145). This growth was significant in the case of Klebsiella pneumoniae, Escherichia coli, and Streptococcus pneumoniae (145, 147) but S. aureus did not multiply over the 4 hr clearance period (145). These results were obtained by comparing clearance rates in tetracycline-treated mice to those in untreated mice.

Few studies of pulmonary bacterial clearance have been done in species other than the mouse. Reported clearance rates of S. aureus for the rat are 77% in 5 hr (90), 78% in 6 hr (183) and 74% in 4 hr (141). S. aureus was killed at about the same rate in guinea pigs, hamsters, rats, and mice, but P. mirabilis was killed more slowly in the mouse and rat than in the other two species (141). Staphylococcus albus (S. epidermidis) was cleared more efficiently than S. aureus from rat lungs, 98% being inactivated in 6 hr (183).

Clearance rates are reported to be independent of inoculum size over a rather wide range (98). However, in recent work (273), a minor but statistically significant difference in the rate of pulmonary clearance of S. aureus was obtained in mice inoculated with different numbers of organisms by aerosol exposure. The 4 hr clearance rate was 84% with an inoculum of 4.4×10^6 CFU, and 75% with an inoculum of 1.56×10^7 CFU. Whether or not the difference of 9% is significant from a practical standpoint is not clear. Pseudomonas aeruginosa was cleared at a rate of 53% when 8×10^4 CFU were inoculated, but intrapulmonary growth occurred after deposition of 6×10^5 CFU. Sixty-five percent of an inoculum of 1.3×10^6 CFU of K. pneumoniae was cleared in 4 hr, but a smaller inoculum of 1.6×10^5 CFU was cleared at a rate of only 28%.

Considerable evidence exists that pulmonary bacterial clearance is a valid measure of lung antibacterial defenses. Sendai virus infection in mice induces a dose-dependent decrease in pulmonary bacterial clearance (134, 138) which parallels an increase in susceptibility to fatal experimental Pasteurella pneumotropica pneumonia (133, 136, 137). Lung macrophages from infected mice have a number of abnormalities, including decreased ingestion (143, 144, 287), probably due to changes in surface receptor-mediated function (144, 289). Decreased intracellular killing (142, 287), possibly resulting from inhibited fusion of phagosomes and lysosomes (143, 255), and increased lysosomal enzyme release (288) are also found in these macrophages.

A number of experimental procedures have been reported to inhibit pulmonary bacterial clearance in mice or rats, including complement depletion by cobra venom factor (103), ethanol intoxication (100), cold

exposure (100), ozone inhalation (90), reovirus infection (170), and experimental renal failure (89). Some of these factors are thought to predispose human patients to bacterial pueumonias (98, 100).

The demonstration of decreased pulmonary bacterial clearance in complement-depleted mice (103) is unexplained, inasmuch as only very small amounts of complement are usually found in lavage fluids (225, 242). However, functionally adequate local concentrations of complement might be present in lower respiratory tract secretions (225). The observations that lung macrophages of many species have complement receptors (44, 111, 241, 292) strengthen this possibility. Complement also enhances the *in vitro* phagocytosis of *S. aureus* and *Ps. aeruginosa* by rabbit alveolar macrophages (206). Further work is needed on the role of complement in respiratory defense mechanisms.

Other reports suggest special adaption of lung macrophages for their environment. Lipid fractions of lavage fluids thought to contain surfactant or alveolar lining material enhanced the killing of *S. aureus* by rat (153, 183) and rabbit (181) alveolar macrophages. Alveolar lining material is also reported to be chemotactic for lung macrophages (250, 251). Lung macrophages have Fc receptors for IgG (44, 111, 240), which appears to be the most important immunoglobulin in the lower tract, being present in considerably greater quantities than IgA (47, 97, 158, 160, 225).

Alveolar macrophages are less well studied than peritoneal macrophages and neutrophils, but it is evident that lung macrophages have significant differences from other phagocytes. Alveolar macrophages depend on both aerobic and anaerobic metabolic pathways for optimal function (97, 111). They have a higher resting metabolic rate than any

other phagocytic cell and undergo a limited respiratory burst during phagocytosis (111). Lung macrophages also differ from peritoneal macrophages in surface antigens, although this apparently is due to differences in distribution of antigens rather than population-specific antigens (25).

The microbicidal mechanisms of lung macrophages are incompletely understood. However, they generate reactive oxygen metabolites such as superoxide anion, hydrogen peroxide and hydroxyl radical (11, 26) but apparently do not contain myeloperoxidase (20, 21, 23, 97, 111). Rabbit alveolar macrophages contain highly cationic microbicidal proteins (233).

Lung macrophages have important functions other than phagocytosis of infectious and inert particles. They are active secretors of many substances including lysozyme (22, 23), proteolytic enzymes (47), prostaglandins (125, 126), complement components (47, 164), interferon (47), and chemotactic and colony-stimulating factors for neutrophils (47, 124, 164). Lung macrophages also can participate in cell-mediated immune reactions in the lung (97, 148, 158), and may have an immunoregulatory role (10, 61, 113-116, 234). Additional functions of lung macrophages include cytotoxicity against virus-infected cells, surfactant catabolism, and possibly, antigen processing (47).

Respiratory tract immune system. Lymphoid tissue is found throughout the respiratory tract (158, 164). BALT consists of nodules, aggregates, and diffuse collections of lymphoid cells, which are overlaid by specialized lymphoepithelium (17, 19). Lymphoepithelium may provide interaction between inhaled antigen and lymphocytes (164).

The function of BALT is unclear (158). It may be part of a common mucosal immune system (246), and is probably a source of IgA-bearing lymphocytes (18). Although BALT contains IgA-, IgM-, and IgG-bearing lymphycytes and T cells (18), specific antibody-containing cells were not found in BALT after local immunization (18, 46). Even so, current evidence indicates the existence of both humoral and cell-mediated mechanisms in the respiratory tract, which are at least partially independent of systemic immunity (14-16, 164, 239, 283, 284).

Bronchoalveolar lavage has been used extensively to study respiratory tract immunity although whole lung lymphocytes also have been examined (65, 122, 123). The cells recovered generally include a majority of macrophages and a lesser number of lymphocytes, and may contain a few neutrophils. The proportions of macrophages and B and T cells vary among species and the state of immunization (47, 60, 158, 162, 165, 225). The exact anatomic source of these cells is unclear, but it is assumed that they come from the air spaces because they can be obtained without red blood cells, indicating a lack of tissue damage (158). The host defense functions of bronchoalveolar lymphocytes and immunoglobulins are unclear (158). Recent work is summarized in the following paragraphs.

The respiratory tract has mostly IgA cells in the lamina propria, and secretory IgA is present in bronchial and nasal secretions (19). Resistance to infection, at least with certain organisms, correlates better with secretory IgA than with serum antibody (19, 225, 283). Even so, the functions of secretory IgA are not well understood (158, 164, 225). It apparently does not have a critical role in host defense because many patients with selective IgA deficiency remain healthy (158).

Others, however, may have an increased susceptibility to respiratory infections (225). Possible functions of secretory IgA include virus and toxin neutralization, bacterial growth inhibition, prevention of bacterial adherence to host epithelia, agglutination of bacteria, and limiting antigen absorption (97, 158, 225). While IgA does not fix complement by the classical pathway, complement fixation by aggregated IgA via the alternative pathway, perhaps acting in concert with lysozyme, might result in significant bactericidal activity (97, 225). Most secretory IgA appears to be locally synthesized (158), but present evidence indicates that other immunoglobulins in respiratory secretions can come from the blood as well as from local synthesis (45, 97, 158).

Differences exist between humoral mechanisms of the ciliated airways and those of the respiratory membrane. Lavage fluids from the distal respiratory tract contain IgG in much greater relative amounts than those from the proximal airways (47, 97, 158, 160, 225). The relative importance of secretory IgG is unclear (158), but it is probably functionally more important in the respiratory spaces than IgA. IgA apparently has little or no opsonic activity for lung macrophages 239, 240, 244). IgG in respiratory secretions can originate either in local secretion, particularly after local immunization (225), or from serum (164, 243, 245). This immunoglobulin is probably important in enhancing alveolar macrophage function in immune animals, inasmuch as IgG Fc receptors have been demonstrated on the alveolar macrophages of several species (44, 111, 240). Also, pulmonary bacterial clearance can be enhanced by immunization (135, 139, 182). Antibody-mediated mechanisms are probably involved because lung washings from immunized

animals contained opsonic activity (182) and passive immunization also enhanced clearance (135).

Many investigators have not found IgM in respiratory secretions (229, 243), but others have found small amounts at all levels of the respiratory tract (160). The function of IgM, if any, in the lower respiratory tract is unknown (97, 158, 164). Nevertheless, the capacity for local IgM production does exist. IgM and IgG plaque-forming cells have been demonstrated in lavaged lymphocytes from dogs (14-16, 158) and humans (158). The function of these cells is uncertain, but specific antibody-forming cells were found after local immunization in dogs (16, 33, 158) and rabbits (158). Evidence has been presented that the source of these cells is the blood, following their production in local lymph nodes (14-16, 33). Systemic immunization can also result in specific antibody-forming cells in bronchoalveolar spaces (161, 275).

There is substantial evidence for the existence of cell-mediated immune mechanisms in the respiratory tract that are, like humoral mechanisms, somewhat independent of systemic cell-mediated immunity (83, 97, 158, 225). This evidence includes production of migration inhibition factor by antigen-stimulated lavage lymphocytes (79, 109, 284), the activation of alveolar macrophages by supernatants from antigen-stimulated lower respiratory tract lymphocytes (148), and the demonstration of cytotoxic effector cells in lavages from hamsters infected with parainfluenza 3 virus (108). Systemic immunization with Listeria monocytogenes enhanced lung microbicidal activity against aerosol challenge with that organism (274), suggesting that stimulated

cell-mediated immune responses can enhance pulmonary defensive capacity as can humoral immunity (135, 139).

Interactions of *Mycoplasma pulmonis* and Host Defenses

Epithelial cells and mucociliary transport. Attachment of the mycoplasma to the surface of host epithelial cells is characteristic of most mycoplasmal infections (37). How this contributes to disease is unclear. Mycoplasmas require cholesterol, fatty acids, and nucleic acid precursors for growth (238). Presumably these are obtained from host cells but the mechanism by which this occurs is unknown. It is possible that attachment allows toxic host cell damage, but no true exotoxin has been demonstrated (238). Many mycoplasmas produce hydrogen peroxide, but this alone cannot account for pathogenicity because hydrogen peroxide production *in vitro* does not correlate with pathogenicity (238).

M. pulmonis (177, 228) and other respiratory mycoplasmas (238, 266, 293) attach to ciliated respiratory epithelial cells strongly enough that they are not removed by ciliary action (238, 293). Ciliostasis is associated with *M. pulmonis* attachment *in vitro* (178), but rats with bronchial lesions of MRM are reported to have increased mucus and particulate transport rates (132).

Humoral immune defenses. The role of immune mechanisms in mycoplasmal disease also is unclear (37). Immunity to mycoplasmas does occur and can be demonstrated in various ways (37, 119, 293). Recovered animals are resistant to reinfection (293). Mice (269) and rats (36) appropriately vaccinated against *M. pulmonis* are resistant to challenge. Resistance is also passively transferable (41, 270) and local

immunization is apparently more effective than parenteral (36, 119, 269). However, there is some evidence that the immune response can be detrimental. For example, mice depleted of T cells are reported to develop less severe disease after inoculation with M. pulmonis (270). Lewis rats develop more severe lesions than do Fischer rats (66, 67). In Lewis rats, lung T cells, B cells, and plasma cells proliferated to a considerably greater extent than in Fischer rats, although this could represent differences in responses to nonspecific mitogenicity of M. pulmonis rather than specific immune responses (67). Lewis rats also had somewhat more vigorous serum antibody responses as measured by ELISA (67). The close association between host cells and mycoplasmas could result in "innocent bystander" damage to the cells by immune mechanisms (37).

Another factor shared by most mycoplasmal diseases is chronicity, despite demonstrable immune responses (37, 293). This apparently results from the ability of the organisms to evade host immune effector mechanisms (37). How this is accomplished is not understood. It is possible that the close association with surfaces of ciliated cells affords protection, but other factors may well be involved (37). It is also possible that some mycoplasmas can "disguise" themselves by selectively acquiring host antigens (295).

