MYCOPLASMA PULMONIS INFECTION OF THE RESPIRATORY TRACT AND MIDDLE EAR IN THE RAT

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ABSTRACT

MYCOPLASMA PULMONIS INFECTION OF THE RESPIRATORY TRACT AND MIDDLE EAR IN THE RAT

By

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Research was conducted to reproduce *Mycoplasma pulmonis* infection in rats reared conventionally, exgermfree, and germfree. Ninety-six rats were used in 5 sequential experiments.

Chronic respiratory disease was reproduced in rats exposed to broth cultures of *M. pulmonis*. The clinical signs and lesions were characteristic of the natural disease.

Clinical signs were observed in the rats by 3 weeks after exposure to *M. pulmonis*. The most severe clinical signs were seen in the germfree rats. These rats developed dyspnea and became depressed. The young conventionally reared rats, exposed to *M. pulmonis* at 45 days of age, developed only mild clinical signs. The mild signs were often followed by apparent recovery.

Tracheitis, rhinitis, and otitis media were present in rats exposed to *M. pulmonis* regardless of age and method of rearing. The tracheitis and rhinitis were most severe in rats exposed at 45 days of age to both *M. pulmonis* and ammonia fumes. Also, upper respiratory lesions and purulent exudate in the middle ear were observed in the conventional rats exposed to only ammonia fumes.

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Bronchopneumonia with pulmonary solidification (consolidation) was a consistent finding in conventionally reared, exgermfree, and germfree rats exposed to *M. pulmonis* at 3 months of age or older. Conventional raised rats exposed to *M. pulmonis* alone and to *M. pulmonis* and ammonia fumes at 45 days of age developed fewer lung lesions. Upper respiratory and middle ear changes but no significant lung lesions were observed following exposure of 45-day-old conventional rats to ammonia fumes only.

Mycoplasma pulmonis microorganisms were reisolated from previously exposed rats. The reisolation procedures were complicated by the presence of contaminating microorganisms in the conventional and exgermfree rats.

Chronic respiratory disease in the rats was primarily due to *M. pulmonis*. Ammonia fumes and contaminating microorganisms may contribute to the disease as it occurs naturally in rat colonies used in research.

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By

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INTRODUCTION

Chronic respiratory disease in rats is an important problem and seriously restricts their usefulness in a wide variety of research. The disease is difficult to detect clinically in a rat colony. Experimental results obtained using rats afflicted with the disease would be unreliable (Lindsey et al., 1971).

Viruses, bacterial agents, and combinations of pathogens have been suggested as causes of chronic respiratory disease (CRD) in rats (Brennan et al., 1971). In recent years, however, *Mycoplasma pulmonis* has been demonstrated to be the primary cause of CRD (Kohn and Kirk, 1969; Lindsey et al., 1971; Whittlestone et al., 1972; Jersey et al., 1973). Research workers have recently suggested that factors altering pulmonary clearance may also play a part in the pathogenesis of CRD in the rat (Cassell et al., 1973).

The significance of this disease in rat colonies used for research, the importance of the mycoplasmal microorganisms in diseases of man and livestock, and the possibilities of the rat as a model for respiratory disease research emphasize the need for additional investigations on the role of *M. pulmonis* pneumonia in rats. Respiratory disease in rats has been referred to by a variety of terms and, for consistency, in this thesis the infection will be called chronic respiratory disease.

LITERATURE REVIEW

The literature on CRD in the rat has been recently reviewed rather extensively by other workers (Nelson, 1967; Brennan et al., 1969; Giddens et al., 1971a,b; Cassell et al., 1973). Therefore, this review is focused primarily on the cause, factors that contribute to susceptibility, the immune response, clinical signs and lesions, pathogenesis of infection, and methods of prevention and control of *M. pulmonis* in rats. Germfree techniques that enhance research on the pathogenesis and in control and prevention of the disease are also included.

Mycoplasmal Diseases of Man and Animals

The determination that *Mycoplasma mycoides* was the cause of contagious bovine pleuropneumonia by Nocard and Roux in 1898 brought about a systematic study of mycoplasmal diseases in man and animals. During the next six decades only a few diseases caused by mycoplasmas were identified. Increased interest was stimulated in the early 1960's when *Mycoplasma pneumoniae* was found to be the cause of primary atypical pneumonia in man. Since that time, important diseases caused by mycoplasmas have been identified in numerous domestic species. Presently mycoplasmas are associated with important diseases in cattle, swine, sheep, goats, fowl, and laboratory animals. Mycoplasmas have been isolated from horses, dogs, and cats, but proof of

pathogenicity is lacking (Carter, 1975; Whittlestone, 1975). Since it is likely more mycoplasmas will be discovered, further association of these microbes with animal disease will probably occur.

Mycoplasmas are the smallest known organisms able to grow and multiply autonomously. These organisms have no cell wall but are bounded by a unit membrane. They can pass through pores and penetrate into spaces much smaller than their usual diameter. The mycoplasmas have rather fastidious growth requirements (Whittlestone, 1975).

The order Mycoplasmatales can be separated into three genera. The first genus, the true mycoplasmas, requires cholesterol as a growth substance. The second genus, the acholeplasmas, does not require cholesterol. The third genus requires urea as an essential metabolite, and members of this genus are called T-mycoplasmas or ureaplasmas. The T-mycoplasmas produce very small colonies. Most of the known pathogenic mycoplasmas belong to the genus of true mycoplasmas (Whittlestone, 1975).

Several of the mycoplasmas inhabit the upper respiratory tract, genital tract, and upper digestive tract of animals without causing injury to the host. Pathogenic organisms may involve the respiratory tract, joints, mammary glands and other body systems as extracellular parasites. Infections caused by mycoplasmas are often associated with stresses and are usually low grade and chronic (Carter, 1975).

Mycoplasma pulmonis Infection of Rats

While *Mycoplasma pulmonis* is now considered to be the primary etiologic agent of rhinitis and otitis media commonly associated with the upper respiratory tract of the rat, the role of this organism in the development of lower respiratory infection has been investigated, but a role has not been fully established (Cassell et al., 1973).

Klieneberger and Steabben (1937) at the Lister Institute, London, first reported the isolation of a pleuropneumonia-like organism (PPLO) from the lungs of rats with pneumonia. This was named the L_1 organism. In a subsequent report (Klieneberger and Steabben, 1940), the L_3 organism (changed from the previously named L_1 organism) was closely correlated with bronchiectatic lesions in laboratory rats, although this organism did not produce bronchiectasis following experimental inoculation. The L_3 organism later became known as *M. pulmonis*.

Klieneberger-Nobel and Chang (1955) and later Venture and Domaradzki (1967), physicians at St. Joseph of Rosemont Hospital, Montreal, were able to experimentally produce pneumonia and bronchiectasis by ligating bronchi of young rats that were naturally exposed to *M. pulmonis*.

Nelson (1940, 1967), at the Rockefeller Institute, New York, described a relationship between "coccobacilliform bodies" from gram-stained exudates and disease of the upper respiratory tract, middle ear, and infrequently the lungs. The "coccobacilliform bodies" were subsequently identified as *M. pulmonis*. Nelson (1967), in a review summarizing three decades of research on chronic respiratory disease in rats, concluded that the condition consisted of two entities.

These were infectious catarrh caused by *M. pulmonis* and enzootic bronchiectasis caused by a virus. The infectious catarrh resulted in rhinitis, otitis media and less often pneumonia, and the enzootic bronchiectasis was responsible for rhinitis and chronic pneumonia.

Brennan et al. (1969), at Argonne National Laboratory, concluded that the usual causes of murine pneumonia (any pneumonia in rats) were *M. pulmonis* and *Pasteurella multocida*. They also believed that the two causative organisms together produced an explosive pneumonia and death.

Using *M. pulmonis* cultures, Kohn and Kirk (1969), at the West Virginia University Medical Center, experimentally produced lung lesions like those of CRD in conventionally reared progeny of gnotobiotic Sprague-Dawley rats, monocontaminated with lactobacilli. Repeated inoculations were required. Lindsey et al. (1971) successfully produced the lesions of natural CRD with various inocula containing only *M. pulmonis* in inbred Fischer rats raised under rigid disease-free conditions. Of the five strains of organisms used, the most severe lesions were produced by strain M/J69, isolated earlier and supplied to Dr. Lindsey's group by Dr. George Jersey at Michigan State University.

Whittlestone et al. (1972), a pioneering group of researchers on mycoplasmal diseases at Cambridge University, reported that *M. pulmonis* by itself could produce pneumonia and bronchiectasis. They used Sprague-Dawley, cesarean derived, barrier sustained rats. A cloned inoculum was used to eliminate possible carryover of another pathogen. The course of the disease was chronic with lung lesions

first being observed at 85 days after exposure and persisting to 715 days.

Jersey et al. (1973), at Michigan State University, using germfree rats, concluded that *M. pulmonis* was the primary cause of chronic respiratory disease in rats and that an appropriate name for the disease was respiratory mycoplasmosis. The disease developed fairly rapidly with typical respiratory lesions occurring within 2 to 6 weeks following exposure of adult germfree rats.

Cassell et al. (1973) emphasized that Koch's postulates for producing all respiratory tract lesions characteristic of CRD have been satisfied using pure cultures of *M. pulmonis*. They suggested murine mycoplasma respiratory disease as an appropriate name for the disease.

