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PORCINE IMMUNE SYSTEM

Iowa State University

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Interactions between Mycoplasma hyopneumoniae
and the porcine immune system

by

Serge Jacques Messier

A Dissertation Submitted to the
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GENERAL INTRODUCTION

Mycoplasmal pneumonia of swine (MPS) is a chronic respiratory disease of pigs characterized by a nonproductive cough, retarded growth and poor feed utilization. The etiological agent of the disease is Mycoplasma hyopneumoniae (syn. Mycoplasma suis pneumoniae) (1,2). The disease has a worldwide distribution and is an important economic burden on the swine industry, especially in countries with intensive rearing practices. Serological studies have revealed that antibodies to M. hyopneumoniae were present in 60% of Iowa swine herds (3). Lesions similar to those of MPS have been found in 45% to 75% of slaughtered pigs (4,5).

Attempts to control MPS by inducing immunity to M. hyopneumoniae have given positive (6,7,8,9,10,11) and negative (12,13,14) results. Both cell-mediated and humoral immune responses to the organism developed following vaccination (13,14) and experimental infection (15), but the exact roles of these immune responses in protection or pathogenesis of MPS have not been determined. Studies conducted in other animal species with mycoplasmal pneumonias similar to MPS indicated that the local humoral response was important in control of the disease (16,17,18,19).

Apart from the presence of glycocalyx-like material on the surface of virulent M. hyopneumoniae (20) and the in vitro cytopathic effect exhibited by M. hyopneumoniae membranes (21) no other pathogenicity factor has been associated with M. hyopneumoniae. Various nonspecific interactions between mycoplasmas and lymphocytes have been reported and their possible roles as factors in development of lesions in mycoplasmal

diseases investigated (22,23,24,25,26,27).

In an effort to help elucidate the pathogenesis of MPS, the following approach was taken to study interactions between M. hyopneumoniae and the porcine immune system: i) the nonspecific mitogenic activity of M. hyopneumoniae membranes for swine lymphocytes was evaluated; ii) membrane preparations of M. hyopneumoniae given intratracheally to pigs were tested for their ability to either induce lesions of pneumonia similar to those seen in natural cases of MPS, or to induce some protection against subsequent challenge with live M. hyopneumoniae; iii) the systemic and local humoral and cell-mediated immune responses of pigs following experimental infection with M. hyopneumoniae were monitored in order to compare them with the effects of M. hyopneumoniae membranes on the immune system.

This dissertation was written in the alternate format. It consists of a general introduction, a review of the literature, three separate manuscripts, and a general discussion. The references cited in the introduction, literature review, and the general conclusion are listed following the general discussion. Each separate manuscript is written according to the guidelines of the American Journal of Veterinary Research. A list of references cited in each manuscript follows the manuscript. The doctoral candidate, Serge Messier, was the principal investigator and is the senior author of each manuscript. Each manuscript will be submitted to the American Journal of Veterinary Research.

LITERATURE REVIEW

The peribronchiolar and perivascular lymphoid hyperplasia observed in microscopic lesions of MPS and other similar mycoplasmal pneumonias occurring in various animal species strongly suggests that the immune system is involved in the development of lesions. This involvement may lead to the mounting of a specific immune response, cell-mediated, humoral, or both, against the organism involved. The mycoplasmas have evolved various virulence factors that may facilitate colonization of the respiratory tract. The motility (28) and attachment properties (29,30,31) of Mycoplasma pneumoniae, Mycoplasma pulmonis, and Mycoplasma gallisepticum could help these organisms penetrate the mucus layer covering the bronchial epithelium and allow them to attach to the epithelial cells. Ciliostatic effects observed in vitro in tracheal organ cultures infected with M. pneumoniae (32), Mycoplasma dispar (33), or M. pulmonis (34) would be an important virulence factor if expressed in vivo. A cytopathic effect was produced in cell cultures by Mycoplasma bovis (35) and Mycoplasma hyopneumoniae (21) but the mechanism leading to cell damage is unknown. Hemolysins are produced by many mycoplasmas and in some cases the hemolysin has been found to be hydrogen peroxide; the in vivo role of these cell products is still obscure (36). The presence of glycocalyx-like material has been demonstrated on M. dispar (37), M. pulmonis (30), and M. hyopneumoniae (20) by ruthenium red staining. The role of capsular substance as an anti-phagocytic virulence factor in bacteria is very well-documented. Development of autoantibodies against red blood cells (cold agglutinin), nervous

and cardiac tissues following M. pneumoniae infection has been linked to similarities in membrane components of the mycoplasma and host tissues (38,39). These similar components may permit escape from the immune response or induce autoimmune diseases.

In the absence of specific antibodies, mycoplasmas can survive quite well in cultures of alveolar macrophages (40,41,42,43), as well as in the presence of neutrophils (41,44) but addition of specific antisera will lead to ingestion of the mycoplasmas. This ability to survive could well be of importance in establishment of infection in unsensitized animals.

Mycoplasmas have been found to interact nonspecifically with lymphocytes and induce several responses. The nonspecific interactions, or their consequences, between mycoplasmas and lymphocytes might modulate the immune system in such a way that it results in development of lesions. It is quite possible also that both specific and nonspecific interactions between mycoplasmas and the immune system are involved in the development of lesions.

Mycoplasma-Lymphocyte Interactions

Mitogenic activity of mycoplasmas

Ginsburg and Nicolet (22) reported in 1973 that a mycoplasma species, M. pulmonis, induced blastogenesis of rat lymphocytes in vitro. The transforming activity of the mycoplasmas was believed to be nonspecific. Additionally, the transformation factor was found to be heat labile (56°C, 30 minutes), and was associated with the organisms because it was lost after filtration of the culture fluid. Growth of the mycoplasmas in the

lymphocyte cultures was necessary for the transforming activity to be expressed.

Following this report, numerous species of Mycoplasma were tested in order to determine if they possessed mitogenic properties. M. pneumoniae was found to be mitogenic for mouse (24,45), guinea pig (24), and human lymphocytes (46). Rat (47,48,49) and mouse (45,47) lymphocytes were stimulated by M. pulmonis; Mycoplasma neurolyticum could also stimulate lymphocytes of rat (48,50) and mouse (47,51) origin. Mycoplasma arthritidis induced transformation of lymphocytes obtained from rats (48,52,53) and humans (52,54). Acholeplasma laidlawii was found to stimulate murine lymphocytes (45,55). Mycoplasma fermentans was mitogenic for mouse (45) as well as human (46) lymphocytes. Other species reported to have mitogenic activity for mouse lymphocytes included: Mycoplasma canis, Mycoplasma felis, M. gallisepticum, Mycoplasma synoviae, Mycoplasma hyorhinis, Spiroplasma citri, Mycoplasma sp. GS-1, Mycoplasma hyosynoviae, Mycoplasma hominis, and Mycoplasma arginini (45).

The mitogenic activity exhibited by the numerous mycoplasma species mentioned was associated with live mycoplasmas (48), nonviable organisms (45,55), suspensions of disrupted organisms obtained by ultrasonication (24,45,46) or by osmotic lysis (47,48,49,50,51), and culture supernate (52, 53,54). Membrane preparations of mycoplasmas were found to stimulate lymphocytes to the same extent as live cultures of the organisms (48).

Either T- or B-lymphocytes or both are stimulated by mycoplasmas indicating heterogeneity exists in mitogenic activity produced by the various mycoplasmas species. Guinea pig (24) and human (46) T- and

B-lymphocytes were both activated by M. pneumoniae. Stimulation of T- and B-lymphocytes also occurred when cell preparations of rat and mouse origin were exposed to M. pulmonis (47,49). Activation of human T- and B-lymphocytes by M. fermentans was also reported (46). Selective stimulation of B-lymphocytes was noted in rat and mouse cells exposed to M. neurolyticum (47,50,51), and in mouse lymphocytes stimulated by A. laidlawii (45,55). On the other hand, human and murine T-lymphocytes were stimulated by M. arthritidis (53,54).

Heat-treatment of the various mitogens, at 56°C for periods of time ranging from 0.5 to 2 hours, resulted in loss of the mitogenic activity (53,54,56), no alteration of the mitogenic activity (24,45,46), or expression of heat-resistant mitogen following inactivation of an inhibitory factor (45). The nature of this inhibitory factor was not further documented.

The mitogenic activity exhibited by the mycoplasma species mentioned previously was found to result from nonspecific stimulation of lymphocytes and meant that the positive response was not dependent on previous sensitization to antigen. This was confirmed by using experimental subjects without serological evidence of previous infection caused by the mycoplasma species tested (22,45,46,52,53) or specific-pathogen free animals (24,47,48,49,50,51,53) and getting positive stimulation. On the other hand, activation of human lymphocytes by A. laidlawii could not be ruled out as antigen specific stimulation because the immune status of the individuals tested was unknown (55).