Cellular immune defenses. Two studies indicate that mouse peritoneal macrophages ingest and kill M. pulmonis only in the presence of specific antibody (149, 151). Experiments with Mycoplasma pneumoniae and guinea pig macrophages gave similar results (31, 74, 236, 237). In all of these experiments rabbit antiserum was used. Others have found that mouse antibody is ineffective in promoting phagocytosis in

comparison to rabbit antibody (64, 268). These and other studies (127) indicate the importance of using homologous antisera in future investigations.

It has been reported that mouse peritoneal macrophages do not support the growth of M. pulmonis as well as fibroblasts (150), but others (64) have found that mouse alveolar macrophages do not inhibit the growth of M. pulmonis in vitro. In contrast, Fischer rat alveolar macrophages are capable of significant inhibition (64). M. pulmonis may possess an antiphagocytic surface protein (152), and Howard and Taylor (121) reported that the virulence of 5 strains of M. pulmonis appeared to be related to resistance to in vivo killing by mouse peritoneal macrophages. These reports suggest that the ability to resist attack by macrophages may be a determinant of pathogenicity.

Many mycoplasmas or preparations thereof have mitogenic activity for the lymphycytes of several species (3, 13, 48, 207-210) and may inhibit the mitogenic response to other mitogens (258). M. pulmonis is mitogenic for murine B and T cells (207, 209, 210) although it apparently does not induce cytotoxicity (207). It has been suggested that a relationship may exist between pathogenicity and in vitro mitogenicity (207).

Several mycoplasmal infections have been associated with immunosuppression (1), and injection of mycoplasmas may result in reduced humoral responses (155).

Many mycoplasmas, including M. pulmonis, induce interferon production in human and ovine lymphocytes (50). M. pulmonis and M. arthritidis cells and cellular components induce interferon production in mice and inhibit interferon induction by other interferon inducers (49).

Effects of Atmospheric Ammonia

The known effects of ammonia on animals and man have been extensively reviewed (262). Exposures to several hundred ppm are associated with irritative effects on the eyes and respiratory mucosa (27, 252, 263, 285). Massive exposures can result in burns, pulmonary edema, and death in humans (252, 285). However, little information exists on the effects of long term exposure to low concentrations.

Ammonia long has been associated with a keratoconjunctivitis in chickens confined in poultry houses in which ventilation is insufficient to prevent ammonia accumulation from excreta (35, 296). Ammonia concentrations of 100 ppm or less have been reported to reduce weight gains, feed efficiency, and egg production in chickens (42, 43, 175). Similar concentrations of ammonia also were reported to reduce the rate of gain in swine (55, 261). Exposure to 20 or 50 ppm ammonia increased the rate of seroconversion to Newcastle disease virus in chickens (5) although it did not influence mortality (6). Ammonia and dust exposure are reported to increase the incidence of airsacculitis and pneumonia in turkeys (8) which are the predominant lesions of Mycoplasma meleagridis disease. In chickens, more severe airsacculitis from aerosol exposure to E. coli resulted from exposure to 100 ppm ammonia (231). Drummond et al. (71) described decreased pulmonary bacterial clearance in pigs exposed to 50 and 75 ppm ammonia.

Several investigators have described morphologic changes in the respiratory tract with exposures to low concentrations of ammonia. Unfortunately, in many cases the descriptions are fragmentary or the possibility that infectious diseases could account for some changes was not recognized. Tracheal epithelial changes including an increase in

epithelial thickness and acute inflammation were described in rats exposed to 200 to 400 ppm (80), but M. pulmonis infection was not excluded and the reported lesions resemble those of MRM. Another report describes 90 to 98% mortality in rats exposed to about 650 ppm (51), but the descriptions of lesions in these animals and in others exposed to various concentrations of ammonia were unclear. Guinea pigs exposed to 140 to 200 ppm did not develop lung lesions (290); proximal structures were not examined. In swine, 50 and 75 ppm did not produce any lesions (54), but 100 ppm reportedly resulted in increased thickness of the tracheal epithelium and decreased numbers of goblet cells (70). Ciliary loss in chickens exposed to ammonia was described in the nasal and tracheal epithelium (6). Broderson et al. described changes limited to the nasal passages in gnotobiotic rats, including epithelial cellular hypertrophy and degeneration and submucosal edema. These changes were more severe anterodorsally.

Ammonia apparently is almost completely absorbed in the nasal passages (52, 59). It induces ciliostasis (52, 58, 59), but ambient ammonia concentrations over 500 ppm were required to induce ciliostasis in the trachea in rabbits (52, 59). Morphologic changes in the lungs have been observed in rabbits and cats after inhalation of several thousand ppm ammonia (27).

Ammonia is thought to damage cells by inhibiting energy metabolism (189, 281). Ammonia combines with α-ketoglutarate from the Krebs cycle to form glutamic acid under the influence of glutamic acid dehydrogenase. It was recently reported that ammonium salts caused enhanced secretion of lysosomal enzymes by fibroblasts in vitro (92). A similar response in vivo might result in a variety of effects. Ammonia has been reported

to alter hepatic microsomal enzyme activity (279, 280), and may affect cell turnover rates (281, 282).

It is evident from the preceeding discussion that, given the present state of knowledge concerning the pathogenesis of MRM and the effects of inhaled ammonia, an extremely wide variety of hypotheses can be advanced concerning the role of ammonia in the pathogenesis of MRM, and in particular in the development of lung lesions. Among the possibilities considered were: (a) direct enhancement of the pathogenicity of M. pulmonis through increased growth or metabolic activity; (b) inhibition of any of the respiratory defense mechanisms including mucociliary transport, pulmonary macrophages, and specific immunity; (c) effects on respiratory tract physiology, for example quantitative or qualitative changes in secretions which might favor the growth of M. pulmonis or its aspiration into the distal respiratory tract; (d) increased availability of essential mycoplasmal nutrients, such as nucleic acid components and cholesterol, as a result of ammonia-induced cell damage; and (e) altered attachment of the organism to respiratory epithelium through changes in the interaction of the organism with cell surfaces.

Several lines of evidence suggested that investigations into the effect of ammonia on alveolar macrophages and pulmonary bacterial clearance might be productive. First, mice are susceptible to acute pneumonic lesions in experimental MRM, but rats are not (41). Immunofluorescence studies showed that M. pulmonis quickly became associated with alveolar macrophages in rats after intranasal inoculation and were to a large extent cleared from the alveoli and bronchi in 48 hr, and completely cleared by 72 hr (41). However, in mice, organisms persisted

free in the alveoli for several days and fewer were associated with alveolar macrophages. These studies suggested that alveolar macrophages may be important in the resistance of rats to the development of lung lesions in MRM. Second, HMPA given orally to rats greatly enhanced the development of bronchopneumonia in rats with natural and experimental MRM (230). Preliminary immunofluorescence studies suggested that the HMPA-treated rats were unable to clear M. pulmonis from the alveoli and bronchi (41), although no data were obtained regarding the possible mechanism for this lack of clearance. Finally, young pigs continuously exposed to 50 to 75 ppm ammonia were reported to have reduced rates of pulmonary clearance of E. coli after aerosol inoculation (71).

Other questions addressed were whether ammonia exposure resulted in differences in populations of M. pulmonis in the respiratory tracts of rats after intranasal inoculation, as compared to rats not exposed to ammonia, and whether any changes resulted directly from the effects on the growth of the organism or indirectly through effects on the host.

MATERIALS AND METHODS

Production and Quality Control of Rats

Procedures for rearing and maintaining rats and monitoring for rodent pathogens were as previously described (32, 41, 193). A disease-free breeding colony of Fischer rats was maintained in 150 x 60 x 60 cm Trexler type plastic film isolators (StaSafe, Germ-Free Supply Division, Standard Safety Equipment Co., Palatine, IL). Rats were housed in polycarbonate cages, and provided with autoclaved water, food (Wayne Lab-Blox Sterilizable, Chicago, IL), and hardwood chip bedding (Beta-Chip, Northeastern Products Corp., Warrensburg, NY). The colony was monitored quarterly for infection by detailed post mortem examination and bacteriologic and serologic tests on randomly selected weanling and retired breeder rats. Sera were tested (by Microbiological Associates, Bethesda, MD) for 11 rodent viruses: pneumonia virus of mice, reovirus type 3, Theiler's encephalomyelitis virus, Sendai virus, minute virus of mice, Kilham rat virus, Toolan H-1 virus, rat coronavirus, lymphocytic choriomeningitis virus, mouse adenovirus, and mouse hepatitis virus. In all experiments, rats were used when they were between 6 and 8 weeks old.

Method of Ammonia Exposure

Rats were exposed to 100 ppm ammonia by a previously described technique (32). Gaseous ammonia from a tank of liquid anhydrous ammonia (PB & S Chemical Co., Henderson, KY) was introduced into the air supply

of an isolator through a specially constructed regulator-filter apparatus. Control rats were housed in an identical isolator without ammonia. Special 19 x 41 x 25 cm suspended stainless steel wire mesh cages were used. The bedding beneath was changed daily to prevent exposure to any naturally produced ammonia, although there were no ammonia-producing organisms in the rats' intestinal flora. These cages also allowed better air exchange than conventional cages.

Ammonia concentration in the isolators was monitored daily with a Drager Multi Gas Detector (Dragerwerk, Lubeck, Federal Republic of Germany), or continuously by a Honeywell Ammonia Detector (Honeywell, Ft. Washington, PA). The concentration of 100 ppm was used because it is well above the lowest concentration, 25 ppm, used by Broderson et al. (32) and because this concentration is commonly encountered naturally (253; J. R. Lindsey, unpublished).

Measurement of Pulmonary Bacterial Clearance

Method. The rate of pulmonary clearance of an organism is dependent on the rates of physical removal from the lungs by the mucociliary system, intrapulmonary killing of the organism, and growth of the organism in the lung (99, 145, 247). By using radiolabelled organisms in clearance measurements, the role of physical removal can be assessed simultaneously with and independent of the effects of intrapulmonary killing and growth of the organism (90, 96, 99, 145, 247). A decline in lung radioactivity more rapid than spontaneous loss of radiolabel from the organisms in vitro would indicate that a decrease in numbers of bacteria in the lungs was due to physical removal from the lungs, although in previous studies, (90, 96, 99, 145, 247), physical removal

contributed little to clearance as measured by this method. A decrease in the ratio of CFU to radioactivity in the lungs would indicate that the rate of intrapulmonary killing exceeded that of bacterial growth, and an increase in the ratio would indicate the reverse (90, 99, 247). Use of this ratio allows evaluation of net increases or decreases in viable organisms independent not only of physical removal, but also of variation among animals in numbers of organisms inoculated (247). However, the individual contributions of the rates of intrapulmonary growth and killing cannot be distinguished by this method (145). Therefore, if differences in net clearance rates were found, additional studies would be necessary to determine whether the differences were due to changes in rates of intrapulmonary killing or in bacterial growth.

In the present study, pulmonary bacterial clearance was measured by a method similar to that of Green and Kass (99) and the "0 hr group mean method" of Ruppert et al. (247). The ratios of CFU to radioactivity in lung homogenates of rats killed after the clearance period were compared to the mean ratio from a group of rats killed immediately after inoculation of all rats was completed. Inoculation of 20 to 40 rats required about 30 min. The percent bacteria cleared after 4 or 6 hr was determined from these individual ratios by the formula:

$$\text{Percent clearance} = 100\left[1 - \left(\frac{\text{individual ratio CFU/ml/CPM/ml at 4 or 6 hr}}{\text{mean ratio CFU/ml/CPM/ml at 0 hr}}\right)\right]$$

Clearance was determined in groups of rats exposed to 0 and 100 ppm ammonia.

Organisms, media, and labelling. For the staphylococcal inocula, S. aureus (American Type Culture Collection No. 25923) and S. epidermidis (American Type Culture Collection No. 12228) were labelled with ^{32}P (as phosphoric acid, HCl-free, carrier-free, No. NEX053, New England Nuclear, Boston, MA) in a medium adapted from one previously described (262). The medium contained 1.0 g ammonium sulfate, 0.10 g hydrated magnesium sulfate, 0.5 g ammonium citrate, 2.5 g casamino acids (Difco, Detroit, MI), one 10 ml vial lyophilized yeast extract dialysate (Baltimore Biological Laboratories, Cockeysville, MD), 2.5 g glucose, 3.0 g tris buffer, 0.07 g monopotassium phosphate, and 500 ml deionized water. This was sterilized by filtration. The organisms were grown overnight in a shaker bath in 50 ml of the medium containing 1 mCi ^{32}P, then separated from unincorporated label by centrifugation at 1500 G for 30 min followed by washing and centrifuging twice in phosphate-buffered saline (PBS). They were resuspended in PBS, and frozen at -70°C in 5 ml aliquots. Before use, thawed aliquots were resuspended to remove any label lost in freezing and thawing.

