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Effects Of Host Age, Nonspecific Lymphocyte Responses, And Organism Adherence On Pathogenesis Of Murine Respiratory Mycoplasmosis.

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EFFECTS OF HOST AGE, NONSPECIFIC LYMPHOCYTE RESPONSES, AND ORGANISM ADHERENCE ON PATHOGENESIS OF MURINE RESPIRATORY MYCOPLASMOSIS

by

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A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title Effects of Host Age, Nonspecific Lymphocyte Responses, and Organism Adherence on Pathogenesis of Murine Respiratory Mycoplasmosis

The pathogenesis of murine respiratory mycoplasmosis (MRM), caused by Mycoplasma pulmonis, is poorly understood because complex interactions of host, organism, and environmental factors contribute to disease severity. Several of these factors, including exposure to environmental ammonia, ingestion of hexamethylphosphoramide, concurrent viral respiratory infections, deficiencies of vitamins A and E, and host genotype have been studied sufficiently to determine that they do exacerbate MRM severity.

These studies were undertaken in an attempt to determine whether or not age of the host at time of infection influences MRM disease severity, degree of nonspecific lymphocytic responses, or adherence of M. pulmonis to respiratory epithelium. The effect of host age at time of infection on disease severity was determined by inoculating M. pulmonis intranasally into F344 rats at either 5, 8, 12, 20, or 40 weeks of age, exposing the rats to constant concentration of ammonia, and, after 28 days, killing them, doing quantitative cultures on nasal passages, tracheas, and lungs, and semi-quantitative histologic analysis of changes in these organs and middle ears. To detect possible effects of increasing host age on nonspecific lymphocyte responses, mitogenic responses to M. pulmonis membranes of splenic lymphocytes from F344 rats 5, 8, 12, 16, or 20 weeks old were measured in a 48-hour microculture assay. The effect of host age

on adherence of M. pulmonis was evaluated using tracheal organ cultures from F344 rats 8 and 40 weeks old, in the presence of an agent known to enhance MRM severity, either ammonia or concurrent Sendai virus infection.

Increasing host age at time of infection correlated directly with increasing MRM severity. Rats 20 weeks and older developed more severe pulmonary disease than younger rats, and older rats had more mycoplasma organisms per gram of lung tissue than younger rats. These results were statistically significant at $p = 0.05$. However, mitogenic response of splenic lymphocytes to M. pulmonis membranes and adherence of M. pulmonis to respiratory epithelium in tracheal organ cultures were not affected by host age. Neither exposure of organ cultures to ammonia nor presence of concurrent Sendai virus infection increased the number of M. pulmonis organisms adherent to respiratory epithelium as determined by quantitative culture. Although there are limitations in sensitivity of the methods used, these findings suggest that while MRM severity clearly increases with increasing host age, neither nonspecific lymphocyte responses nor organism adherence significantly increases disease expression.

Abstract Approved by: Committee Chairman J. Russell Lindsey

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LIST OF ABBREVIATIONS

ANOVA . . . . . analysis of variance

CFU . . . . . colony-forming units

CPM . . . . . counts per minute

CVA . . . . . co-factors, vitamins, and amino acids

DPM . . . . . disintegrations per minute

ELISA . . . . . enzyme-linked immunosorbent assay

F1 . . . . . first filial generation

F2 . . . . . second filial generation

FCRF . . . . . Frederick Cancer Research Facility

F344 . . . . . Fischer 344 rat

HEPES . . [4-(2-hydroxyethyl)-1piperazinethanesulfonic acid]

HMPA . . . . . hexamethylphosphoramide

^{3}H-Tdr . . . . . tritiated thymidine

IgA . . . . . immunoglobulin A

IgG . . . . . immunoglobulin G

LEW . . . . . Lewis rat

µCi . . . . . microcurie

µM . . . . . micromolar

mCi . . . . . millicurie

mM . . . . . millimolar

M . . . . . molar

MPM . . . . . Mycoplasma pulmonis mitogen

LIST OF ABBREVIATIONS (continued)

MRM . . . . . murine respiratory mycoplasmosis

NK . . . . . natural killer

PBS . . . . . phosphate-buffered saline

PHA . . . . . phytohemagglutinin

PPLO . . . . . pleuropneumonia-like organism

SDAV . . . . . sialodacryoadenitis virus

USP . . . . . United States Pharmacopeia

LITERATURE REVIEW

Introduction

Mycoplasmas have been recognized as pathogens in the respiratory tract of a wide variety of animals, including humans (87). Mycoplasma pulmonis is the cause of murine respiratory mycoplasmosis (MRM), a major disease of laboratory rats and mice. This infection is important of itself because while it is usually clinically "silent," it is widespread and results in morbidity and shortened life span (41, 130). In addition, it subtly to severely alters respiratory histology, physiology, and immunology (12, 41, 130, 131) and is thus capable of seriously confounding the results of biomedical research using rats in widely divergent fields including respiratory disease, gerontology, nutrition, toxicology, carcinogenesis, and behavior (41, 130, 131).

Etiology

The first descriptions of MRM were reported in the early 1900s, although the syndrome was not called MRM at that time (89). The cause of MRM in rats was for many years a controversial subject (15). There were numerous conflicting reports concerning the agent or agents involved in respiratory disease in rats, and the syndrome was given a wide variety of names, one of the commonest being "chronic respiratory disease." The controversy centered on the difficulty

encountered in reproducing the entire syndrome with one or a combination of agents (12, 110, 122, 149, 151, 163). These difficulties were due in part to the variability of disease expression in rats, to obstacles encountered in obtaining and maintaining animals free of naturally occurring MRM for use in experimental infections (101, 152), and to the fastidiousness of M. pulmonis which hampered its isolation and propagation (54, 141, 211). By the late 1930s and 1940s, M. pulmonis was believed to be the cause of the upper respiratory tract disease and was designated "infectious catarrh" by Nelson (153, 154, 156). He believed that the frequently concurrent lower respiratory tract disease was caused by a filterable agent, probably a virus. He called the lower respiratory tract disease "enzootic viral bronchiectasis," even though the filtration test results could as easily have defined a mycoplasma, and this was known at that time (150, 154-157). By the middle of the 1960s, "chronic respiratory disease" of rats was firmly believed to have a complex etiology involving a variety of agents (15, 82, 83, 90, 100, 157).

In the late 1960s and early 1970s, the entire spectrum of "chronic respiratory disease" in rats was determined to be caused by M. pulmonis, Koch's postulates having been fulfilled (104, 123, 130, 228). Confidence in this work increased with the development and use of gnotobiotic and pathogen-free rats. The preferred term for the syndrome now is "murine respiratory mycoplasmosis."

Taxonomy

Like other mycoplasmas, M. pulmonis is a member of the Class Mollicutes. These organisms differ from most other bacteria in a variety of ways. They are the smallest and simplest free-living microorganisms known, 0.6-0.8 μm in diameter (158, 208). They contain only minimal organelles for growth and reproduction, their genome is smaller than that of other bacteria, and they lack a cell wall (179-182). They contain both DNA and RNA and thus differ from viruses; they are free-living and thus differ from rickettsia and chlamydia (71). Because of their minimal biosynthetic capability, they require a source of purine and pyrimidine bases (181). Most also require cholesterol and other long-chain fatty acids (181). The parasitic members of the Class must also be provided with sugars or arginine as an energy source, depending on whether they are fermenters or non-fermenters, respectively (179, 181).

Epizootiology and Pathology

It is believed that natural infection with M. pulmonis is acquired most frequently early in life from infected dams (22, 25-27, 131). The nasal passages, nasopharyngeal duct, middle ear, larynx, and trachea may be colonized naturally by 3 weeks of age (M. K. Davidson, M. S. thesis, University of Alabama at Birmingham, 1981). Horizontal cage-to-cage transmission is slow; adult rats with clinical signs transfer the organism more readily than rats with subclinical infections (90). The importance of fomites in transmission is not clear;

conflicting reports have appeared in the literature (111, 222). Wild rats are a natural reservoir of the disease (91).

MRM is noted for lack of uniformity of morphologic changes in rats of the same age and colony and even rats from the same cage (104). It is known that besides producing disease inconsistently, recognized mycoplasmal pathogens can remain in immunocompetent hosts for long periods of time as commensals (23). Manifestations of MRM are strongly influenced by a variety of environmental, host, and organism factors (17, 23, 25, 26, 104, 130, 131, 197). As with other mycoplasmal diseases, the interaction of these factors contributes to the difficulty in understanding the pathogenesis of MRM.

MRM in rats can affect any part of the respiratory tract, but it most commonly affects the nasal passages and middle ears (26). The changes most often seen are rhinitis, otitis media, laryngotracheitis, and a slowly progressive pneumonia (25, 26). Characteristic microscopic changes occurring throughout the respiratory tract are accumulation of neutrophils in airways, epithelial hyperplasia, and submucosal lymphoid cell infiltration. Perivascular, peribronchial, and peribronchiolar accumulation of lymphoid cell is the most characteristic feature of the pneumonia. Other changes include increased mucus production, goblet cell hyperplasia, squamous metaplasia of airwary epithelium, atelectasis and bronchiectasis with accumulation of inflammatory cells in airways and alveoli (130). Clinical signs are usually absent

until late stages of the disease when there may be weight loss, rough hair coat, dyspnea, nasal and ocular discharge, head tilt, or any combination of these signs (131).

Pathogenesis

Pathologic manifestations of MRM are affected by a variety of environmental, host, and organismal factors which probably interact among one another. Environmental factors including exposure to ammonia from soiled bedding (17, 25, 26, 131, 132, 197), deficiencies of vitamins A, E, or both (221), and ingestion of hexamethylphosphoramide (HMPA) (161) have been shown experimentally to enhance MRM expression. Host genotype is known to affect MRM expression in rats and mice (49, 51, 53, 164); other host factors suspected of doing so are host age at time of infection (25, 26, 50, 104, 127) and nonspecific lymphocyte responses (26, 35, 52, 53, 146, 147).

A microbial factor shown to affect disease expression is the synergism between M. pulmonis and concurrent infection with either Sendai virus or sialodacryoadenitis virus (25, 198, 199). Other proposed factors include adherence of M. pulmonis to respiratory epithelium (24, 213, 214) and induction by the organism of ciliostasis and loss of cilia (23, 37). The number of factors capable of affecting expression of the disease has made understanding the pathogenesis of MRM difficult. Further, the mechanisms by which these factors act are poorly understood.

Ammonia Exposure

Exposure to ammonia was the first naturally occurring factor to be shown to exacerbate MRM. A number of mechanisms have been suggested to explain this effect. These include reduction of ciliary activity which may decrease mycoplasmal clearance (44), release of growth-promoting nutrients, and loss of an inhibitory factor by ammonia-damaged respiratory epithelial cells (171). Ammonia also adversely affects a variety of immune responses. In guinea pigs, it has been reported to inhibit delayed type hypersensitivity in vivo and bactericidal activity of alveolar macrophages in vitro (209). In vitro, ammonia inhibits mitogenic responses of peripheral blood lymphocytes in guinea pigs and cattle and of bronchial lymphocytes in guinea pigs (209, 210), antibody-dependent cell-mediated cytotoxicity in human lymphocytes (58), macrophage receptor-mediated endocytosis (217), murine natural killer (NK) cell activity (192), and macrophage phagosome-lysosome fusion (86).

In studies done to define the mechanism(s) of action of ammonia, it was found that the concentration of ammonia capable of enhancing disease in vivo was in the range routinely found in cages of conventionally housed rats (17, 132). Ammonia increased the frequency of isolation of M. pulmonis from infected rats (17). Disease severity throughout the respiratory tract was greater in animals exposed to ammonia and in the lungs was roughly proportional to the concentration of this promoting agent (17). In vitro, the

number of organisms recoverable from tracheal organ cultures increased when M. pulmonis was grown in rat tracheas previously exposed to 50 mM ammonium chloride (NH_4Cl) (171). It is known that the nasal passages of rats can absorb up to 380 µg/ml (500 ppm) environmental ammonia (45, 197). This fact supports the hypothesis that in naturally occurring disease the increased numbers of organisms found in organs distal to the ammonia-exposed nasal passages are not directly attributable to the effect of ammonia on the organism, but rather that ammonia increases numbers of organisms indirectly by its effect on the host (197).

Hexamethylphosphoramide (HMPA) Ingestion

This chemical was once a widely used organic solvent in industry and research laboratories. In 1968 it was reported to cause MRM-like pulmonary changes as a toxic effect in rats (121). In 1976 it was demonstrated that HMPA exacerbated pneumonia resulting from pre-existing M. pulmonis infection but was not itself responsible for the lesions. In M. pulmonis-free rats this compound produced thinning and microulceration of respiratory epithelium in the nasal passages and lung without causing inflammation (161). Recently, it was postulated that the mechanism by which HMPA exacerbates MRM may be impairment of mucociliary clearance resulting from the destruction by HMPA of respiratory epithelium (125).

Host Genotype

The existence of MRM disease-resistant and disease-susceptible strains of mice and rats has been reported since the 1930s. Freundenberger reported that Long-Evans and Wistar rats housed in the same room had differences in susceptibility to MRM (69, 70). Broderson et al. (17) reported that when Sherman and F344 rats were housed under identical conditions, the F344 rats resisted disease better than the Sherman rats; however, differences in the backgrounds of the rat strains made interpretation of these observations uncertain. Saito et al. reported similar findings for pulmonary changes in outbred mice of the ICR and ddY strains (189). More recently, conclusive studies by Davis and Cassell have confirmed that F344 rats are more MRM disease- resistant than LEW rats (49). M. pulmonis-infected LEW rats developed more severe and more rapidly progressive upper and lower respiratory tract disease than F344 rats when environmental and microbial factors were held constant (49). There were major differences between these rat strains in responding pulmonary lymphocytes, and the differences in rate of progression and in severity of pulmonary changes directly correlated with changes in pulmonary lymphocyte populations in the two rat strains (53). In LEW rats all classes of lymphocytes proliferated throughout the 120-day study, but in F344 rats lymphocytic proliferation peaked at 28 days. In F344 rats only T lymphocytes, IgA-bearing B cells, and IgA- and IgG-producing plasma cells proliferated, and pulmonary changes were resolving by the end

of the study. Although LEW and F344 rats apparently have different genetic backgrounds, they share the same major histocompatibility antigens and many of the same lymphocyte surface antigens (63, 129). Nevertheless, F344 rats have much weaker immunologic responses to various antigens and mitogens than do LEW rats (42). Thus, it has been suggested that the differences in disease susceptibility of F344 and LEW rats are related to differences in host response to M. pulmonis and that intrinsic, possibly regulatory, differences between the two rat strains in nonspecific lymphocyte activation may be the source of the difference in host response (53).