Some responses of the immune system to stimulation are known to be

under control of genes encoded within the major histocompatibility complex (MHC) (57). Regulation by the MHC was believed to occur in the response of murine (53) and human (54) lymphocytes to stimulation by M. arthritidis, as well as in the transformation of murine splenic lymphocytes by M. hyorhinis (58). Differences noted in susceptibility of 2 different strains of rats to M. pulmonis infection, or lymphocyte responsiveness to stimulation by M. pulmonis were not explained (59,60,61). Although a genetic mechanism probably regulated the strain susceptibility noted, dissimilarity in number of T-helper lymphocytes was mentioned as a factor that might influence differences in susceptibility to disease (62).

The biochemical composition of the mitogenic factor(s) associated with the various mycoplasma species mentioned above is in most cases not well-defined. It was clearly demonstrated (56) that the mitogen present in membrane preparations of M. pulmonis was proteinaceous in nature, and exposed on the outer surface of the membrane. Recent data (47), indicated that carbohydrate-containing membrane components of M. pulmonis might also be involved in the mitogenic activity. In the same study (47) it was reported that M. neurolyticum mitogen was present in the membrane of the organism and composed mainly of substances containing carbohydrates. Membrane proteins and lipids were also found to have some mitogenic activity. Indirect evidence suggests that the mitogen present in culture supernate of M. arthritidis is protein or contains protein, since heating the supernate at 56°C for 60 minutes abrogated the mitogenic effect (53,54). Heat-resistance of mitogens from M. pneumoniae (24,46), M. fermentans (46), A. laidlawii (55), M. hyosynoviae (45), M. hominis (45),

and M. arginini (45) indicates that proteins are not present, or are unimportant in the make-up of the mitogenic factors of these species.

Evidence that the stimulatory effect of mycoplasma mitogen observed in vitro correlates with involvement of the mitogen in the pathogenesis of the corresponding disease has been reported only for M. pulmonis. Rats inoculated intranasally with membrane preparations of M. pulmonis, known to be mitogenic for rat lymphocytes, developed tracheitis and interstitial pneumonia (63). Lung lesions were similar to those developing during active infection with the organism and initially consisted of alveolar thickening and edema, interstitial infiltration of lymphocytes, macrophages, and plasma cells; followed by peribronchiolar and perivascular lymphocytic cuffing and lymphocytic nodule formation. It was thus concluded that mitogenicity of M. pulmonis contributed to production of pneumonia in rats.

The identical nature of the mitogenic factor(s) and the lesion inducing factor(s) has not been proven. But, decrease in the lesion inducing capacity (63), and mitogenic activity (56) of M. pulmonis following heat-treatment or proteolysis of membrane preparations would suggest that they are related.

Polyclonal activation of antibody production

In addition to inducing blastogenesis, measurable by incorporation of radioactive nucleotide or enumeration of blast cells, a few mycoplasma species have also been found to induce polyclonal antibody production, resulting from the transformation of B-lymphocytes into plasma cells. The transformation of B-lymphocytes into antibody producing cells occurred

with (24,46,49,50) and without (64) synthesis of DNA. Detection of antibody producing cells, done by a modified haemolytic plaque assay, revealed that lymphocytes isolated from various anatomical sites or animal species were induced by M. pneumoniae (24,64), M. fermentans (46), M. pulmonis (49), and M. neurolyticum (50). Also reported was the fact that human lymphocytes exposed to killed suspensions of M. pneumoniae started producing antibodies to measles, rubella, and/or herpes simplex virus (65). The phenomenon was believed to be T-cell dependent and probably resulted from the release of nonspecific helper factors by M. pneumoniae stimulated cells.

Mycoplasma capping by lymphocytes

In studies of murine lymphocytes culture infected with M. hyorhinitis the organism has been found to selectively acquire host cell membrane antigens (23). Association of M. hyorhinitis with the surface of cells was demonstrated by complement-dependent cytotoxicity (23), and by immunofluorescence assays (66). Mycoplasma hyorhinitis acquired host-membrane antigens following capping and shedding from the surface of the lymphocytes (23,66,67,68). Host antigens identified on the surface of M. hyorhinitis after infection of lymphocytes included Thy-1 antigen (23,67,69), H-2^k antigen (23,67), but not gp70 antigen (67). The possibility that some antigens act as receptors for M. hyorhinitis was mentioned (23). Thy-1 bearing cells were noted to be heavily infected by M. hyorhinitis after 24 hours while cells not possessing Thy-1 antigen were only lightly infected after 72 hours (58).

Capping of mycoplasmas from lymphocytes generally led to blast

transformation in these cells (58,66,68). The consequences of non-specific stimulation of lymphocytes would depend on the type and sub-population of lymphocytes activated.

Besides capping of host cell antigen, translocation of lipid molecules from the host membrane to M. hyorhinis could be demonstrated (69). Both capping and translocation of host molecules to mycoplasmas might induce modifications in the composition and conformation of the host membrane and possibly lead to immunopathologic disease. Another possible mechanism leading to immunopathologic manifestations could be the presentation of modified host membrane antigens to the immune system which recognizes them as foreign.

Induction of interferon production

The first successful in vitro induction of interferon production by mycoplasmas was obtained when ovine leukocytes growing in mixed culture with fetal lamb kidney cells produced substantial levels of interferon, which were attributed to infection of the kidney cells by A. laidlawii (25). Eleven other species of mycoplasmas were also capable of inducing interferon production in ovine leukocyte cultures (70). Live organisms were required for interferon production to occur (25,70).

Interferon was produced by ovine lymphocytes, but not by polymorphonuclear cells (PMN), following in vitro exposure to M. pneumoniae, A. laidlawii, M. arthritidis, and M. pulmonis (71); it was also found that human lymphocytes produced interferon following exposure to M. pneumoniae (71,72) and M. synoviae (71). Interferon production was not detected in

murine peripheral leukocyte, spleen cell, and peritoneal cell cultures infected with A. laidlawii, M. pneumoniae, or M. arthritidis (73); but M. arginini was found to cause interferon production in murine spleen cell cultures and the data suggested that interferon was produced by B-lymphocytes (74). Human tumor cell lines known to be producing interferon stopped doing so as soon as they were rid of a Mycoplasma orale 1 infection (75). Reinfection of the cell lines with the same organism restored interferon production.

Peak serum levels of interferon were detected 6 hours following intraperitoneal injections of mice with A. laidlawii, M. pneumoniae, or M. arthritidis (73); but other investigators (72) failed to do so after intraperitoneal inoculation of M. pneumoniae.

The potency of mycoplasmas as an interferon inducer in vitro was found to be less than that of bluetongue virus (25,70,71), but comparable to that of Newcastle disease virus (72). In vivo studies revealed that Newcastle disease virus was a more potent interferon inducer than M. arthritidis (73).

Interferon induced by mycoplasmas has had no deleterious effect on the organisms (25,70,72), and in fact growth of the mycoplasmas, up to optimal levels, generally resulted in increasing titers of interferon (25,70).

Cole and Thorpe (76) have indicated that, according to the characteristics of the interferon produced in early studies (25,70,71,72,73,74,75) it was most probably interferon α . Mitogen(s) present in culture supernate of M. arthritidis induced human lymphocytes to produce interferon γ (76); while hydrogen peroxide released by M. pneumoniae cocultured with human

lymphocytes was responsible for production of β and γ interferon (77).

Other responses

Recently, new evidence of interaction between mycoplasmas and lymphocytes has been presented. It was found that rat lymph node lymphocytes stimulated by M. pulmonis membranes produced significant levels of interleukin-2 (IL-2), while M. neurolyticum stimulated cultures contained negligible amounts of IL-2 (27). IL-2 is produced by T-lymphocytes following antigen specific interaction, or stimulation by a T-cell mitogen such as concanavalin A (78), and is required for the continued growth of activated T-cells (79,80). It seems possible then that a nonspecific helper factor released by M. pneumoniae stimulated T-cells (65), inducing polyclonal activation of human blood lymphocytes, was in fact some type of interleukin.

Evidence of yet another aspect of mycoplasma-lymphocyte interaction was provided when it was demonstrated that viable M. arthritidis and M. hominis induced normal mouse lymphocytes to become cytotoxic for allogeneic and syngeneic mouse embryo fibroblasts (26). These findings led to the suggestion that such activation of lymphocytes in vivo could result in cell damage and initiate an inflammatory reaction.

Humoral Immunity

The majority of studies dealing with the local response of various animal species to the respective mycoplasmal pneumonia has documented mainly the humoral response (16,18,81,82,83,84,85,86). Mycoplasma hyopneumoniae is considered a porcine respiratory tract pathogen (36) and

conceivably, local immunity would be of importance in response of the host to this aggression.