Factors Contributing to the Susceptibility of Rats to Respiratory Mycoplasmosis

Reports have indicated that dietary factors, environmental influences, and genetic factors affect the susceptibility of rats to chronic respiratory disease. Lindsey et al. (1971) mentioned the importance of proper nutrition. Tvedten et al. (1973) reported germfree rats fed deficient vitamin A and vitamin E rations were more susceptible to *M. pulmonis* infection than were those fed adequate levels of both vitamins and that high levels of vitamin supplementation may be a means of reducing the disease incidence in rat colonies. Kasali (1974) reported that conventionally reared rats fed a high lard diet were more susceptible to *M. pulmonis* than those fed a standard commercial diet.

Giddens et al. (1971b) noted that environmental factors such as air flow, temperature and humidity of animal rooms, overcrowding, sanitary standards, and concentration of ammonia fumes influence the establishment of tracheobronchial infections in rats. The rapid respiratory rate of the rat, the anatomical structure of the rat bronchus, and genetic susceptibility were mentioned as additional influencing factors.

There is evidence that age affects the susceptibility of rats to respiratory mycoplasmosis. Nelson (1967), Giddens et al. (1971b), and Jersey et al. (1973) all noticed that young rats were more resistant to CRD and seldom developed significant lung lesions. Adult rats, on the other hand, were highly susceptible to experimental CRD and rapidly developed an extensive pneumonia.

Cassell et al. (1973) explained that the pulmonary clearance of organisms from the lower respiratory tract is important in the prevention of pulmonary lesions in rats with chronic respiratory disease. They administered hexamethylphosphoramide (HMPA), a compound which interferes with normal pulmonary clearance, and markedly enhanced lower respiratory lesions due to *M. pulmonis*. High levels of sulfur dioxide and ammonia were suggested as playing a part in lowering resistance of rats to *Mycoplasma*, since both compounds interfere with pulmonary clearance in the rat.

Clinical Signs and Lesions

Chronic respiratory disease in laboratory rats has been described in the literature since the early 20th century. Klein (1903) gave a brief description of CRD. He described extensive pulmonary

consolidation with a fibrinous exudate in the bronchi and alveoli of affected lungs. A diphtheroid bacterium was isolated from the diseased lungs.

Hektoen (1916), at the Memorial Institute of Infectious Diseases, Chicago, provided one of the first detailed morphological descriptions of CRD. Bronchitis and bronchopneumonia were observed in laboratory rats examined for lung lesions. Two types of pulmonary consolidation were identified. Suppuration was observed in the first type and a mucoid type of degeneration was seen in the second. Unthriftiness, listlessness, and dirty hair coats were the main clinical signs noted. A streptothrix was isolated from the lungs.

McCordock and Congdon (1924), at the Buffalo General Hospital in New York, outlined the clinical and historical manifestations of suppurative otitis media in laboratory rats. The affected animals tilted their heads to one side and circled in the direction of the tilt. In these rats the squamous epithelial lining of the middle ear was replaced by fibrous granulation tissue, and pus filled the cavity. Osteitis was present in bone surrounding the cavity.

Klieneberger and Steabben (1937) reported considerable variation in the gross appearanace of lung lesions in CRD-affected rats. They emphasized that microscopic lesions may exist without the development of detectable gross lesions. The initial microscopic lung lesions were visualized as a complicated group of simultaneous changes involving the bronchi. The significant changes were proliferation of bronchial epithelium, increased mucus production, peribronchial lymphocytic infiltration, bronchial dilatation, alveolar collapse, neutrophilic infiltration through the bronchial epithelium

into the lumen, and accumulation of mononuclear cells in the submucosa and lamina propria. As the disease progressed, a bronchial exudate containing neutrophils blocked the lumen. Finally the bronchial epithelium and neutrophils were destroyed leaving a purulent mass surrounded by fibrous connective tissue.

Innes et al. (1956) and Newberne et al. (1961) have provided a detailed and rather complete description of the gross and microscopic lesions of naturally occurring CRD in rat research colonies. Innes and associates observed that most of the affected rats lacked visible clinical signs although in the advanced disease state weight loss, rough hair coat, and dyspnea were noticeable. Entire lung lobes in affected rats were indurated, rubbery in consistency, and cobbled at the surface. Despite the often diffuse lung involvement, only a low incidence of emphysema and pleural adhesions was observed. An accumulation of inflammatory exudate in the alveolar walls near affected bronchi was noted. This process often accompanied the concurrent bronchial changes. Peribronchial collapse was an accompanying finding. Foamy macrophages were often seen in the affected alveoli. Newberne and co-workers emphasized a squamous metaplasia of the affected lung bronchi. Additional pulmonary lesions noted were induration, bronchiectasis, lymphoid proliferation, pneumonia and hyperplasia of bronchial epithelium. Nelson (1967) observed a compensatory hypertrophy of unconsolidated portions of affected lungs.

Giddens et al. (1971a,b) described the morphologic characteristics of respiratory systems of germfree, conventional and CRD affected rats. The morphology of the lungs, middle ear, trachea and nasal

cavity of rats under the three conditions was described. In the natural infection the rhinitis was purulent in nature with exudation in the nasal cavity, a marked proliferation of lymphocytes beneath the epithelium and changes in the mucosal surface which sometimes included necrosis.

Lindsey et al. (1971) described in considerable detail the sequential appearance of disease signs in a CRD affected rat colony. Signs of the disease were not observed until the rats were at least 1 month of age. At this time, small encrustations around the external nares were seen and snuffling was noticed. Clinically inapparent otitis media had developed and varying numbers of rats had acquired pneumonia. Most of the lesions up to this time required histologic examination for detection. By 2 months of age, there was polypnea, inactivity, humped posture, rough hair coat, and diminished weight gains. However, in many rats the clinical signs were difficult to detect. Mortality normally remained low. Examples were cited in which the incidence of inapparent infection in adult rats ranged from 50 to 100%.

Jersey et al. (1973), in previous research in this department, were able to readily reproduce CRD by using germfree rats. The clinical signs were essentially the same as reported by previous researchers. Rats exposed under 21 days of age did not develop a rapidly progressive respiratory infection. The lung lesions observed included bronchiectasis, mucopurulent bronchial exudation, peribronchial and perivascular lymphoid hyperplasia, and pulmonary atelectasis. The lung lesions were accompanied by a tracheitis with hyperplasia of the epithelium and an infiltration of lymphocytes,

plasma cells, and neutrophils beneath the epithelium. The epithelium reached several times its normal thickness. Rats affected with otitis media rapidly accumulated purulent exudate in the tympanic cavity. Proliferative connective tissue eventually filled the tympanic cavities. The tympanic membranes also became markedly thickened due to proliferation of epithelial cells and connective tissue. The rhinitis produced the same morphological changes as described previously by Giddens et al. (1971a).

Methods of Control and Prevention

Nelson (1967) reported that attempts have been made to control CRD by biological means and by chemotherapy. He (Nelson, 1951) established a colony of CRD-free rats from parent stock by cesarean-section delivery and foster suckling the young on germfree mothers. Chronic respiratory disease, which had been present in the original colony, was eliminated in the cesarean-derived colony. The CRD-free colony and subsequent generations were maintained in rigidly isolated quarters. Outside rats were not introduced into the colony. Nelson (1967) stated that this colony was kept for several years in a CRD-free state before being discontinued. Foster (1958) developed a commercial CRD-free colony beginning with cesarean-section delivered pups and foster-nursed by germfree mothers. The CRD colony was maintained within barrier facilities that prevented the introduction of microbial pathogens. Many CRD-free rat colonies have since been developed utilizing this general protocol.

Kappel et al. (1974) outlined steps to be followed for establishment and maintenance of a large-scale commercial rat breeding colony free from mycoplasmas. Defined flora foster parents without mycoplasmal type of infection in germfree isolators were used to raise young derived by cesarean section from *M. pulmonis*-infected conventional donors. The offspring, who were free of mycoplasmas, were used to establish a breeding nucleus within a smaller barrier room. The breeding nucleus was in turn used to establish a production colony which was housed in a second larger barrier room. Colonies were monitored by routine diagnostic methods and by nasal washing techniques.

Kappel et al. (1974) further reported that previous efforts to rid laboratory rats of mycoplasmal microorganisms by therapeutic or prophylactic means were generally not successful. Haberman et al. (1963) eliminated CRD in rats by oral administration of sulfamerazine at the rate of 5 mg/20 gm of feed. Respiratory disease was eliminated in the third through tenth generations. Chlortetracycline given orally at the same rate did not eliminate respiratory disease. Gannaway and Allen (1969), at the National Institutes of Health (NIH), housed rats in filtered-air cages and gave them sulfamerazine in the drinking water at a rate of 1 gm/gallon of water. They concluded that the sulfamerazine prophylaxis was responsible for eliminating respiratory disease. In contrast to the findings at NIH, Lindsey et al. (1971) reported that rats exposed to CRD and given sulfamerazine at a rate of 0.25 mg/ml of water developed severe rhinitis, otitis media, and lung lesions. However, when tetracycline was given at the rate of 0.25 mg/ml, the rats

failed to develop rhinitis or otitis media and only an occasional rat had suggestive hyperplasia of lymphoid tissue.

Lamb (1975) reported that early histological screening, sampling of animals of all ages at frequent intervals, and further research into the problem of respiratory disease are necessary to provide ultimate solutions to the control of CRD in rats.

Immune Response of Rats to *Mycoplasma pulmonis*

Little is known about the immune response of rats to *M. pulmonis*. Lemecke (1961), at the University of Cambridge, demonstrated that in the early stages of the natural disease, when the organisms were confined to the nasopharynx, complement-fixing (CF) antibody values were very low. As the disease progressed and the organisms invaded the lungs, circulating antibody titers increased. Whittlestone et al. (1972), working in the same laboratory as Lemecke, reported a correlation between the extent of pneumonia in rats and CF titers and pointed out that CF antibody may be a helpful indicator in determining *M. pulmonis* infection in rats. They, however, found that both *M. pneumoniae* and *M. pulmonis* organisms persisted in the presence of high titers of cirulating CF antibodies, therefore suggesting that circulating antibody does not play an important role in protection or recovery from mycoplasmal infection.