In mice experimentally infected with M. pulmonis, gross and microscopic pulmonary changes in the C3H/HeN strain were significantly more severe than those occurring in C57BL/6N mice (51). Unlike the differences in severity reported to occur in all parts of the respiratory tract of F344 and LEW rats (49), significant differences in lesion severity between the mouse strains occurred only in the lungs (51). These differences in strain susceptibility were believed to be haplotype related with the H-$2K^b$ haplotype conferring resistance (51). In further studies, resistance of C57BL/6N mice to pulmonary changes correlated with increased mycoplasmal clearance from the lungs. Actual physical clearance of mycoplasma from the lungs did not differ significantly between the two strains; clearance was associated with mycoplasmacidal activity. This activity was not dependent upon neutrophils or specific antibody (164).

As with the rats, the differences between the mouse strains were thought to be the result of nonspecific host defense mechanisms (164).

Host Age

Host age at time of infection has been proposed to affect MRM expression, but the evidence has been based on incidental observations in studies of other aspects of M. pulmonis infection, including vaccination efficiency (50), antibody responses (127), and MRM disease induction (104). Conclusive evidence of increased susceptibility to disease in "young" versus "old" rats has not been reported. Neither is it known whether or not aging effects on the immune system alter response to M. pulmonis infection in the rat.

Interactions Between Viral and Bacterial Infections

Exacerbation of bacterial respiratory disease by concurrent respiratory viral infections is a well recognized phenomenon in human influenza epidemics in which there is an increased risk of pulmonary, as well as systemic, infection caused by such secondary bacterial invaders as Staphylococcus aureus, Hemophilus influenza, Escherichia coli, and Neisseria meningitidis. In fact, mortality in influenza epidemics usually is associated with secondary bacterial pneumonia (103, 143). In 1931 Shope demonstrated that swine influenza could be precipitated by intramuscular injections of Hemophilus influenzae suis in animals infected with swine influenza virus, but that alone neither organism could produce clinical disease (201). Concurrent infectious bovine rhinotracheitis

virus and Pasteurella hemolytica infections in calves results in longer duration of illness than that seen in calves given either agent alone (40).

Mechanisms proposed to explain this phenomenon include (a) virally induced cell changes, including virally induced formation of bacterial receptors on host cell surfaces (11, 60, 103, 143, 173); (b) virally induced suppression of specific immunity, especially cell-mediated responses; and (c) virally induced defects in bactericidal activity of alveolar macrophages and of neutrophils (102, 103, 136, 143). All of these mechanisms promote bacterial colonization (102, 103, 136, 143). Two or more of these mechanisms may act simultaneously; there is some evidence to suggest that they do in studies of influenza virus and cytomegalovirus infections (103, 143). Viral suppression of cell-mediated immunity has been noted in influenza virus infections of mice and guinea pigs, adenovirus and measles infections of human beings, and reovirus infection of mice (136). In humans most of the supporting data are epidemiologic.

Evidence to support virally mediated increases in bacterial adherence is well represented in the literature. Influenza A virus infection of cell monolayers resulted in significantly increased adherence of six strains of Staphylococcus aureus (191), and influenza A virus infection of human pharyngeal cells in vivo increased adherence of S. aureus, H. influenzae, and Streptococcus pneumoniae type 1 (60). In infant rats, influenza A virus, Sendai virus, and rat coronavirus increased

nasal titers of H. influenzae type b up to 10,000 fold during the first week after viral infection. Colonization by the bacterium was not only greater, but also prolonged after viral infection (142).

Similarly, synergism occurs between respiratory viral and mycoplasmal infections. Co-infection with porcine rhinovirus and Mycoplasma hyorhinis (an organism of low pathogenicity) enhanced the numbers of mycoplasmas detectable in porcine tracheal organ cultures (185). In bovine fetal tracheal cultures, prior infection with rhinovirus, influenza, or parainfluenza virus enhanced colonization and growth of M. hyorhinis. This mycoplasma ordinarily does not associate closely with normal epithelium, but easily colonizes virally damaged cell surfaces (185). Concurrent swine influenza virus and M. hyorhinis infections increased the amount of respiratory epithelial damage over that seen with either agent alone (184). Co-infection with avian influenza A virus strain meleagrium and Mycoplasma gallisepticum caused more severe disease in turkeys than that occurring with either agent alone (178). Likewise, co-infection with Sendai virus and M. pulmonis enhanced the growth of M. pulmonis in the lungs of mice and increased the severity of pulmonary changes in mice compared to those seen with either agent alone (93, 190). Concurrent infections with M. pulmonis and influenza A/PR-8 virus in mouse tracheal organ cultures caused more rapidly developing and more severe epithelial changes than did either organism alone (227). Finally, concurrent infection with

Sendai virus or sialodacryoadenitis virus (SDAV) increased the severity of MRM lesions in rats and increased the number of recoverable mycoplasmas in nasal passages, trachea, and lungs compared to rats inoculated with M. pulmonis alone (199).

Effects of Age on Susceptibility to Disease

Aging humans and animals of other species have an increased incidence of infectious diseases, as well as autoimmune and neoplastic conditions (77, 99, 117, 119, 137, 196, 224). During the past 50 years, it has been well documented that coincident with aging and increased incidence of these diseases there is a decline in the immune system of humans and many other species (6, 224). It also is generally accepted that T cell functions decline more dramatically than B cell functions (31, 59, 77, 118-120, 135, 138, 226). Although the decline in immune function is not the only theory proposed to explain the increase in disease with advancing age, it is probably the most widely accepted (7, 116, 119, 135, 224). An interesting alternative proposal is that decline in immune function is not a result of aging, but is rather the underlying cause of aging and of the changes associated with it (223, 225). In any case, both extrinsic, or microenvironmental, and intrinsic factors apparently contribute to immunologic deterioration (119, 138, 176, 177, 225) as determined by lymphocyte transfer studies. When lymphocytes from aged mice are transplanted into irradiated young mice providing the old cells with a "young" milieu, normal function is not restored, suggesting that intrinsic

cellular defects contribute to immunologic deterioration (177). When lymphocytes from young mice are transplanted into irradiated old mice subjecting the young cells to an "old" milieu, function is impaired, suggesting that extrinsic cellular defects also contribute to immunologic decline (177). Kay and Makinodan have estimated the responsibility for immune aging to be 10% extrinsic factors and 90% intrinsic changes (116, 119, 138).

Contradictory reports concerning the effects of aging on specific and nonspecific systemic immunity have accumulated in scientific literature because experimental results are affected by a large number of variables that have not always been identified and controlled, making comparison among studies difficult. Among the most important factors to consider when comparing studies on the effect of age on immunity are (a) the criteria used to define "old" and "young," especially with regard to comparison of animal strains having different life expectancies, (b) the species studied, (c) health status of the animals, (d) the immune functions studied and the comparability of the techniques used, and (e) the compartments of cells studied.

On the other hand, the effects of aging on mucosal immunity are controversial (187, 196, 206, 207). Part of the controversy appears to be that the changes noted may be species specific, tissue specific, or both. The effects of aging on the mucosal immune system have not been studied as thoroughly as effects on the systemic immunity, and most of

the work that has been done has concentrated on gut rather than pulmonary mucosal immunity.

Aging humans are especially susceptible to infections of the respiratory system (77, 144). Such diseases as tuberculosis, influenza, and bacterial pneumonia cause more deaths in people over 75 years old than in younger age groups (55, 77). Reasons proposed to explain this susceptibility include physiologic, physical, and environmental, as well as immune, phenomena (55, 77, 144).

Age-related susceptibility to infectious diseases in humans is well recognized and is thought by many to be related to immune dysfunction. It is known, for example, that elderly individuals are less able to form protective antibody after immunization against the viral antigens of Japanese B encephalitis (188) or influenza (96, 169). These data suggest that age-related immune system decline could contribute to the increased susceptibility to infectious disease seen in the elderly. However, most evidence for an association between age-related changes in immune function and disease susceptibility is epidemiologic, and a cause and effect relationship has not been established experimentally. A relationship between infectious diseases and immune dysfunction unassociated with aging has been noted in clinical studies of children born with immunodeficiencies (2, 32, 72), graft recipients given immunosuppressive therapy (13, 167), and individuals with acquired immune deficiency syndrome (2, 32, 139).

In animal studies, increased susceptibility to Salmonella typhimurium (57, 168), murine sarcoma virus (166), Trichinella spiralis (43), Toxoplasma gondii, and Listeria monocytogenes (57, 78, 165) in aging animals has been shown to correlate with decline in immunologic functions. For T. gondii and L. monocytogenes, the increase in susceptibility was detectable as early as 9 months of age (78). There was a greater incidence of mortality in "old" (17 to 22 months of age) mice inoculated either intramuscularly or intraperitoneally with S. aureus than in "young" (1.5 to 2 months of age) mice (134). Resistance to S. typhimurium in old mice was returned to that seen in young mice by giving the old mice spleen cells obtained from young mice before inoculation of the bacterium (78). A similar effect was noted in old mice given serum from young mice before inoculation with T. gondii (78). In another study it was noted that while amounts of specific antibody to T. gondii decreased, the number of tissue encysted organisms increased with age (79). Also, macrophages in old mice had delayed activation during acute infection as compared to young mice (80). Both decreased antibody response and delayed macrophage activation may contribute to increased susceptibility in old mice. Despite the fact that cell-mediated immunity declines more significantly in aging animals than humoral immunity, infectious diseases resisted by host mechanisms other than cell-mediated immunity have just as marked an increase in prevalence in aging populations (77).

It should be stressed that changes in immune functions are not limited to aged individuals. Immunity is also altered in the young, although the mechanisms are probably different. For example, natural killer cell (NK) activity in mice peaks between 6 and 10 weeks of age and is low or undetectable in infant and elderly mice. In infant mice there are fewer precursor cells in the spleen, indicating a delay in development of the NK population, while in older mice the low NK activity is related to regulatory factors rather than cell numbers (186). In rats it is known that for some immune functions, maximal competence occurs at the onset of puberty (about 4 weeks of age) and declines thereafter (14).

In studies of streptococcal adherence to oral mucosal epithelium in humans, it was found that group A streptococci bound poorly during the first 2 to 3 days of life, but group B streptococci type III, considered the most virulent strain in neonates, adhered significantly better to cells of 1- to 6-day-old infants than to adult cells (19). This adherence was significantly greater in those infants known to be infected with group B streptococci, suggesting an increase in host cell receptors in infected babies (19). These findings strongly suggest a correlation between adherence and age. However, in a rat model it was found that neonatal Wistar rats have deficient myeloid storage pools and a lag in myeloid response to group B streptococcal infection compared to older rats (230), suggesting that increased binding to cells is not the only virulence factor for this neonatal disease.

The incidence of clinical pneumonia caused by M. pneumoniae is greatest in late childhood and during adolescence with a peak incidence at 10 years of age; clinical disease is unusual, or at least undetected, before 4 years of age (66); however, subclinical infections are known to occur in young children (61).

Host age at time of infection has been suggested to modulate expression of MRM in rats, but there have been no conclusive studies of this apparent increase in susceptibility with increasing age. One intriguing explanation is the possibility that nonspecific immune responses contribute to disease expression in these mycoplasmal diseases (26, 52, 53, 146, 147). In general, diseases in which host immune responses contribute to expression of disease severity are less severe in the very young and very old, because immaturity and senescence of the immune system, respectively, prevent expression of disease as seen in fully immunocompetent individuals (144).

Mycoplasma-Induced Nonspecific Lymphocyte Responses

The lesions of MRM are not adequately explained by direct injury of host epithelial cells by M. pulmonis (23). In experimental studies of M. pneumoniae infection in hamsters, the lymphoid cell accumulation in the lungs was greater and occurred more quickly upon re-infection (33). Antithymocyte serum reduced the severity of the pneumonia (212). Clinically, four humans with B cell dysfunction and M. pneumoniae infection had disease symptoms more severe and of

longer duration than those commonly occurring in immunocompetent individuals, but they did not have radiographic evidence of pneumonia (67). As a result of such studies and clinical observations, the host immune response is believed to contribute indirectly to disease expression. One mechanism of indirect injury proposed to explain the lymphoid hyperplasia occurring in the respiratory tract, as well as cell injury and autoimmune phenomena, is mitogenic stimulation of lymphocytes (23, 26, 35). Many mycoplasmal species are capable of initiating lymphocyte blast transformation in vitro and, in some instances, in vivo (35). There is evidence that this nonspecific polyclonal lymphocyte response contributes to disease expression in MRM and in M. pneumoniae infections of humans (35, 52, 53, 87, 146, 147).

Proposed mechanisms whereby mitogenic activity could contribute to pathogenicity include (a) nonspecific activation which could result in the production of such regulatory cells as helper and suppressor T cells, capable of enhancing or suppressing specific responses; (b) release of inflammatory mediators by mitogen stimulated cells which could produce toxic effects on host cells; and (c) mediator-stimulated differentiation of the activated lymphocyte subpopulations which could produce autoimmune clones (35).

Naot et al. inoculated mitogenic membrane preparations of M. pulmonis intranasally into "specific pathogen-free" rats, which subsequently developed tracheitis and interstitial pneumonia with vascular and bronchial lymphocytic cuffs (146).

These inflammatory lesions were mitogen dose-dependent, and severity of disease in the two rat strains used directly correlated with the degree of nonspecific lymphocyte response to the mitogen (147). These studies were claimed to demonstrate *in vivo* a pathogenetic role for nonspecific lymphocyte responses in MRM, but important questions concerning these studies remain unanswered. These include (a) Were there control animals and were their lungs free of the described changes? (b) What does the production of interstitial pneumonia mean when this is not a feature of naturally occurring or experimentally induced MRM? (c) How does the instillation of large amounts of crude membrane preparation relate to this airway oriented disease?

In other more carefully controlled studies of experimentally induced disease in disease-resistant and disease-susceptible rat strains, Davis et al. found that the amount of specific anti-*M. pulmonis* IgG was not significantly different in the two strains, but there were many times more IgG-producing lymphocytes in the lungs of the disease-susceptible rats (53). These findings suggest that the differences in lesion severity are, at least in part, the result of nonspecific responses.

There is considerable variation in the capacity of various strains and species of mycoplasmas to induce nonspecific lymphocyte responses among various species and inbred strains of animals (9, 35, 62, 148). Some mycoplasmas can induce these nonspecific responses in species of animals for which

the organism is not a known pathogen (9, 35, 87, 148), and there are strains of M. pulmonis that are mitogenic for mice but are not pathogenic (48, 75). This is also true in rats (M. K. Davidson, personal communication). These findings indicate that mitogenicity may contribute to, but is not the sole determinant of, pathogenicity in MRM.