The humoral immune response of pigs to infection by M. hyopneumoniae has generally been evaluated using level of serum antibodies. These antibodies can be detected by the complement-fixation test (CF) (87,88), indirect hemagglutination test (IHA) (83,89,90), tube agglutination test (91,92), and enzyme-linked immunosorbent assay (ELISA) (93). Comparative studies indicated that there was a good correlation between results obtained with CF and ELISA tests (94), and the CF, ELISA, and IHA tests (95,96). Differences in test sensitivity were noted.

Antibodies in lungs

Natural or experimental respiratory mycoplasmal infections of various animal species have elicited development of local antibodies to these organisms. Antibodies were detected in bronchial (81), nasal (16), and tracheobronchial secretions (82,97,98,99), sputum (16), and lung washings (18,86). The predominant class of antibodies present was IgA (16,81,82, 86,97). Antibodies of the IgM (81,82) and IgG (16,81,82,86) classes were also detected.

Presence of local antibodies correlated better than systemic antibodies with resistance to M. pneumoniae (16,17), M. bovis (18,100) or M. pulmonis infection (19). Furthermore, studies on M. pulmonis infection in mice indicated that antibodies of any immunoglobulin isotype could mediate resistance to the infection (101). However, in the case of pulmonary disease due to M. pneumoniae, the organism could be isolated from sputum

specimens of more than 50% of the patients even if these specimens contained specific antibodies (102).

Antibodies to M. hyopneumoniae were found in lung and bronchial lymph node extracts (83), and in tracheobronchial secretions (84,85) of infected pigs. Antibodies could be detected by IHA in tracheobronchial secretions but not in serum of pigs killed 2 and 4 weeks post-infection, and were tentatively identified as secretory IgA (84). Use of the ELISA allowed antibodies of the IgM, IgG, and IgA classes, specific for M. hyopneumoniae, to be detected in tracheobronchial secretions by 2 weeks post-infection (85). Maximal values were attained by 5 weeks post-infection for both IgM and IgG antibodies in tracheobronchial secretions; titers of IgA antibodies increased over the 8 week period of the experiment (85). Titers of antibodies in sera, due exclusively to the IgG isotype, reached a maximum at 7 weeks post-infection (85). Tracheobronchial secretions collected 13 weeks post-infection contained IHA antibodies of both IgA and IgG classes, but IgG antibodies were thought to be serum antibodies transuded into the airways (84).

Preferential involvement of the local response over the systemic response following infection of pigs with M. hyopneumoniae has been suggested when it was found that the percentage and total number of B-lymphocytes in lungs increased following infection (103). Percentage of B-lymphocytes in peripheral blood did not vary following infection.

Antibody producing cells in lungs

Direct visualization of immunoglobulin-bearing cells in lesions of respiratory mycoplasmal infection has been done by indirect immunofluorescence (82,104,105) and by the avidin-biotin technique (85). Peribronchiolar lymphoid hyperplasia in lungs of hamsters infected with M. pneumoniae consisted mainly of IgM-positive cells; detection of IgG-positive cells varied throughout the experiment and number of IgA-positive cells was minimal (105). In mice infected with M. pulmonis, IgM-positive cells appeared early in the perivascular and peribronchiolar spaces and were followed within a week by IgG- and IgA-positive cells. Cells of this latter isotype predominated by 3 weeks post-infection and remained so until the end of the experiment (82). Characterization of lymphocyte populations in lungs of rats infected with M. pulmonis revealed that the only population of immunoglobulin-bearing cells to increase in the F344 strain were those of the IgA isotype (99,106), whereas in LEW rat strain all B-cell isotypes were increased significantly (99). During the first 2 weeks post-infection, pigs infected with M. hyopneumoniae had minimal stimulation of immunoglobulin-containing cells in the lungs; but by 3 weeks post-infection, IgA- and IgG-positive cells had increased 20 times and a rapid decrease in number of immunoglobulin-positive cells occurred at 5 weeks post-infection (85). The number of cells producing antibodies specific for the antigen involved were estimated to be about 1% of the total number of immunoglobulin-positive cells present in the lung in the case of M. hyopneumoniae (85), and for M. pulmonis between 50 to 80% of the plasma cells in the lungs (82). The number of plasma cells of each immunoglobulin class, IgM, IgG, and IgA,

increased as a consequence of M. pulmonis infection in the rat (99). Other investigators (104) reported that plasma cells were not detected by histopathologic examination in lungs of mice infected with M. pulmonis. When hamsters having recovered from M. pneumoniae infection were rechallenged with the same strain of organism and their lungs examined for immunoglobulin-positive cells, it was found that only a few IgA-positive cells were present. The bulk of lymphocytes in the peribronchiolar and perivascular areas were nonimmunoglobulin producing cells (105).

Changes taking place in paratracheal lymph nodes of mice infected with M. pulmonis (82), and in bronchial lymph nodes of pigs infected with M. hyopneumoniae (85) were generally similar. Increased numbers of immunoglobulin-containing cells of all classes were noted 2 weeks post-infection and lasted for about 3 to 4 weeks. Following this period, the number of plasma cells gradually diminished.

Cell-Mediated Immunity

Clear evidence of the pathogenic or protective roles for cell-mediated immunity (CMI) in mycoplasmal respiratory infections have not yet been demonstrated. Increase in number of T-lymphocytes in lungs following infection of rats with M. pulmonis has been reported (99,106) but no functional analysis of these lymphocytes was done. Investigators have used indirect methods to study the role of CMI in the lungs.

Suppression of CMI has been the most common method used to study its roles in various processes. Immunosuppression has been achieved by thymectomy (104,107), administration of drugs (108), or antithymocyte

serum (107,109,110).

Animals immunosuppressed and subsequently infected with mycoplasma species known to cause pneumonia have developed less severe microscopic lesions of pneumonia than nonimmunosuppressed infected animals. This phenomenon was observed in mice infected with M. pulmonis (104), hamsters infected with M. pneumoniae (109,110), and pigs infected with M. hyopneumoniae (107). A consistent finding in immunosuppressed infected animals was the marked reduction in the number of lymphocytes around bronchioles and blood vessels (104,107,109,110). Bronchiolar and alveolar exudate of immunosuppressed infected hamsters (109,110) and pigs (107) consisted mainly of macrophages with little PMN infiltration, whereas the exudate found in immunologically normal infected animals consisted predominantly of PMNs. Immunosuppressed mice infected with M. pulmonis (104) had fewer alveolar macrophages in the exudate than infected control mice and no apparent difference in PMN numbers was noted.

Although immunosuppression reduced the severity of pneumonia, it did allow mycoplasmas to multiply and reach titers 10 to 1000 times higher in the lungs (104,107,108,110). Another consequence of immunosuppression was that dissemination of mycoplasmas from the lungs to other organs (liver, spleen, and brain) occurred at least twice as frequently as in immunocompetent mice (104), and titers of mycoplasmas in these organs were consistently higher (108). Immunosuppression induced by a combination of cyclophosphamide treatment and M. pulmonis infection of mice resulted in greater mortality rate than could be accounted for by M. pulmonis or cyclophosphamide treatment alone (108).

Reconstitution with spleen cells from mice previously infected with M. pulmonis significantly decreased mortality rate in mice immunosuppressed with cyclophosphamide and infected with M. pulmonis (108). However, transfer of spleen cells from immune to nonimmune recipient mice did not confer protection against M. pulmonis pneumonia (111). The ability of spleen cells to confer immunity in the respiratory tract was questioned by the authors and appears unlikely in light of what is known today about cell migration at mucosal surfaces. In a recent review (112), apparent extensive traffic of immunocompetent cells between secretory sites was cited as support for the existence of a mucosal-associated lymphoid tissue (MALT). Tissues and organs involved included the gut, lungs, mammary glands, salivary and lacrimal glands, and possibly the urinary and genital tracts.

Because systemic CMI is easier to evaluate, it has generally been the way by which possible CMI to respiratory mycoplasmal infection has been assessed. Exposure of peripheral blood lymphocytes (PBL) to antigen and measurement of the incorporation of radiolabeled nucleotide has been the most widely used procedure. The majority of early published reports dealt with M. pneumoniae infections in humans (113,114,115,116). Sensitized PBL were generally detected within a month following onset of disease and persisted for several years. Recent or active infection with M. pneumoniae in children was found to be better indicated by the response of lymphocytes from adenoid vegetations than PBL (117). This observation added support to the suggestion that repeated asymptomatic infections due to M. pneumoniae resulted in increasing numbers of locally sensitized lymphocytes reaching the systemic circulation, as evidenced by the fact that as the age of the

patients increased so did their stimulatory response to M. pneumoniae antigens (118). In a guinea pig model used to study M. pneumoniae infection, sensitized spleen cells were detectable 2 weeks after infection and remained so up to 6 weeks post-infection; these responses were taken as an indication of CMI (97).