Kohn and Kirk (1969) detected plate agglutination titers in rats inoculated with *M. pulmonis* and in rats exposed indirectly to infected animals. Attempts by these investigators to demonstrate antibody titers by the metabolic inhibition test were essentially negative.

Cassell et al. (1973) reported that rats inoculated intranasally with tracheal aspirates and tested 4 weeks later developed a positive delayed hypersensitive skin reaction following intradermal administration of washed *M. pulmonis* cells. The skin reaction was characterized by infiltration into the dermis of lymphocytes, macrophages, and a few plasma cells.

The mere demonstration of hypersensitivity did not prove its importance in the pathology of the disease or its role in protection against the disease. Therefore, it is apparent that further studies are needed to determine the role of hypersensitivity in *M. pulmonis* infections in the rat.

Germfree Principles

Pleasants (1974), at the Lobund Laboratory at the University of Notre Dame, has extensively reviewed the general literature on germfree methodology. Trexler and Reynolds (1957) and Trexler (1959) reported that plastic germfree isolator units offer general advantages over previously used rigid materials. Transparency, flexibility of the units, lack of excess weight, multiplicity of sizes and shapes, inexpensiveness, and the fact that plastic units are partially sustained by enclosed air pressure were the primary advantages cited. The major disadvantage described was the possibility of puncture.

Newton (1965), at the National Institutes of Health, reported that the basic germfree system consists of a sterile airtight structure to house the experimental animals and methods for passing air, water, food, bedding, cages, and other needed supplies in and out of the isolator through some sterilizing device. He also mentioned that

plastic isolators are sterilized by chemicals usually applied directly to disinfect both the air space and the surface of the unit. Peracetic acid in a 2% aqueous solution is the most widely used disinfectant for plastic isolators. Jaworski and Miller (1963) developed supply cylinders which can be autoclaved and then attached to the plastic unit for delivery of materials by way of a germicide connection.

Sacquet (1968), in France, outlined a specific set of procedures for maintaining germfree animals. The importance of carefully following the principles for sterilization and keeping positive air pressure to prevent entry of microorganisms from the outside were strongly emphasized.

Fuller (1968), in England, described procedures to follow when testing isolators for sterility. In routine control, materials from the isolator must be cultured and examined microscopically for evidence of contamination.

Pleasants (1974) reported further that persons contemplating the use of germfree animals for research should consider the importance of the microbial variable to their study and the technical requirements for the research. Technical factors outlined included: (1) availability of required animal species and strains, (2) availability of animals to be used as controls, (3) technical skills required to complete the research, (4) availability of isolators and accessory equipment to maintain the animals, (5) interior equipment needed, (6) air supply and filters, (7) diets for the germfree animals, (8) water and bedding to be used, (9) sterilization of the isolators and supplies, (10) husbandry, and (11) microbial monitoring.

The rat is one of the many animals available for germfree research. Sprague-Dawley, Wistar, and Fischer strains of rats at Lobund have been extensively tested and reported to have no bacterial, fungal, protozoan, or viral contamination (Pollard and Kajima, 1967). Newton (1965) reported that weight gains of germfree rats compare favorably with those of conventional rats but that reproduction has been somewhat erratic. Infertility and ignoring, eating, or inability to nurse the young have been difficulties encountered. Dietary deficiencies due to oversterilization, too much inbreeding, and excessive noise or other disturbances may be contributing factors to poor reproduction.

Summary of Literature Review

The information in this literature review indicates that CRD is an important disease that complicates research using the rat. The clinical signs and gross and microscopic lesions characteristic of the natural disease are fairly well established. *Mycoplasma pulmonis* is believed to be the primary etiologic agent of CRD, but additional information is needed as to the nature and pathology of the disease, especially factors that enhance or prevent infection. Germfree rats can be utilized for these studies, since they are free of mycoplasmas and other known pathogenic agents and therefore supply information uncomplicated by microbial contamination.

OBJECTIVES

The objectives of this research were:

1. To reproduce *Mycoplasma pulmonis* infection in the rat with a known agent isolated from rats with the disease.

2. To reisolate and identify the agent producing the disease.

3. To compare the susceptibility of conventional, exgermfree, and germfree rats of different ages.

4. To determine the role, if any, of ammonia fumes in susceptibility to *M. pulmonis* infection.

5. To continue previous research started in this department on the pathogenesis, etiology and general nature of *M. pulmonis* infection in rats used in research.

MATERIALS AND METHODS

This research was initiated in September 1975. It was conducted in the Pathology Laboratories, Barn 5, at the Veterinary Research Farm, and at the Clinical Microbiology Laboratory.

Sources and Care of Experimental Animals

Exgermfree (born and reared germfree and later changed to a conventional environment), conventional (reared in open room conditions in association with other rats of the same type), and germfree (free from demonstrable forms of associated life) rats were used for this research. The exgermfree rats were obtained from the Lobund Laboratories, University of Notre Dame. The conventional rats were first and second generation offspring of the original exgermfree rats. The germfree rats were obtained as weanlings from a commercial source (A. R. Schmidt Co., Inc., Madison, WI). All rats were of the Sprague-Dawley strain. The exgermfree rats were maintained in filter-topped "shoebox" cages, initially containing up to 6 rats each. Male and female rats were separated except during breeding. The conventional rats were maintained similarly to the exgermfree rats except that air filter-topped cages were not used. The germfree rats were kept in flexible plastic isolator units measuring 24 x 24 x 60 inches (Trexler, 1959). Standard germfree procedures were

followed (Sacquet, 1968). Autoclavable, plastic "shoebox" cages fitted with stainless steel wire covers housed the rats within the isolator.

The exgermfree and conventional rats were fed a commercially prepared pelleted ration (Peerless Laboratory Diet, Peerless Pet Foods of Battle Creek, Inc., Battle Creek, MI) formulated for laboratory rats and mice. Originally the germfree rats were fed the same ration (autoclaved) as the other rats, but later they were fed a commercially formulated autoclavable ration (Tekled Mouse/Rat Autoclavable Diet [L485], Mogul Corporation, Winfield, IA) when nutritional deficiencies developed due to sterilizing the diet.

The cages were cleaned at weekly intervals. The germfree isolators were entered approximately 3 times monthly to replenish food, water, and litter and to remove waste material. Isolators were tested periodically for sterility according to the method of Fuller (1968). Water, litter, and feces were collected in screw-top tubes, removed from the isolator, and taken to the Pathology Laboratories for cultures and microscopic examination. When germfree rats were necropsied, intestinal contents were cultured to further measure their microbial status.

Necropsy and Tissue Preparation Procedures

Rats were killed with an overdose of sodium pentobarbital given intraperitoneally. The skin was then reflected from the abdomen, thorax, and ventral cervical region. The trachea was located and severed at the laryngotracheal junction. Material for culture was collected from the trachea and lungs by tying a cannula securely into

and trachea and injecting 2.0 ml of *Mycoplasma* medium into the lung and withdrawing a minimum of 0.5 ml back into the syringe. The lung perfusion technique has worked well because mycoplasmal microorganisms are located almost entirely on the surface of the bronchial epithelial cells (Taylor-Robinson et al., 1972). Next, the thoracic cavity was opened and the trachea and lungs were removed intact. Enough fixative was infused into the tracheal cannula to slightly distend the lungs. The trachea and lungs were fixed in 10% buffered formalin.

The skin was reflected from the head, and 0.4 ml of broth was injected and withdrawn from the tympanic cavity of the right ear. The head was removed at the atlanto-occipital joint and placed in formalin solution for later decalcification. The intact heads were demineralized in R.D.O. solution (DuPage Kinetic Laboratories, Inc., Downers Grove, IL). Sectioning of the heads included a transverse section midway between the external nares and the medial canthi, and a transverse section of the middle ear through the external auditory meatus.

Selected tissues for microscopic examination were paraffin embedded, cut at 6 microns thickness, and stained with hematoxylin and eosin. Techniques used were essentially those suggested by the Armed Forces Institute of Pathology (Luna, 1968).

Experimental Exposure of the Rats

The exgermfree and conventional rats were exposed to *M. pulmonis* by aerosol inhalation. Fourth passage, 72-hour broth culture was used. A total of 5 ml was given per cage of 5 or 6 rats. The aerosol was placed in a glass nebulizer (Model 640 Nebulizer, The DeVilbiss

Co., Toledo, OH) using a low pressure stream of compressed nitrogen gas. The spray was directed through a hole cut in the filter top cage caps. After all of the broth was nebulized, the hole in the filter cap was sealed with plastic tape to trap the mist inside the cages. To expose the germfree rats, screw-cap vials containing 5 ml of *M. pulmonis* in broth culture were first washed in 70% ethyl alcohol and then passed into the germfree isolator through the entry lock. The peracetic acid fumes were allowed to dissipate from the isolator for 45 minutes, after which the isolator's air inlet and outlet were sealed. A portion of the broth culture was applied to the nostril area of the rats using a 1 ml tuberculin syringe. The remaining broth culture was placed on the feed and bedding within the cages.

Microbiologic Techniques

Mycoplasma pulmonis strain M/J 69 (Jersey et al., 1973) was used in this research. The organisms had been stored at -64 C, were thawed and then passed 3 times in exgermfree rats (experiment 1) to enhance their virulence.