Mycoplasma broth was prepared from Frey mycoplasma broth base (Grand Island Biological Co., Grand Island, NY) so as to contain 0.05% glucose, 10% yeast extract (Grand Island Biological Co.), 20% gamma globulin-free horse serum (Grand Island Biological Co.), 500 units/ml penicillin, 1:2000 thallium acetate, 0.002% phenol red, and deionized water. The pH was adjusted to between 7.8 and 8.0 with sodium hydroxide. Solid medium was made by adding 1.0% Noble Agar (Difco) to these ingredients. M. pulmonis (stock culture No. 5782 C4, isolated from a

rat with natural MRM) was grown by inoculating 10 ml of broth, then daily increasing the volume with fresh broth to 100, 250, 500, and 1000 ml. The organisms were then separated from the broth by centrifugation at 8000 G for 30 min. Iodine-125 (^{125}I, as sodium iodide, HCl-free, protein iodination grade; No. NEZ 0331, New England Nuclear) labelling was done with lactoperoxidase (Sigma Chemical Co., St. Louis, MO) and hydrogen peroxide according to the method of Marchalonis (200).

Inoculation. Labelled organisms were inoculated directly into the trachea in 0.5 ml PBS, rather than by aerosol exposure. This modification was made primarily to eliminate the problems of dealing with apparatus contaminated with radioisotopes and pathogenic microorganisms, but there were other reasons as well. The aerosol exposure method can result in variation in exposure to the aerosol among animals within the exposure chamber and in decreased viability of bacteria during and after aerosolization (247). In a chamber large enough to contain sufficient rats for an experiment, more variation in exposure would be expected than generally occurs in small chambers for mice. Further, M. pulmonis would probably be particularly vulnerable to viability loss during aerosolization.

In preparation for intratracheal inoculation of organisms, rats were given by intramuscular injection 0.10 ml of Innovar-Vet (Pitman-Moore, Inc., Washington's Crossing, NJ) diluted 1:10 in sterile distilled water with 1.0 ml (0.5 mg) of atropine sulfate added and the pH adjusted to 3.0. With the aid of an otoscope, inoculations were done with tuberculin syringes and 1.5 inch 20 gauge needles tipped with 3 cm of small diameter polyethylene tubing (No. PE90, Clay-Adams, Parsippany, NJ). This apparatus allowed deposition of the inoculum at the bifurcation of

the major bronchi. Examination of sections of lungs from rats thus inoculated, which were prepared with tracheal ligation rather than the usual infusion of fixative, showed that the organisms were delivered to the alveoli. The distribution of organisms seemed reasonably uniform, and no acute inflammatory changes resulted.

Quantitative cultures and determination of lung radioactivity. After inoculation, rats were killed by cervical dislocation followed immediately by exsanguination via a severed femoral artery. The fur of each rat was wetted with absolute ethanol and the lungs were removed with scissors and forceps flamed in absolute ethanol. The heart, thymus, esophagus, and mediastinal tissue were trimmed away and the major bronchi transected.

The lungs were homogenized in individually autoclaved ground glass homogenizers (Duall #23, Kontes Biomedical Products, Vineland, NJ) driven at 800 rpm by a constant-speed, constant-torque electric motor (Master Servodyne, Cole-Parmer Instrument Co., Chicago, IL). In the experiments with staphylococci, the homogenization was done in sterile distilled water to help break up the lung cells, but PBS was used with M. pulmonis. Homogenates were brought to a uniform volume of 3 ml. Homogenization in this manner did not completely break up all cells as judged by examination of stained smears of homogenates. Inasmuch as clumping of viable bacteria, either free or associated with cells, might result in erroneously low colony counts (29), the homogenates were briefly sonicated in a cup horn containing ice water (Model W220F Sonicator, Heat Systems-Ultrasonics, Inc., Plainview, NY) at settings of sufficient power and duration to disrupt cells without destroying the bacteria or mycoplasmas. The settings were 2 min at full power for

the staphylocci and 30 sec at full power for M. pulmonis. The CFU/ml of both staphylocci and mycoplasma in the homogenates were determined by serial 10-fold dilution and plating. At each step of the dilution procedure, the tube was vigorously vortexed (Deluxe Mixer, Scientific Products, McGraw Park, IL). The homogenates in the experiments with staphylococci were diluted in PBS, and 100 µl spots of each dilution were plated in duplicate on trypticase soy agar plates with 5% sheep blood (Baltimore Biological Laboratories).

Homogenates containing M. pulmonis were diluted in broth. Duplicate serial dilutions of each homogenate were made. Each dilution was applied in 25 µl spots to duplicate agar plates. Spots containing 30 to 300 colonies were counted. Inhibition of M. pulmonis growth by tissue inhibitors (156, 157) was usually apparent only in the 10^{-1} dilution tubes. The organisms grew well on the plates even at low dilutions but not as well in spots of undiluted homogenate.

After considerable trial and error, the following was found satisfactory for preparation of lung homogenates for determination of ^{32}P activity by liquid scintillation counting (LSC). To 1.0 ml of homogenate in a scintillation vial, 3.0 ml of tissue solubilizer (Protosol, New England Nuclear) was added, and the mixture was incubated at 37°C for several hours. This resulted in a yellow-brown viscous liquid to which was added 5 drops 30% hydrogen peroxide as a bleach. After several hours, 15 ml of liquid scintillation cocktail (Aquasol-2, New England Nuclear) was added. The cocktail and solubilized tissue were mixed by vigorous agitation. To reduce chemiluminescence and further reduce color quenching, the pH was adjusted to 6.0 - 7.0 by addition of 8 drops of concentrated hydrochloric acid

diluted 1:1 with distilled water. Construction of a curve of counting efficiency vs. the ratio of counts in the tritium channel to total counts showed that only when more than 15% of the total counts were in the tritium channel was efficiency decreased. This degree of quenching was rare in the experimental samples. Samples were counted in a Beckman LS-150 liquid scintillation counter.

Gamma counting of homogenates containing ^{125}I was done in a Picker Autowell II solid crystal scintillation counter.

In these experiments, the rate of loss of label from the organisms was checked by taking 3 samples of 0.05 ml for scintillation counting before the experiment, then centrifuging the inoculum and resuspending in PBS after the experiment and again taking samples. Ratios of CFU/CPM were also determined.

Statistical analysis. CFU/ml and ratios of CFU to radioactivity were transformed to common logarithms before analysis. Results of preliminary 2-group experiments were analyzed by Student's t-test (256). Two-way analysis of variance (256) and Duncan's multiple range test (72) were used in multigroup, factorial experiments. CFU and CPM data were analyzed in addition to CFU/CPM ratios. In these and all subsequent experiments, results of statistical tests were considered significant when $P < 0.05$.

Effects of Ammonia on Respiratory Tract Populations of *Mycoplasma pulmonis*

Methods. Rats were tranqulized with Innovar-Vet without atropine and inoculated intranasally with *M. pulmonis* in 50 µl of PBS. Half of the inoculated rats were exposed to 100 ppm ammonia. Two

different doses of M. pulmonis were used, 1 to 4 x 10^4 and 4 to 5 x 10^6 CFU. Exposure to 100 ppm ammonia was begun either 1 week before or simultaneously with inoculation as described with the results of these experiments. Five rats each from the control and ammonia-exposed groups were killed at each time post inoculation (PI). Originally, intervals of 0, 2, 7, 14, 21, and 28 days were planned, but for some experiments there were not enough rats of the same age available in the breeding colony. Modifications are described with the results.

Quantitative cultures of the lungs, larynxes, and tracheas were done by homogenization in PBS as described above. The 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 in a dental drill. The soft tissues and teeth were removed and the remaining bony structures fragmented with small bone cutters. The resulting fragments of bone and mucous membrane were sonicated for 30 sec in PBS, which was cultured as described in preceeding sections. Serum samples were collected from each rat for ELISA of anti-M. pulmonis IgM and IgG antibodies (118).

Statistical analysis. CFU were transformed to common logarithms and analyzed by 2-way analysis of variance (256). Comparisons among means of log CFU were made by the least significant difference test (256). A computer analysis of the data from all experiments by a general linear models procedure was used to evaluate effects of dose and pre-exposure to ammonia.

Effect of Ammonia on *In Vitro* Growth of *Mycoplasma pulmonis*.

Liquid medium was prepared as described above, except that concentrated ammonium hydroxide was added so that the final concentration in the medium was 1 mM and the pH 7.9 - 8.0. Control medium was prepared as usual with pH adjusted to 7.9 - 8.0 with sodium hydroxide. Colony-forming units were quantitated by serial dilution and plating as described above.

Results were analyzed after log transformation by 2-way analysis of variance (256).

Ammonia Inhalation Studies

A face mask for exposure of rats outside the isolator was constructed from the barrel of a 50 ml plastic syringe cut off about 5 cm from the needle end. The Leuer lock was drilled out and a hole was drilled in the side of the barrel about 2 cm from the needle end. A length of 3/8 inch plastic tubing was attached to the drilled-out Leuer lock and a second piece was inserted into the hole in the side. One piece of tubing was connected to the isolator containing 100 ppm ammonia and the other to the room air exhaust vent, providing a constant flow through the mask. The cut off end of the barrel was covered with rubber cut from a surgical glove and held in place by a rubber band. A small hole was made in the center of this rubber diaphragm.

Rats were anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) diluted 1:10 in sterile PBS and injected intraperitoneally at a dosage of 40 mg/kg. They were then exposed to ammonia via the face mask. Tracheas were surgically exposed

and connected to the detector tube of the Drager Multi Gas Detector by a short length of plastic tubing.

Results were analyzed by the paired t-test (256).

Determination of Nucleic Acids in Nasal Washings

Collection of nasal washings. Rats were anesthetized with intraperitoneal pentobarbital as described above, and exsanguinated by severing a femoral artery to reduce blood contamination of washings. The lower jaw was removed along with attached skin and salivary glands by severing the rami of the mandible with scissors. The head was then cut off with scissors and the nares held in the mouth of a small plastic tube. Three ml of PBS were passed through the nasal passages with a blunted 20 gauge needle attached to a 3 ml plastic syringe and inserted into the nasopharyngeal duct.

Measurement of nucleic acids. The technique used was that of LePecq and Paoletti (191) for fluorescent determination of DNA and RNA. Measurements were made in an Aminco SPF-125 Spectrofluorometer (American Instrument Co., Silver Spring, MD) with an emission wavelength of 590 nm and excitation wavelengths of 365 and 540 nm. Both slits were set at 4 mm. The concentration of ethidium bromide was 5 µg/ml.

Concentration of samples. Washings from some rats were pooled and concentrated by ultrafiltration (Amicon Ultrafiltration Cell No. 5106, Amicon Corp., Lexington, MA) through a filter retaining molecules larger than about 10,000 molecular weight (Diaflo PM 10 ultrafiltration membrane, Amicon).

RESULTS

Pulmonary Bacterial Clearance

Previous studies of pulmonary clearance of mycoplasmas inoculated intratracheally or by aerosol exposure have not been reported. Also, it was not known whether or not different results would be obtained with organisms inoculated intratracheally rather than by aerosol exposure. Therefore, measurements of staphylococcal clearance were made, allowing comparisons with the results of other investigations. In addition, the staphylococci grow much more rapidly than M. pulmonis, giving results more quickly and aiding the development of the laboratory procedures involved. Promising preliminary results were obtained with both S. aureus and S. epidermidis; therefore, the effect of ammonia on clearance of both organisms was tested.

Clearance of Staphylococcus aureus. A preliminary experiment of a simple 2-group design was done to test whether or not pulmonary clearance could be demonstrated with the described technique. Ten rats were inoculated with 7×10^8 CFU ^{32}P-labelled S. aureus in 0.05 ml PBS. Four were killed immediately PI and 6 at 4 hrs PI. The ratios of CFU/ml to CPM/ml in lung homogenates were significantly different between the 2 groups ($P < .001$), and the calculated mean percent bacteria cleared in the 4 hr group was 97% (Table 1). The spontaneous loss of label from the bacteria in vitro was 35%, somewhat more than approximately 20% as

has been reported (96). However, in subsequent experiments the *in vitro* loss of label was less; therefore, no modifications were made.