Detection of positive stimulation of PBL from pigs infected with M. hyopneumoniae was first noted from one week post-exposure (14) up to 15 weeks post-infection (15), and remained detectable up to 44 weeks post-infection (15). Vaccination of pigs with killed M. hyopneumoniae induced the appearance of sensitized PBL 3 to 4 weeks after the first immunization (13,14). Infection of vaccinated pigs with M. hyopneumoniae resulted in marked increases in the lymphocyte stimulation index of PBL (13,14).

Although histological examination of bronchial lymph nodes from pigs infected with M. hyopneumoniae revealed cell proliferation in the thymus-dependent area (107,119) lymphocytes isolated from bronchial lymph nodes of vaccinated challenged pigs were not stimulated by M. hyopneumoniae antigens (14). The fact that hyperplasia of germinal centers was also observed in infected pigs (107,119) might suggest that local proliferation of antibody-producing cells (85) is more important than cell-mediated immunity. Systemic CMI to M. hyopneumoniae has been associated with the recovering stage of mycoplasmal pneumonia (15), but increased CMI induced by vaccination could not be correlated with protective activity (14), or reduction in gross lesions of pneumonia (13).

The leukocyte migration inhibition test was found useful for detection of CMI to M. pneumoniae (120), Mycoplasma mycoides var. mycoides (121),

and M. hyopneumoniae (122,123), but was judged less sensitive than the lymphocyte stimulation test (120,121,123). Intradermal allergic reaction to mycoplasmal antigens has also been used to study CMI in cattle (121, 124), guinea pigs (97), and pigs (15,123), and was also found less sensitive than the lymphocyte stimulation test (15,121,123).

Immunosuppression

The lengthy course of various mycoplasmal diseases, respiratory infections included, has prompted the suggestion that mycoplasmas might have an immunosuppressive effect on their host. This would occur even in presence of developing systemic and local immunity.

Simultaneous injections of M. arthritidis and viral (125) or bacterial antigens (126) resulted in complete or partial suppression of the humoral response to the antigens. However, injection of mycoplasmas 10 days after immunization with the viral antigen did not affect the antibody response (125). Both viable cells (126) and membrane preparations (125) of M. arthritidis could exert the suppressive effect on the humoral response. Mycoplasma pneumoniae also appeared to have some effect on humoral immunity when a transitory depression of serum IgG was noted in patients with pneumonia caused by this organism (127).

Several investigators reported that following infection with mycoplasmas there was depression of CMI. This depression was detected in vitro by loss of responsiveness by lymphocytes to stimulation by the mitogen PHA (121, 124,125,127,128), or by purified protein derivative (PPD) of Mycobacterium tuberculosis (129). Inhibition of the mitogenic effect of PHA or PPD was

variable, ranging from partial to complete (121,124,125,127,129).

Decrease in uptake of radiolabeled nucleotide by nonstimulated lymphocytes was also noted (121). Attempts to induce responsiveness, by lymphocytes, to the specific antigen during the period of depressed CMI yielded negative results (121). Additionally, transient anergy to tuberculin, noted early after onset of pneumonia associated with M. pneumoniae, was thought to reflect possible depression of CMI (102,129,130). Major reduction in the percentage of blood T-lymphocytes has been recorded in a patient suffering from acute mycoplasmal pneumonia (131).

Pathology of Mycoplasmal Pneumonia of Swine

Mycoplasmas causing respiratory infections can produce lesions of fibrinous pleuropneumonia as exemplified by bovine and caprine contagious pleuropneumonia, or a cuffing pneumonia as seen in swine and other animal species (35). The main characteristic of this latter type of pneumonia is the peribronchial, peribronchiolar, and perivascular accumulation of lymphocytes. Although the interstitial lesions found in MPS and similar mycoplasmal pneumonias are often described as characteristic of the disease, they are nonetheless due to a nonspecific inflammatory reaction in the lungs of affected animals.

Gross lesions of MPS were detected for the first time from 7 (132) to 14 days (133) following experimental infection of pigs with M. hyopneumoniae. Small lesions of pneumonia were seen by 3 days post-infection in pigs inoculated with a lung suspension which contained both M. hyopneumoniae and M. hyorhinis (134). The lesions consisted of dark red to

pale gray areas of consolidation, and catarrhal exudate present mainly in bronchi was noted (132,134). During the period from 2 to 6 weeks post-infection, the gross lesions became more extensive, occasionally affecting complete lobes, and were more clearly demarcated from the adjacent normal tissue; the affected areas generally became firmer and less exudative (132). In the late stage of the disease, from 69 to 262 days post-infection, small areas of consolidation caused the lungs to have a fissured appearance (132). Pneumonic lesions were found to occur most frequently in the apical and cardiac lobes (133,134,135), as well as in the intermediate and cranio-ventral portions of the diaphragmatic lobes (134,135). Differences in distribution of lesions throughout the lungs have been attributed to anatomical factors and facility of mycoplasma removal by the mucociliary clearance mechanisms of the bronchial epithelium (132,133).

Enlargement of bronchial and mediastinal lymph nodes were noted by the second week post-infection (134) and would probably result from cell proliferation observed both in germinal centers (85,119) and in the paracortical area (107,119).

Microscopic examination of lung lesions revealed that lymphocytes started accumulating between 5 to 7 days post-infection, and were seen first in perivascular (132,134) or peribronchiolar tissues (133). Increase of lymphocyte proliferation occurred in the perivascular and particularly in the peribronchiolar tissues, and was the most severe between 2 and 6 weeks post-infection (132,133,126). Proliferation of lymphoid tissue close to the airways often resulted in formation of nodules that occasionally invaded the lamina propria of bronchioles (132,136). In late stages of the

disease, collapsed alveoli, areas of alveolar emphysema, and hyperplastic lymphoid nodules associated with bronchioles were observed (132). Obliteration of airways has been found to occur due to the extensive lymphoid proliferation around bronchioles (137). The exact mechanism by which M. hyopneumoniae induces lymphocytes to accumulate and proliferate around airways and blood vessels is still unresolved. As mentioned previously in this review, total numbers of B-lymphocytes in lungs increased following infection (103) and cells of IgA and IgG isotypes were found to have proliferated the most (85). The possibility that T-lymphocytes are also involved in development of MPS lesions was raised when it was found that pigs with suppressed T-cell response had reduced peribronchiolar and perivascular lymphoid hyperplasia (107). Both T- and B-lymphocytes have also been found to be increased following M. pulmonis infection in rats (99,106). Cell proliferation in the T- (107,119) and B-dependent (85,119) areas of regional lymph nodes from infected pigs lends additional support for the involvement of both T- and B-cell response in development of lesions.

Initially, the exudate found in the bronchi and in some alveoli consisted mainly of PMNs and macrophages (132,134). As the disease progressed more plasma cells were present in the exudate (132,136) and fewer PMNs were seen (132). A small number of lymphocytes has also been observed in the exudate (136), as well as sloughed alveolar cells (132). In the recovering stages, the exudate eventually disappeared (132).

Colonization of the bronchial epithelium by M. hyopneumoniae (132, 133,135) or M. hyopneumoniae/M. hyorhinis (134,136) generally resulted in

partial or complete loss of cilia. Loss of cilia has also been observed in tracheal ring explants infected with M. hyopneumoniae (138). In late stages of the disease (10 weeks or more post-infection), ciliated epithelial cells returned to normal (132).

SECTION I. ROLE OF MYCOPLASMA HYOPNEUMONIAE MEMBRANES IN THE
PATHOGENESIS OF MYCOPLASMAL PNEUMONIA OF SWINE

Role of Mycoplasma hyopneumoniae membranes in the
pathogenesis of mycoplasmal pneumonia of swine

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SUMMARY

The possible interactions of cell membranes from Mycoplasma hyopneumoniae with the porcine immune system and their involvement in the pathogenesis of mycoplasmal pneumonia of swine (MPS) were investigated. Non-specific mitogenicity for swine lymphocytes, lesion inducing potential, and immunizing properties of M. hyopneumoniae membranes were studied.

On a few occasions a positive response in the lymphocyte stimulation test was obtained when peripheral blood lymphocytes (PBL) of control or uninoculated pigs were used. Following intratracheal (IT) inoculations of M. hyopneumoniae membranes, no detectable effect was noted in the response of PBL from membrane-inoculated pigs to stimulation by M. hyopneumoniae antigens. Sensitized bronchial lymph node lymphocytes (BLNL) could be detected 7 and 14 days post-inoculation. Heating of the antigens at 56°C for 60 minutes reduced the response of the BLNL by about 50%. Pigs given M. hyopneumoniae membranes IT developed mild lesions resembling those seen in MPS. Exposure of pigs to membranes before challenge with live M. hyopneumoniae resulted in a decrease in the severity of microscopic lesions of MPS.