The media used for isolation and purification of *M. pulmonis* and for preparation of inoculation material was constituted from commercially purchased dehydrated products as suggested by the suppliers. Broth (Bacto-PPLO Enrichment Broth, Difco Laboratories, Detroit, MI) and agar (Bacto-PPLO-Agar, Difco Laboratories, Detroit, MI) media were supplemented with horse serum, yeast extract and inhibitory levels of thallium acetate and penicillin to retard bacterial growth (BBL Mycoplasma Enrichment 11865, Becton, Dickinson, and Co., Cockeysville, MD). The general procedures followed were

essentially those of Jersey et al. (1973). *Mycoplasma* agar was plated with 0.1 ml broth from the original 72-hour cultures, and the plates were checked at 3 and 6 days.

Mycoplasma pulmonis was purified generally as outlined by Whittlestone et al. (1972). Verification of the inoculum as *M. pulmonis* was made by the growth inhibition (GI) test as described by Clyde (1964) and later modified by Stanbridge and Hayflick (1967). In this test anti-*M. pulmonis* and anti-*M. pneumoniae* sera were purchased from a commercial laboratory (Microbiological Associates, Bethesda, MD) and used for identification. *Mycoplasma pulmonis* was identified on the basis of an area of reduced growth surrounding a paper disc impregnated with the specific antiserum. The *M. pneumoniae* antiserum produced no such inhibition.

Experiments

Sequential experiments were conducted to reproduce CRD in rats, to identify the causative agent, and to determine the effect of ammonia fumes on the susceptibility to *M. pulmonis* infection. For convenience in presentation of data, some details of the procedure will be included in the results.

Experiment 1

Fifteen of 25 originally obtained exgermfree rats, 3 to 5 months of age, were used to enhance the virulence of *M. pulmonis* M/J 69. The original culture had been frozen in vials for 2 years. Five rats were exposed at a time with the 3 exposures being approximately 1 month apart. The 10 additional rats in this group were used as controls. Two of the rats were killed and examined before the

experiment to be sure they were free from infection. Eight rats were maintained for the duration of the experiment as colony controls.

Experiment 2

Twenty-two, 3-month-old, conventional rats were used to study the course of CRD infection. Two rats were killed and examined before the experiment to be sure they were free from infection. Twenty animals were placed, 5 per cage, in 4 filter-top boxes and exposed to *M. pulmonis*. They were killed at approximately weekly intervals starting 21 days following exposure.

Experiment 3

Five, 3-month-old, conventional rats were used to study the long-term course of CRD infection. The animals were placed in a filter-top box and exposed to *M. pulmonis*. One rat died 162 days following exposure and the remaining rats were killed 190 days after exposure.

Experiment 4

Thirty-eight conventionally reared rats approximately 45 days of age were used to evaluate the influence of ammonia fumes on the course of CRD. Two rats were killed and examined before the experiment to be sure they were free from infection. Thirty-six were placed, 6 per cage, in 6 filter-top boxes. Twelve rats were exposed to *M. pulmonis* only. The second group of 12 rats was exposed to *M. pulmonis* plus ammonium hydroxide in open bottles 1 inch in diameter. The final group of 12 rats was exposed to ammonium hydroxide only.

Experiment 5

Six germfree rats were used. Two were killed and examined before the experiment to make sure they were free of identifiable microorganisms.

Four rats were placed in a single germfree isolator and inoculated intranasally with a broth culture of *M. pulmonis*. Following inoculation the rats were housed in a single clear plastic "shoebox" cage. Rats were killed when severe dyspnea developed. The number and sex of the rats used in this experiment were determined by their availability at the time the experiment was conducted.

Colony Controls

Eight, exgermfree rats (from Experiment 1) were maintained in a separate room at Barn 5 throughout the duration of this research. This room also maintained a guinea pig colony. These rats were killed and examined for evidence of respiratory disease at the termination of the research.

RESULTS

Chronic respiratory disease was produced in the experimental rats. The susceptibility to infection, clinical nature of the disease, and lesions varied somewhat among the rats in the different experiments. These differences were apparently related to the age of the rats, the methods of rearing, the presence of ammonia fumes, and other factors. The results of each experiment are tabulated (Table 1). A narrative summary of each experiment and a composite description of the clinical nature, pathology, and microbiology of the disease in the different experiments follows.

Summary of Experiments

Experiment 1

The results were similar in each consecutive passage. The clinical signs, gross lesions and microscopic lesions were those described later in the composite description of CRD. The first clinical signs were noticed 10 days after exposure to *M. pulmonis*. The gross lung changes were most often diffuse, and the apical and dependent portions of the lungs were frequently affected. Histologic lesions were present in the lung, nasal cavity, and middle ear tissues from all of the rats. Tracheitis was observed in 14 of the 15 rats.

Table 1. Experimental design, lesions and *Mycoplasma pulmonis* isolations

Experiment Number	Treatment	No.	Age (mo.) **	Sex M	Sex F	Rearing	Killed or died of exposure (days)
1	*M. pulmonis*	15	3-5	15	-	Exgermfree	14-49
	Controls	2	3	2	-	Exgermfree	---
2	*M. pulmonis*	20	3	10	10	Conventional	21-50
	Controls	2	3	1	1	Conventional	---
3	*M. pulmonis*	5	3	5	-	Conventional	162-190
4	NH_3	12	1.5	6	6	Conventional	20-57
	M. pulmonis	12	1.5	6	6	Conventional	20-57
	M. pulmonis + NH_3	12	1.5	6	6	Conventional	20-57
	Controls	2	1.5	1	1	Conventional	---
5	*M. pulmonis*	4	3	-	4	Germfree	10-41
	Controls	2	3	-	2	Germfree	---
CC*	Controls	8	12	2	6	Exgermfree	---

*Colony controls.

**Age exposed or killed if not exposed.

Site and Frequency of Lesions					Site and Frequency of *M. pulmonis* Isolations	
	Microscopic Lesions					
Gross Lesions Lungs	Lungs	Trachea	Nasal cavity	Middle ear	Tracheo-bronchial washings	Middle ear
10/15	12/15	14/15	15/15	15/15	7/15	9/15
0/2	0/2	0/2	0/2	0/2	0/2	0/2
17/20	18/20	20/20	20/20	20/20	8/20	13/20
0/2	0/2	0/2	0/2	0/2	0/2	0/2
5/5	4/5	5/5	5/5	5/5	2/4	2/4
0/12	0/12	12/12	12/12	12/12	0/12	0/12
1/12	6/12	12/12	12/12	12/12	5/12	1/12
2/12	7/12	12/12	12/12	12/12	4/12	1/12
0/2	0/2	0/2	0/2	0/2	0/2	0/2
4/4	4/4	4/4	4/4	3/4	4/4	2/4
0/2	0/2	0/2	0/2	0/2	0/2	0/2
3/8	4/8	5/8	6/8	4/8	3/8	3/8

Mycoplasmas were isolated from the tracheobronchial washings from 7 of the 15 exposed rats. Overgrowth of bacteria on the agar media from the washings of the remaining 8 rats was present. Bacterial colonies were noted on the media in 3 of the plates on which mycoplasmas were identified. Mycoplasmal microorganisms were isolated from middle ear cultures from 9 of the 15 rats. Four additional plates were overgrown with bacteria, and 2 were without mycoplasmal or bacterial growth.

No clinical signs or lesions of the respiratory tract and middle ear were present in the 2 control rats. Mycoplasmas were not isolated from the rats, but bacterial colonies grew from the tracheobronchial washings from 1 rat.

Experiment 2

Clinical signs, gross lesions, and microscopic lesions were essentially those described later. The onset of clinical signs was noticed 12 days after exposure to *M. pulmonis*. Gross lung lesions, observed in 17 of the 20 rats, were generally diffuse and affected all portions of the lungs. Microscopic lung lesions were present in lungs of 18 of the 20 rats exposed to *M. pulmonis*. Tracheitis, rhinitis, and otitis media were present in all of the rats.

Mycoplasmas were isolated from tracheobronchial washings of 8 of the 20 exposed rats. Overgrowth of bacteria was present from the washings of 9 rats and no microorganisms were isolated from washings of 3 rats. Mycoplasmal microorganisms were identified from middle ear cultures of 13 of the 20 exposed rats. Bacterial overgrowth

was observed in cultures from 4 rats. Results from the middle ear cultures from the remaining 4 rats were negative.

No clinical signs or respiratory tract and middle ear lesions were present in the 2 control rats killed prior to the beginning of the experiment. Mycoplasmas were not isolated from these rats. Bacterial growth occurred from the tracheobronchial washings, but not from the middle ear cultures.

Experiment 3

The clinical signs, gross lesions, and microscopic lesions were basically those described later. The onset of clinical signs was as described in Experiment 2. Except for the rat that died, all rats developed relatively mild clinical signs which persisted throughout the experiment. Gross and microscopic lesions were seen in the lung tissues from 4 of the 5 rats. The lung lesions were both focal and diffuse, and areas resembling abscesses were noted on the lung surface of 2 rats. Middle ear, tracheal, and nasal cavity lesions were observed in tissues from all 5 rats.

Mycoplasmal microorganisms were isolated from the tracheobronchial washings of 2 of the 4 rats cultured and from the middle ears of the same 2 rats. Bacterial growth was present on the media in all of the plates examined. The fifth rat in the experiment died during the weekend and microbiological work was not done.

Experiment 4

Lesions of the middle ear, nasal cavity, and trachea were present in the 12 rats exposed to only *M. pulmonis*. The lung changes, when present, were generally a marked lymphoid hyperplasia around large

bronchi. In 1 rat a small solid lesion was observed and the lung changes were more diffuse than in the other rats. Mycoplasmas were recovered from the tracheobronchial washings of 5 of the 12 rats. Bacteria overgrew the media in the remaining 2 plates. Mycoplasmal organisms were isolated from the middle ear of 1 of the 12 rats. Cultures from the remaining rats were overgrown with bacteria.