The age of animals used in mitogenicity assays can affect the results of the studies. For example, it is well documented that the response of splenic lymphocytes to phytohemagglutinin (PHA), a T cell mitogen, decreases with aging in a variety of species including some mouse strains (84, 92, 107, 140, 162), beagle dogs (81), hamsters (140), humans (18, 34, 88, 172), and F344 rats (114, 159). In dogs, humans, mice, and rats mitogenic responses rise to a peak in young adults and decline with increased age (64, 81, 88, 107, 114, 159, 172). Prepubertal subjects have a low mitogenic response (81, 114, 159, 172). The effect of age on B cell responses to mitogens and on response to mitogens that stimulate both B and T cells is less certain; some investigators report a decline with age (107, 140, 215), while others report that no decline occurs (115). The uncertainty may result from the fact that mitogens stimulate different cell populations in different species. For example, while PHA is accepted to be a pure T cell mitogen in mice, it probably stimulates both B and T cells in humans (140). M. pulmonis mitogen (MPM) has been considered to be mitogenic for both B and T cells of rats (35). However, there is more recent

evidence that MPM exerts a direct effect only on rat B cells; any effect on T cells is indirect (S. E. Ross, Ph.D. dissertation, University of Alabama at Birmingham, 1988).

Role of Bacterial Adherence in Disease

"Attachment" is the general term describing the complex bacterium-substrate interaction whereby the microorganism binds itself to a surface; in the case of many pathogens the surface is a host cell. This bacterium-host cell interaction has both nonspecific and specific phases. In the nonspecific, reversible phase the bacterium and the host cell overcome their mutual negative charges through attractive forces between hydrophobic molecules on both their surfaces. This phase in which bacteria localize in close proximity to host cell surfaces is sometimes called "association" (1, 8, 68). In the specific phase multiple bonds form between molecules called "adhesins" on the bacterium and specific receptors on the host cell (1, 10, 11); these bonds are collectively irreversible (10, 11). This relatively stable phase is interchangeably called "adherence" or "adhesion" (8, 68). The presence or absence of specific receptors on epithelial cells explains, at least in part, tissue tropisms of bacteria. For some of these receptors presence or absence is known to be genetically determined (1). "Adhesin" is the term now preferred to describe any bacterial structure or molecule that binds in a stereospecific way with complimentary molecules on the surface of host tissue cells (8, 10, 11). These molecules were formerly called "ligands." This term usually denotes a

small molecule, and bacterial binding molecules are more often large and complex (11).

Adherence of microorganisms to host cells and implanted prosthetic devices has been recognized as an important factor in the pathogenesis of diseases caused by a wide variety of infectious agents. There are numerous studies demonstrating that adherence to natural and artificial surfaces in the body has an important, if not crucial, role in disease development for most pathogens (10, 11, 95, 160, 183). Occurrence of staphylococcal vasculitis involving prosthetic vascular devices is believed to be related to graft and bacterial properties that enhance bacterial adherence (195). Microbial adherence to constituents of nonbacterial thrombotic endocarditis is a critical step in development of infective endocarditis. This factor was studied in rabbits and it was found that those microorganisms frequently isolated from endocarditis in people, including S. aureus, Candida tropicalis, Candida albicans, Streptococcus faecalis, and Streptococcus sanguis, adhered significantly better to constituents of nonbacterial thrombotic endocarditis than microorganisms rarely implicated in this disease (193).

Adherence of bacteria to mucosal surfaces enables them to resist normal mucosal cleansing mechanisms. These include flow of secretion and luminal contents, ciliary action, coughing, sneezing, swallowing, peristalsis, and excretion (1, 10, 11). Adherence also allows them to apply their toxic products directly to the host cell membrane in high

concentration, circumventing the enzymes in host secretions that would otherwise rapidly inactivate these toxins (1, 11).

Adherence of gonococci to mucosal epithelial cells (218), of enterotoxigenic strains of Escherichia coli to enterocytes in humans and swine (108, 128), of E. coli to urogenital tract epithelium (205), and of Vibrio cholerae to enterocytes (204, 216) is a critical step in establishment of infection and disease. In a study of yeast vaginitis, it was determined that a spontaneous mutant of C. albicans with reduced ability to adhere to human vaginal epithelium was also less virulent in a mouse model than the wild type strain (126).

In the respiratory tract, as on other mucosal surfaces, adherence confers advantages on bacterial pathogens. By helping to safeguard the microorganism from elimination by coughing, sneezing, ciliary action, or secretory flushing, adherence promotes colonization, as well as allowing direct, unchecked application of toxic bacterial products to the host cell membrane. Postoperative increases in adherence of Pseudomonas aeruginosa and other gram-negative bacilli to human oropharyngeal cells in vitro correlate with increase in postoperative susceptibility to gram-negative bacillary pneumonia (105). Adherence to ciliated respiratory epithelium is known to be a crucial step in the pathogenesis of disease caused by Bordetella pertussis (219).

It is unlikely that ability to adhere to host cells is the only determinant of virulence for respiratory tract pathogens. This is borne out by S. pneumoniae and H. influenzae; not all

pathogenic strains of these organisms adhere well to host epithelium (85, 115, 174, 200). It has been reported that adherence to pharyngeal epithelium by S. pneumoniae is important to strains causing otitis media (4, 5), while strains of S. pneumoniae and H. influenzae causing systemic diseases, such as meningitis and sepsis, adhere poorly to epithelial cells in vitro (4, 115). Thus, it appears that adherence correlates better with virulence in infections of mucosal surfaces, rather than systemic infections. A possible explanation for this observation is that ability to adhere is not a useful property for invasive microorganisms because they will encounter receptors for their adhesins on host phagocytic cells and be quickly engulfed and eliminated (1, 11, 220). Thus, strains of bacteria which adhere well to epithelial cells may revert to nonadherent forms once they have breached the mucosal barrier (3, 5, 11).

Mycoplasmas are surface parasites of host epithelial cells. Their pathogenic potential seems closely tied to organism-host cell surface interactions (38, 39, 73, 98, 112, 133, 213). In addition to the above listed advantages of adherence for bacterial pathogens, mycoplasmal adherence presumably places the organism in a crucial location to acquire the needed requirements for growth--cholesterol, fatty acids, and nucleotide precursors--which they lack the ability to make for themselves (M. K. Davidson, M.S. thesis, University of Alabama at Birmingham, 1981).

Mycoplasmal adherence to host cells is apparently complex. Kahane (112) proposed three mechanisms of mycoplasmal attachment: (a) interactions of mycoplasmal lectins (carbohydrate-binding proteins) with specific sialoglycoconjugate receptors on the outer surface of the host cell membrane, (b) interactions of proteins exposed on the mycoplasmal surface with receptors on host cell membranes, and (c) hydrophobic interactions between membrane constituents of the mycoplasmal and host cells. It is likely that more than one mechanism is employed by any mycoplasma.

Using a red blood cell model of mycoplasmal attachment, Minion et al. found that M. pulmonis attachment, like that of Mycoplasma pneumoniae and M. gallisepticum, appears to be multiphasic (145). One phase, expressed in the model as adherence of M. pulmonis to red cells, is a recognition event and is not proteolysis sensitive. The other phase, hemagglutination, is sensitive to treatments which alter membrane proteins, suggesting involvement of a mycoplasmal protein. The existence of a proteolysis-resistant protein cannot be ruled out in the adherence phase. The interactions in both phases were hydrophobic (145). Using the same model, several investigators have found that attachment of M. pulmonis to red blood cells is unaffected by neuraminidase (113, 145, 203). Minion et al. could not inhibit attachment of M. pulmonis to red cells with the red cell sialoglycoprotein, glycophorin (145), although glycophorin does inhibit attachment of M. pneumoniae (113). Their

interpretation of these findings was that unlike some other pathogenic mycoplasmas including M. pneumoniae, sialic acid is not involved in receptor sites for M. pulmonis (113, 145, 203). In a study using mouse peritoneal macrophages, neither neuraminidase, trypsin, chymotrypsin, nor glutaraldehyde inhibited surface attachment by M. pulmonis (109).

Although carbohydrate residues are the receptors for the adhesins of many bacteria (1), the role of lectins in M. pulmonis adherence is unclear. In one study strong agglutination of M. pulmonis occurred in the presence of lectins from Vicia cracca, Ricinus communis, and Phaseolus vulgaris, which react, respectively, with N-acetyl-D-galactosamine, D-galactose, and D-galactose-N-acetyl-D-glucosamine-mannose and N-acetyl-D-galactosamine on the surface of the mycoplasma. Weaker agglutination occurred in the presence of Canavalia ensiformis, a lectin reacting with D-mannose and D-glucose (194). Addition of the above carbohydrates inhibited the agglutination reactions. Pronase treatment of the mycoplasma reduced or eliminated agglutination with most of these lectins, suggesting that glycoproteins on the mycoplasmal surface are involved in adherence (194). Minion et al. were unable to inhibit either M. pulmonis-induced hemagglutination or adherence of M. pulmonis to red cells with the above and other carbohydrate compounds, or with glycophorin leading them to the conclusion that these activities were not lectin related (48).

Some mycoplasmas have specialized attachment structures, notably M. pneumoniae and M. gallisepticum (16, 38, 175). M. pulmonis lacks such a specialized structure and is believed to interact in a more generalized way with the host cell membrane (24). Loss of ability to attach has been correlated with loss of virulence in M. pneumoniae in several systems. Treatment of M. pneumoniae with monoclonal antibodies to its attachment tip proteins resulted in significantly decreased pulmonary lesion scores in experimentally infected hamsters (20). A mutant strain of M. pneumoniae which lacked several proteins, including the P1 protein believed to mediate attachment, could not adhere to epithelial cells and was avirulent (97, 124).

There is also evidence that attachment is an important determinant of M. pulmonis pathogenicity, but the exact mechanism is still unknown (24). Loss of the ability of strain JB to hemadsorb correlated with loss of ability to produce pneumonia in mice (213). Loss of the ability of this same strain of M. pulmonis to hemadsorb or to attach to vaginal epithelial cells correlated with loss of ability to produce genital infections in mice (214). Strain PG34(ASH) is avirulent in mice (47, 94); Davidson et al. studied pulmonary clearance of this strain of M. pulmonis using radiolabelled organisms and found that this strain apparently was unable to attach to respiratory epithelium (46).

Ciliostasis as a consequence of adherence of M. pulmonis has been demonstrated in vitro in tracheal organ cultures

(37). Lack of ciliary action with the resultant inability to clear respiratory exudates could contribute to pulmonary changes in chronic MRM.

SIGNIFICANCE AND RATIONALE

MRM is a well recognized complicating factor in many different types of studies using rats. Popular opinion to the contrary, M. pulmonis has not been eliminated and is still a common infection in both conventional and some barrier-maintained rats (28). Consequently, overt MRM remains a common problem in rats used in research. More knowledge of the pathogenetic mechanisms of this disease are needed to control and eliminate it.

Because there is a well-documented increase in susceptibility to infectious respiratory disease with increasing age in humans (77, 144) and because there have been anecdotal reports of increases in severity of MRM with increasing age in rats (50, 104, 127), I hypothesized that severity of MRM increases as host age at time of infection increases. Studies were done to determine the relationship of disease severity to age at inoculation with M. pulmonis in 5-, 8-, 12-, 20- and 40-week-old F344 rats. Understanding the effect(s) of age on host susceptibility to infection could lead to finding methods for earlier detection of M. pulmonis, a serious problem at present because the best detection method available rarely detects infected animals before they reach breeding age. Even in some older rats, infections can be difficult to detect by routine cultural and serologic methods.

Results of the first series of experiments led me to examine the effect of nonspecific lymphocyte responses on disease severity. Because the pulmonary changes occurring in MRM are not adequately explained by direct injury to host cells by M. pulmonis (23), several mechanisms of indirect injury have been proposed. One such mechanism is nonspecific stimulation of lymphocytes (23, 26, 35). Initially, I had hypothesized that disease susceptibility and degree of nonspecific lymphocyte response were strongly correlated, linked genetically, or both. Because both nonspecific lymphocyte responses and disease susceptibility are greater in LEW than in F344 rats and because a genetic component to disease susceptibility already has been demonstrated for F344 and LEW rats, it was felt that if differences in nonspecific lymphocyte responses could have been demonstrated to be genetically determined in the two rat strains, there would have been stronger support for the hypothesis that the pulmonary changes of MRM are produced, at least in part, by nonspecifically activated lymphocytes than demonstration of a relationship between disease and nonspecific responses in only one rat strain.

In preliminary studies, attempts were made to match nonspecific responses of individual rats to MPM with severity of MRM disease using F344, LEW, and F1 (uniformly heterozygous at every locus at which the inbred parents differ) and F2 (randomly heterozygous) hybrids of the two parental strains. These experiments met with limited success because genetic

sorting patterns of the individual rats, if they were present, were masked by the variability inherent to the mitogenicity assay. In addition, there also was evidence that all substrains of identified disease-susceptible rats may not respond identically in mitogenicity assays. For example, lymphocytes from LEW/Tru rats seem to have a consistently greater response to MPM and to PHA than do F344/NCr rats (J. K. Davis, personal communication). This difference has not always been borne out when LEW/NCr or other substrains have been used (106, 229). Another factor that may have confounded the results of these experiments was the suppressor substance recently demonstrated by Dr. S. E. Ross in splenic cultures containing both OX-8+ lymphocytes and macrophages. This spontaneously produced substance inhibited both B and T cell responses in F344 rats (S. E. Ross, Ph.D. dissertation, University of Alabama at Birmingham, 1988).

It was at this point, when the original studies failed to demonstrate a relationship between disease severity and nonspecific lymphocyte responses, that I decided to approach the problem somewhat differently and hypothesized that the age-related differences in severity of MRM would be associated with age-related differences in nonspecific lymphocyte responses to MPM in rats. This hypothesis was supported by our own preliminary data and by evidence from the work of many investigators in a variety of animal species indicating that there is a low response in pre-pubertal animals, a peak response in the young adult period, followed by a decline with

aging (64, 81, 88, 107, 114, 159, 172). It seemed reasonable that information gained from studying the role of nonspecific polyclonal immune responses to M. pulmonis was not only important in understanding MRM, but would also help in understanding the effects of mycoplasmal contamination of cell cultures, a common problem in continuously maintained T and B cell lines for hybridoma and other immunologic work (141). In addition, it was hoped that while the use of the relationship of age of the host at the time of infection to both disease severity and nonspecific lymphocyte responses could not be used to demonstrate a cause and effect relationship, it could demonstrate indirectly a positive relationship between disease severity and nonspecific lymphocyte responses that I had failed to demonstrate using rat genotype. It was anticipated that because the mitogenicity assay used was better-suited to establish the existence of trends or group correlations rather than comparisons between individuals animals and because the variables could be tested in one rat strain, the potentially confounding substrain differences and variability inherent to the mitogenicity assay would be avoided by this approach. The hypothesis was tested by determining the splenic lymphocyte responses of rats 5, 8, 12, 16, and 20 weeks of age using the lymphocyte microculture assay performed in the laboratory of Dr. G. H. Cassell (52).