The decrease in mean CPM/ml of homogenate was 20% from the 0 hr group to the 4 hr group. This can be interpreted as either physical transport of labelled organisms or *in vivo* loss of label. In view of the *in vitro* loss of label, significant transport of bacteria from the lungs probably did not occur. This is in agreement with published clearance studies (96, 99, 145).

Comparison of the calculated total CPM in the 0 hr lung homogenates with the CPM in 0.05 ml of inoculum shows that an average of 58% of the inoculated activity was retained, although this retention varied considerably among individual rats. Much of the remainder of the inoculum was found in the stomach.

The results of the preliminary experiment were erroneous because of inaccurate CFU determination but the results did suggest that it would be possible to demonstrate pulmonary clearance of *S. aureus*. The second experiment was of 2 x 2 factorial design with 8 rats assigned to each of the 4 groups: 0 hr, 0 ppm ammonia; 0 hr, 100 ppm ammonia; 4 hr, 0 ppm ammonia; and 4 hr, 100 ppm ammonia. Because of the time required to complete inoculations and lung homogenization, 0 hr groups were actually killed about 30 min PI and 4 hr groups at 4.5 hr. Rats were inoculated with 3×10^7 CFU labelled *S. aureus*.

In this experiment many of the rats died shortly after inoculation, apparently of respiratory and cardiac arrest. Thereafter, atropine was added to the Innovar-Vet. Deaths of this nature were much less of a problem in subsequent experiments.

Statistical analysis of the calculated ratios indicated that there was no significant difference among the 4 groups (Table 1). The *in vitro* loss of label from the inoculum was 26%. There was essentially no calculated decrease in radioactivity in the control rats from 0 to 4 hr. In the rats exposed to ammonia the calculated decrease was 10%.

The mean clearance rates for control and ammonia-exposed rats calculated from the combined results of these two experiments were 10% and 16%, respectively. These were not statistically different. There was sufficient variation in clearance among individual rats that only much larger differences would have been detectable.

These results are different from those of previous studies using aerosol-inoculated *S. aureus* (90, 141, 183), in which clearance rates in rats were between 70% and 80% in 4 to 6 hr. Therefore, a histologic study of the interaction of *S. aureus* and alveolar macrophages was done, using a method similar to that of Green and Kass (99) and Goldstein *et al.* (90). Ten rats were inoculated with 3×10^7 CFU *S. aureus*; 5 were killed 10 min PI and 5 at 4 hr PI. The tracheas were ligated, then the lungs removed and fixed in alcoholic formalin (10% formalin in 80% ethanol). The tissue was processed routinely and stained by the method of Brown and Brenn (221). Five randomly selected oil immersion fields were examined in each rat and the numbers of intracellular bacteria and total bacteria in each field were determined (Table 2). The mean percentages of intracellular bacteria were significantly different ($P < .01$). The mean percentage at 10 min PI was 3.8%. At 4 hr PI it was 35.2%. A considerably smaller percentage was phagocytized than in a previous report (90). All visible macrophages contained large numbers of bacteria, which was interpreted to indicate that the macrophages may

Table 1. Pulmonary Clearance of ^{32}P-Labelled Staphylococcus aureus

Experiment	ppm NH_3	Lung Homogenates 0 Hr PI				Lung Homogenates 4 Hr PI				Percent Clearance
		n	Log CFU/ml	CPM/ml	Ratio	n	Log CFU/ml	CPM/ml	Ratio	
1	0	4	8.735 ±0.684	72046 ±36375	10740 ±5609	6	6.795 ±0.713	57525 ±28639	275 ±464	97 ± 5
2	0	3	7.185 ±0.035	79644 ± 3901	193 ± 23	7	6.924 ±0.313	55623 ±19249	178 ± 77	8 ±40
	100	3	6.745 ±0.332	56338 ±34397	127 ± 45	5	6.249 ±0.867	39313 ±31033	112 ± 57	12 ±45
3	0	5	6.307 ±0.156	79873 ±17783	26 ± 5	5	6.233 ±0.146	79444 ±22795	23 ± 3	14 ±12
	100	5	6.348 ±0.230	61139 ±32822	44 ± 10	5	6.018 ±0.963	54885 ±32496	41 ± 36	7 ±83

^{32}P = phosphorus-32, PI = post inoculation, ppm NH_3 = parts per million atmospheric ammonia, n = number of rats, Log CFU/ml = log colony-forming units per milliliter, CPM/ml = counts per minute per ml, Ratio = CFU/ml/CPM/ml, and Percent Clearance = $100\left[1 - \left(\frac{\text{individual ratio at 4 hr PI}}{\text{mean ratio at 0 hr PI}}\right)\right]$.

Figures are means ± standard deviations.

Table 2. *In Vivo* Phagocytosis of *Staphylococcus aureus*

	Percent Intracellular Bacteria					
	Rat					
	1	2	3	4	5	Mean ± SD
10 Min PI	0.7	7.7	4.3	4.6	1.9	3.8 ± 2.7
4 Hr PI	33.4	40.7	34.4	43.1	24.4	35.2 ± 7.3

Percent Intracellular Bacteria = number of intracellular bacteria in 5 fields at 1000x magnification divided by the total number of bacteria in those fields, x 100; SD = standard deviation; and PI = post inoculation.

have been unable to phagocytize a larger percentage of the staphylococci, perhaps because too many were inoculated.

The possibility was considered that sonication dispersed clumps of phagocytized but still viable organisms in the 4 hr rats, resulting in higher CFU determinations than if sonication had not been done, and consequently, lower 4 hr clearance rates. Similar efforts to assure dispersion of bacterial clumps were not incorporated in previous studies of pulmonary clearance. To test this possibility, 5 rats were inoculated with 3×10^7 CFU of S. aureus and 4 hr later the CFU determined in the lung homogenates before and after sonication (Table 3). There was no significant difference by the paired t-test. Thus, sonication of the homogenates could not account for the slow clearance rates for S. aureus obtained in the present study.

Clearance of Staphylococcus epidermidis. LaForce et al. (183) reported substantially more rapid clearance of S. albus (S. epidermidis) than of S. aureus in rats. A 2-group experiment was done to determine whether or not clearance could be demonstrated with unlabelled S. epidermidis. Six rats were inoculated with 10^8 CFU and the experiment conducted as before, other than the LSC. The CFU/ml of the lung homogenates were significantly different by the t-test. The average decrease was 83% in 4 hr (Table 4). In a trial of clearance of ^{32}P-labelled S. epidermidis, 5 rats each were used in 0 and 6 hr groups. The longer interval was used in this and subsequent experiments to allow direct comparison with previously reported results (183). Rats were inoculated with 6×10^6 CFU. The ratios of the 0 hr and 6 hr rats were significantly different ($P < .001$), with a calculated mean clearance of 96% (Table 5). These experiments indicated that pulmonary clearance

Table 3. Effect of Sonication on Colony-Forming Units of Staphylococcus aureus in Lung Homogenates

	Lung Homogenate					
	1	2	3	4	5	Mean ± SD
Log CFU/ml Before Sonication	7.114	7.613	7.613	7.748	7.602	7.538 ± 0.245
Log CFU/ml After Sonication	7.255	7.681	7.708	7.690	7.544	7.576 ± 0.191

SD = standard deviation and Log CFU/ml = log colony-forming units per milliliter.

Table 4. Pulmonary Clearance of Unlabelled *Staphylococcus epidermidis*

Log CFU/ml in Lung Homogenates		Percent
10 Min PI	4 Hr PI	Clearance
7.699	6.785	88
7.748	6.832	87
7.681	7.114	75
7.709	6.911	83
±0.035	±0.178	± 7

Log CFU/ml = log colony-forming units per milliliter, PI = post inoculation, and Percent Clearance = $100\left[1-\left(\frac{\text{individual CFU/ml at 4 hr PI}}{\text{geometric mean CFU/ml at 10 min PI}}\right)\right]$.

Means ± standard deviations are at the bottom of each column.

could be demonstrated easily with S. epidermidis. The results agreed well with those of previously reported studies (183).

To test whether 100 ppm ammonia affected the clearance rate, a 2 x 2 factorial design was used as described above. The 8 rats in each group were inoculated with 2×10^8 CFU. There was no statistically significant difference between the control and ammonia-exposed rats. The calculated mean clearance in 0 ppm rats was 93%, and in the 100 ppm rats, 94% (Table 5). The loss of label from the inoculum in vitro was 56%. Therefore, the inoculum was washed once by centrifugation before use in the next experiment. This loss was not quite so severe in vivo, however. The calculated loss of label in the control rats was 20% and in the ammonia-exposed rats, 32%. Calculation of the amount of inoculum retained in the lungs of the 0 hr rats from the radioactivity of the homogenates was 47% in the controls and 51% in the group exposed to ammonia.

This experiment was repeated with 9 rats per group given 2×10^7 CFU S. epidermidis. The results are shown in Table 5. The statistical analysis again showed that there was no significant difference between the control and ammonia-exposed rats, in which the calculated mean rates of clearance were 87% and 88% respectively.

The calculated loss of label from the inoculum in vitro was 24%, and the decrease in mean radioactivity of the lung homogenates was 25% in the control group and 19% in the ammonia-exposed group.

The results of these 2 experiments clearly show that 100 ppm ammonia did not affect pulmonary clearance of S. epidermidis.

Clearance of Mycoplasma pulmonis. The design of the next experiment was as above, except that the lung homogenates were sonicated for

Table 5. Pulmonary Clearance of ^{32}P-Labelled Staphylococcus epidermidis

Experiment	ppm NH_3	Lung Homogenates 0 Hr PI				Lung Homogenates 6 Hr PI				Percent Clearance
		n	Log CFU/ml	CPM/ml	Ratio	n	Log CFU/ml	CPM/ml	Ratio	
1	0	4	5.120 ±0.628	13696 ±13247	18 ± 5	5	3.442 ±0.231	16254 ± 8301	0.66 ±1.08	96 ±6
2	0	8	7.143 ±0.261	91709 ±15426	177 ±99	8	5.880 ±0.253	73204 ±23926	13 ± 6	93 ±3
	100	8	7.167 ±0.258	99326 ±17492	166 ±74	8	5.733 ±0.297	67378 ±28027	9 ± 3	94 ±2
3	0	9	6.916 ±0.731	42382 ±17674	297 ±99	9	5.920 ±0.599	31731 ±15628	38 ±17	87 ±6
	100	9	6.527 ±0.919	28467 ±24068	208 ±67	9	5.866 ±0.296	35503 ± 3318	25 ±13	88 ±6

^{32}P = phosphorus-32, PI = post inoculation, ppm NH_3 = parts per million atmospheric ammonia, n = number of rats, Log CFU/ml = log colony-forming units per milliliter, CPM/ml = counts per minute per ml, Ratio = CFU/ml/CPM/ml, and Percent Clearance = $100\left[1-\left(\frac{\text{individual ratio at 6 hr PI}}{\text{mean ratio at 0 hr PI}}\right)\right]$.

Figures are means ± standard deviations.

30 sec rather than 2 min because longer periods resulted in a decrease in viability of M. pulmonis. The calculated mean clearance in the 0 ppm rats was -265% and in the 100 ppm rats, -377%. The difference was not statistically significant.

These results seemed to indicate intrapulmonary growth of M. pulmonis, but further analysis of the data indicated that this was not the case. The CFU/ml of the lung homogenates were not significantly different among the 4 groups, indicating neither clearance nor growth (Table 6). The CPM/ml of the lung homogenates of the 6 hr rats were significantly ($P < .05$) lower than those of the 0 hr rats (Table 6), the decreases being 40% in the control group and 45% in the ammonia-exposed group.

Inasmuch as CFU of M. pulmonis in the lungs did not decrease, the organisms could have been physically transported from the lung with the rate of multiplication approximately matching the rate of removal, or the ^{125}I label could have been lost from the organisms in vivo. It would have been helpful to know the in vivo generation time of M. pulmonis in interpreting these results, but this information is not available. Because the thawed inoculum lost 76% of its label during washing by centrifugation, and because previous reports (90, 96, 99, 145, 247) indicate that physical removal contributes little to pulmonary bacterial clearance, it seemed most likely that the label was being lost from the organisms.