Tracheitis, rhinitis, and otitis media were observed in the 12 rats exposed to *M. pulmonis* plus ammonia fumes. The tracheitis and rhinitis were more severe than noted in those rats exposed to *M. pulmonis* only. The lung changes, which were observed in 3 of the 12 rats, resembled those in the rats exposed to only *M. pulmonis*. Mycoplasmas were recovered from the tracheobronchial washings of 4 of the 12 rats. Bacterial overgrowth was noted from the washings in an additional 6 rats, and tracheobronchial cultures from the remaining 2 rats were negative. Mycoplasmas were isolated from the middle ear of 1 of the 12 rats. Six middle ear cultures were overgrown with bacteria, and no growth was noted on the media in the remaining 5 plates.

The 12 rats exposed to ammonia fumes alone developed a tracheitis, an otitis media, and a severe rhinitis. A slight lymphocytic infiltration of questionable significance was noticed around the lung bronchi in 4 of the 12 rats. Mycoplasmas were not isolated from tracheobronchial or middle ear washings from these rats. Bacterial overgrowth was noted on the media in several of the plates.

No clinical signs, respiratory tract lesions, or middle ear lesions were noted in the 2 rats killed prior to the beginning of the experiment. Mycoplasmas were not isolated from the respiratory

tract or middle ear of these rats. Bacterial overgrowth was observed on the media in 1 plate cultured from the middle ear.

Experiment 5

The 4 rats given an intranasal inoculation of *M. pulmonis* developed dyspnea and became depressed in 9 to 24 days. Diffuse lung lesions, tracheitis, rhinitis, and otitis media were observed in the 4 exposed rats. The changes are described later. Mycoplasmas were recovered from the tracheobronchial washings from all exposed germfree rats and from the middle ear cavities of 2 of the 4 rats. No growth of bacteria was present.

The 2 rats killed before the experiment were free of identifiable microorganisms. No lesions suggestive of CRD were seen in the lung tissue, bronchi, trachea, nasal cavity, or middle ear of either rat.

Colony Controls

All of the rats appeared to be healthy at the end of the experiments, except that 2 of the 8 rats were smaller than the others. Gross lung lesions were present in 3 of the 8 rats, and microscopic lesions were present in 4 of the 8 rats. Tracheitis was observed in 6 of the 8 rats, but the inflammation was mild. A mild rhinitis was present in 6 of the 8 rats. Middle ear involvement was present in 4 of the 8 rats. The lesions were of the nature of those described later.

Mycoplasmal microorganisms were isolated from the tracheobronchial washings of 3 of the 8 rats. Bacterial overgrowth was present on the media plates from 4 additional rats. One plate was negative

microbial growth. Mycoplasmas were isolated from the middle ear of 2 rats. The plates from the middle ears of 6 rats were overgrown with bacteria.

Composite Description of CRD

Clinical Signs

Clinical signs were not readily apparent in many of the exgerm-free or conventional rats exposed to *M. pulmonis*. It was generally not possible to predict the severity of respiratory lesions based on clinical signs. The clinical signs tended to be variable among rats even within individual cages.

The first clinical signs appeared within 3 weeks after exposure. An infrequent sniffling and rubbing the nose and face with the front paws were noted. This sneezing was accompanied by a general depression, fast respirations, and the appearance of a dirty hair coat. Many of the 45-day-old rats (Experiment 4) exposed to only *M. pulmonis* developed these signs and later appeared to recover. The more pronounced clinical signs, when present, were a stunted appearance, labored breathing, reduced food consumption, and arching of the back (Figure 1). Terminal signs were gasping with the mouth held open and the neck stretched forward and a tendency to huddle in the corners of the cages. These clinical signs were observed in 3 of the germfree rats (Experiment 5) and 2 other rats that died (Experiments 1 and 2). All rats that were exposed to ammonia fumes almost immediately developed excessive tearing, sniffling, and gasping. These signs generally subsided within 2 to 3 days.

Figure 1. Clinical signs of CRD in a 55-day-old rat reared germfree, 25 days after exposure to a broth culture of *M. pulmonis*. Although rough hair coat, emaciation, and dyspnea were evident in this rat, only an arched back and rubbing of the nose and face with the front paws are clearly recognizable in the photograph.

Figure 2. Dorsal view of lungs in a 9-month-old, conventionally reared rat, 6 months after aerosol exposure to *M. pulmonis*. Note the demarcation between the solidified portions (arrows) and the normal portions of the lungs. Mucopurulent exudate was draining from the trachea but is not discernible in the photograph.

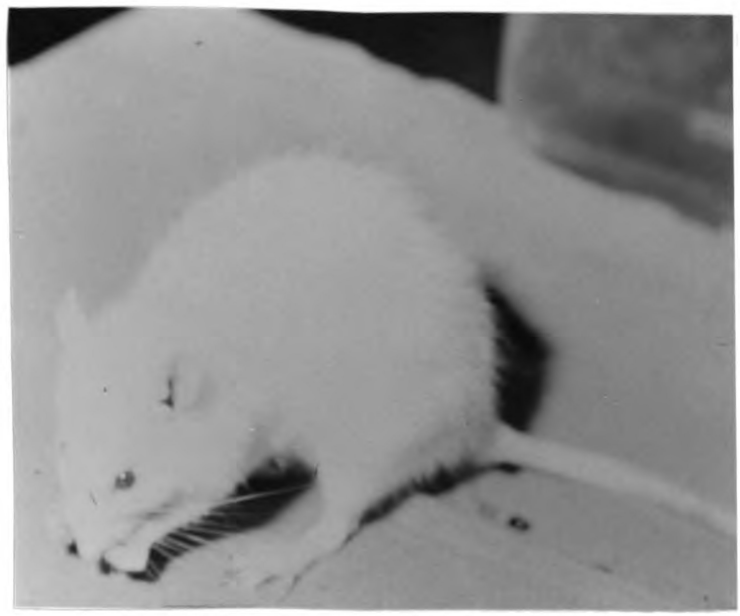

Figure 1

Figure 2

Gross Lesions

The major gross changes were in the lungs (Figure 2) and the trachea. The affected lung tissue was usually sunken, firm, and solid. The solidified (consolidated) areas were dark red initially, later becoming mottled with reddish, grayish, and yellowish areas. The lung solidification was accompanied by the production of a yellowish slimy exudate and dilatation of the bronchi. Focal lesions resembling abscesses were present in some of the lungs. The lung lesions involved 5 to 60% of the total lung mass and affected portions of lung lobes or entire lobes. More than 1 lung lobe was usually involved.

Mucopurulent exudate was frequently observed in the tracheal lumen. The amount was slight to excessive. The tympanic membrane was distended in an occasional rat, due to the accumulation of purulent material in the middle ear.

Microscopic Lesions

Histopathologic changes were observed in the lung tissue, the bronchial tubes, the trachea, the nasal cavity, and the middle ear. Significant bronchial changes were dilatation of the bronchi (bronchiectasis) and proliferation (hyperplasia) of the bronchial epithelium. Other alterations were an epithelial invasion of neutrophils, coagulation necrosis of the bronchial epithelium, and an accumulation of mucus and pus with many neutrophils in the bronchial lumen. This exudate occluded the lumen in many sections.

Changes around the bronchi and bronchioles were evident. Peribronchial and peribronchiolar lymphocytic infiltration of varying degrees

Figure 3. Bronchiole and surrounding tissue of a 3-month-old, exgermfree rat. Note the lymphoid tissue in the wall of the bronchiole (arrow), the lumen of the bronchiole (L), and a blood vessel filled with erythrocytes (V). The breaking off of the alveolar walls was likely from infiltrating the lung with media and/or 10% formalin solution. H & E stain; x 312.

Figure 4. Subacute bronchiolitis showing peribronchiolar lymphocytic infiltration in a 4-month-old, conventionally reared rat, 29 days after exposure to *M. pulmonis*. Note the lumen (L), thickened, hyperplastic bronchiolar wall (W), partial collapse of adjacent alveoli (A), and collection of lymphoid cells (C). Atelectasis was also observed surrounding the bronchiole. H & E stain; x 125.