The results of the second series of experiments led me to examine the effect of age-associated changes in susceptibility

to disease on adherence of the organism to respiratory epithelium. Ability to adhere to host cells is a virulence factor for many pathogenic bacteria (10, 11, 160) including mycoplasmas in which pathogenicity is closely linked to organism-host cell surface interactions (38, 39, 73, 98, 112, 133, 213). Loss of ability to adhere to host cells has been related to loss of virulence by M. pneumoniae and M. pulmonis (20, 46, 97, 124, 213, 214). Inasmuch as ammonia exposure and co-infections of M. pulmonis and murine respiratory viruses are factors known to enhance the expression of MRM in rats and these factors increase the number of M. pulmonis organisms recoverable in vivo, in vitro, or both in rats (17, 170, 185), I hypothesized that factors which exacerbate MRM, increase adherence of M. pulmonis to respiratory epithelium. The study of the effect on organism adherence of factors that enhance MRM disease should be useful in understanding the effects of exogenous agents on the expression of respiratory mycoplasmosis in many species other than rats.

To determine the effect of NH_4Cl treatment, Sendai virus infection, and host age on M. pulmonis adherence to respiratory epithelium, a tracheal organ culture system was used. The use of an organ culture system to study adherence of M. pulmonis allows the effects on respiratory epithelium of known and suspected disease enhancers to be examined without the confounding effects of immune and inflammatory responses. The perfusion block system used by Gabridge and Hoglund for study of M. pneumoniae infection of guinea pig

tracheas (76) was chosen because its use for study of pathogenesis of mycoplasmal diseases is well established (21, 30, 36, 37, 39), and it already had been adapted for use with M. pulmonis and rat tracheas by Pinson et al. (170). Tracheas were chosen as the source of respiratory epithelium to study because they are easy to obtain, and vary little in length and diameter in F344 rats of a given age and sex; thus, they can be standardized easily. Nasal mucosal epithelium might be preferable, inasmuch as the nasal mucosa is the initial site of M. pulmonis colonization, but it has a more varied cell population and is difficult to obtain and, thus, is not as amenable to the production of standard cultures. NH_4Cl exposure causes microscopic and ultrastructural changes in tracheal epithelium in vitro similar to those observed in vivo in nasal epithelium of rats exposed to gaseous ammonia (171), and both rhinitis and tracheitis occur consistently in experimental Sendai virus and SDAV infections (198, 199). Thus, the effects of ammonia and of these viruses on tracheal epithelium should be representative of their in vivo effects on respiratory epithelium in general regardless of the tissue of origin. Quantitative culture of M. pulmonis is a well-established technique. This technique would be expected to be more accurate in experimentally inoculated organ cultures than in whole animals, and in preliminary work it had given precise and consistent results.

MATERIALS AND METHODS

Animals and Housing

All animals used in these studies were pathogen-free and were housed at the University of Alabama at Birmingham (UAB) in Trexler-type plastic film isolators (Germ-free Supply Division, Standard Safety Equipment Co., Palatine, IL) or microisolator cages (Lab Products, Inc., Syracuse, NY). For disease expression studies, isolators were modified to provide a continuous concentration of 19 to 38 µg/l commercial grade ammonia (PB & S Chemical Co., Henderson, KY) by a previously described method (16). Ammonia concentration in the isolators was monitored by gas detector tubes using a multigas detector (Dragerwerk, AG, Lubeck, Federal Republic of Germany). The monitoring was done daily or more often for the first week of each experiment until it was ascertained that the above ammonia concentration range was being maintained in the isolators. In no instance did the concentration exceed 76 µg/ml (100 ppm) for more than 24 hours. Once the ammonia flow was well regulated, monitoring was done weekly. Bedding was changed as often as necessary to prevent production of ammonia in soiled bedding from increasing the ammonia concentration in the isolators. The frequency determined to accomplish this goal was 3 to 4 changes per week. These animals were housed in hanging wire bottom cages to facilitate frequent removal

of soiled bedding (Teriwipers, Kimberly Clark Corp., Roswell, GA) and exposure to uniform concentrations of ammonia. Rats in microisolator cages were bedded on hardwood chips (Sanichips, P. J. Murphy Forest Products, Rochelle Park, NJ). Bedding, water, and food (Agway, Syracuse, NY) were autoclaved.

Male F344/NCr rats were obtained from the Frederick Cancer Research Facility (FCRF), Frederick, MD. Animals at this facility are housed in barrier rooms and monitored monthly to assure absence of viral, parasitic, and bacterial pathogens. Animals from FCRF were obtained at 4 weeks and at retired breeder age at intervals appropriate for each study and were housed at UAB until they reached the ages desired.

Male and female F344 and LEW rats used in preliminary mitogenicity assays were obtained originally from FRCF and they and the F1 and F2 hybrids of these strains were bred and maintained at UAB in plastic film isolators by personnel in the laboratory of Dr. Gail H. Cassell. The pathogen-free status of these colonies was monitored by serological and cultural techniques for viral, fungal, mycoplasmal, and other bacterial pathogens as previously described (130). Experimental animals were also determined to be M. pulmonis-free by ELISA (28). In addition, M. pulmonis was not detected in animals randomly selected from each disease expression control group by culture for M. pulmonis. M. pulmonis also was not detected by quantitative culture of control tracheas in tracheal organ culture experiments.

Lymphocyte Suspensions

Animals were anesthetized to a surgical plane with a ketamine HCl (Ketalar 50 mg/ml, Parke-Davis, Morris Plains, NJ) and xylazine HCl (Rompun 100 mg/ml, Haver-Lockhart, Shawnee, KS) combination given intramuscularly (10 ml ketamine/1.5 ml xylazine). Spleens and lymph nodes were removed aseptically, minced, pressed through a sterile nylon screen (mesh size, 250 μm), and suspended in cold sterile RPMI 1640 (GIBCO Laboratories, Chagrin Falls, OH) with 2 mM L-glutamine (GIBCO Laboratories), 40 mg/ml gentamicin sulfate (Elkins-Sinn, Inc., Cherry Hill, NJ), 10 mM HEPES buffer as the sodium salt of [4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid] (Research Organics, Inc., Cleveland, OH), and 2.5% fetal calf serum (GIBCO Laboratories) per ml, at pH 7.2.

Spleen cell suspensions were washed and centrifuged on a medium-Ficoll (sp. gr. = 1.077 ± .004) discontinuous density gradient (Ficoll-Paque, Pharmacia Fine Chemicals, Piscataway, NJ). Lymph node cell suspensions were not separated on a gradient, but were washed and centrifuged. Cells were then washed in sterile cold medium, counted with a Model FN Coulter counter (Coulter Electronics, Inc., Hialeah, FL), and resuspended in lymphocyte culture medium consisting of sterile RPMI 1640 with L-glutamine, gentamicin, HEPES buffer, 0.2% sodium bicarbonate (Fisher Scientific Co., Fair Lawn, NJ), and 2.5% mycoplasma antibody-free (tested by ELISA) pooled rat serum to make a final suspension of $10^{6.6}$ lymphocytes/ml. Cell

suspensions were kept on ice between steps and until each step was completed on all samples.

Peripheral blood cells were obtained by collecting 3 ml of blood by cardiac puncture from rats lightly anesthetized with ketamine/xylazine. The blood was collected into syringes containing 2 ml of sterile RPMI 1640 with 10 units of sterile heparin. This resultant suspension was gently mixed to prevent clotting of the blood, diluted to 2 to 2.5 times its original volume by the addition of sterile medium, and then centrifuged. After discarding the supernatant, the cells were resuspended in sterile medium and separated on a medium-Ficoll gradient of sp. gr. 1.081 ± .001. Cells collected at the medium-Ficoll interface were then washed and counted as for spleen cells.

Mitogens

The method of preparing M. pulmonis mitogen (MPM) was one performed in the laboratory of Dr. G. H. Cassell (48). Dose-response curves were done on each batch of MPM to determine concentration in µg of protein/ml giving the peak response. Protein concentration of mitogen was determined by the Bio-Rad protein assay (Bio-Rad Laboratory, Richmond, CA). In our experience, the peak response was usually 5 µg/ml. The concentration giving the peak response was included among those used in all experiments. MPM was stored at -70°C until needed. PHA (Phytohemagglutinin M, Difco Laboratory, Detroit, MI) was used at 0.1 and 0.2 mg/ml as a positive control. All mitogen dilutions were prepared fresh on the day of use.

Mitogenicity Assay

In each well of 96-well flat-bottom-type tissue culture plates (Linbro, Flow Lab., Inc., McLean, VA), 100 µl of appropriate mitogen dilution was mixed with an equal volume of cell suspension (4 X 10^5 or $10^{5.6}$ cells/well). Twenty-four hours before harvesting, 0.5 µCi tritiated thymidine (^{3}H-Tdr) (Amersham, Arlington Heights, IL) was added to each well. After incubation for 48 hour at 37°C in water-saturated air with 5% CO_2, cells were harvested (Titertek, Skatron, Lilrbyen, Norway) on glass fiber filter strips (Gelman Sciences, Inc., Ann Arbor, MI), strips were allowed to air dry for at least 24 hours, and incorporation of ^{3}H-Tdr was determined as counts per minute (CPM) on individual filter discs in liquid scintillation fluid (Econofluor, New England Nuclear Research Products, Boston, MA) using a liquid scintillation counter (6895 BetaTrac Liquid Scintillation Systems, TM Analytic, Elk Grove Village, IL). Samples were done in triplicate except for preliminary peripheral blood assays in which duplicate samples were done.

For the parallel studies of the effect of age of the host at time of infection on disease expression and on nonspecific lymphocyte responses, results were expressed and evaluated as "stimulation indices" (the ratios of the mean counts from triplicate cultures of mitogen stimulated lymphocyte to the mean counts of triplicate unstimulated or background control cultures) and as "change (delta) in counts per minute" (the mean of counts per minute from triplicate control cultures

subtracted from mean counts from triplicate cultures of mitogen-stimulated lymphocytes). Transforming data in either of these ways is done with the assumption that proliferation in both treated and control cultures is subjected to the same external variables and that responses of both types of cultures to these variables are proportional. The stimulation index enhances the effect of variable background counts, whereas change in counts per minute tends to dampen this effect. Background counts in these experiments were moderately variable, so both methods of analyzing the data were used. By removing the background counts from the calculation, both techniques make the net results directly comparably to each other for statistical analysis. Also, with stimulation indices the resulting data are in smaller numbers and so are more easily manipulated statistically.

Mycoplasma Inocula

A stock culture of M. pulmonis (seventh passage of UAB strain 5782C), originally isolated from a naturally infected rat, was used in all experiments except for adherence studies in which freshly grown stocks of M. pulmonis strain UAB-5782C were used. The original isolate of this strain had been examined for species identity and purity by immunofluorescence (R. A. Del Giudice, Frederick Cancer Research Facility, Frederick, MD). Stock inoculum for these experiments was grown by placing 2.5 ml of fourth passage UAB 5782 stock culture into 25 ml of mycoplasma medium and incubating it until the pH was 7.3 to 7.4. The 25 ml was inoculated into

100 ml of fresh medium and the 100 ml inoculated into 400 ml of fresh medium, with incubation and growth to pH 7.3 to 7.4 repeated each time. The 400 ml of stock culture was tested for bacterial contamination and was stored in 1 to 5 ml aliquots in cryotubes at -70°C until needed. The stock contained $10^{8.33}$ CFU/ml after thawing. This organism has been used extensively in our laboratory, and this passage of M. pulmonis UAB-5782C, like earlier passages of the organism, was virulent for F344 rats at 10^7 CFU/rat. As each vial was used, a 0.1 ml sample was quantitatively cultured to ensure uniform dosage in both live animals and tracheal organ cultures.

For growth of stock cultures, each 200 ml of mycoplasma broth medium contained 4 g of Frey's mycoplasma broth base (GIBCO Laboratories, Madison, WI), 0.04 g DNA (Sigma Chemical Co., St. Louis, MO), 0.4 ml of 1% phenol red (Fisher Chemical Co., Springfield, NJ), and 150 ml of distilled water. After the pH was adjusted to between 8.0 and 8.1 with 10 and 1 M NaOH, and the medium had been autoclaved, the medium was supplemented with 40 ml of heat-inactivated (30 minutes at 56°C) gamma globulin-free horse serum (GIBCO Laboratories, Grand Island, NY), 2.5 ml of CVA supplement (GIBCO Laboratories), 2.5 ml of filter-sterilized 50% dextrose (Sigma Chemical Co., St. Louis, MO), and 40 µl of 250 mg/ml stock solution of cefoperazone sodium (Cefobid, Pfizer, Inc., New York, NY). The pH was adjusted to between 7.8 and 8.0 with NaOH, and the medium filter-sterilized, stored in sterile bottles, and refrigerated until needed. For agar plates, 2.5

g Noble agar (Difco Laboratories, Detroit, MI) was added to the above medium before autoclaving; after autoclaving and addition of supplements as above, the medium was allowed to cool slowly in a 50°C water bath and then was dispensed aseptically into sterile disposable Petri dishes. Both the broth and agar media were tested for sterility and ability to support growth of M. pulmonis prior to use in experiments.