Two possible explanations for this loss of label were readily apparent. First, the mycoplasmas could have had proteins from the horse serum in the medium adsorbed to their surfaces, which were labelled but later dissociated from the organisms' surfaces. Second, freezing and

Table 6. Pulmonary Clearance of ^{125}I-Labelled Mycoplasma pulmonis

Experiment	ppm NH_3	Hr PI	n	Log CFU/ml	CPM/ml	Ratio
1	0	0	8	5.144 ±0.362	1448 ± 642	118 ± 49
		6	8	5.472 ±0.188	864 ± 241	431 ± 276
	100	0	7	5.194 ±0.197	1679 ± 763	109 ± 38
		6	8	5.469 ±0.473	829 ± 372	520 ± 303
2	0	0	5	4.375 ±0.166	8387 ±2918	3 ± 0.4
	0	6	3	4.328 ±0.139	1930 ± 247	12 ± 2.5
	0	24	3	4.392 ±0.462	704 ± 278	79 ± 709

^{125}I = iodine-125, PI = post inoculation, ppm NH_3 = parts per million atmospheric ammonia, n = number of rats, Log CFU/ml = log colony-forming units per milliliter, CPM/ml = counts per minute per ml, Ratio = CFU/ml/CPM/ml.

Figures are means ± standard deviations.

thawing could have disrupted many of the organisms. Accordingly, another attempt was made to label the organisms, after more extensive washing in PBS. After freezing and thawing, only 28% of the label was removed in one washing, but the viability of the organisms was so reduced as to make the inoculum useless. A more extensive analysis of the loss of label and viability of the inoculum was then done. A third batch of organisms was labelled after 3 washes in PBS. After labelling, 65% of the ^{125}I was removed in the first wash in PBS, but only 9% in the second and 6% in the third. The washed organisms were then frozen at -70°C and thawed, and after washing by centrifugation only 23% of the label present before freezing remained. It seemed then that if this labelling procedure were to be usable at all, the organisms could not be frozen but would have to be labelled and washed immediately before use. The rate of loss of label from unfrozen organisms was such that about 60% of the label was lost during a 12 hr incubation in broth. This rate was great enough that clearance probably would be difficult to demonstrate by this method unless the CFU in the lung could be shown to decrease rapidly. Freezing and thawing resulted in an 86% decrease in CFU/ml of the inoculum, suggesting that there was considerable destruction of organisms which might account for the loss of much of the label in addition to spontaneous loss. Other means of labelling were not tried because, in the following experiments, no decrease in numbers of M. pulmonis and thus, no pulmonary clearance, was demonstrated, even at intervals of up to 48 hr.

The next experiment was done to determine whether or not clearance could be measured at 6 and 24 hr using mycoplasmas that had been labelled immediately before use. Fifteen rats were inoculated with

3×10^5 CFU ^{125}I-labelled M. pulmonis. Five were killed at 0 hr, 3 at 6 hr and 3 at 24 hr PI. The mean ratios of the 6 and 24 hr groups were not significantly different, but the 0 hr mean was significantly less than the other two (Table 6). This experiment also failed to demonstrate clearance. The CFU in the lungs were not significantly different, but the means of the CPM/ml of all 3 groups were significantly different one from another ($P< .05$). There was a calculated loss of activity of 78% from 0 to 6 hr and 92% from 0 to 24 hr. Therefore, it seemed that surface labelling with ^{125}I by the lactoperoxidase technique (200) was not feasible.

Other experiments with unlabelled M. pulmonis were done to determine whether or not a decrease in CFU/ml in lung homogenates could be shown at one or more of several intervals after intratracheal inoculation. These experiments tested the change in CFU/ml in lung homogenates 2, 4, 6 and 18 hr after intratracheal inoculation of M. pulmonis. Results are shown in Table 7. In the first experiment 9 rats were inoculated with 1.5×10^7 CFU. Four were killed at 0 hr and 5 at 6 hr. In a second experiment 9 rats were also given 1.5×10^7 CFU. Four rats were killed at 0 hr and 5 at 18 hr. Ten rats were inoculated with 10^8 CFU in a third experiment. Three were killed at 0 hr, 3 at 2 hr, and 4 at 4 hr PI. No statistically significant change was found except at 18 hr, when there was a significant increase in CFU/ml compared to 0 hr ($P< .01$). Finally, an experiment was done to test whether or not ammonia influenced the numbers of intratracheally inoculated unlabelled M. pulmonis at intervals up to 48 hr. Thirty rats were inoculated with 4×10^7 CFU unlabelled M. pulmonis. Half had been kept at 0 ppm and half at 100 ppm ammonia for 1 week. Five rats from each group were killed at

Table 7. Pulmonary Clearance of Unlabelled Mycoplasma pulmonis

Experiment	ppm NH_3	Hr PI	n	Log CFU/ml
1	0	0	4	7.084 ± 0.552
	0	6	5	6.330 ± 0.807
2	0	0	4	5.841 ± 1.407
	0	18	5	8.514 ± 0.299
3	0	0	3	7.473 ± 0.158
	0	2	3	7.125 ± 0.318
	0	4	4	7.334 ± 0.061
4	0	0	5	5.132 ± 0.566
	0	24	5	3.055 ± 1.973
	0	48	5	5.293 ± 1.900
	100	0	5	4.306 ± 2.409
	100	24	5	3.276 ± 0.787
	100	48	5	3.448 ± 0.651

ppm NH_3 = parts per million atmospheric ammonia, PI = post inoculation, n = number of rats, and Log CFU/ml = log colony-forming units per milliliter given as mean ± standard deviation.

0, 24, and 48 hr. Those not killed at 0 hr were returned to the isolators. Although there was an apparent trend in both groups for the CFU in the lungs to decrease with time, the differences among groups were not statistically significant.

Effects of Ammonia on Respiratory Tract Populations of *Mycoplasma pulmonis*

Inasmuch as ammonia was not shown to affect pulmonary clearance, a second hypothesis, that ammonia could affect respiratory tract populations of *M. pulmonis* was tested.

The original protocol called for 60 rats per experiment, all within 1 week of the same age. Five ammonia-exposed and 5 control rats were to be killed at each of 6 PI intervals: 0, 2, 7, 14, 21, and 28 days. However, the weekly production of the breeding colony was usually not sufficient to meet this requirement. Modifications to the design of the experiments included dropping either the ammonia-exposed or both 0 day groups and substituting a 17 day PI interval for 14 and 21 days. In 3 experiments an intranasal inoculation dose of 4 to 5 x 10^6 CFU *M. pulmonis* was used (high dose) and in 3 others, a dose of 1 to 4 x 10^4 CFU (low dose). With each dose, ammonia exposure was begun immediately after inoculation in 2 experiments and 1 week before inoculation in the third. These conditions are indicated with results from individual experiments in Tables 8 through 11. Statistical analyses were done on the results of individual experiments and on combined data from all 6.

Quantitative cultures of respiratory tracts. Results within dose were similar, although specific temporal relationships varied from experiment to experiment. The effect of ammonia pre-exposure could not be distinguished from variation among experiments, so the results of

Table 8. Growth of *Mycoplasma pulmonis* in Nasal Passages After Intranasal Inoculation

Experiment	ppm NH_3	Log Total Colony-Forming Units at Post Inoculation Day						
		0	2	7	14	17	21	28
1	0	ND	0*	6.910* ±0.275	5.949* ±0.425	ND	6.246* ±0.369	6.043 ±0.306
	100	ND	6.107* ±0.511	8.019* ±0.628	7.602* ±0.628	ND	7.867* ±0.527	6.654 ±1.095
2	0	4.170 ±0.676	1.578* ±2.176	5.389* ±0.256	ND	5.535 ±0.358	ND	5.858 ±0.339
	100	ND	2.982* ±0.651	7.885* ±0.612	ND	7.291* ±0.714	ND	6.414 ±0.613
3	0	3.277 ±0.511	3.664* ±0.192	5.706* ±0.433	ND	5.794* ±0.492	ND	5.882* ±0.490
	100	3.716 ±0.452	5.368* ±0.313	8.067* ±0.424	ND	6.917* ±0.475	ND	7.444* ±0.650
4	0	2.953 ±0.192	3.098 ±0.341	5.270 ±0.346	ND	OG	ND	4.998* ±0.327
	100	ND	3.673 ±0.314	5.917 ±0.300	ND	OG	ND	5.781* ±1.190
5	0	0	0	0	ND	0*	ND	1.048* ±2.343
	100	ND	0	0	ND	7.098* ±0.159	ND	7.397* ±0.658
6	0	0*	0*	1.357* ±1.253	3.156* ±2.285	ND	4.055* ±2.451	2.435* ±2.575
	100	1.940* ±1.136	3.650* ±0.450	8.034* ±1.033	7.960* ±0.945	ND	7.013* ±0.894	7.991* ±0.878

ppm NH_3 = parts per million atmospheric ammonia, ND = not done, OG = overgrown by bacteria. Figures are means ± standard deviations. For each group n = 5.

*Indicates significant difference ($P < 0.05$) between the two means at each post inoculation time in each experiment.

Inoculation dose = 4-5 x 10^6 colony-forming units in experiments 1-3, and 1-4 x 10^4 in experiments 4-6.

Ammonia-exposed rats in experiments 3 and 6 pre-exposed for 1 week.

Table 9. Growth of *Mycoplasma pulmonis* in Larynxes After Intranasal Inoculation

Experiment	ppm NH_3	Log Total Colony-Forming Units at Post Inoculation Day 0	2	7	14	17	21	28
1	0	ND	0	4.238 ±0.457	2.953* ±2.720	ND	5.274 ±0.798	5.333 ±0.868
	100	ND	0	5.023 ±0.527	6.767* ±1.296	ND	6.548 ±1.265	6.495 ±0.418
2	0	1.364 ±1.295	0	4.402 ±0.334	ND	4.635* ±0.088	ND	5.311* ±0.126
	100	ND	0	4.572 ±0.167	ND	7.082* ±0.830	ND	6.719* ±0.075
3	0	0	0*	4.587 ±0.095	ND	5.078* ±0.498	ND	4.874* ±0.369
	100	0	2.302* ±1.325	5.004 ±0.508	ND	6.462* ±0.849	ND	7.514* ±0.403
4	0	0	0.985 ±1.579	4.227 ±0.277	ND	OG	ND	3.980* ±0.423
	100	ND	0.922 ±1.449	3.491 ±0.446	ND	OG	ND	5.550* ±0.830
5	0	0	0	0	ND	0*	ND	0.999* ±2.233
	100	ND	0	0	ND	5.495* ±0.653	ND	6.913* ±1.307
6	0	0	0	0*	1.673* ±2.313	ND	4.159* ±2.402	1.982* ±2.020
	100	0	0	5.189* ±0.452	6.399* ±1.144	ND	6.323* ±1.120	6.681* ±0.122

ppm NH_3 = parts per million atmospheric ammonia, ND = not done, OG = overgrown by bacteria. Figures are means ± standard deviations. For each group n = 5.

*Indicates significant difference ($P < 0.05$) between the two means at each post inoculation time in each experiment.

Inoculation dose = 4-5 x 10^6 colony-forming units in experiments 1-3, and 1-4 x 10^4 in experiments 4-6.

Ammonia-exposed rats in experiments 3 and 6 pre-exposed for 1 week.

Table 10. Growth of *Mycoplasma pulmonis* in Tracheas After Intranasal Inoculation

Experiment	ppm NH_3	Log Total Colony-Forming Units at Post Inoculation Day						
		0	2	7	14	17	21	28
1	0	ND	0	0	0*	ND	3.810* ±3.594	4.338* ±2.941
	100	ND	0.506 ±1.132	1.232 ±1.239	6.195* ±3.546	ND	8.615* ±0.428	8.312* ±0.856
2	0	0	0	0.295 ±0.661	ND	2.163* ±1.455	ND	4.205* ±4.071
	100	ND	0	0.295 ±0.661	ND	8.388* ±0.420	ND	8.752* ±0.112
3	0	0	0	0*	ND	4.380* ±3.176	ND	1.765* ±2.451
	100	0	0	2.842* ±1.974	ND	8.749* ±0.184	ND	8.721* ±0.073
4	0	0	0	2.464 ±2.251	ND	OG	ND	3.476* ±2.032
	100	ND	0	0.948 ±1.365	ND	OG	ND	6.972* ±1.527
5	0	0	0	0	ND	0*	ND	0.516* ±1.154
	100	ND	0	0	ND	1.323* ±1.407	ND	7.575* ±2.480
6	0	0	0	0	0.567* ±1.267	ND	1.658* ±2.854	0.369* ±0.825
	100	0	0	0.931 ±0.854	6.898* ±3.857	ND	8.355* ±0.503	8.579* ±0.120

ppm NH_3 = parts per million atmospheric ammonia, ND = not done, OG = overgrown by bacteria. Figures are means ± standard deviations. For each group n = 5.