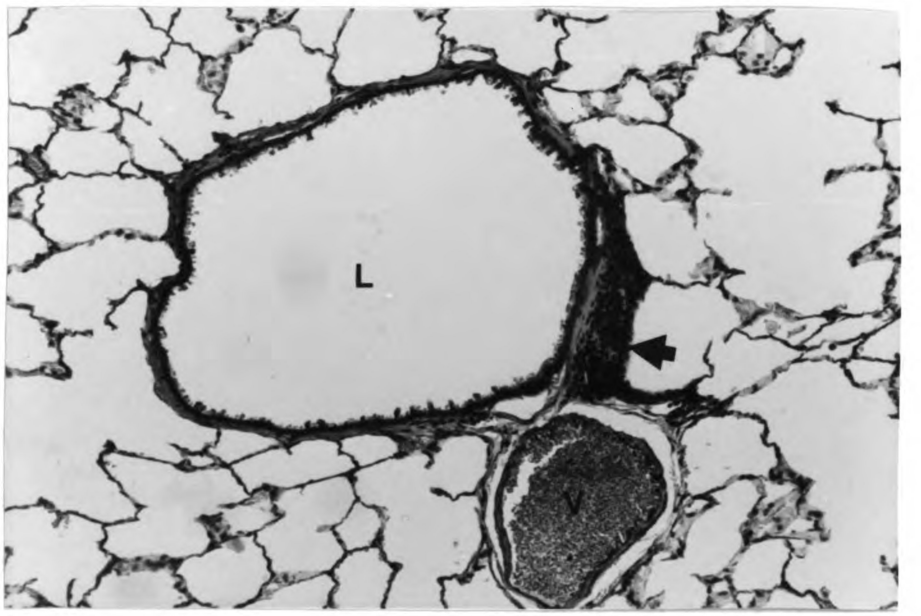

Figure 3

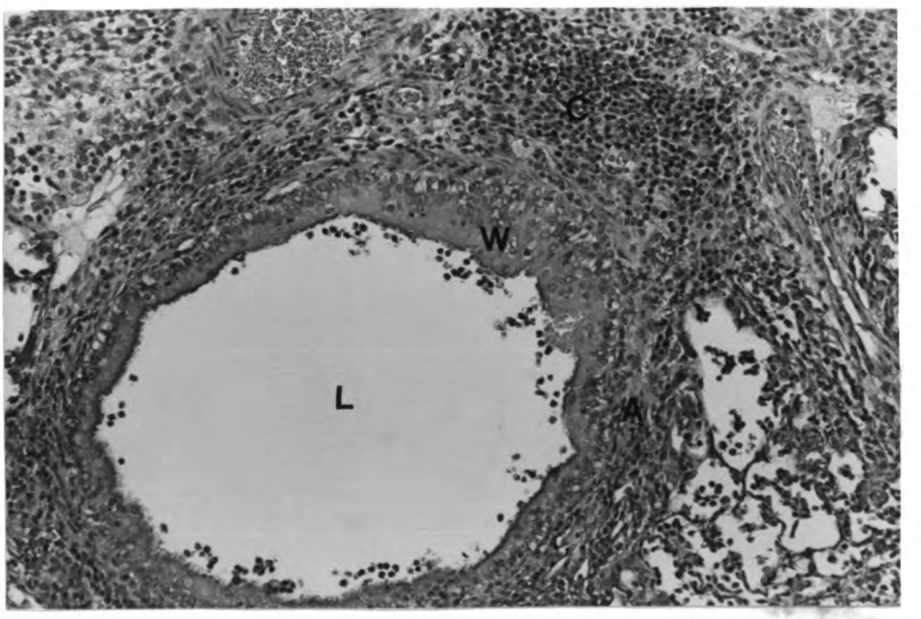

Figure 4

was noted. Similar changes were observed around blood vessels. In rats with a chronic infection, connective tissue proliferation was noted around the bronchi and bronchioles and in collapsed and solidified (consolidated) areas of the lung. The degree of pulmonary involvement was variable, and portions of lung free from noticeable microscopic changes were always present. The overall lung changes were those of bronchopneumonia (Figures 3, 4 and 5).

Subacute tracheitis (Figures 6 and 7) was a consistent finding. The tracheitis was characterized by a hyperplastic increase in the number of tracheal epithelial cells. In a few of the rats (especially those exposed to ammonia fumes) necrosis of the epithelial surface of the trachea was present. Cyst-like invaginations of the mucous membranes were observed. In areas of epithelial hyperplasia, the mucus-forming glands of the trachea were hyperplastic. The tracheitis was more severe in the rats exposed to ammonia fumes alone and ammonia fumes plus *M. pulmonis* than in those rats exposed to *M. pulmonis* alone.

Subacute rhinitis (Figures 8, 9 and 10) with exudation into the nasal passage, infiltration of lymphocytes below the epithelium, and an increased number of goblet cells were the major changes observed in the nasal cavity. Necrosis of the nasal epithelial lining was seen in a few instances. The mucus-forming glands of the submucosa were hyperactive.

Otitis media (Figures 11, 12 and 13) with an accumulation of purulent exudate within the tympanic cavity was present. Connective tissue proliferation and formation of cystic spaces were commonly

Figure 5. Bronchiolitis showing a respiratory bronchiole, dilated and filled with exudate, 40 days after exposure of an exgermfree rat to *M. pulmonis*. Note the purulent exudate with many neutrophils (E), the somewhat thickened epithelium (T), and accumulation of lymphocytes, macrophages, and neutrophils around the bronchiole (B). H & E stain; x 312.

Figure 6. Tracheitis in a 95-day-old, conventionally reared rat exposed to *M. pulmonis* and ammonia fumes at 45 days of age. Note the loss of epithelium with a deep invagination (arrow), the mucinous degeneration of the glands of the lamina propria (D), the infiltration of lymphocytes and plasma cells into the lamina propria (L), and submucosa (S). H & E stain; x 125.

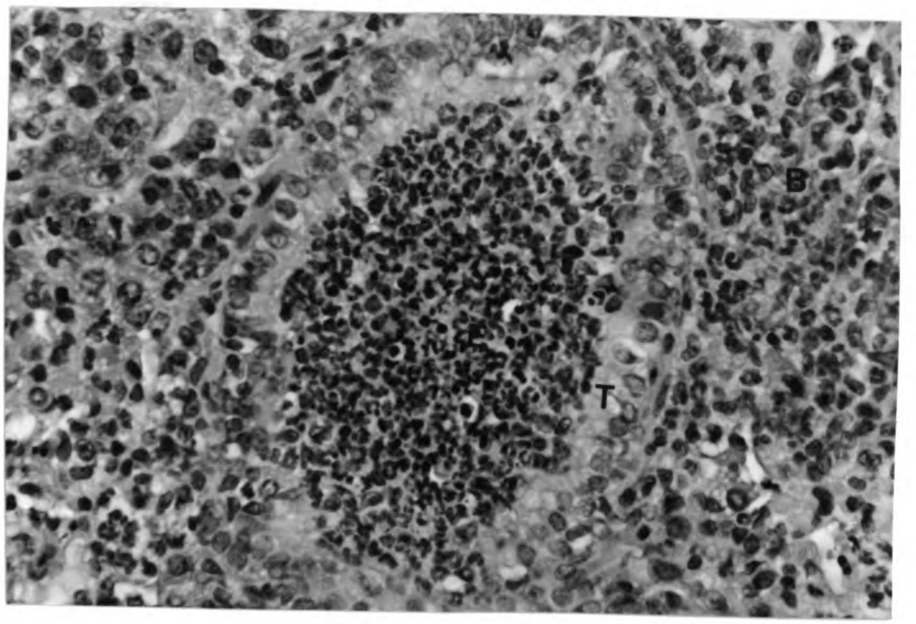

Figure 5

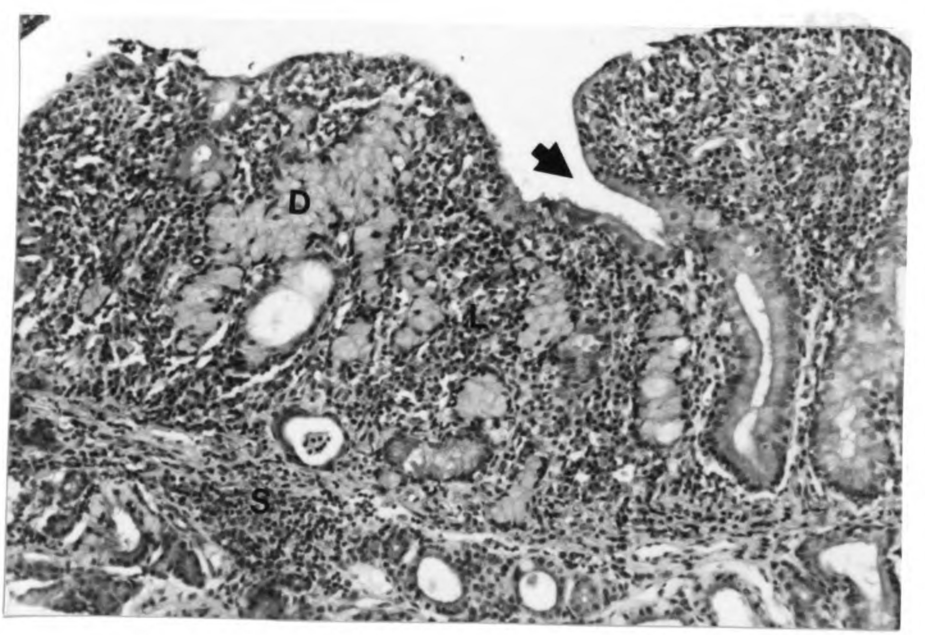

Figure 6

Figure 7. Higher magnification of Figure 6. Note the glandular degeneration and cellular infiltration into the lamina propria. H & E stain; x 312.

Figure 8. Purulent rhinitis in an exgermfree rat that was exposed to *M. pulmonis* at 3 months of age and died 42 days later. Note the purulent exudate with neutrophils in the lumen (E), the epithelium with an increased number of goblet cells (G), and the subepithelial infiltration of inflammatory cells (I). H & E stain; x 125.