Quantitative Mycoplasma Cultures

For disease-expression studies, quantitative mycoplasma cultures of soft tissues were done by aseptically collecting and homogenizing each tissue in individually sterilized ground glass tissue grinders (Duall #23, Kontes Biomedical Products, Vineland, NJ). Grinders were driven at 800 rpm by a constant-speed, constant-torque electric motor (Master Servodyne, Cole-Parmer Instrument Co., Chicago, IL). All cultures were done in a standard volume of sterile mycoplasma culture medium. Lung homogenates were brought to a volume of 3.0 ml; 1 ml was used for nasal mucosa and trachea. To disrupt clumps of M. pulmonis that could result in erroneously low colony counts, the tissue homogenates were sonicated (Model W220F Sonicator, Heat Systems-Ultrasonics, Inc., Plainview, NY) in a cup horn in ice water for 30 seconds at 100% output. Because the bony tissue of the nasal passages could not be homogenized, nasal tissues were crushed with bone rongeurs and sonicated as above to disrupt cells and clumps of M. pulmonis. Serial 10-fold dilutions of 0.1 ml of tissue homogenate were made in mycoplasma culture broth, and 0.02 ml of each dilution was

inoculated onto mycoplasma culture agar. After 5-7 days, the mycoplasma colonies were counted and expressed as CFU/organ for nasal mucosa, CFU/g of tissue for lungs, and CFU/std length or CFU/mm^2 for tracheas. The lungs were weighed and the CFUs in the lung were calculated as CFU/g of tissue to eliminate the confounding factor of the considerable size difference between the lungs of the 40-week rats and those of the 8-week-old rats. Tracheas were evaluated by unit area for the same reason. Tracheal length was standardized to 8 mm, the inner diameter of the trachea measured in mm with a caliper, and the area of the mucosal surface calculated. CFUs were then expressed as CFU/mm^2. This was necessary because the diameter of the trachea of a 40-week-old rat was about 1 mm greater than that of an 8-week-old rat. Within each age group the diameter of the tracheas varied little; the mean diameter for 8-week-old rats was 3.0 ± 0.0 mm and for 40-week-old rats, 3.8 ± 0.37 mm.

For adherence studies, the tracheas in organ culture were treated as stated above except that tissue culture medium (described under Tracheal Organ Cultures) was substituted for mycoplasma culture medium in the homogenization procedure, and the tissues were not sonicated. For quantitative cultures in disease-expression studies, the mycoplasma culture medium used was that described under Mycoplasma Inocula for growth of stock mycoplasma cultures. In adherence studies, quantitative cultures were done in mycoplasma culture medium modified by the absence of the CVA supplement and the agar used was

SeaPlaque agarose (FMC Corporation, Rockland, ME). The CVA supplement did not enhance growth of M. pulmonis UAB-5782C and, in some instances, apparently inhibited growth (M. K. Davidson, personal communication). SeaPlaque agarose is a clearer agar than Noble agar and, therefore, makes counting of colonies easier and more accurate.

Evaluation of MRM Disease Expression

Twenty-eight days after inoculation, animals were killed and respiratory organs including nasal passages were removed and fixed in 80% ethanol:10% formalin. Lungs were infused via the airways with fixative to approximately normal distention. Bony tissue was demineralized for 48 hours in 0.7% tetrasodium ethylenediamine tetraacetate in hydrochloric acid. After routine processing for paraffin embedded tissue, sections were evaluated microscopically for lesion severity. The method used was a semi-quantitative scoring system devised in this laboratory (51, 171, 198). This method involves the preparation and examination of standard transverse sections through the skull which include all three types of nasal epithelium and the middle ears, longitudinal sections of larynx, and trachea, and individual sections through each lung lobe cut to obtain a longitudinal section through the major bronchus. This method of scoring respiratory tract lesions has been shown to correlate well with the results of computerized morphometry (51, 171).

Histologic sections were evaluated in random order. For nasal passages and middle ears inflammatory exudates,

epithelial changes, and submucosal lymphoid aggregates were evaluated together and given a score of 0-3 to reflect normal tissue (0), mild changes (1), moderate changes (2), or severe changes (3). In lungs and tracheas inflammatory exudates, epithelial changes, and submucosal lymphoid aggregates were evaluated separately. Tracheas were scored 0-3 to reflect normal tissue to severe changes, as above. Lungs were scored 0-5 to represent normal tissue (0); mild (1), moderate (2), and severe (3) bronchitis; severe bronchitis with suppurative pneumonia (4); and severe bronchitis, suppurative pneumonia, and bronchiectasis (5). Also, lesion scores in individual lung lobes were weighted to reflect the respective contribution of each lobe to total lung volume. Scores for the right anterior, right middle, and azygos lobes were multiplied by 1. Scores for the right caudal lobe were multiplied by 2, and those for the left lobe were multiplied by 3. For each organ in each animal the scores were summed, divided by the maximum possible score for that organ, and expressed as lesion indices.

Tracheal Organ Cultures

After rats were anesthetized to a surgical plane with ketamine/xylazine, their tracheas were collected aseptically and placed in perfusion blocks according to the method of Gabridge and Hoglund (73, 76). Tracheas were placed in the central trough of a sterile Teflon (DuPont, Wilmington, DE) block device having two wells connected via a central trough. After sterile Teflon plugs were placed in the wells snugly

against the trough openings to seal the ends of each trachea, liquid agar was added to the trough. After the agar hardened, the plugs were removed and one well was filled with liquid culture medium to flow back and forth through the tracheal lumen when the blocks were placed on a rocker platform (Bellco, Vineland, NJ). To maintain sterility and to improve ease of handling, the blocks were placed in sterile disposable Petri dishes and immobilized in the dish with sterile Noble agar (Difco Laboratories, Detroit, MI). The entire system was then incubated at 37°C in a water-saturated air environment. Liquid culture medium was replaced every 24 hours. The culture medium was modified McCoy's 5A medium with 5% fetal calf serum, 250 mg/ml of stock of cefoperazone sodium (Cefobid, Pfizer, Inc., New York, NY), and HEPES buffer (170). The agar medium was the same except that 1.5% SeaPlaque agarose (FMC Corporation, Rockland, ME) was added.

The tracheas were cultured overnight, then stock culture of M. pulmonis was added to 4 ml of culture medium in the wells, resulting in an inoculation dose of $10^{6.8}$ CFUs/trachea. After 1 hour of incubation, tracheas were removed from the perfusion blocks, rinsed with sterile medium to remove nonadherent organisms, trimmed to a uniform length of 8 mm, homogenized, and quantitatively cultured.

Virus Infection of Tracheas

Sendai virus was obtained from the American Type Culture Collection, grown in BHK-21 cells known to be free of viral and mycoplasmal contaminants, titrated by hemadsorption with

guinea pig erythrocytes, and frozen in 1 ml aliquots at -70°C. As determined by the hemadsorption assay, there were $10^{4.5}$ 50% tissue culture infective doses/ml. To produce virus-infected tracheas, rats at 8 and 40 weeks of age were inoculated with 25 µl of undiluted stock culture of Sendai virus in each nostril using an adjustable pipettor. This is a dose of $10^{3.2}$ 50% tissue culture infective doses. Rats were killed at 5 days post-inoculation, the time previously reported to be that of peak lesion severity (29), and their tracheas collected aseptically for organ culture.

Ammonia Exposure of Tracheas

NH_4Cl treatment was done by adding NH_4Cl to double strength modified McCoy's 5A culture medium described above and reconstituting to normal strength by addition of distilled water to obtain 50 mM ammonium ion. This concentration of ammonium ion was determined in previous studies to be the maximum allowing survival of the tracheal epithelium for 72 hours (171). The pH was adjusted to 7.4. Tracheas were exposed overnight to the medium containing NH_4Cl.

Statistical Analyses

Data for all experiments except those mitogenicity assays comparing F344, LEW, and hybrid rats were analyzed for significance by multifactorial analysis of variance (ANOVA) (202), using the ANOVA II software (Human Systems Dynamics, Northridge, CA). When F tests were significant, Duncan's multiple range test (56) was used to compare means. Data from the above excepted experiments were analyzed using the

Statistical Analysis Systems (SAS^{tm}, Cary, NC) program with the assistance of Dr. D. C. Hurst of the Department of Biostatistics.

RESULTS

Effect of Host Age at Time of Infection on Severity of MRM

To determine the possible effect of host age at time of infection on disease expression in MRM, two experiments (nos. 1 & 2) were done using 7 to 10 male F344 rats per age group, at 5, 8, 12, and 20 weeks of age. This age range was chosen because it included the range that had been used in previous mitogenicity experiments in which it seemed that age affected the numbers of transforming lymphocytes. Rats in each age group were randomly assigned to M. pulmonis-inoculated and control groups. Lightly anesthetized rats were inoculated with either 25 µl of M. pulmonis stock culture or sterile medium in each nostril. The dose of M. pulmonis per rat was $10^{7.3}$ CFUs. Controls were housed in separate isolators for the duration of each experiment.

In experiments 1 and 2, splenic lymphocytes were collected from littermates of the M. pulmonis-inoculated animals. This was done to compare changes in nonspecific lymphocyte responses in mitogenicity assays to changes in disease expression at each age. Because mitogenic responses vary among individuals even in inbred rat strains, it would have been best to evaluate both response to M. pulmonis mitogen and to M. pulmonis infection in the same individuals. However, because splenectomy alters immune responses making results of

such experiments invalid (162) and because of difficulties encountered in attempts to use lymphocytes from other organs, the best compromise was to use littermates. Also, because male rats were chosen for use in mitogenicity assays to eliminate any physiologic variation in lymphocyte responses that might have resulted from estrual cycling of female rats, only male rats were used to make results as comparable as possible.

After completing experiments 1 and 2, it appeared that satisfactory results could be obtained with fewer age groups, and weanling (about 4 weeks old) and retired breeder (about 36 weeks old) rats were more readily available from our source. Therefore, rats at 8 and 40 weeks of age were used in experiments 3 and 4. In these experiments the rats were each given $10^{7.3}$ CFUs of M. pulmonis as in the earlier experiments. Twenty-eight days post-infection 4 to 5 from each age group were used for histologic evaluation of disease expression and the other 4 to 5 animals in the same age group were used for quantitative cultures. Quantitative cultures were done to determine whether or not older rats with more severe pulmonary lesions of MRM, as previously noted in experiments 1 and 2, would have more CFUs of M. pulmonis in their respiratory tissues.

In all disease-expression experiments (nos. 1 - 4) the animals were subjected to a continuous concentration of ammonia maintained between 19 and 38 µg/l (25 to 50 ppm) by regulated flow of commercial grade ammonia into the isolators.

This concentration range of ammonia is no higher than that commonly found in the intracage environment of conventional rats and is known to hasten disease production (17).

It had been anticipated that there would be an increase in disease severity with age, as suggested by others (26, 50, 104, 127). The result of statistical analysis of combined data from experiments 1 and 2 was that 20-week-old rats had significantly more severe pulmonary disease than rats in any younger age group (Table 1). In experiments 3 and 4, 40-week-old rats had significantly more severe pulmonary disease than 8-week-old rats (Table 1). There were no age-related differences in lesion indices in nasal passages or tracheas in any experiment, whether the data were analyzed separately or combined (Tables 2 and 3). Mean lesion indices for middle ears were not consistently significantly different in the age range examined (Table 4).

In the two disease-expression experiments (nos. 3 and 4) in which quantitative cultures were done, the mean CFUs of M. pulmonis recovered from the lungs of 40-week-old rats were significantly greater than those from the 8-week-old rats (Table 5). However, mean CFUs of M. pulmonis in nasal passages and tracheas did not differ between 8- and 40-week-old rats (Table 5).

Effect of Host Age on Splenic Lymphocyte Response to Mitogens

I had initially anticipated detecting a relationship between disease susceptibility and nonspecific lymphocyte responses by comparing the lymphocyte responses to MPM of the

Table 1. Effect of Age of the Host at Time of Inoculation on Severity of Lung Lesions of MRM[a]

Experiments	Age in Weeks			
	5	8	12	20
1 and 2	0.07 ±0.13 n = 9	0.03 ±0.07 n = 9	0.10 ±0.13 n = 8	0.34[b] ±0.25 n = 8

Experiments	Age in Weeks	
	8	40
3 and 4	0.02 ±0.02 n = 9	0.22[c] ±0.20 n = 10

[a]Results are expressed as mean lesion indices; data are combined from experiments 1 and 2 and from experiments 3 and 4.

[b]Mean lesion indices of 20-week-old rats are significantly different from those of all younger age groups ($p = 0.001$).

[c]Mean lesion indices of 40-week-old rats are significantly different from those of all younger age groups ($p = 0.009$).

Table 2. Effect of Age of the Host at Time of Inoculation on Severity of Nasal Passage Lesions of MRM[a]

Experiments	Age in Weeks			
	5	8	12	20
1 and 2	0.68 ±0.12 n = 9	0.69 ±0.10 n = 9	0.65 ±0.09 n = 8	0.58 ±0.15 n = 8

Experiments	Age in Weeks	
	8	40
3 and 4	0.75 ±0.19 n = 9	0.56 ±0.25 n = 10

[a]Results are expressed as mean lesion indices; results are combined from experiments 1 and 2 and from experiments 3 and 4; there are no significant differences among means.

Table 3. Effect of Age of the Host at Time of Inoculation on Severity of Tracheal Lesions of MRM[a]

Experiments	Age in Weeks			
	5	8	12	20
1 and 2	0.48 ±0.20 n = 9	0.47 ±0.15 n = 9	0.44 ±0.20 n = 8	0.51 ±0.09 n = 8

Experiment	Age in Weeks	
	8	40
3 and 4	0.63 ±0.29 n = 9	0.59 ±0.19 n = 10

[a]Results are expressed as mean lesion indices; results are combined from experiments 1 and 2 and from experiments 3 and 4; there are no significant differences among means.

Table 4. Effect of Age of the Host at Time of Inoculation on Severity of Middle Ear Lesions of MRM[a]

Experiments	Age in Weeks			
	5	8	12	20
1 and 2	0.53[b] ±0.19 n = 9	0.25 ±0.34 n = 8	0.29 ±0.22 n = 8	0.45 ±0.26 n = 8

Experiments	Age in Weeks	
	8	40
3 and 4	0.45 ±0.28 n = 17	0.47 ±0.30 n = 18

[a]Results are expressed as mean lesion indices; results are combined from experiments 1 and 2 and from experiments 3 and 4.

[b]Five-week-old rats have lesion indices significantly different from those of 8- and 12-week-old rats ($p = 0.02$), but not from those of 20-week-old rats.

Table 5. Effect of Age of the Host at Time of Inoculation on Numbers of Recoverable CFUs of *Mycoplasma pulmonis*[a]

Organ	Age in Weeks	
	8	40
Nasal passages	6.74 ±0.26 n = 8	6.89 ±0.52 n = 8
Trachea (length)	7.22 ±1.08 n = 8	7.55 ±1.28 n = 7
Trachea (area)	5.45 ±0.74 n = 4	5.89 ±1.25 n = 4
Lung (/g)	4.72[b] ±1.01 n = 8	6.59[b] ±1.44 n = 7

[a]Results are expressed as mean $\log_{10}$ CFUs.