INTRODUCTION

Since the first report by Ginsburg and Nicolet (1) that mycoplasmas could induce nonspecific proliferation of lymphocytes, several mycoplasma species have been shown to stimulate lymphocytes derived from various anatomical sites or animal species (2,3,4). The mitogenic activity is generally associated with the cell membranes of the organisms (2,3,5) although cell-free culture supernate of Mycoplasma arthritidis also contains a mitogen (6). Only limited information is available about the biochemical composition of these mitogenic factors and variability in specificity and biochemical nature has been reported among mitogens from different mycoplasma species (7,8). The possibility that M. hyopneumoniae might be mitogenic for swine lymphocytes has been mentioned (9,10). In vitro mitogenicity of membranes from M. pulmonis has been shown to correlate with ability of similar membrane preparations to induce pneumonia when instilled intranasally in rats; lesions of pneumonia were similar to those developing in rats infected spontaneously with the organism (11,12).

Pigs exposed to M. hyopneumoniae develop both cell-mediated and humoral immune responses. Procedures used for detection of cell-mediated immunity (CMI) to M. hyopneumoniae have included the lymphocyte stimulation test (9,10,13,14,15), the leukocyte migration inhibition assay (9,13,15), and intradermal reactivity test (9,13). Humoral responses to the organisms are detectable by means of the complement-fixation test (CF) (16), indirect hemagglutination test (IHA) (17), and enzyme-linked immunosorbent assay (ELISA) (18). The role of humoral and CMI responses to M. hyopneumoniae in

protection or pathogenesis of MPS has not been fully resolved.

The following experiments were undertaken in order to investigate the possible role(s) of membranes from M. hyopneumoniae cells in the pathogenesis of MPS. The in vitro ability of membranes to nonspecifically stimulate lymphocytes as well as their potential to induce lesions of MPS in pigs were evaluated. Possible interactions between M. hyopneumoniae membranes and swine lymphocytes were further investigated by inoculating pigs intratracheally with membranes 1 or 2 weeks before challenge with live M. hyopneumoniae.

MATERIALS AND METHODS

Experimental Animals

Yorkshire pigs, 7 to 10 weeks old, were obtained from a barrier-maintained, closed herd at the animal resource station at Iowa State University. The herd was originally established from Cesarean-derived, isolation-reared animals. The pigs were fed a 16% protein swine grower ration without any growth promoters or other antibacterial substances. Each experimental group was housed in separate isolation rooms.

Preparation of Cell Membranes

The 1262 strain (field isolate, passage 40-42) of M. hyopneumoniae was used for preparation of cell membranes. The organisms were grown in Friis mycoplasma broth (19,20) with acid adjusted swine serum instead of whole serum (21). Mycoplasmas were grown in flasks by shaking (50 rpm) at 37°C for 3 days in a waterbath.^a Following incubation, the number of color changing units (CCU) was determined in order to estimate the number of viable organisms.

Membranes of mycoplasmas were obtained by slight modification of the osmotic lysis technique described by Razin (22). Briefly, cells were pelleted by centrifugation at 12,000 X g for 15 minutes and washed once with a large volume of 0.02M Tris-HCl buffer, pH 7.0. The cells were then resuspended in prewarmed (37°C) 2M glycerol (in 0.25M NaCl) solution and

^aAquatherm Waterbath Shaker, New Brunswick Sci. Comp. Inc., Edison, N.J.

incubated at 37°C for 15 minutes. The cell suspension was then transferred into 50 volumes of warm (37°C) deionized water and incubated at 37°C for 15 minutes. The membranes were collected by centrifuging at 26,000 X g for 40 minutes and washed once with 0.05M NaCl-0.01M phosphate buffer, pH 7.0. The cells were resuspended in diluted buffer [1/20 dilution of 0.15M NaCl-0.05M tris (hydroxymethyl) aminomethane-0.01M 2-mercaptoethanol, pH 7.4] and the protein concentration determined according to Lowry et al. (23) with bovine plasma proteins^b as the standard.

For use in the lymphocyte stimulation test, M. hyopneumoniae membranes were diluted to 200 µg of protein/ml and stored at -70°C in 1-ml aliquots. For intratracheal inoculation of pigs, the membranes were resuspended in diluted buffer to give a suspension equivalent to 0.5 to 1.0 x 10¹¹ ccu/ml.

Nonviability and sterility of membrane preparations were checked by inoculating samples on both Friis mycoplasma agar and broth. Furthermore, to ensure complete nonviability of the membranes administered to pigs formaldehyde was added to give a final concentration of 0.25%.

Experimental Design

Experiments 1 and 2

Both experiments had the same experimental design, the only difference being the number of pigs in each experiment; 10 in experiment 1 and 20 in experiment 2. In each experiment the pigs were divided into two groups

^bLab-Trol Chemistry Control, Dade Diagnostics Inc., Aguada, Puerto Rico.

of equal size. The feed given to animals in these two experiments was soaked in water for 5 minutes to obtain a mash and then fed to the pigs. This was done to prevent pigs from inhaling feed particles. Pigs in one group were inoculated intratracheally (IT) (24) three days in a row with 5 ml of M. hyopneumoniae cell membrane suspension. The second group of pigs was inoculated in the same manner with the buffer in which the membranes were resuspended. In experiment 1, 2 pigs from each group were euthanized at 7 days post-inoculation (DPI) and the remaining pigs at 14 DPI. In experiment 2, 5 pigs from each group were euthanized at 7 and 14 DPI, respectively.

Experiment 3

This experiment was done to investigate the effects of exposure to M. hyopneumoniae cell membranes before challenge with viable M. hyopneumoniae. Challenge consisted of 5 ml of a 10% pneumonic lung suspension obtained from a pig infected with the field strain M. hyopneumoniae 194 (isolated at the Veterinary Medical Research Institute, Iowa State University). Twelve pigs were divided into 4 groups of equal size. One group of pigs (M2) was inoculated three days in a row with M. hyopneumoniae membranes and challenged two weeks later. A second group of pigs (M1) was inoculated the same way but one week before challenge. The third group (PC) was challenged only with the pneumonic lung suspension. Finally, the fourth group (NC) received placebo inoculations. All inoculations were done IT. All pigs were euthanized and necropsies done on all pigs 14 days post-challenge (DPC).

Isolation of Lymphocytes

Blood samples were obtained by cranial vena cava puncture from the pigs before any inoculation was made and at various DPI and/or DPC. Heparinized blood samples (20 U heparin/ml of blood) were diluted 1:2 with phosphate buffered saline solution containing 1% glucose (PBSS-glucose). The diluted blood was layered on a Ficoll-sodium diatrizoate solution (Histopaque-1077^c) and centrifuged at 400 X g for 30 minutes at room temperature. The peripheral blood lymphocyte (PBL) rich band was collected and washed once with PBSS-glucose by centrifuging at 150 X g for 10 minutes. Contaminating erythrocytes were lysed by incubating the PBL in 0.83% NH₄Cl-0.01M phosphate buffer (pH 7.2) for 5 minutes at room temperature. The PBL were then washed twice with PBSS-glucose. Following the final wash, the PBL were resuspended in RPMI-1640^d medium supplemented with fetal bovine serum^d (15%), penicillin^d (50 IU/ml), streptomycin^d (50 µg/ml), kanamycin^d (50 µg/ml), L-glutamine (2mM) and HEPES (10mM). The PBL were counted and the concentration adjusted to 2 X 10⁶ lymphocytes/ml. Viability as determined by trypan blue dye exclusion test was >98%.

On days of necropsy, bronchial lymph nodes were aseptically collected and placed in Hanks' balanced salt solution without Ca++ or Mg++^d (HBSS). The lymph nodes were minced using a pair of forceps and a scalpel. The cell suspension was transferred to a tube and the large pieces allowed to

^cSigma Chemical Co., St. Louis, Mo.

^dFlow Laboratories Inc., McLean, Va.

sediment. The supernate was removed and centrifuged at 150 X g for 10 minutes at room temperature. Cells were resuspended in NH_4Cl -phosphate buffer and incubated as above. Bronchial lymph node lymphocytes (BLNL) were washed twice with PBSS-glucose and resuspended in supplemented RPMI-1640. Cell concentration was adjusted to 2×10^6 lymphocytes/ml. Viability of BLN was >90%.