*Indicates significant difference ($P < 0.05$) between the two means at each post inoculation time in each experiment.

Inoculation dose = 4-5 x 10^6 colony-forming units in experiments 1-3, and 1-4 x 10^4 in experiments 4-6.

Ammonia-exposed rats in experiments 3 and 6 pre-exposed for 1 week.

Table 11. Growth of *Mycoplasma pulmonis* in Lungs After Intranasal Inoculation

Experiment	ppm NH_3	Log Total Colony-Forming Units at Post Inoculation Day						
		0	2	7	14	17	21	28
1	0	ND	0	0.889 ±1.247	2.216* ±1.340	ND	4.264* ±2.077	2.554* ±2.758
	100	ND	0	2.708 ±1.591	5.024* ±1.040	ND	6.583* ±1.658	7.690* ±0.760
2	0	0	0	0	ND	0*	ND	0.489* ±1.094
	100	ND	0	0	ND	5.985* ±1.303	ND	7.238* ±0.798
3	0	0	0	1.196 ±1.669	ND	2.499* ±1.684	ND	0.468* ±1.048
	100	0	0	1.896 ±1.118	ND	7.900* ±0.976	ND	7.948* ±0.573
4	0	0	0	0.416 ±0.930	ND	OG	ND	0*
	100	ND	0	1.435 ±1.971	ND	OG	ND	3.341* ±1.874
5	0	0	0	0	ND	0	ND	0*
	100	ND	0	0	ND	1.067 ±1.590	ND	5.536* ±2.073
6	0	0	0	0	0*	ND	0.489* ±1.094	0*
	100	0	0	0.489 ±1.094	3.326* ±2.308	ND	7.781* ±1.633	7.787* ±1.166

ppm NH_3 = parts per million atmospheric ammonia, ND = not done, OG = overgrown by bacteria. Figures are means ± standard deviations. For each group n = 5.

*Indicates significant difference ($P < 0.05$) between the two means at each post inoculation time in each experiment.

Inoculation dose = 4-5 x 10^6 colony-forming units in experiments 1-3, and 1-4 x 10^4 in experiments 4-6.

Ammonia-exposed rats in experiments 3 and 6 pre-exposed for 1 week.

all high dose inoculations were combined as were those of low dose inoculations. These combined results are depicted in Figure 1. Statistically significant differences between ammonia-exposed and control rats in mean log CFU at each post inoculation interval are indicated for each site in the tables of results from each experiment (Tables 8 through 11).

Data from 14 and 21 day PI intervals were combined with those from 17 day PI times so that the 17 day points in the graphs represent results from at least 15 rats. Other points also include data from 15 rats except the point comprising data from the ten 0 day high dose control rats, and the points including results from ammonia-exposed rats at day 0. Each of the 2 latter points represent data from 5 rats pre-exposed to ammonia.

Day 0 results from the nasal passages from both ammonia-exposed and control rats are nearly the same in the high dose experiments, but not in the low dose experiments (Figure 1). This difference is likely the result of variation among experiments, inasmuch as the mean log CFU in the nasal passages of the 0 day controls in one low dose non-pre-exposure experiment was higher than the mean in the rats exposed to ammonia. In the other low dose experiments, including the pre-exposure experiment, the mean of the 0 day controls was lower (Table 8).

In all experiments, increases in CFU of M. pulmonis were greater, in many instances by several orders of magnitude, in the rats exposed to ammonia. In both ammonia-exposed and control rats, the numbers of M. pulmonis CFU increased first in the nasal passages and larynxes, then in the trachea and lungs. In the analysis of the combined data, a striking difference between the high dose and low dose rats was evident

Figure 1. Growth of Mycoplasma pulmonis in respiratory tracts of rats after intranasal inoculation. Graphs show combined data from 6 experiments in which 295 rats were given 1-4 x 10^4 (---) or 4-5 x 10^6 (——) colony-forming units Mycoplasma pulmonis and exposed to 100 (●) or 0 (○) ppm ammonia.

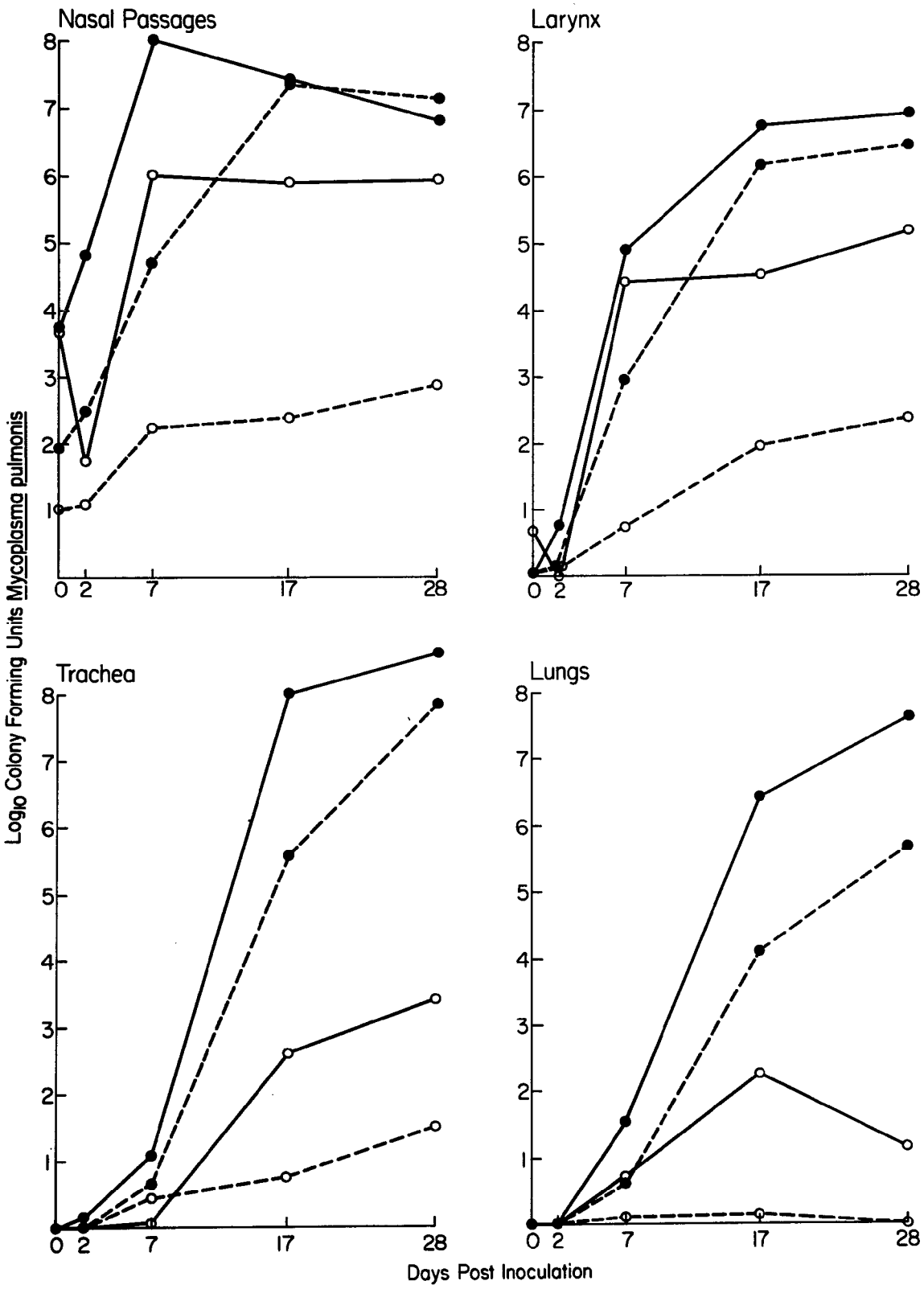
Nasal Passages
Larynx
Trachea
Lungs
Log10 Colony Forming Units Mycoplasma pulmonis
Days Post Inoculation
0 2 7 17 28
1 2 3 4 5 6 7 8

Figure 1.

in the response of M. pulmonis populations to ammonia. This is visible in the graphs of results from the nasal passages and larynxes (Figure 1). In these structures, similar maximum numbers of M. pulmonis CFU were reached in the ammonia-exposed rats regardless of inoculation dose, although this number was attained somewhat later in the rats inoculated with the low dose. In contrast, inoculation dose had a profound effect on the subsequent progression of infection in the control rats. In the tracheas and lungs this interaction is less evident although it was statistically significant at all sites.

ELISA of IgG and IgM anti-M. pulmonis serum antibody. IgG and IgM antibody responses generally paralleled the numbers of M. pulmonis CFU recovered from the tissues, but were not highly correlated with populations at any one site (Figure 2, Tables 12 and 13). IgM antibody reached a peak or plateau before IgG, as would be expected. No significant decreases in mycoplasmal populations were associated with rising serum antibody concentrations.

Effect of Ammonia on In Vitro Growth of Mycoplasma pulmonis

An important question and a determinant of the course of subsequent investigations was whether the increased populations of M. pulmonis in ammonia-exposed rats resulted directly from enhancement of the growth of the organism, indirectly from effects on the host, or perhaps from both. Therefore, an experiment was designed to determine the effect of ammonia on growth of M. pulmonis in liquid medium, separated from any benefits the organism could derive from effects on the host.

Medium containing 1 mM ammonium ion and control medium were inoculated with M. pulmonis. Determinations of CFU were done immediately

Figure 2. Enzyme-linked immunosorbent assay of serum IgG and IgM antibodies against Mycoplasma pulmonis. Graphs show combined data from 6 experiments in which 295 rats were inoculated intranasally with 1-4 x 10^4 (---) or 4-5 x 10^6 (——) colony-forming units M. pulmonis and exposed to 100 (●) or 0 (○) ppm ammonia.

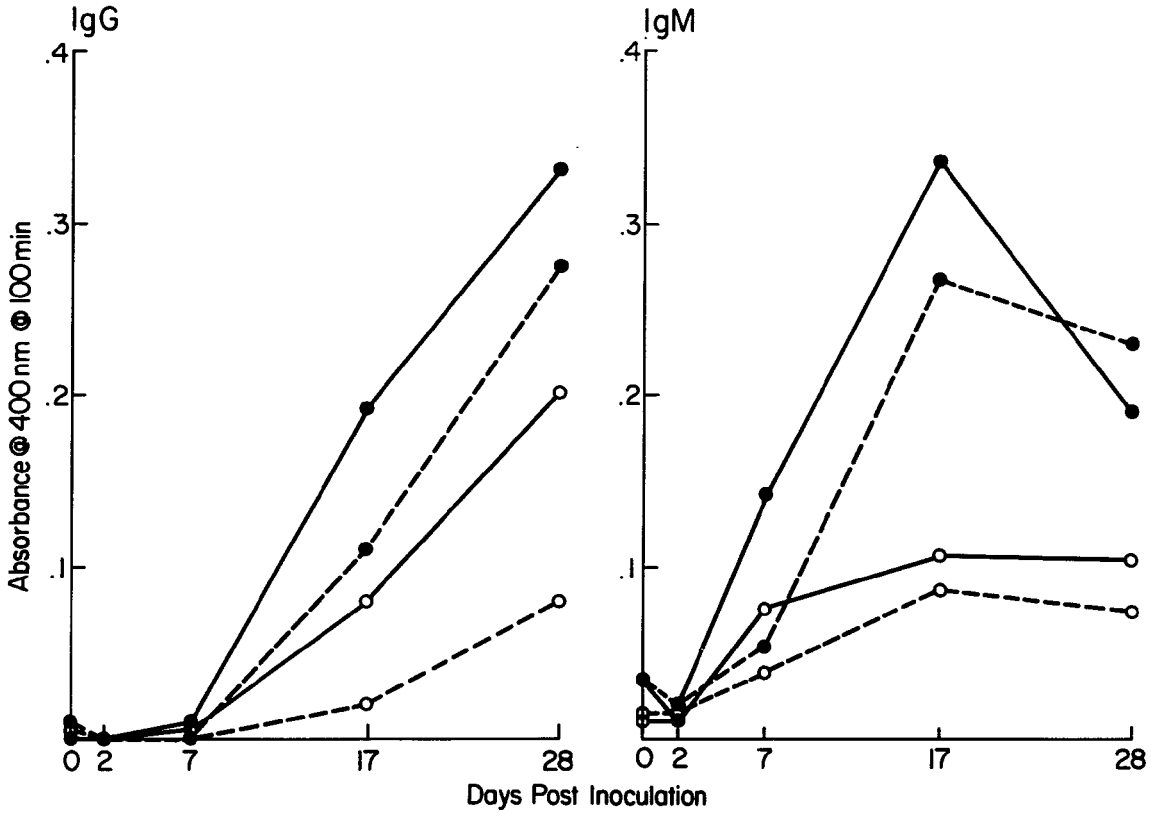
IgG
IgM
.4
.3
.2
.1
Absorbance @ 400nm @ 100min
0 2 7 17 28
Days Post Inoculation

Figure 2.