[b]Means are significantly different ($p = 0.05$).

relatively resistant F344 rats and the relatively susceptible LEW rats. First, the response of a uniformly heterozygous cross of the inbred parents, the F1 hybrids, would be tested to determine whether their inherited mitogenic response was high, low, or intermediate relative to the parental strains. F1 hybrids would then be randomly mated to produce a randomly heterogenous F2 generation. The individual responses of these rats would be used to determine the frequency of high, low, and intermediate mitogenic responses and, possibly, to determine the mode of inheritance, if it followed a recognizable pattern. These experiments would require that adequate numbers of lymphocytes be obtained from rats that would then have to survive to be inoculated with M. pulmonis and develop MRM. Therefore, lymphocytes would have to be collected from either peripheral blood or lymph nodes, or by hemisplenectomy. Development of a technique for the use of the same rats in both series of experiments was vital to the use of the F2 generation rats which would be randomly heterozygous. It also was anticipated that such a technique would eliminate or diminish animal-to-animal variation as a confounding variable in the mitogenicity assay.

Unfortunately, sufficient peripheral blood lymphocytes could not be collected from individual rats to perform even duplicate mitogen-stimulated and positive and negative control cultures, especially at the lower end of the chosen age range. To determine whether or not this problem could be circumvented by using lymphocytes from blood samples pooled from several

animals, pooled and individual samples were compared. The resulting pooled stimulation indices were quite different from the mean stimulation indices of the individual animal's mitogenic responses (Table 6). For MPM, pooled stimulation indices were similar to the lowest values obtained from the individual rats. In addition, the mitogenic responses of lymphocytes from different sources were examined. Because spleen had been the source of lymphocytes used previously in our laboratory to evaluate F344 and LEW responses, and it was on these studies that my hypothesis was based, it was necessary to determine whether or not the responses of peripheral blood and lymph node lymphocytes parallelled those of the spleen. However, no significant correlations were found among responses of lymphocytes from the three different sources (Tables 7, 8, and 9). Thus, splenic lymphocytes were used. It was anticipated that the alterations in the immune responses following splenectomy noted by Pachciarz and Teague would not rule out the use of splenic lymphocytes nor the use of the same animal in both mitogenic assays and in disease-expression studies because hemisplenectomy could be performed to circumvent the problem. However, variability inherent in the mitogenicity assay masked any gene assortment that might have been present, and these studies were abandoned.

At this point, when the genetic studies failed to demonstrate a relationship between disease severity and nonspecific lymphocyte responses, the studies were redirected to test whether or not age-related differences in disease

Table 6. Effect of Pooling of Peripheral Blood Lymphocytes on Results of Lymphocyte Transformation Assays in Rats[a]

Experiment 1	Mitogen[b]			
	MPM (μg/ml)		PHA (mg/ml)	
Animal Number	2.0	5.0	0.1	0.2
268	8.8	12.6	323.6	528.8
269	26.5	28.8	523.4	779.6
270	21.3	28.1	473.2	682.6
272	33.8	40.5	517.4	886.0
273	20.2	32.6	550.3	886.6
274	18.0	31.5	354.8	479.0
275	23.4	26.1	632.3	833.7
276	22.2	25.0	693.5	1165.7
Mean Stimulation Index for Individual Rats	21.8	28.2	508.6	780.2
Stimulation Index for Pooled Sample	8.6	10.5	665.7	862.2

Experiment 2	Mitogen			
	MPM (μg/ml)		PHA (mg/ml)	
Animal Number	2.0	5.0	0.1	0.2
292	2.8	2.7	184.5	357.8
293	7.2	---	77.5	----
375	1.7	2.1	54.3	26.0
Mean Stimulation Index for Individual Rats	3.9	2.4	105.4	191.9
Stimulation Index for Pooled Sample	.4	1.3	58.9	-----

[a]Results are expressed as mean stimulation indices.

[b]MPM = M. pulmonis mitogen; PHA = phytohemagglutinin.

Table 7. Results of Lymphocyte Transformation Assays Using Splenic, Lymph Node, and Peripheral Blood Lymphocytes from the Same F344 Rats[a]

Mitogen[b]	Rat Number	Source of Lymphocytes		
		Spleen	Lymph Node	Blood
MPM 2 µg/ml	839	2.6	5.8	1.4
	840	6.2	2.8	3.4
	841	5.7	1.7	2.1
	842	5.6	3.1	2.3
MPM 5 µg/ml	839	5.1	3.1	2.3
	840	12.3	3.4	7.5
	841	10.7	2.6	3.1
	842	12.8	3.9	4.8
PHA 0.1 mg/ml	839	5.0	30.1	55.3
	840	30.0	19.8	176.5
	841	10.4	20.1	57.8
	842	13.5	19.4	94.9
PHA 0.2 mg/ml	839	2.9	32.2	53.9
	840	17.0	15.3	275.7
	841	5.9	21.3	26.4
	842	5.0	20.5	116.7

[a]Results are expressed as mean stimulation indices. There are no significant correlations among responses of lymphocytes from the three different sources.

[b]MPM = M. pulmonis mitogen; PHA = phytohemagglutinin

Table 8. Results of Lymphocyte Transformation Assays Using Splenic, Lymph Node, and Peripheral Blood Lymphocytes from the Same LEW Rats[a]

Mitogen[b]	Rat Number	Source of Lymphocytes		
		Spleen	Lymph Node	Blood
MPM 2 µg/ml	889	4.9	2.2	1.9
	890	5.4	7.8	2.7
	891	6.6	6.6	2.7
	892	7.2	2.5	6.7
MPM 5 µg/ml	889	4.7	3.3	1.0
	890	5.2	10.1	3.6
	891	6.2	5.8	1.6
	892	6.4	3.0	1.0
PHA 0.1 mg/ml	889	3.6	39.9	11.2
	890	4.6	64.4	73.2
	891	7.8	41.8	43.6
	892	17.9	20.0	27.7
PHA 0.2 mg/ml	889	2.3	45.4	3.3
	890	3.5	74.3	2.1
	891	3.9	55.3	---
	892	7.1	24.0	---

[a]Results are expressed as mean stimulation indices. There are no significant correlations among responses of lymphocytes from the three different sources.

[b]MPM = M. pulmonis mitogen; PHA = phytohemagglutinin.

Table 9. Results of Lymphocyte Transformation Assays Using Splenic, Lymph Node, and Peripheral Blood Lymphocytes from the Same F1 Hybrid (F344 x LEW) Rats[a]

Mitogen[b]	Rat Number	Source of Lymphocytes		
		Spleen	Lymph Node	Blood
MPM 2 µg/ml	883	5.0	0.9	2.0
	884	8.2	1.6	---
	885	3.0	3.3	0.7
	886	5.1	0.6	1.5
MPM 5 µg/ml	883	7.4	0.9	1.2
	884	13.0	2.2	1.0
	885	4.1	5.3	1.4
	886	7.8	0.7	2.2
PHA 0.1 mg/ml	883	8.7	9.7	57.0
	884	23.4	13.0	---
	885	4.1	28.0	---
	886	12.1	9.2	29.6
PHA 0.2 mg/ml	883	5.5	9.5	13.4
	884	15.0	16.1	---
	885	1.8	39.9	0.4
	886	5.5	9.1	3.3

[a]Results are expressed as mean stimulation indices. There are no significant correlations among responses of lymphocytes from the three different sources.

[b]MPM = M. pulmonis mitogen; PHA = phytohemagglutinin.

susceptibility were associated with differences in nonspecific lymphocyte responses to MPM. From results of many mitogenicity assays performed in this laboratory, we had suspected that responses to MPM and PHA mitogens in rats 8 weeks of age or less would be significantly lower than in rats 12 weeks of age or greater. It was felt that the experiment-to-experiment variation in mitogenicity studies caused the standard error to be too great to identify differences in lymphocyte response with age in these many studies, so specific studies were designed to determine the effect of age on lymphocyte transformation by MPM and PHA.

An assay duration of 48 hours was chosen for testing mitogenic response because with MPM there was no statistically significant increase in ^{3}H-Tdr incorporation by splenic lymphocytes in 72-hour assays. Mean stimulation index for F344 rats in 48-hour assays was 20.8 ± 10.5, and in 72 hours was 35.7 ± 6.4. The method of MPM preparation used for this assay was selected because it resulted in the greatest yield of mitogen (measured in µg/ml protein) and required less handling that could have resulted in bacterial contamination of the mitogen than other methods. The glass filter strips on which the triplicate samples were collected were allowed to dry completely to prevent quenching of the soft beta emissions of tritium by water.

Separation medium was tested at several different sp. gr. to determine the sp. gr. giving the best lymphocyte preparations. Separation medium with a sp. gr. of 1.081 ±

0.001 did not result in a statistically significant increase in mean stimulation indices over that obtained with 1.077 ± 0.004 sp. gr. for splenic cell suspensions at the peak MPM dose of 5 µg/ml. Separation medium of sp. gr. of 1.087 ± 0.001 resulted in a significant decrease in stimulation indices, so the commercially available 1.077 sp. gr. Ficoll-Paque was used (Table 10). However, for peripheral blood lymphocytes the greatest stimulation indices were obtained when the sp. gr. of the separation medium was 1.081 (Table 10), even though more cells were collected at sp. gr. 1.087.

Pentobarbital was not used as the anesthetic in these studies because of a report that this anesthetic could decrease mitogenic responses (65). The ketamine/xylazine combination used gave uniformly good anesthesia and recovery with a wide margin of dosage safety from anesthetic overdose. Also, the ratio of ketamine/xylazine could be easily manipulated to give either light, short acting anesthesia for intranasal inoculations in the disease expression studies or deeper, longer lasting surgical anesthesia for splenectomy in mitogenicity assays.

To determine whether or not there was a change in nonspecific lymphocyte responses with increasing age, a mitogenicity assay was done using 3 or 4 male rats/group at 8, 10, 12, and 16 weeks of age. As stated above, this age range was chosen because it encompassed the range commonly used in our mitogenicity assays (8 to 12 weeks). The impression that age affected nonspecific responses was

Table 10. Effect of the Specific Gravity of the Separation Medium on the Results of Lymphocyte Transformation Assays in Rats at Peak MPM Dose[a]

	Medium Specific Gravity		
Source of Lymphocytes	1.077	1.081	1.087
Spleen	8.34[b]	9.33[b]	5.54
Peripheral Blood	3.33	17.60[c]	8.07[c]

[a]Results are expressed as mean stimulation indices at MPM concentration 5 µg/ml; MPM = M. pulmonis mitogen.

[b]Mean stimulation indices for splenic lymphocytes at specific gravity 1.077 and 1.081 are significantly greater than those at 1.087 ($p = 0.019$), but are not different from each other.

[c]Mean stimulation index for peripheral blood lymphocytes at specific gravity 1.081 is statistically greater than that at 1.077, but not that at 1.087 ($p = 0.012$).

demonstrated in this preliminary mitogenicity experiment in which there was a significantly lower mitogenic stimulation index at 8 weeks of age than at any later age tested with both MPM and PHA (Table 11). This relationship also was true for both doses of MPM when data were analyzed as delta CPMs.

Two further mitogenicity experiments were done to confirm these findings. Littermates of these 5-, 8-, 12-, and 20-week-old rats were used in parallel disease expression experiments designed to determine whether or not any relationship that might exist between host age and nonspecific lymphocyte responses would be paralleled by the relationship between host age at time of infection and disease severity. This would strengthen the evidence that there was a relationship between disease severity and nonspecific lymphocyte responses. While the finding of parallel direct relationships would not prove that the factors had a cause and effect relationship, the knowledge gained could indicate a direction in which to steer further studies on the mechanism(s) of increased disease severity in MRM.

However, the preliminary findings were not borne out in subsequent experiments in which no age-related trend in mitogenic response to MPM and no consistent age-related change in mitogenic response to PHA was found when data were analyzed as stimulation indices (Tables 12 and 13). When data were analyzed as delta CPMs, there was no consistent age-related change in response to either mitogen (Tables 12 and 13).

Table 11. Effect of Age on Splenic Lymphocyte Responses in Male F344 Rats-Experiment 1[a]

	Mitogen[b]			
	MPM (μg/ml)		PHA (mg/ml)	
Age (weeks)	2.0	5.0	0.1	0.2
8	2.4[c]	2.9[c]	20.3[c]	10.9[c]
n = 4	1479.8[c]	2039.5[c]	44123.5[c]	69871.8[c]
10	5.9	9.2	69.4	61.9
n = 4	6686.4	11071.0	92356.2	83876.3
12	6.1	8.2	61.2	74.6
n = 3	7696.5	11113.9	90643.9	109623.6
16	5.5	7.7	68.6	86.2
n = 4	5904.3	8674.5	89171.4	112773.0

[a]Results are expressed as mean stimulation indices (upper values) and as mean changes in CPMs (lower values).

[b]MPM = M. pulmonis mitogen, PHA = phytohemagglutinin.

[c]Mean stimulation indices and mean changes in CPMs for 8-week-old rats are significantly less than those of all other age groups for each mitogen and dose ($p = 0.001$).

Table 12. Effect of Age on Splenic Lymphocyte Responses in Male F344 Rats-Experiment 2[a]

Mitogen[b]	Age				
	5 Wk	8 Wk	12 Wk	16 Wk	20 Wk
MPM	13.9[c]	13.6	13.3	12.7	10.8
5 µg/ml	22017.5	32402.2	19989.0	22228.2	25110.4
MPM	15.3	13.4	16.0	14.1	10.3
10 µg/ml	24627.8	34894.7	24584.8	24708.2	23808.8
MPM	11.4	10.3	14.0	11.6	8.9
20 µg/ml	18099.9	26703.5	21531.7	20136.5	20308.9
MPM	7.3	9.2	4.6	5.9	6.7
40 µg/ml	10675.6	8325.0	8105.1	9532.6	11683.7
PHA	20.4	21.1	11.8	22.0	12.6
0.1 mg/ml	14963.6	37193.2	24482.3	40996.6	27984.0
PHA	28.4	21.1	14.8	26.6	13.7
0.2 mg/ml	26369.9	37032.3	28274.1	48383.2	33636.9

[a]Results are expressed as mean stimulation indices and as changes in CPMs; n = 3 for each group.

[b]MPM = M. pulmonis mitogen; PHA = phytohemagglutinin.

[c]There are no significant age-related differences among means for either mitogen at any dose.