Lymphocyte Stimulation Test

Lymphocytes were dispensed in 100- μl volumes in wells of microtiter plates^d with equal volumes of concanavalin A type IV^c (ConA 4 $\mu\text{g}/\text{ml}$), phytohemagglutinin A type V^c (PHA 10 $\mu\text{g}/\text{ml}$), pokeweed mitogen^c (PWM 5 $\mu\text{g}/\text{ml}$), heated (60 minutes at 56°C) or nonheated M. hyopneumoniae cell membranes (4 $\mu\text{g}/\text{ml}$) or control medium (HBSS). All cultures were set up in triplicate and incubated for 3 days (ConA, PHA, PWM and HBSS), or 5 days (ConA, M. hyopneumoniae and HBSS), at 37°C in an atmosphere of 5% CO_2 in air and high humidity. Eighteen hours before harvesting the respective plates, 1 μCi of [³H]-thymidine^e (specific activity 6.7 Ci/mmol) was added to each well. The cells were collected on glass fiber paper strips^f using a cell harvester (Microharvester^f). After air-drying, glass fiber disks were placed in 4 ml of scintillation cocktail [42 ml of PPO-POPOP concentrate (Liquifluor^e) plus 1000 ml toluene] and [³H]-thymidine

^eNew England Nuclear, Boston, Mass.

^fBellco Glass Inc., Vineland, N.J.

incorporation counted using a liquid scintillation counter.⁸ Results were expressed as a stimulation index calculated as follows: $SI = \text{cpm in stimulated cultures} / \text{cpm in control cultures}$. Because great variations were seen in cpm of control cultures, a SI of 3 or greater was considered positive.

Necropsy

Following deep anesthesia with pentobarbital sodium, or electrocution, pigs were exsanguinated. After a piece of the right cardiac lobe was taken for microbiologic examination, lungs were removed and examined for gross lesions of MPS. The number of lobes with lesions was recorded and the lesions were sketched.

Histopathology

In experiments 1 and 2, two pieces of lung tissue from the 7 lobes of each pig were collected and fixed in 10% buffered formalin. For each pig in experiment 3, samples were taken from 4 lobes with lesions when possible. Tissues were processed according to standard procedures and 5 μm sections stained with hematoxylin and eosin. Using light microscopy, the tissues were examined for presence of lesions characteristic of MPS (25). The extent of peribronchiolar and perivascular lymphocytic hyperplasia was scored on a scale of 0 to 3; 0=absent; 1=focal or mild; 2=multifocal or moderate; 3=diffuse or severe. The degree of infiltration of the

⁸LS-230, Beckman Instrument, Inc., Fullerton, Ca.

bronchiolar lamina propria by lymphocytes was also scored on a scale of 0 to 3 based upon the amount of cells.

In experiment 3, the extent of nodular lymphoid formation, adjacent or embodied in the peribronchiolar and perivascular lymphocytic tissues, as well as the amount of exudate were both scored on a scale of 0 to 3. For the nodular formation the same scoring system as above was used. For each section, the amount of exudate present was estimated by examining the entire section under 40X magnification and scoring the amount of exudate as follows: 0=absent; 1=25% or less of the section; 2=25 to 50% of the section; 3=more than 50% of the section.

A total mean lesion score per group was derived by adding the score of each criterion evaluated and dividing the total score by the number of lobes examined.

Bacteriologic and Mycoplasmal Cultural Procedures

All bacteriologic and mycoplasmal cultural procedures used in these experiments were similar to those described by Ross et al. (26). In experiments 1 and 2, a piece of right cardiac lobe was ground in Friis liquid media (without added antibiotics) using a TenBroeck tissue grinder. Serial tenfold dilutions of the lung homogenate in Friis broth with methicillin and bacitracin were carried out to 10^{-4} . In experiment 3, similar procedures were followed except that the lung homogenates were diluted through 10^{-7} .

Samples of secretions from the nasal cavity, trachea, and bronchus (left cardiac for experiments 1 and 3; right cardiac for experiment 2) were collected with sterile cotton tipped applicators.

Serology

Blood samples were obtained from all pigs at the start of each experiment, and at weekly intervals after IT inoculation of membranes and/or challenge. Sera were assayed for presence of antibodies to M. hyopneumoniae by a modified (16) direct microtiter complement fixation test (CF) (27).

Immunofluorescence

In experiment 3, lung samples collected from 4 lobes were evaluated for presence of M. hyopneumoniae or its antigens using a direct immunofluorescence procedure (28). Samples were collected contiguous to samples collected for histopathology and embedded immediately in a glycol-based medium (OCT^h).

Statistical Analysis

Analysis of variance was used to compare the mean stimulation indices of the various groups in each experiment as well as to compare the mean microscopic lesion scores in experiments 1 and 2. In experiment 3 the mean lesion scores were compared using the t-test.

^hLab-Tek Products, Division of Miles Laboratories, Inc., Naperville, Ill.

RESULTS

Lymphocyte Stimulation Test

Positive SI with M. hyopneumoniae membranes were obtained in lymphocyte stimulation tests before as well as after exposure of the pigs to membranes. In experiment 1, the response of PBL from pigs in both groups was low and varied from day to day (Figure 1). Exposure of pigs to M. hyopneumoniae membranes did not appear to influence the response of PBL to M. hyopneumoniae antigens.

Marked differences between the groups BLNL responsiveness to membranes were noted. The BLNL of membrane-inoculated pigs killed 7 DPI had a mean SI of 75.6 compared to 1.7 for control pigs. At 14 DPI, differences between the 2 groups were still present but to a lesser degree, 36.1 compared to 2.7. Heat-treatment of M. hyopneumoniae membranes decreased the response of BLNL of membrane-inoculated pigs by about 50% both at 7 and 14 DPI (Table 1). Although substantial, this reduction was not statistically significant ($p>.05$).

Slight increases in SI of PBL to M. hyopneumoniae membranes were noticed at DPI 6 and 13 in both groups following heat-treatment of membrane preparations (Table 1).

Results in experiment 2 were similar to those in experiment 1 except for the response of BLNL from membrane-inoculated pigs (Table 2). Only low SI were obtained both at 7 and 14 DPI with BLNL from this group of pigs.

In experiment 3, exposure to M. hyopneumoniae membranes before challenge did not influence the response of PBL at 13 DPC (Table 3).

Figure 1. Response of PBL from control and membrane-inoculated pigs to stimulation by heated (top panel) and nonheated (bottom panel) M. hyopneumoniae membranes (exp. 1)

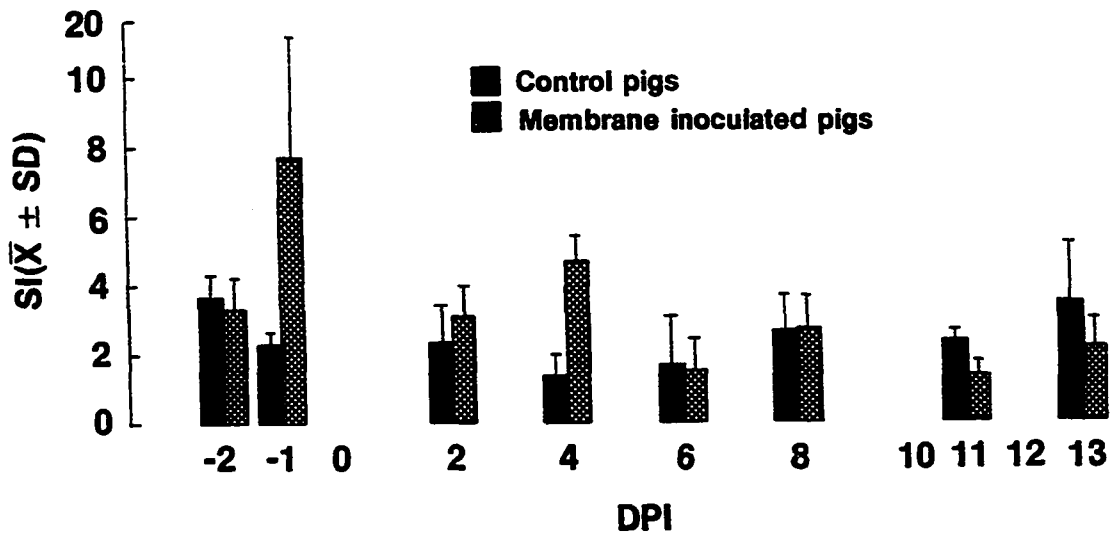
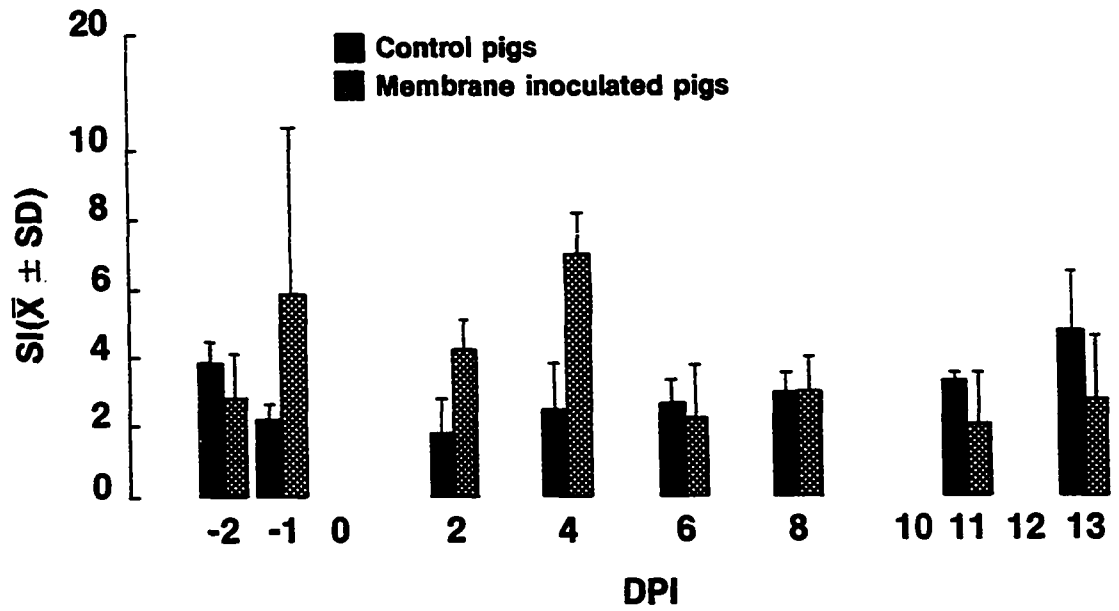