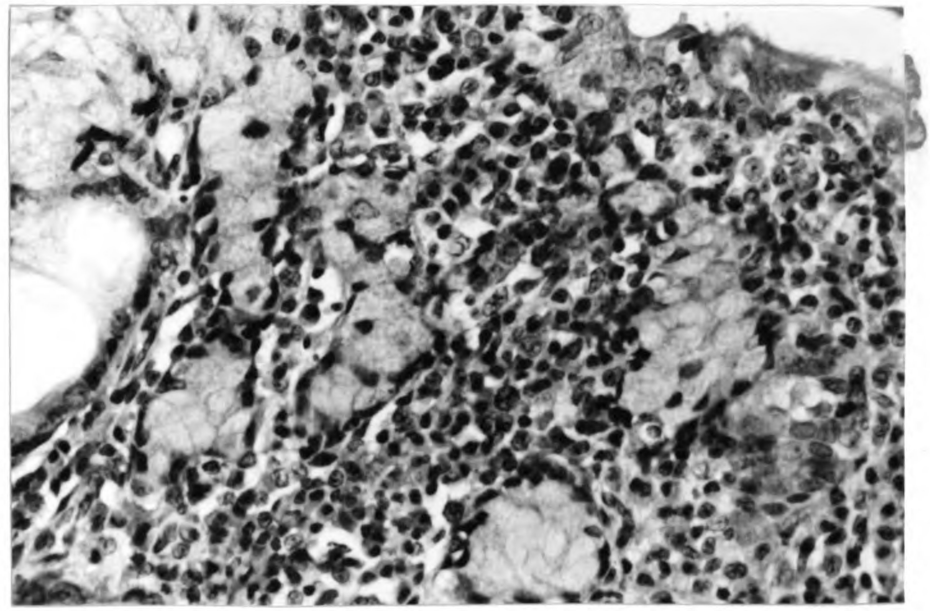

Figure 7

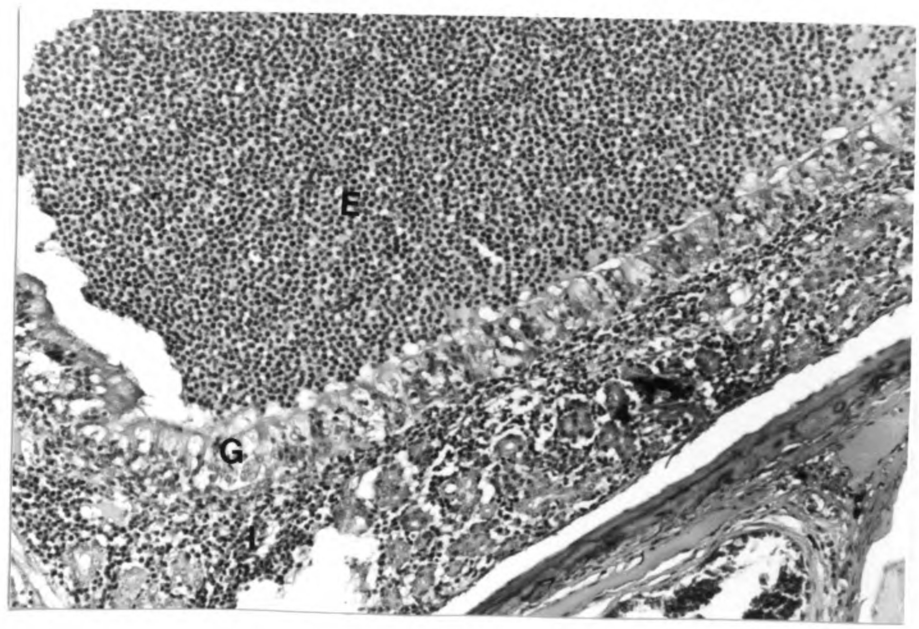

Figure 8

Figure 9. Purulent rhinitis in a 102-day-old, conventionally reared rat that was exposed to *M. pulmonis* and ammonia fumes at 45 days of age. Note the purulent exudate in the nasal cavity (N), the epithelium (E), and the accumulation of inflammatory cells in the lamina propria (C). The space between the cartilaginous median septum (S) and the lamina propria is an artifact. H & E stain; x 125.

Figure 10. Higher magnification of Figure 9. Note the neutrophils which have infiltrated into the epithelium. H & E stain; x 312.

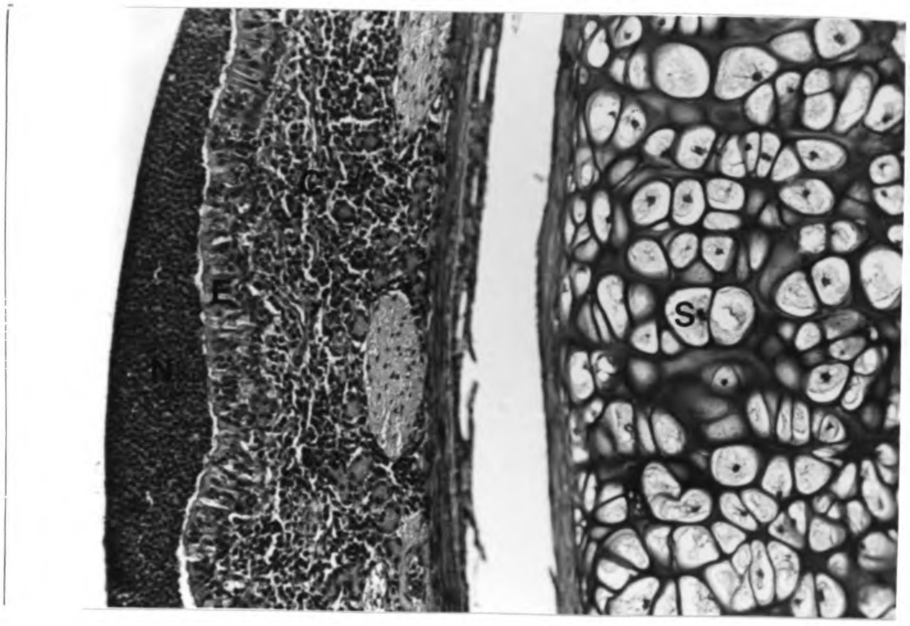

Figure 9

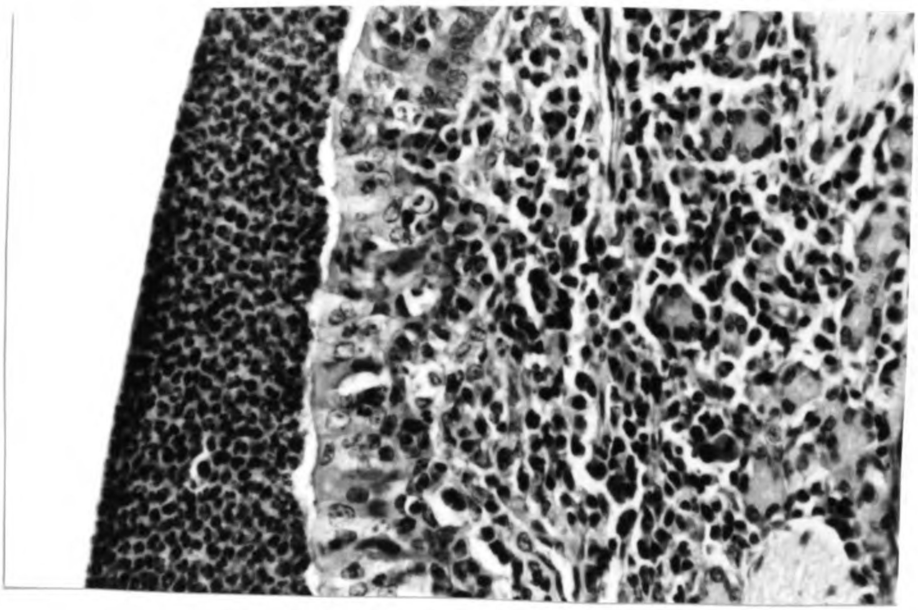

Figure 10

Figure 11. Otitis media in a 4-month-old rat that was reared germfree and exposed to *M. pulmonis* at 3 months of age. Note the large space filled with tympanic exudate (P), the smaller space filled with inflammatory cells and exudate (C), the tissue proliferation around the abscessation (T), and the osseous tympanic wall (O). H & E stain; x 50.

Figure 12. Higher magnification of the tympanic cavity of the same ear as in Figure 11. Note the neutrophils (N) and cystic space (S). H & E stain; x 125.