Table 12. Enzyme-Linked Immunosorbent Assay of IgG Antibody After Intranasal Inoculation of *Mycoplasma pulmonis*

Experiment	ppm NH_3	Post Inoculation Day 0	2	7	14	17	21	28
1	0	ND	0.006 ±0.009	0.005 ±0.005	0.056* ±0.019	ND	0.146* ±0.038	0.202* ±0.055
	100	ND	0.007 ±0.009	0.030 ±0.015	0.117* ±0.015	ND	0.300* ±0.041	0.338* ±0.110
2	0	0.000	0.000	0.000	ND	0.055* ±0.034	ND	0.204* ±0.053
	100	ND	0.000	0.000	ND	0.146* ±0.035	ND	0.305* ±0.018
3	0	0.000	0.000	0.000	ND	0.061* ±0.013	ND	0.199* ±0.050
	100	0.000	0.000	0.000	ND	0.205* ±0.032	ND	0.350* ±0.036
4	0	0.000	0.000	0.000	ND	0.062 ±0.039	ND	0.178* ±0.029
	100	ND	0.000	0.004 ±0.008	ND	0.116 ±0.049	ND	0.297* ±0.031
5	0	0.003 ±0.006	0.003 ±0.006	0.004 ±0.008	ND	0.000	ND	0.008* ±0.018
	100	ND	0.001 ±0.003	0.000	ND	0.013 ±0.021	ND	0.184* ±0.092
6	0	0.006 ±0.009	0.006 ±0.001	0.000	0.003* ±0.006	ND	0.017* ±0.016	0.057* ±0.077
	100	0.008 ±0.009	0.000	0.000	0.062* ±0.036	ND	0.255* ±0.012	0.343* ±0.062

ppm NH_3 = parts per million atmospheric ammonia, ND = not done. Figures are means ± standard deviations of ELISA values (absorbance at 400 nm at 100 min) from serum samples.

*Indicates significant difference ($P < 0.05$) between the two means at each post inoculation time in each experiment.

Inoculation dose = 4-5 x 10^6 colony-forming units in experiments 1-3, and 1-4 x 10^4 in experiments 4-6.

Ammonia-exposed rats in experiments 3 and 6 pre-exposed for 1 week.

Table 13. Enzyme-Linked Immunosorbent Assay of IgM Antibody After Intranasal Inoculation of *Mycoplasma pulmonis*

Experiment	ppm NH_3	Post Inoculation Day 0	2	7	14	17	21	28
1	0	ND	0.012 ±0.006	0.100 ±0.015	0.150* ±0.108	ND	0.138* ±0.023	0.119* ±0.020
	100	ND	0.020 ±0.020	0.197 ±0.112	0.517* ±0.111	ND	0.346* ±0.080	0.288* ±0.148
2	0	0.006 ±0.013	0.000	0.054 ±0.029	ND	0.072* ±0.043	ND	0.133 ±0.025
	100	ND	0.000	0.099 ±0.069	ND	0.191* ±0.034	ND	0.174 ±0.060
3	0	0.010 ±0.012	0.022 ±0.013	0.071* ±0.019	ND	0.069* ±0.036	ND	0.058* ±0.047
	100	0.036 ±0.034	0.010 ±0.010	0.130* ±0.038	ND	0.282* ±0.044	ND	0.104* ±0.038
4	0	0.003 ±0.007	0.006 ±0.007	0.062* ±0.021	ND	0.122* ±0.042	ND	0.094* ±0.069
	100	ND	0.011 ±0.016	0.102* ±0.019	ND	0.216* ±0.050	ND	0.213* ±0.101
5	0	0.018 ±0.016	0.024 ±0.014	0.039 ±0.036	ND	0.030* ±0.018	ND	0.046* ±0.037
	100	ND	0.021 ±0.013	0.023 ±0.019	ND	0.127* ±0.072	ND	0.240* ±0.055
6	0	0.024 ±0.018	0.013 ±0.005	0.015 ±0.009	0.072* ±0.051	ND	0.118* ±0.055	0.079* ±0.046
	100	0.035 ±0.018	0.029 ±0.007	0.036 ±0.023	0.325* ±0.147	ND	0.399* ±0.067	0.231* ±0.056

ppm NH_3 = parts per million atmospheric ammonia, ND = not done. Figures are means ± standard deviations of ELISA values (absorbance at 400 nm at 100 min) from serum samples.

*Indicates significant difference ($P < 0.05$) between the two means at each post inoculation time in each experiment.

Inoculation dose = 4-5 x 10^6 colony-forming units in experiments 1-3, and 1-4 x 10^4 in experiments 4-6.

Ammonia-exposed rats in experiments 3 and 6 pre-exposed for 1 week.

after inoculation and 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 54, 60, 66, 72, and 84 hr later.

The selection of 1 mM was based on an estimate of the concentration of ammonium ion that might be attained in nasal secretions. Calculations based on the average respiratory rate and tidal volume for rats (24) indicated that a rat breathing 100 ppm ammonia in air would inhale about 8×10^{-4} mole ammonia in 24 hr. If all ammonia were absorbed in the nasal passages and none was locally metabolized, 100 ml of nasal secretion in 24 hr would be required for the concentration of ammonium 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 greatly exceed 100 ml/24 hr. One mM ammonium hydroxide also conveniently adjusted the pH of the medium to the required 7.8 - 8.0.

This experiment was done twice. The results were nearly identical and were combined for statistical analysis and representation in Table 14. It is readily evident that 1 mM ammonium ion strongly inhibited the growth of M. pulmonis, rather than enhancing it or having no effect. These results indicate that ammonia probably does not directly enhance M. pulmonis growth.

Ammonia Inhalation Studies

Ammonia promoted changes in numbers of M. pulmonis first in the nasal passages, followed sequentially by the larynx, trachea, and lungs. It seems more likely that the larger nasal populations resulted in a more rapid spread of the organism to the distal parts of respiratory tract than that ammonia had a direct but delayed effect in the trachea and lungs, because ammonia is reported to be absorbed in the nasal

Table 14. Growth of Mycoplasma pulmonis in Liquid Medium

Hour	Log Colony-Forming Units Per Milliliter	
	Control	1 Millimolar NH_4^+
0	4.289 ± 0.047	4.155 ± 0.107
3	4.216 ± 0.056	4.040 ± 0.056
6	4.159 ± 0.064	4.190 ± 0.020
9	4.703 ± 0.030	4.216 ± 0.056
12	4.949 ± 0.184	4.136 ± 0.394
15	5.283 ± 0.111	4.192 ± 0.213
18	5.512 ± 0.009	4.211 ± 0.240
21	5.460 ± 0.064	4.228 ± 0.162
24	6.172 ± 0.082	4.385 ± 0.337
30	6.567 ± 0.499	4.305 ± 0.320
36	6.594 ± 0.070	4.257 ± 0.388
42	7.606 ± 0.372	4.356 ± 0.392
48	7.423 ± 0.012	4.486 ± 0.575
54	8.338 ± 0.084	4.679 ± 0.566
60	7.943 ± 0.370	4.613 ± 0.974
66	8.484 ± 0.030	4.467 ± 0.235
72	7.710 ± 0.054	4.623 ± 0.592
84	7.831 ± 0.095	4.537 ± 0.027

Figures are means ± standard deviations; for each, n = 2.

passages (52, 59). However, studies of ammonia absorption in the respiratory tract of rats have not been reported. Therefore, it was important to determine whether or not ammonia could be detected distal to the nasal passages.

After anesthetization, the head of the rat was inserted up to the eyes in the specially constructed face mask. Before tracheal ammonia measurements were made, the ammonia concentration of the air within the face mask was measured. Samples were obtained in 2 ways. First, samples were withdrawn through a 27 gauge needle inserted into the trachea. This gave an air flow through the proximal airways of the rat not greatly different from the average flow through the nasal passages of an unanesthetized rat. This was estimated at 127.5 ml/min and was calculated from published values for rats of a tidal volume of 1.5 ml and a respiratory rate of 85/min (24). Because the gas detector takes in 100 ml per stroke, about 10 min were needed to complete a measurement with the required 10 strokes. Ammonia concentrations in the face mask measured through the small needle were about 70% of those obtained with unrestricted flow through the detector tube. A second measurement was made on each rat after transection of the trachea. The distal segment was cannulated to permit the rat to continue breathing. Measurements were made through a second cannula inserted into the proximal section of the trachea.

No ammonia was detected in the tracheal air of 5 rats exposed to 100 ppm ammonia, nor in 3 other rats which had been pre-exposed to ammonia for 1 week. In each rat, ammonia was detected in the trachea only during exposure to 500 ppm, and then only in small amounts, 5 to 10 ppm.

Nucleic Acids in Nasal Washings

Inasmuch as ammonia was found to inhibit, rather than to enhance, the growth of M. pulmonis in vitro, it appeared that the growth enhancement in vivo was the result of an effect on the host. Of several reasonable hypotheses, one was that ammonia-induced nasal epithelial cell damage (32) resulted in increased availability of nutrients, such as nucleic acids or their precursors, essential to mycoplasmal growth (238). To explore this possibility, attempts were made to determine whether ammonia exposure resulted in increased amounts of nucleic acids in nasal secretions.

Rats were housed in the control and ammonia isolators for 1 week prior to the collection of nasal washings. In a preliminary experiment, 10 adolescent and 10 retired breeder rats were assigned to equal ammonia-exposed and control groups. No fluorescence over that of the blank was detected in nasal washings from any of these rats. In a second experiment, washings from 10 rats exposed to ammonia for 1 week and 10 control rats were pooled and concentrated by ultrafiltration. The relative intensity of fluorescence was up to 18 fold greater than the blank in the concentrated washings, but there was little difference in relative intensity between the control washings and those from the ammonia-exposed rats at either wavelength (365 or 540 nm) of excitation light.

DISCUSSION

The data obtained in the clearance experiments with S. epidermidis clearly showed that 100 ppm ammonia did not affect clearance of this organism. Results were comparable to those of previously published studies (183) and were consistently reproducible. The results also indicate that intratracheal inoculation can be used successfully in pulmonary bacterial clearance measurement.

In the experiments with S. aureus, clearance of the organisms in both the control and ammonia-exposed rats was very slow. There was considerable variation among clearance rates in individual rats, and thus only large differences would have been significant. It is possible that differences among strains of S. aureus and among strains of rats could account for differences in reported clearance rates (90, 141, 183) and for the apparent lack of clearance of S. aureus by our known pathogen-free Fischer rats. However, this seems unlikely because clearance rates for S. epidermidis in these experiments were consistent and were similar to those previously reported (183). For the same reason, decreased activity of macrophages in gnotobiotic rats (106, 154), also seems unlikely. The probable explanation suggested by the results of the study of in vivo phagocytosis, is that too large an inoculum was used, exceeding the bactericidal capacity of the alveolar macrophages. It has been reported that inoculum size can affect the rate of pulmonary clearance of S. aureus in mice (273), and about 5 to 10 times more CFU

were deposited in the lungs in these experiments than in those of Goldstein et al. (90).

In the study of in vivo phagocytosis, only 35% of the visible bacteria were intracellular 4 hr PI but all of the alveolar macrophages contained large numbers of bacteria. In contrast, others (90) have found up to 92% intracellular organisms at 5 hr PI with an inoculum of about one-tenth as many CFU. In the present study, the inoculum also may have contained many dead staphylococci, inasmuch as the organisms were incubated long enough to be well past the logarithmic phase of growth.