Table 1. Effect of intratracheal inoculation of M. hyopneumoniae membranes on lymphocyte responsiveness to the organism (Exp. 1)

Animals	No. of pigs sampled	Days post inoculation	Type of cells ^a	<u>M. hyopneumoniae</u> membranes	
				Nonheated	Heated ^b
Control	2	6	PBL	1.6±1.5 ^c	2.6±1.1
	2	7	BLNL	1.7±0.7	1.8±1.5
	3	13	PBL	3.4±1.7	4.8±1.7
	3	14	BLNL	2.7±1.1	4.1±4.5
Membrane inoculated	2	6	PBL	1.4±1.0	2.3±1.5
	2	7	BLNL	75.6±40.5	36.2±21.6
	3	13	PBL	2.2±0.4	2.8±1.9
	3	14	BLNL	36.1±34.3	15.7±19.1

^aPBL: peripheral blood lymphocytes; BLNL: bronchial lymph node lymphocytes.

^b56°C, 60 minutes.

^cStimulation index: $\bar{X} \pm \text{SD}$. No significant differences were observed ($p > .05$).

Table 2. Effect of intratracheal inoculation of M. hyopneumoniae membranes on lymphocyte responsiveness to the organism (Exp. 2)

Animals	No. of pigs sampled	Days post inoculation	Type of cells ^a	<u>M. hyopneumoniae</u> membranes	
				Nonheated	Heated ^b
Control	5	6	PBL	2.2±1.0 ^c	3.2±1.2
	5	7	BLNL	1.1±0.4	1.5±1.1
	5	13	PBL	3.0±2.0	3.7±3.4
	5	14	BLNL	3.0±4.9	4.0±4.2
Membrane inoculated	5	6	PBL	2.9±2.0	3.6±2.2
	5	7	BLNL	5.5±4.9	3.8±2.9
	5	13	PBL	3.5±5.4	4.0±4.2
	5	14	BLNL	8.1±9.8	4.2±3.8

^aPBL: peripheral blood lymphocytes; BLNL: bronchial lymph node lymphocytes.

^b56°C, 60 minutes.

^cStimulation index: $\bar{X} \pm SD$. No significant differences were observed ($p > .05$).

Table 3. Effect of intratracheal inoculation of M. hyopneumoniae membranes on the responsiveness of lymphocytes from challenged pigs

Group ^b (n=No. of pigs)	<u>M. hyopneumoniae</u> membranes			
	Nonheated		Heated ^a	
	Source of cells ^c		Source of cells	
	PBL	BLNL	PBL	BLNL
NC(n=3)	2.6±1.6 ^d	0.9±0.5	2.6±1.6	1.3±1.2
PC(n=3)	2.7±0.7	29.1±35.0	2.7±0.2	10.6±10.9
M1(n=3)	2.1±0.7	63.0±56.1	2.5±0.9	18.9±14.4
M2(n=3)	4.2±2.9	14.5±12.8	3.6±1.6	3.9±3.9

^a56°C, 60 minutes.

^bNC: negative control; PC: positive control; M1: inoculated with M. hyopneumoniae membranes 1 week before challenge; M2: inoculated with M. hyopneumoniae membranes 2 weeks before challenge.

^cPBL: peripheral blood lymphocytes, 13 days post-challenge; BLNL: bronchial lymph node lymphocytes, 14 days post-challenge.

^dStimulation index: $\bar{X} \pm \text{SD}$.

Large differences were detected in the SI of BLNL from the three groups of pigs infected with M. hyopneumoniae. Heating of the membranes reduced the SI of the BLNL by about 65%.

In all 3 experiments, the SI obtained when lymphocytes were stimulated with ConA, PHA, and PWM were almost always >40 and very often >100.

Bacteriological and Mycoplasmal Isolation

Very few bacteria were isolated from the lung homogenates. In experiment 1, 5 to 10 colonies of Streptococcus equisimilis were isolated from 2 pigs, 20 colonies of an alpha-hemolytic streptococcus from 1 pig and about 40 colonies of Pseudomonas sp. from 1 pigs. In experiment 2, 3 colonies of an unidentified gram-positive diptheroid were isolated from 1 pig, and 10 colonies of Staphylococcus sp. from one other pig. All lung homogenates from pigs in experiment 3 were negative for bacteria.

No M. hyopneumoniae was isolated from the lung homogenates of pigs from experiments 1 and 2. Mycoplasma flocculare was isolated from 4 pigs in experiment 1 and from 2 pigs in experiment 2. In experiment 3, M. hyopneumoniae was reisolated from all pigs in the PC and M2 groups and from 2 pigs in the M1 group.

CF Antibody Testing

Complement-fixing antibodies were not detected in any pre- or post-inoculation sera from pigs in experiment 1. In experiment 2, complement-fixing antibodies were detected in one pig at 6 DPI and in one different pig at 13 DPI. In experiment 3, antibodies were detected in sera from

Table 4. Effect of prior intratracheal inoculation of M. hyopneumoniae membranes on development of lesions following challenge with live M. hyopneumoniae

Group [@] (n=No. of pigs)	Inoculation	Challenge exposure (day 0)	Gross lesions	
			No. of pigs positive	No. of lobes positives/No. examined
NC(n=3)	None	None	2	6/21
PC(n=3)	None	5 ml of 10% pneumonic lung suspension	3	21/21
M1(n=3)	<u>M. hyopneumoniae</u> membranes IT 7 days before challenge		3	16/21
M2(n=3)	<u>M. hyopneumoniae</u> membranes IT 14 days before challenge		3	17/21

[@]NC: negative control; PC: positive control; M1: inoculated with M. hyopneumoniae membranes 1 week before challenge; M2: inoculated with M. hyopneumoniae membranes 2 weeks before challenge.

* Groups with different superscript differ ($p < .05$).

Micro- scopic lesions	Isolation of <u>M.</u> <u>hyopneumoniae</u>	Immuno- fluorescence	Complement fixing antibody		
Mean lesion score*	No. of pigs positive	No. of pigs positive	No. of lobes positive/No. examined	No. of pigs positive (day 13)	Mean titer of positive CF test
1.9±1.6 ^a	0	0	0/12	0	0
11.0±0.7 ^b	3	3	10/12	3	65
7.2±1.0 ^c	2	3	8/12	0	0
9.6±1.0 ^d	3	3	8/12	3	54

3 pigs from both the PC and M2 groups but from none of the pigs from the M1 and NC groups.

Necropsy

No gross lesion typical of MPS were detected in pigs used in experiments 1 and 2, at 7 or 14 DPI. In experiment 3, gross lesions typical of MPS were seen in all pigs that had been challenged with M. hyopneumoniae (Table 4).

Histopathology

In experiments 1 and 2, microscopic lesions resembling those of MPS were detected in pigs inoculated with M. hyopneumoniae membranes (Figure 2). The peribronchiolar and perivascular cuffing occasionally accompanied by lymphocytic infiltration of the lamina propria were generally limited to a few areas in the sections. Inflammatory cells were not observed in alveoli of any section. Mild accumulation of lymphocytes around bronchioles was observed in some of the sections from control pigs. This was assumed to be normal bronchial-associated lymphoid tissue.

In experiment 1, membrane-inoculated pigs killed 7 DPI had significantly more ($p < .01$) microscopic lesions than control pigs killed on the same day (Table 5). Peribronchiolar and perivascular lymphocytic cuffing was generally more pronounced in the former group, as was also infiltration of the lamina propria of airways by lymphocytes.