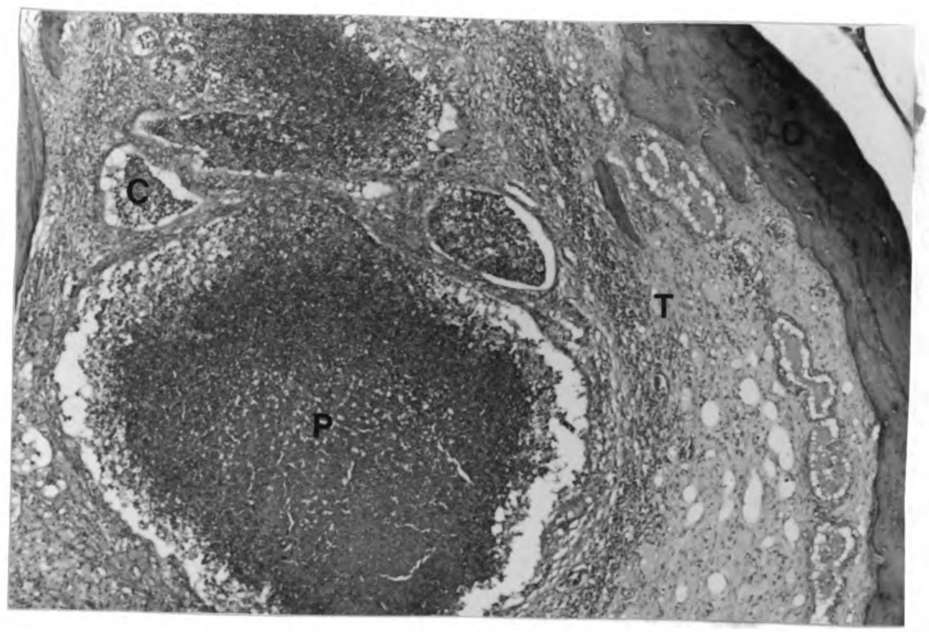

Figure 11

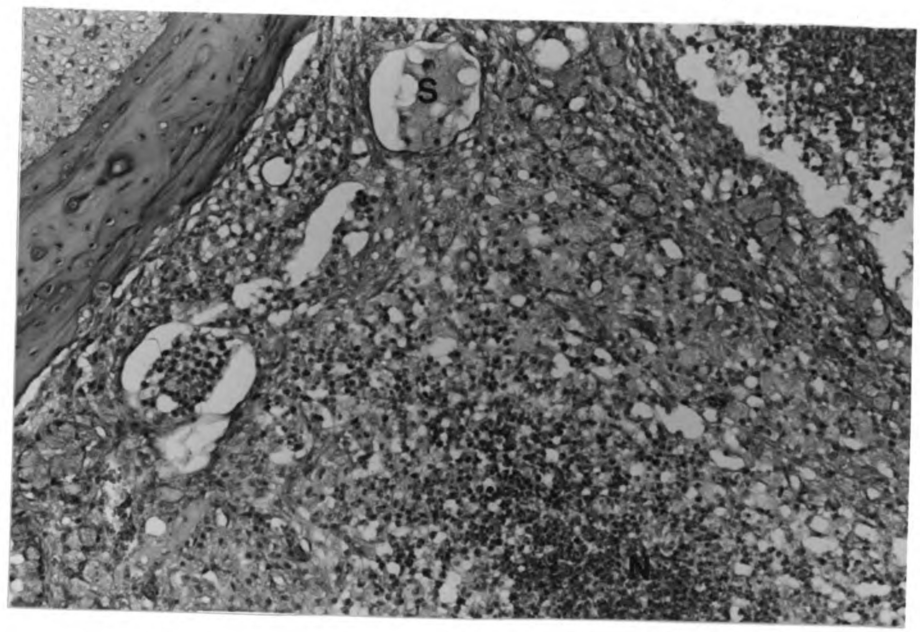

Figure 12

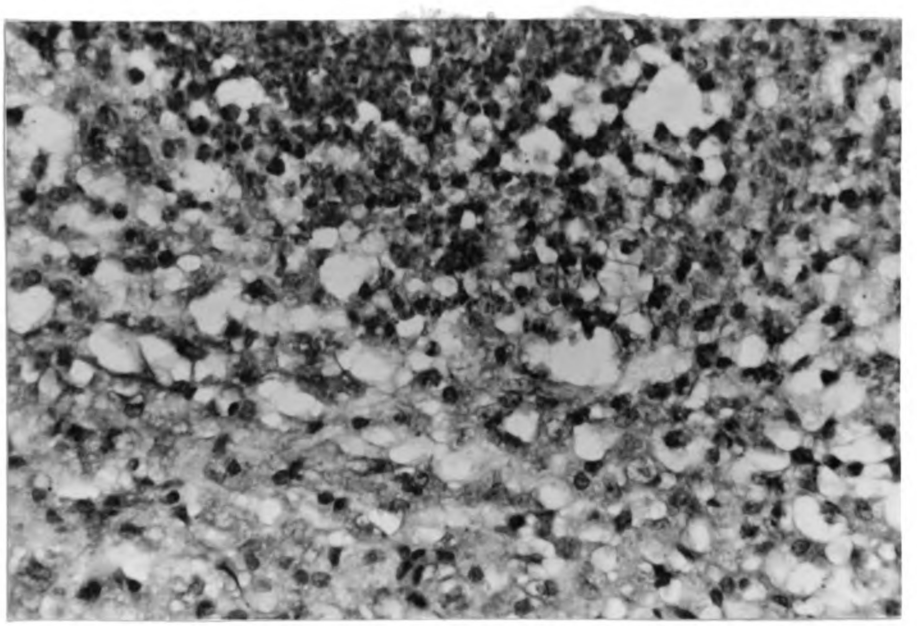

Figure 13. Higher magnification of tympanic cavity of same ear as Figures 11 and 12. Note the inflammatory cells and vacuolated areas. H & E stain; x 312.

observed. Alternate areas of connective tissue with cystic spaces and purulent exudate were present in some of the rats examined. Inner ear involvement was not observed.

Microbiology

Isolation of mycoplasmal microorganisms by sites and frequencies was discussed with each experiment. Colonies of *M. pulmonis* grown on agar medium were detected as early as the day after the plates were inoculated. The optimum time to examine the plates was 72 hours after inoculation. Although the plates were kept for a minimum of 1 week, little additional growth was evident after 72 hours.

For most of the rats, bacterial cultures were not done in parallel with the cultures for mycoplasmal microorganisms. As emphasized in the discussion of each experiment, considerable overgrowth of bacteria on the mycoplasma agar medium was present. This growth was often observable within 12 hours after inoculation.

DISCUSSION

The clinical nature and lesions of CRD were essentially as described by previous researchers (Giddens et al., 1971a,b; Jersey et al., 1973). The lesions of the lungs have been given primary consideration in much of the previous research on CRD. In fact, Innes et al. (1956) and Newberne et al. (1961), in an examination of several hundred rats, failed to mention changes in the middle ear and upper respiratory tract. Rhinitis, tracheitis, and otitis media were more prevalent than were lung lesions in the exgermfree and conventional rats in my experiments. Therefore, I believe that investigators should give more attention to upper respiratory and middle ear lesions in research on CRD.

Considerable difficulty was encountered in identifying *M. pulmonis* from tissues of the exgermfree and conventional rats because of bacterial overgrowth on the mycoplasma media plates. Rather than reliance on the inhibitors in the mycoplasma media, one might consider the possibilities of immunofluorescence and electron microscopy to identify *M. pulmonis* from the respiratory systems and middle ears of exgermfree and conventional rats. *Mycoplasma pulmonis*, on the other hand, was readily reisolated and identified from tracheobronchial washings from the germfree rats.

The germfree rats were most susceptible to CRD infection. They developed a marked respiratory distress, and lesions of the

respiratory tract were extensive. An explanation for the increased susceptibility would be the lack of contact of the germfree rats with agents to stimulate their body defense mechanisms. The pathologic changes in the exgermfree and older conventional rats were similar but developed more slowly than did the lesions in the germfree rats. No appreciable differences in susceptibility to infection or in the course of the disease were noticed when comparing the exgermfree and conventional rats of similar ages. This finding contradicted that of Kasali (1974), in which the exgermfree rats were more susceptible to *M. pulmonis* infection than were conventional rats. The younger conventional rats (Experiment 4) did not readily develop pulmonary lesions but did develop upper respiratory and middle ear lesions. Ammonia fumes did not enhance the susceptibility to *M. pulmonis* lung infection, but they did cause a more severe rhinitis and tracheitis.

The ammonia fumes alone produced lesions in the trachea and nasal cavity closely simulating those of *M. pulmonis* in germfree rats. The rats also unexpectedly developed otitis media. One explanation might be that the ammonia fumes made the rats more susceptible to an incidental bacterial infection. The accumulation of ammonia in animal cases from urine and the effects of this chemical on the respiratory system need to be considered in designing *M. pulmonis* experiments and also inhalant toxicity studies utilizing rats. Husbandry aspects should not be overlooked when designing respiratory experiments using rats. It is the experience of the author that many researchers do not give husbandry due consideration when planning their experiments.

The isolation of *M. pulmonis* and the presence of CRD lesions in the colony controls was not expected. The rats were maintained in a separate room for 9 months and did not have direct contact with other diseased rats. No clinical signs were noted during the 9 months. The infection could have originated from infected rats in an adjacent room, contamination in husbandry, wild rodent carriers, or the guinea pigs housed in the same room.

Judging from the increased susceptibility of the germfree rats, the development of more uniform clinical signs and lesions,and the lack of exogenous contamination, the germfree rat is a suitable model for studying *M. pulmonis* infection. Germfree rats may also be a useful model to conduct research on the role of mycoplasmal infections in a variety of other species, including man, and thus be of value in comparative medicine.

SUMMARY

Research was conducted to reproduce *Mycoplasma pulmonis* infection in rats reared conventionally, exgermfree, and germfree. Ninety-six rats were used in 5 sequential experiments.

Chronic respiratory disease was reproduced in rats exposed to broth cultures of *M. pulmonis*. The clinical signs and lesions were characteristic of the natural disease.

Clinical signs were observed in the rats by 3 weeks after exposure to *M. pulmonis*. The most severe clinical signs were seen in the germfree rats. These rats developed dyspnea and became depressed. The young conventionally reared rats, exposed to *M. pulmonis* at 45 days of age, developed only mild clinical signs. The mild signs were often followed by apparent recovery.

Tracheitis, rhinitis, and otitis media were present in rats exposed to *M. pulmonis* regardless of age and method of rearing. The tracheitis and rhinitis were most severe in rats exposed at 45 days of age to both *M. pulmonis* and ammonia fumes. Also, upper respiratory lesions and purulent exudate in the middle ear were observed in the conventional rats exposed to only ammonia fumes.

Bronchopneumonia with pulmonay solidification (consolidation) was a consistent finding in conventionally reared, exgermfree, and germfree rats exposed to *M. pulmonis* at 3 months of age or older. Conventional raised rats exposed to *M. pulmonis* alone and to *M.*

pulmonis and ammonia fumes at 45 days of age developed fewer lung lesions. Upper respiratory and middle ear changes but no significant lung lesions were observed following exposure of 45-day-old conventional rats to ammonia fumes only.

Mycoplasma pulmonis microorganisms were isolated from previously exposed rats. The reisolation procedures were complicated by the presence of contaminating microorganisms in the conventional and exgermfree rats.

Chronic respiratory disease in the rats was primarily due to *M. pulmonis*. Ammonia fumes and contaminating microorganisms may contribute to the disease as it occurs naturally in rat colonies used in research.

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