There was clearly no effect induced by 100 ppm ammonia on lung populations of M. pulmonis at intervals of up to 48 hr after intratracheal inoculation. No pulmonary clearance of M. pulmonis was demonstrated, in contrast to the results of Thomson and Hill (272) and Cassell et al. (41). The reason for this is unknown. Cassell et al. (41) did not quantitate the numbers of CFU introduced into the alveoli by intranasal inoculation. In the present study it was found during development of the inoculation technique that very little of an intranasal inoculum reached the lungs. With radiolabelled bacteria, the activity in the lungs frequently was indistinguishable from background counts. It is possible that such large numbers of organisms were given as to overwhelm the clearance mechanisms, yet the range of numbers of inoculated CFU included the dose used by Thomson and Hill (272). They, however, injected the organisms directly into the lung through the thoracic wall. Possibly in certain circumstances small numbers of organisms are more readily cleared from the lungs.

It may be that pulmonary clearance as measured in these experiments is not applicable to the study of pulmonary defense against mycoplasmas. Because the organisms adhere to ciliated epithelium (238), quite efficient alveolar clearance could occur but be masked if sufficiently large numbers of mycoplasmas adhered to the airway epithelium after intratracheal inoculation. Inasmuch as respiratory defenses against mycoplasmas are so poorly understood, it also seems possible that alveolar macrophages are not effective against M. pulmonis and that these results simply reflect this. This seems unlikely because alveolar inflammation is not prominent in MRM in rats (40, 195). Davis et al. (64) found that in cultures of lung macrophages, numbers of M. pulmonis neither increased nor decreased. Their results are similar to those of the present in vivo study.

The results of quantitative cultures of rat respiratory tracts showed that ammonia has striking effects on the population dynamics of M. pulmonis in the respiratory tissues of rats. Although the numbers of organisms varied from experiment to experiment in temporal relationships and peak populations, in general the results were very similar in all experiments. It was apparent in both control and ammonia-exposed rats that after intranasal inoculation M. pulmonis populations began to increase first in the nasal passages, followed slightly by the larynx, then in the trachea and lastly the lungs. However, in the ammonia-exposed rats, the increase in numbers of M. pulmonis was more rapid and the peak populations were much higher, in many cases by several orders of magnitude. Thus, ammonia probably contributes to the expression of MRM in some way which results in the appearance of much larger numbers of organisms, and increases in mycoplasmal populations

in the distal respiratory tract appear after increases in the proximal structures. Therefore, it seems reasonable to assume that ammonia exerts a local effect in the nasal passages which results in profound increases in the numbers of M. pulmonis at that site, and that large numbers in the nasal passages are associated with the distal extension or spread of infection.

In the present ammonia inhalation study, ammonia was not detected in the tracheas of rats exposed to ammonia by face mask. These findings are in agreement with those in previous reports (52, 59). Therefore, a direct but delayed effect of ammonia on tracheal and lung populations of M. pulmonis is highly unlikely. It is not definitely disproven, inasmuch as concentrations below the limit of sensitivity of the gas detector used might have a slight effect, although this seems improbable. Furthermore, comparison of the high dose and low dose controls shows that larger populations of organisms appeared in the tracheas and lungs of the rats inoculated with the higher dose and that this occurred after larger numbers had first appeared in the nasal passages and larynxes.

A study of the effect of ammonia on the growth of M. pulmonis in vitro showed that 1 mM ammonium ion did not enhance growth, but instead inhibited it (Table 14). The inhibition was unexpected although not surprising in view of the sensitivity of mycoplasmas to many substances (68, 69, 156, 157, 267). Although it is possible that lower concentrations of ammonium ion might have been stimulatory rather than inhibitory, this is unlikely inasmuch as there is no known metabolic feature of M. pulmonis by which growth enhancement by ammonium ion would be expected (M.F. Barile, personal communication). Therefore, rather than directly enhancing the growth of M. pulmonis, ammonia

probably influences the expression of MRM by affecting respiratory physiology in ways which result in (a) stimulation of growth or attachment of the organism, (b) in a reduction in killing, removal, or growth inhibition by respiratory defenses, or (c) in a combination of these.

One attractive hypothesis is that the degenerative changes in nasal epithelium (32) result in increased availability of cellular components such as nucleic acids and cholesterol which mycoplasmas require as nutrients (238). Measurements of nucleic acids in nasal washings did not show differences between ammonia-exposed and control rats. However, the technique used does not measure fragments of degraded nucleic acid molecules (191). Further studies including measurements of polynucleotides, free nucleotides and other components of respiratory secretions are required to resolve this question.

Other possibilities for fruitful investigations include studies of the effect of ammonia on mycoplasmal attachment to respiratory epithelial cells. Enhanced attachment could result in larger populations. Alternatively, less tenacious adherence might favor spread to distant sites. Quantitative or qualitative changes in respiratory secretions could favor growth of the organism, its aspiration into the distal respiratory tract, or both. Additional posssibilities exist, of course, and it is quite conceivable that ammonia acts by a combination of mechanisms.

Several aspects of the IgG and IgM responses (Figure 2) were probably to be expected. IgM antibody reached a peak or plateau, but IgG continued to increase throughout the experiments. Both IgG and IgM responses paralleled the changes in populations of organisms in the respiratory tracts but were not highly correlated with populations of

organisms at any single site. The lack of any significant decline in mycoplasmal populations with increasing amounts of serum antibody was not surprising, inasmuch as immune responses appear to be ineffective in many mycoplasmal diseases (37, 293), and because systemic immune responses may not accurately reflect local respiratory immunity.

However, these studies do provide quantitative information regarding the course of infection and antibody responses, and emphasize the need for greater understanding of immunity to mycoplasmal infections. The results demonstrate a profound effect of an environmental factor on an immune response, albeit probably indirectly through exposure to increased amounts of antigen. Further, the sera of rats given low doses of M. pulmonis and not exposed to ammonia were usually not positive by either IgM or IgG ELISA (Figure 2) as compared to pooled normal rat serum. In the low dose experiments the numbers of organisms recovered from the respiratory tracts of the control rats were quite low (Tables 8 through 11). These findings suggest that it would be possible for a very well-managed colony to have M. pulmonis infection sufficiently mild to go undetected by both culture and ELISA.

Large numbers of M. pulmonis seldom occurred in the lungs of control rats whereas they were the rule in the ammonia-exposed rats. In view of the results of the studies of Broderson et al. (32), it seems probable that the development of lung lesions is related to the numbers of organisms in the lung. A number sufficient to cause lesions may not develop in rats kept in a low-ammonia environment. That work and the studies described herein both emphasize the importance of defined environmental conditions in research in MRM in particular and in animal experimentation in general. They also indicate that differences

in environmental conditions are a likely reason for the inconsistent results of early investigations of MRM in lesion production and cultural recovery of the organism. Even in the experimental production of MRM by Whittlestone et al. (294), clinical disease and lung lesions were not evident even several weeks after inoculation. They had noted that the clinical condition of rats with natural MRM improved when they were moved into an isolation facility with improved sanitation. Subsequent reduction in frequency of cage cleaning resulted in ammonia accumulation in the room, followed by development of lung lesions of MRM.

Other observations also suggest that rats in well-managed colonies can be infected with M. pulmonis yet have little overt evidence of MRM. Ganaway et al. (82) found only a small proportion of rats with ear or lung lesions of MRM in an infected germ-free colony. Davidson et al. (63) reported that in experimental infections induced by inoculating very low numbers of organisms, most rats did not have cultural or serologic evidence of M. pulmonis infection as long as 28 days after inoculation and that demonstrable infection was not always accompanied by lesions. Longer intervals were not examined. Cassell et al. (unpublished) have identified infected rats by culture and ELISA in many so-called "barrier-maintained" colonies. The majority of these rats had no lesions or minimal lesions limited to the nasal passages, larynxes, or middle ears.

These are significant observations. They indicate that, while modern management techniques have no doubt reduced the incidence of mycoplasmal disease in rats, they have not eliminated mycoplasmal infection, as some may believe (221). Research done with rats from infected colonies is subject to all the complications introduced by

MRM. Even though the rats might appear perfectly healthy, the stress of experimental manipulation, substandard environmental conditions, age, and exposure to other pathogens could result in exacerbations of the disease.

SUMMARY

Murine respiratory mycoplasmosis (MRM), caused by Mycoplasma pulmonis, is one of the most important diseases of laboratory rats. It is a frequent complication of research in which rats are used, (193), and is a useful model for the study of other mycoplasmal diseases (37). It is ubiquitous among conventionally raised rats and also occurs in barrier-maintained colonies. Yet, many investigators fail to appreciate the significance of MRM.

Manifestations of MRM have been identified in the past by a wide variety of terms because early investigators did not recognize a single, common cause. This resulted, at least in part, from the unavailability of M. pulmonis-free rats, and from difficulties in culturing the organism. Potential effects of variations in strains of M. pulmonis, in different strains of rats, in particular, environmental conditions, have only recently been recognized, which undoubtedly contributed to the confusion. Several investigators reproduced all features of MRM and fulfilled Koch's postulates with isolates of M. pulmonis (146, 179, 193, 294). Further, ammonia released from soiled cage bedding was shown to have profound effects on the expression of MRM (32, 196).

The objective of the studies described herein was to obtain information regarding the mechanisms by which ammonia exacerbates MRM. Rats used in these experiments were raised so as to exclude viral, bacterial, and fungal pathogens. Plastic film isolators were used for ammonia

exposure. Ammonia was supplied through a regulating and filtering apparatus from a tank of anhydrous ammonia.

A method modified from that of Green and Kass (99) was used to investigate the effect of ammonia exposure on pulmonary bacterial clearance, which is essentially a measure of intrapulmonary killing by phagocytic cells. Radiolabelled S. aureus, S. epidermidis, and M. pulmonis were inoculated via the trachea into the lungs of 6 to 8 week old rats. Results of quantitative culture and scintillation counting of lung homogenates were used to calculate rates of pulmonary clearance. S. aureus clearance was variable but ammonia exposure clearly did not affect rates of inactivation of S. epidermidis at 6 hr after inoculation. Measurable clearance of M. pulmonis at up to 48 hr PI did not occur, but ammonia exposure did not result in detectable intrapulmonary growth.

In 6 experiments the effect of ammonia on population dynamics of M. pulmonis was examined by quantitative culture of nasal passages, larynxes, tracheas, and lungs of rats at intervals up to 28 days after intranasal inoculation with high (4 to 5 x 10^6 CFU) or low (1 to 4 x 10^4 CFU) doses of M. pulmonis. In all experiments results were similar in that populations of M. pulmonis increased more rapidly and to much higher numbers, in many instances by several orders of magnitude, in the rats exposed to ammonia than in the controls. In both ammonia-exposed and control rats M. pulmonis populations increased first in the nasal passages and larynxes, then in the trachea and lungs. M. pulmonis was not isolated from the lungs of many of the low dose control rats. Serum IgG and IgM ELISA antibody to M. pulmonis generally paralleled mycoplasmal populations but did not correlate highly with populations at any one site.

Ammonia was not detected in the tracheas of anesthetized rats exposed to ammonia by face mask, in agreement with previous reports that ammonia is absorbed in the nasal passages except at very high concentrations (52, 59). Thus, it seems likely that ammonia exerts a local effect in the nasal passages of rats which results in the appearance of large numbers of organisms in the proximal respiratory tract and that this in turn results in increased mycoplasmal populations in the tracheobronchial tree and the appearance of bronchopulmonary lesions.

One mM ammonium ion inhibited rather than enhanced growth of M. pulmonis in liquid artificial medium. Therefore, ammonia probably affects respiratory physiology or defenses of the rat in some fashion rather than directly enhancing the growth of M. pulmonis. Because ammonia alone causes damage to nasal epithelium in rats (32), it seemed possible that such damage might result in increased availability of essential mycoplasmal nutrients such as nucleic acids or their percursors and cholesterol (238). Assay of nasal washings for nucleic acids by an ethidium bromide fluorescence enhancement procedure (191) did not show differences between ammonia-exposed and control rats. However, the technique does not measure individual nucleotides or small polynucleotides. Further studies on the effects of ammonia on upper respiratory physiology and mycoplasma-host cell interactions are needed.

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Dissertation Committee:

J. Russell Lindsey, **Chairman**

Gail W. Cassell

Sanford P. Bishop

Frank M. Griffin Jr.

Director of Graduate Program

Dean, UAB Graduate School

Date

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