Table 13. Effect of Age on Splenic Lymphocyte Responses in Male F344 Rats-Experiment 3[a]

Mitogen[b]	Age				
	5 Wk	8 Wk	12 Wk	16 Wk	20 Wk
MPM	4.5[c]	4.9	3.3	4.4	5.4
5 µg/ml	3084.5	3471.4	2077.0	2485.4	3211.6
MPM	7.5	7.9	6.2	7.1	8.0
10 µg/ml	5874.4	6503.2	4528.5	4387.8	5352.4
MPM	8.9	9.8	7.2	9.0	8.2
20 µg/ml	7134.9	8474.1	5221.6	5957.9	5636.3
MPM	4.8	5.7	4.1	4.4	4.5
40 µg/ml	3359.1	4577.1	2585.2	2336.7	2709.7
PHA	53.2	50.9	52.8	74.0	40.7
0.1 mg/ml	47449.1	48137.3	43952.5	52250.4	31187.4
PHA	49.3	44.7	45.1	88.4	39.5
0.2 mg/ml	44909.1	43228.0	36721.1	61724.0	2910.7

[a]Results are expressed as mean stimulation indices and as mean changes in CPMs; n = 3 for each group.

[b]MPM = M. pulmonis mitogen; PHA = phytohemagglutinin.

[c]There are no age-related significant differences between means for either mitogen at any dose.

Development of a Method for the Study of Adherence of M. pulmonis to Respiratory Epithelium

Because an age-related change in lymphocyte responses to mitogens was not detected, experiments were devised to look for another explanation for the age-associated increase in susceptibility to MRM. The purpose of these experiments was to determine whether or not the increased disease severity and the increased number of CFUs of M. pulmonis in the lungs of 40-week-old rats over that from 8-week-old rats could be related to an increase in adherence of M. pulmonis to respiratory epithelium. Because both environmental ammonia exposure and concurrent Sendai virus infection are known to enhance severity of pulmonary changes in MRM, it was decided to determine whether or not the effect of these factors could be related to increased adherence of M. pulmonis to respiratory epithelium.

I attempted to determine whether or not increased adherence of M. pulmonis could be detected using the tracheal organ culture system developed by Gabridge and Hoglund (73, 76). Modification of the system for use with rats instead of hamsters had already been accomplished by other investigators in our laboratory (168). Attempts were made to radiolabel M. pulmonis with a ^{14}C-L-amino acid hydrolysate (New England Nuclear, Boston, MA) by using a protocol that had been successful for M. G. Gabridge in radioactive labelling of M. pneumoniae (74). One ml of M. pulmonis stock culture ($10^{8.33}$ CFU/ml) was grown in 25 ml of mycoplasma culture medium to which 2.5 ml of the radiolabelled amino acids were added

(specific activity = 0.1 mCi/ml). When the phenol red in the medium indicated that the pH of the medium was decreasing (red beginning to change to orange), Gabridge added fresh medium to permit continued growth of the organism. In this instance, however, additional medium was not added. Instead, the pH was carefully readjusted to 7.8 with sterile 1 M NaOH to allow the mycoplasmas to continue to grow in the radiolabelled medium so as to incorporate as much label as possible while increasing the likelihood of radiolabel incorporation by avoiding dilution of labelled components with additional medium. This pH adjustment was done twice at about 24- to 48-hour intervals as judged necessary by color change in the medium. However, although adequate numbers of organisms attached to the epithelium of tracheal organ cultures as detected by quantitative culture, M. pulmonis did not incorporate sufficient radiolabel to produce disintegrations per minute (DPM) much above the detectable background DPMs. Expressing data as mean incorporation indices, the ratios of the mean DPMs detectable in M. pulmonis attached to epithelium of tracheal organ cultures: the DPMs detectable in uninoculated tracheal organ cultures, the results for three experiments (n = 4 to 5 tracheas/experiment) were 1.72, 1.38, and 1.00. After several such unsuccessful attempts, this approach was abandoned.

At this point, preliminary studies were done to determine the feasibility of using a quantitative culture method for evaluating adherence of M. pulmonis to respiratory epithelium.

Since lag time for proliferation of M. pulmonis strain UAB-5782C is often as short as 4 hours under usual mycoplasmal culture conditions, the duration of adherence studies was planned to be less than 4 hours. Time points of 0.5, 1, 2, and 3 hours were tested in the tracheal organ culture system to determine whether or not proliferation of M. pulmonis occurred within this time span. In these preliminary experiments there was a significant increase ($p = 0.003$) of $10^{0.37}$ to $10^{0.65}$ in recoverable CFUs of M. pulmonis after 1 to 3 hours (Table 14). While this small but statistically significant difference was not thought to be biologically significant, 1 hour was chosen as the time period for adherence experiments in order to avoid any possible confounding effect of this increase in CFUs. Also, organ cultures were maintained overnight before M. pulmonis adherence assays were done to ensure that they were not contaminated with a microorganism that could interfere with M. pulmonis adherence.

To determine whether or not quantitative culture was sensitive enough to detect small numbers of adherent organisms and to determine the saturation dose for the tracheal organ cultures, $10^{4.34}$ to $10^{8.94}$ CFUs of M. pulmonis were placed in tracheal organ cultures of 2 to 5 rats at each dosage point to give actual doses of $10^{3.74}$ to $10^{8.34}$ CFUs/organ culture. M. pulmonis was easily and consistently recovered within this wide dosage range, but saturation was not detected (Table 15). Inasmuch as 100 ml of stock culture had to be centrifuged and

Table 14. Effect of Time on the Numbers of CFUs of *M. pulmonis* Recoverable from Rat Tracheal Organ Cultures[a]

	CFUs Recovered		
	Experiment		
Time (hr)	1	2	3
0.5	5.40 ±0.06 n = 2	---	3.29 ±0.07 n = 3
1	5.79[b] ±0.01 n = 2	5.50[b] ±0.08 n = 3	3.12[c] ±0.01 n = 3
2	5.93[b] ±0.16 n = 3	5.66[b] ±0.09 n = 3	3.62 ±0.09 n = 3
3	---	6.15 ±0.14 n = 3	3.66 ±0.22 n = 3

[a]Results are expressed in $\log_{10}$ CFU.

[b]Mean recoverable $\log_{10}$ CFUs at 1 hour are significantly different from those at 3 hours ($p = 0.001$). Results at 1 and 2 hours are not significantly different from each other.

[c]Mean recoverable $\log_{10}$ CFUs at 1 hour are significantly different from those at 2 and 3 hours ($p = 0.001$).

Table 15. Numbers of CFUs of M. pulmonis Recoverable from Rat Tracheal Organ Cultures after Inoculation of $10^{3.74}$ to $10^{8.34}$ CFUs[a]

Dose of M. pulmonis per Tracheal Culture	Mean CFUs Recovered	Percentage Recovered
3.74	1.72	1.0
4.74	2.52	0.6
5.74	3.74	1.0
6.20[b]	5.27	11.6
6.64[b]	5.52	7.4
7.13	5.94	6.5
8.34	6.98	4.2

[a]Results are expressed as $\log_{10}$ CFU.

[b]Dose chosen for use in tracheal organ culture studies.

resuspended to make 1 ml of inoculum to obtain the highest dose given, and for the next higher dose centrifugation of 1000 ml of stock culture would be required to obtain 1 ml of inoculum, no further efforts were made to achieve saturation. In the dose range used, 1% to 12% of the M. pulmonis organisms placed in the culture system were recoverable by quantitative culture; the dose chosen for use in these studies was that giving the greatest percentage adherence (Table 15). This small percentage of adhering organisms was not unexpected, because adherence of no greater than 10% is usual for M. pneumoniae-inoculated tracheal organ cultures (73).

To determine whether or not the numbers of organisms recovered from the tracheas were adherent or simply retained by capillary action, an experiment was done in which tracheas in organ culture were inoculated with 10^7 CFUs of M. pulmonis and then were quantitatively cultured after being subjected to 1 to 5 thorough washes. Three rats were used for each number of washes of the tracheal cultures. Mean CFUs recovered for 1 to 5 washes did not vary more than $10^{0.14}$ CFUs. This difference was not significant ($p = 0.13$) (Table 16).

The effect of sonication on recovery of M. pulmonis from tracheal organ cultures was investigated. Some investigators working with mycoplasmas recommend sonication of samples to improve the accuracy of quantitative cultures. Sonication separates clumped organisms, thereby permitting the formation of colonies by more individual mycoplasmas. In adherence studies, sonication of tracheal homogenates did not increase

Table 16. Effect of Number of Washes on the Numbers of CFUs of M. pulmonis Recovered from Tracheal Organ Cultures[a]

Number of Washes	CFUs Recovered
1	5.59 ±0.12 n = 3
2	5.73 ±0.04 n = 3
3	5.71 ±0.11 n = 3
4	5.59 ±0.02 n = 3
5	5.61 ±0.04 n = 3

[a]Results are expressed as $\log_{10}$ CFU. Numbers of CFUs recovered were not significantly different after 1 to 5 washes.

consistently the numbers of CFUs recovered from the tracheas. Mean CFUs recovered from 8-week-old male F344 rats were $10^{4.45}$ with sonication and $10^{3.85}$ without. For 40-week-old male F344 rats these numbers were $10^{3.47}$ with sonication and $10^{3.73}$ without. These differences were not statistically significant. In addition, there was the possibility of sonication killing mycoplasmas, especially those recently thawed, causing erroneously low colony counts. Therefore, sonication was not done in these experiments. Tissue culture medium was used in the homogenization and dilution steps of these experiments because it did not alter numbers of recoverable CFUs from that obtained by use of mycoplasma culture medium, and it was more readily available. In the trimming process the ends of the tracheas were removed to eliminate nonspecific attachment of M. pulmonis to exposed connective tissue, a confounding factor in quantitative culture.

To investigate the possibility that nonviable organisms from the frozen stocks might be adhering to the epithelium in tracheal organ cultures, a tracheal organ culture experiment using freshly grown M. pulmonis organisms was done. Because it is usual to lose one logarithmic unit of organisms after freezing, and because viability is essential for detection of organisms by quantitative culture, but not necessarily for adherence, these nonviable organisms could, by adhering, mask any age-associated effects. In this experiment the mean recoverable CFUs for 8-week-old rats were $10^{5.64}$ with frozen organisms ($n = 5$) and $10^{6.24}$ with freshly grown ones ($n = 4$).

For 40-week-old rats the mean recoverable CFUs were $10^{5.69}$ with frozen organisms (n = 5) and $10^{6.39}$ with fresh ones (n = 4). Although the mean recoverable CFUs increased by a factor of $10^{0.65}$ with freshly grown M. pulmonis, the freshly grown stocks contained a factor of $10^{1.42}$ more CFUs/ml than the frozen stocks. The percentage of adherent organisms was about 1% for freshly grown organisms, and about 6% for frozen stocks for both 8- and 40-week-old rats. Thus, when differences in numbers of CFUs/ml recovered were evaluated with differences in numbers of CFUs/ml in fresh and frozen stock cultures taken into account, the mean numbers of CFUs attached actually were slightly less for fresh than for frozen stocks by a factor of $10^{0.09}$ to $10^{0.22}$. There were no significant age-related differences in results whether the M. pulmonis stocks used had been frozen or freshly grown ($p = 0.16$).

Effect of NH_4Cl, Sendai Virus Infection, and Host Age at Time of Infection on Adherence of M. pulmonis to Respiratory Epithelium

Previous studies in our laboratory had shown that exposure to ammonia and concurrent Sendai virus infection strongly exacerbate MRM (17, 197, 198). It seemed likely that these two agents, which cause epithelial damage, would affect M. pulmonis attachment and, thus, might serve as positive controls for testing the effect of age. In addition, these agents are important factors in expression of natural MRM.

In these experiments changes in M. pulmonis adherence with age of the host were first tested in experiments of 2 X 2 factorial design. In later experiments M. pulmonis adherence

was tested in a 3-factor experimental design against the variables of host age and either presence of ammonia in the medium or concurrent Sendai virus infection. Three or four rats/group at 8 and 40 weeks were used in each tracheal organ culture studies. The ages were chosen to match those in the disease-expression studies. Also, it was determined that unless the trachea culture blocks were altered, it was not feasible to use rats less than 7 to 8 weeks of age because their tracheas were too short to fit snugly against the Teflon plugs in the organ culture blocks.

For adherence of M. pulmonis in the presence of concurrent Sendai virus infection, tracheas were collected for use in organ cultures 5 days after intranasal inoculation of Sendai virus, because this was a previously reported peak response time for Sendai viral changes in the respiratory tract (29) and was the time span used in previous Sendai virus experiments in our laboratory (168). For ammonia-exposure studies, the maximum concentration of NH_4Cl that would allow survival of the tracheal epithelium had been determined to be about 50 mM by previous work done in this laboratory by Dr. D. M. Pinson (170).

The mean numbers of CFUs of M. pulmonis recovered from tracheal organ cultures of 8- and 40-week-old rats were not significantly different when the means were analyzed as number of CFUs/standard (8 mm) length of trachea (Table 17). In the experiments in which the results were significantly different, the mean numbers of CFUs had been analyzed according to area,

Table 17. Effect of Age at Time of Inoculation on Numbers of Recoverable CFUs of M. pulmonis from Tracheal Organ Cultures[a]

Experiment	Age (wks)	Mean CFUs/ 8 mm	Mean CFU/sq mm	n
1	8	4.88	3.13	3
	40	5.00	3.04	3
2	8	3.85	2.09	3
	40	3.73	1.77	5
3	8	5.17	3.42	5
	40	5.18	3.22	5
4	8	5.28	3.52[b]	5
	40	5.33	3.36	5
5	8	5.64	3.89	5
	40	5.69	3.73	5
6	8	6.24	4.49	4
	40	6.39	4.41	4
7	8	5.63	3.87	3
	40	5.79	3.82	3
8	8	6.25	4.50	3
	40	6.38	4.42	3

[a]Results are expressed as $\log_{10}$ CFU. There are no significant age-related differences between mean recoverable CFUs of M. pulmonis when results are evaluated by standard tracheal length.

[b]The only experiment in which the numbers of M. pulmonis CFUs recovered were different between the two age groups was experiment 4.

and 8-week-old rats had more recoverable mean CFUs than 40-week-old rats. The means were different by a factor of $10^{0.08}$ to $10^{0.32}$ (Tables 17 and 18). These slight differences were of questionable biological significance. However, when ammonium ions were present in the medium, this effect was more pronounced. In these experiments, 8-week-old rats had more recoverable mean CFUs of M. pulmonis than 40-week-old rats by a factor of $10^{0.31}$ to $10^{1.2}$ (Table 18). Increases in this range are more likely to be biologically significant.

In addition, in 40-week-old rats, mean CFUs of M. pulmonis recovered from tracheal organ cultures exposed to ammonium ions were consistently significantly fewer by a factor of $10^{0.23}$ to $10^{0.67}$. This phenomenon was observed whether the results were analyzed by numbers of CFUs/standard length of trachea or by numbers of CFUs/per tracheal mm^2 (Table 18).

In studies testing the effect of concurrent Sendai virus infection on adherence of M. pulmonis to tracheal epithelium, there was no significant difference in mean numbers of recoverable CFUs between 8- and 40-week-old rats (Table 19). There were, however, statistically fewer mean recoverable CFUs of M. pulmonis from tracheal organ cultures of rats infected with the virus. The significant differences ranged from a factor of $10^{0.75}$ to $10^{0.96}$ and, thus, were 0.75 to 1 logarithmic unit greater for tracheas from uninfected rats (Table 19). This occurred in both 8- and 40-week-old rats whether the results were evaluated by standard tracheal length or by area. Preliminary data from another investigator in our laboratory

Table 18. Effect of Ammonium Ions on Numbers of Mean Recoverable CFUs of M. pulmonis in Tracheal Organ Cultures[a]

Experiment	Age in Weeks	Mean CFUs/ 8 mm without ammonia	n	Mean CFUs/ 8 mm with ammonia	n
1	8	3.85	3	4.05	5
	40	3.73	5	3.06	3
2	8	5.64	5	5.56	5
	40	5.69[b]	5	5.46[b]	5
3	8	6.24	4	6.38	4
	40	6.38[b]	4	6.07[b]	4

Experiment	Age in Weeks	Mean CFUs/ sq mm without ammonia	n	Mean CFUs/ sq mm with ammonia	n
1	8	2.09	3	2.30	5
	40	1.77[c]	5	1.10[c]	3
2	8	3.89	5	3.81	5
	40	3.73[b,c]	5	3.50[b,c]	5
3	8	4.49	4	4.63	4
	40	4.41[b,c]	4	4.11[b,c]	4

[a]Results are expressed as $\log_{10}$ CFU.

[b]In tracheas prepared from 40-week-old rats, significantly fewer CFUs were recovered from tracheas exposed to ammonium chloride than from control tracheas, whether results were standardized according to tracheal length or to tracheal area.

[c]Tracheas from 40-week-old rats had significantly fewer CFUs than tracheas from 8-week-old rats, when the results were standardized according to tracheal area.

Table 19. Effect of Concurrent Sendai Virus Infection on Numbers of Mean Recoverable CFUs of M. pulmonis in Tracheal Organ Cultures[a]

Experiment	Age in Weeks	Mean CFUs/ 8 mm without virus	n	Mean CFUs/ 8 mm with virus	n
1	8	5.63	3	5.33	3
	40	5.79[b]	3	5.03[b]	3
2	8	6.25[b]	3	5.74[b]	4
	40	6.38[b]	3	5.96[b]	4

Experiment	Age in weeks	Mean CFUs/ sq mm without Virus	n	Mean CFUs/ sq mm with Virus	n
1	8	3.87	3	3.58	3
	40	3.82[b]	3	3.07[b]	3
2	8	4.50[b]	3	3.98[b]	4
	40	4.42[b]	3	3.99[b]	4

[a]Results are expressed in $\log_{10}$ CFU.

[b]Significantly fewer CFUs of M. pulmonis were recovered from tracheas from rats with Sendai virus infection than from control tracheas in both age groups, whether results were standardized according to tracheal length or to area.

supports this finding in that fewer M. pulmonis CFUs also attached to tracheas of rats infected with SDAV, another agent that exacerbates MRM, than to control tracheas.

DISCUSSION

Although it previously had been proposed that rats become more susceptible to MRM with age, this proposition was based on clinical impressions or incidental findings rather than experimental evidence. This is the first study specifically designed to determine whether or not severity of MRM varies with host age at the time of infection. The results showed conclusively that more severe bronchopulmonary lesions developed in rats infected when 20 weeks old and older than in rats infected when 8 weeks old. Lesions in the nasal passages, middle ears, larynges, and tracheas, however, did not differ. Experiments testing the effect of age on possible mechanisms of the increased susceptibility to lung lesions did not support a role either for changes in nonspecific lymphocyte responses to M. pulmonis or for increased adherence of the organism to respiratory epithelium.

One proposed mechanism of host injury in mycoplasmal infections is nonspecific activation of lymphocytes by mycoplasmal membrane components. The activated cells could damage host cells and tissues by secreting toxic lymphokines, by activating macrophages to secrete injurious lysosomal enzymes or other substances, or by disrupting immune regulation. Although the importance of nonspecific lymphocyte activation in mycoplasmal disease has not been definitely

established, there is a modest amount of evidence supporting a pathogenetic role. First, a correlation exists between polyclonal lymphocyte responses and disease severity in resistant F344 rats, whose lymphocytes respond relatively weakly to mitogens, and susceptible LEW rats, whose lymphocytes respond more strongly (52, 53). Second, mitogenic membrane preparations of M. pulmonis caused interstitial pneumonia in rats (146). Third, nude mice with M. pulmonis infection tend to have less severe pulmonary disease than normal immunocompetent mice (22). Finally, in instances of spontaneous or induced immunosuppression there is a decrease in pulmonary manifestations of M. pneumoniae disease (67, 211).

Using two different approaches, I attempted to obtain more direct experimental support for a pathogenetic role of nonspecific lymphocyte responses. The first approach was to determine whether or not lymphocyte responses and susceptibility to MRM followed the same pattern of inheritance by assessing lymphocyte mitogenicity and susceptibility to MRM in F1 and F2 hybrids of F344 and LEW rats. In contrast to previously reported findings (52, 53), however, results of these experiments consistently failed to indicate a difference in lymphocyte responsiveness between the two strains. The reason or reasons for the lack of agreement with previous results was not apparent, and various manipulations of the assay protocol did not alter the results. A partial explanation could be that variability among animals and among

individual experiments in results of lymphocyte mitogenicity assays masked any genetic sorting patterns that may have been present. In addition, the substrain of LEW rats used by others (52, 53) was no longer available, and it is possible that, due to minor genetic differences or unrecognized variations in microbial flora, the responses of the substrain of LEW rats I used differed from those of the previously used substrain (106, 229). Furthermore, a different lymphocyte culture system was used in the previous study (53).

The second experimental approach to testing the link between nonspecific lymphocyte responses and disease expression was to determine whether or not lymphocyte mitogenic responses and MRM lesion severity correlated directly with the age of the host at the time of infection. To diminish animal-to-animal variability, I used littermates within each age group for both mitogenicity assays and lesion evaluations, and to diminish experiment-to-experiment variability, I used sufficient animals in each age group to evaluate each experiment separately. Nonetheless, a correlation between polyclonal responses and increasing age was not evident. These results could indicate that polyclonal responses do not have an important role in the age-related increase in MRM lung disease severity. However, it is possible that in these studies, variability in assay results prevented a relationship from being detected. A possible source of variability in mitogenicity assay results that was not recognized until some time after these experiments were

done is release of regulatory substances occurring within short time spans after cells are removed from the animal. It is likely that the attempt to use larger numbers of rats in each age group in each experiment to avoid the effect of experiment-to-experiment variability was a serious confounding factor contributing to within-experiment variability, because it increased the length of time required to process the cells from all of the rats. It recently was demonstrated that F344 rat spleen cells spontaneously can produce a substance that inhibits both B and T cell responses, even if the cells are kept on ice during collection and processing (S. E. Ross, Ph.D. dissertation, University of Alabama at Birmingham, 1988). Because cells from some rats were processed much less rapidly than those from others, there was ample time for production of regulatory substances in some cultures. The finding that responses of pooled samples of peripheral blood lymphocytes tended to be similar to the lowest responses of cells from individual rats supports this possibility. On the other hand, polyclonal lymphocyte responses also did not correlate with expression of acute MRM induced with various strains of M. pulmonis in C3H/HeN mice (48). In sum, it appears that although some evidence suggests that the host's response to the mitogenicity of M. pulmonis could be a pathogenetic factor, it probably is less important than other mechanisms and probably does not contribute to increased severity of lung lesions in older rats. The finding that lesions differed with age only in the lungs suggests that

perhaps nonspecific pulmonary defenses against M. pulmonis, such as intrapulmonary killing or mucociliary transport, could deteriorate with age. In addition, it is known that specific immune responses decline with age in rats (14); thus, it also is possible that specific anti-M. pulmonis immune responses could be weakened in older rats, although no evidence to support this has been reported. These possibilities should be addressed in future studies, and the relationships between age and nonspecific lymphocyte responses and between host genotype and lymphocyte responses should be re-examined in studies with appropriate controls for spontaneous induction of suppressor factors.

Adherence to epithelial cells is an important virulence factor for many bacteria; it allows them to resist mucosal cleansing mechanisms such as the flow of secretions and luminal contents, swallowing, coughing, and sneezing. Most pathogenic mycoplasmas are parasites of the surface of host epithelial cells, and their survival, as well as their pathogenic potential, probably is intimately related to organism-host cell surface interactions (38, 39, 73, 98, 112, 133, 203). Although most of the studies concerning the role of adherence in mycoplasmal disease have been done with M. pneumoniae, there is evidence that adherence also is a virulence factor for M. pulmonis. Correlation between loss of adherence to epithelium and loss of ability to cause pneumonia or genital infections in mice has been demonstrated for the JB strain of M. pulmonis (212, 213). M. pulmonis

strain PG34(ASH) is avirulent; it neither attaches to respiratory epithelium nor causes pneumonia in mice (46). Because in my experiments older rats had larger numbers of CFUs of M. pulmonis/g of pulmonary tissue, it seemed likely that increased adherence to host respiratory epithelium could be at least a partial explanation for the increase in pulmonary lesions with increasing age. However, no increase with age in adherence of M. pulmonis to tracheal epithelium was evident in in vitro experiments. Nor was there an increase in adherence with exposure to ammonia or with concurrent Sendai virus infection, two factors known to increase severity of expression of M. pulmonis infection in rats (17, 132, 197, 198). In fact, when tracheas were evaluated by area, the older rats had slightly fewer CFUs/mm^2 than did younger rats in several experiments, and the effect was somewhat greater in the presence of ammonium ions and Sendai virus infection. Thus, increased disease severity associated not only with age but also with ammonia exposure and Sendai virus infection probably is not related to increased adherence of M. pulmonis to respiratory epithelium. However, it is possible that adherence of M. pulmonis in vivo, in which attachment occurs at a tissue-air interface, is not duplicated in tracheal organ cultures in which attachment takes place at a tissue-fluid interface. Also, any contribution to attachment made by the mucus layer will not be detected in tracheal organ cultures, because mucus is washed from the tracheal mucosa by the continuous movement of

the organ culture medium across the surface of the tracheal epithelium. Because adherence is not dependent upon organism viability, nonviable organisms attaching to the respiratory epithelium might have masked age-associated changes; however, use of freshly grown M. pulmonis did not alter the results. Another factor may be that quantitative cultures may not be sensitive enough to detect small differences in attachment. A technique which positively identifies attaching organisms but which does not depend upon ability of the organism to form a colony, would be useful in confirming the results of these studies. For this reason, efforts should continue to develop a radiolabelling method or an immunohistochemical assay for M. pulmonis, such as an avidin-biotin technique, that could to be used in conjunction with computer morphometry or autoradiography to assess attachment. Further efforts also should be made to evaluate attachment in vivo, which could be done in experiments measuring pulmonary clearance of radiolabelled M. pulmonis as has been described for studies in mice (46).

SUMMARY

MRM, caused by M. pulmonis, is an important disease of laboratory rats and mice because of its insidious nature and its ability to confound the results of studies in respiratory histology, physiology, immunology, aging, and carcinogenicity. Although MRM has been recognized since the early 1900s, and Koch's postulates were fulfilled with M. pulmonis in the early 1970s, its pathogenesis remains poorly understood. Disease expression varies from mild rhinitis and otitis media to severe disease of the entire respiratory tract, including bronchopneumonia characterized by perivascular, peribronchial, and peribronchiolar lymphoid cell accumulation. Disease expression is strongly influenced by environmental, host, and organism factors and by interactions among these factors. Environmental factors known to promote MRM disease expression include exposure to ammonia from soiled cage bedding, deficiencies of vitamins A and E, and ingestion of hexamethylphosphoramide. Host genotype and concurrent viral infections of the respiratory tract also are known to affect disease expression.

Several investigators have, in the course of studying other aspects of MRM, reported that disease expression was influenced by age of the host. Experiments were done to test the hypothesis that MRM in rats increases in severity as the

age of the host at the time of infection increases. M. pulmonis was inoculated intranasally into 5-, 8-, 12-, 20-, and 40-week-old rats, which were then exposed to a uniform concentration of environmental ammonia for 28 days. At that time the rats were killed. Changes in the respiratory organs were evaluated by a semiquantitative scoring system, and the numbers of M. pulmonis in each organ were determined by quantitative culture. The results of these experiments demonstrated conclusively that older rats (20 and 40 weeks old) are significantly more susceptible to pulmonary disease than rats in younger age groups. Also, in experiments in which quantitative cultures were done, the mean CFUs of M. pulmonis recovered from the lungs of 40-week-old rats were significantly greater than those from the 8-week-old rats. Age-related differences in lesions did not occur in nasal passages, middle ears, or tracheas, and mean CFUs of M. pulmonis in nasal passages and tracheas did not differ between 8- and 40-week-old rats.

The ability of M. pulmonis to stimulate lymphocytes nonspecifically is thought to be a factor in the pathogenesis of MRM, and lymphocyte responses are known to change with age. To test whether or not there was a correlation between disease expression and nonspecific lymphocyte responsiveness, lymphocyte mitogenic responses to M. pulmonis were examined in MRM-resistant F344 rats and MRM-susceptible LEW rats and in hybrids of these strains. No difference in nonspecific lymphocyte responsiveness between the two strains was found.

I also tested whether or not there was a relationship between host age and the degree of nonspecific lymphocyte transformation induced by M. pulmonis in splenic lymphocytes from rats at 5, 8, 12, 16, and 20 weeks of age. No correlation between host age and splenic lymphocyte responsiveness to MPM was detected. Failure to find relationships of lymphocyte responses to either age or disease expression could have resulted from the variability inherent in the mitogenicity assays, from differences in LEW rat subline from that used previously, from technical differences in the mitogenicity assay, or from spontaneously produced suppressor substances.

In a final set of experiments, I tested the hypothesis that factors that enhance MRM disease expression do so by increasing the adherence of M. pulmonis to respiratory epithelium. Host age at time of infection, exposure to ammonia, and concurrent Sendai virus infection were the factors selected. The influence of each of these factors on adherence was tested in tracheal organ cultures, in which the numbers of M. pulmonis adhering to tracheal epithelium were determined by quantitative culture. No increase in adherence of M. pulmonis was detected with any of the factors studied.

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