In experiment 2, membrane-inoculated pigs killed on DPI 14 had a significantly higher ($p < .05$) mean microscopic lesion score than control pigs

killed on the same day (Table 5). Increased lymphocytic proliferation around bronchioles and blood vessels accounted for the major difference between the two groups.

Microscopic evaluation of lung sections from pigs used in experiment 3 revealed significant differences in mean microscopic lesion scores obtained with the 4 groups (Table 4). Pigs inoculated with membranes 2 weeks (M2) or 1 week (M1) before challenge had significantly less ($p < .05$) microscopic lesions of MPS than the positive control group (PC). Reduction in severity of lesions was mainly due to decreased amount of inflammatory cells in alveoli and bronchioles, and slight reduction of peribronchiolar and perivascular lymphocytic proliferation.

Immunofluorescence

Fluorescent-antibody technique was done only on lung samples from pigs of experiment 3. All animals that had been challenged with M. hyopneumoniae were FA positive (Table 4). Both the M1 and M2 groups had a slightly lower number of positive lobes than the PC group (Table 4). No difference in the intensity of positive response was noted between the 3 groups.



Figure 2. Peribronchiolar lymphoid hyperplasia in lungs from membrane-inoculated pig, 7 DPI. 51X

Table 5. Individual and mean microscopic lesion score of pneumonia of pigs inoculated intratracheally with M. hyopneumoniae membranes

Experiment	Days post-inoculation	Group	Microscopic lesion score					
			Individual pigs				Mean	
1	7	Control	0.57	2.57				1.57±1.22 ^{**, @}
		Membrane-Inoculated	4.00	3.42				3.71±1.22 ^{**}
	14	Control	2.86	2.00	4.00			2.95±1.47
		Membrane-Inoculated	3.86	2.00	1.43			2.43±1.36
	7	Control	0.57	0.29	1.71	1.43	1.57	1.11±1.32
		Membrane-Inoculated	0.71	0.80	1.29	1.57	1.14	1.11±1.20
2	14	Control	0.71	0.71	1.57	2.14	2.29	1.48±1.22 [*]
		Membrane-Inoculated	4.29	3.29	1.71	1.57	0.86	2.34±1.62 [*]

^{**} p < .01.

^{*} p < .05.

[@] $\bar{X} \pm SD$.

DISCUSSION

Nonspecific mitogenic activity for lymphocytes from various animal species or anatomical sites has been demonstrated with several Mycoplasma species (1,2,3,4). Results presented here indicated that M. hyopneumoniae membranes stimulated porcine PBL only slightly and somewhat inconsistently (Figure 1). A possible nonspecific mitogenic activity associated with M. hyopneumoniae has been reported previously (9,10). The authors found that inactivated whole organisms produced a positive SI with PBL from noninfected pigs. However, Kishima et al. (14) were unable to detect a nonspecific stimulatory effect when using sodium azide killed whole M. hyopneumoniae. In the present study, heat-treatment of the membranes used as antigens in the lymphocyte stimulation test generally resulted in a slight increase in nonspecific stimulation of PBL. Heat-stable mitogenic factors have been reported in M. arthritidis, M. hyosynoviae, M. hominis, and M. arginini (4).

Response of BLNL to M. hyopneumoniae membranes in all three experiments tended to indicate that a population of sensitized lymphocytes develops in the regional lymph nodes following intratracheal inoculation of killed or live organisms. Substances responsible for stimulation of the lymphocytes in sensitized pigs appeared to be proteinaceous or to contain protein material since heating of the membranes reduced the SI by approximately 50% (Tables 1, 2, and 3). It should be noted however, that heat-treatment of membranes did not completely abolish their ability to stimulate the BLNL. The SI of BLNL from pigs given membranes or

infected with M. hyopneumoniae were generally higher than those obtained with BLNL from control pigs. This response was probably primarily in response to the heat-resistant antigen(s) on the membranes.

Kristensen et al. (10) found no evidence that lymphocytes from bronchial lymph nodes of M. hyopneumoniae infected animals were sensitized to the organism, although PBL from the same pigs were responsive to stimulation by the antigen. Length of time since exposure to M. hyopneumoniae antigens might account for the differences observed in responsiveness of regional lymphocytes in the above study and the present work. In the present study, BLNL were stimulated either 1 or 2 weeks after IT inoculation of membranes, or 2 weeks after challenge with live M. hyopneumoniae. Kristensen et al. (10) on the other hand, assayed BLNL 5 weeks after pigs had been contact-exposed to M. hyopneumoniae infected pigs. It is possible that the number of sensitized lymphocytes present in the lymph nodes of their swine was too low to be detected.

Kishima et al. (14) reported that heating of M. hyopneumoniae antigen preparation at 60°C for 30 minutes greatly enhanced stimulation of PBL. As noted above, in the present study heat-treatment of M. hyopneumoniae antigen resulted in a decrease of the SI of BLNL. The initial route of inoculation of antigen, and use of dextran sulfate in the former study might have induced sensitization to different antigens and could explain the differences noted in heat stability of these antigens.

Contrary to the BLNL, no highly sensitized PBL could be detected following IT inoculation of M. hyopneumoniae membranes and/or live M. hyopneumoniae. These results were not surprising for experiments 1

and 2, since the animals were euthanized early following the inoculations and sensitized lymphocytes might not have entered the pool of circulating lymphocytes. Results obtained in experiment 3 gave no clear evidence that PBL were sensitized but it was noted that pigs in contact with M. hyopneumoniae antigens the longest (group M2) had the highest SI. These SI were in the range of values obtained with PBL from M. hyopneumoniae infected pigs in other studies.

The origin of M. flocculare isolated from pigs in experiment 1 and 2 is unknown. M. flocculare is generally considered nonpathogenic for swine (29). In our experiments, it was isolated from pigs in both groups and although no significant difference in the various responses of pigs within a group could be noted, the possibility that interference with one or more of the tests performed still exists.

M. hyopneumoniae was reisolated from 2 out 3 pigs in the M1 group. Also, all pigs in that group were seronegative (Table 4), and the BLNL of this group had the highest SI (Table 3). This might suggest that a local population of lymphocytes temporarily impeded growth of M. hyopneumoniae in the lung without complete clearing the infection. This was evidenced by detection of mycoplasmal antigens in lung sections by FA. This effect appears transient since no difference in seroconversion could be seen between the PC and M2 groups. The possibility of temporary interference with growth of the organisms in the lungs was further supported by the results of the microscopic evaluation of the lungs (Table 4), where pigs inoculated with membranes before challenge had a significant reduction in severity of lesions. Induction of local humoral immune factors following

IT inoculation of M. hyopneumoniae membranes might have prevented or impeded development of lesions normally seen in MPS. Tajima et al. (30) reported that suppression of cell-mediated immune mechanisms of pigs by thymectomy and treatment with antithymocyte antisera reduced the severity of microscopic lesions of MPS. Following suppression of cell-mediated immunity, similar observations were made in mice infected with M. pulmonis (31) and hamsters infected with M. pneumoniae (32,33).

Naot et al. (11,12) correlated the mitogenic activity of M. pulmonis membranes with pathogenicity of the organism in vivo after they demonstrated that intranasal administration of membranes to rats induced lesions similar to those occurring in natural infection. Naot et al. (12) indicated that the severity of microscopic lesions of pneumonia induced in Hooded and Lewis rats by administration of M. pulmonis membranes was directly related to the degree of in vitro responsiveness of lymphocytes from these rat strains to similar membrane preparations of this organism.

Our preparations of M. hyopneumoniae membranes had weak mitogenic activity for pig lymphocytes and might explain why only mild microscopic lesions resembling those of MPS could be induced in pigs having received membranes IT.

Another possible explanation for the presence of only mild lesions is that an insufficient amount of membranes was administered in relation to the volume of the porcine lung. Also, the size of the area evaluated by microscopic examination is so minimal in regards to the whole lung tissue that it might not be an accurate representation of the total lung response.

REFERENCES

1. Ginsburg, H., and J. Nicolet. 1973. Extensive transformation of lymphocytes by a mycoplasma organism. *Nature New Biol.* 246:143-146.
2. Biberfeld, G., and E. Gronowicz. 1976. Mycoplasma pneumoniae is a polyclonal B-cell activator. *Nature* 261:238-239.
3. Naot, Y., J. G. Tully, and H. Ginsburg. 1977. Lymphocyte activation by various Mycoplasma strains and species. *Infect. Immun.* 18:310-317.
4. Cole, B. C., K. E. Aldridge, and J. R. Ward. 1977. Mycoplasma-dependent activation of normal lymphocytes: mitogenic potential of mycoplasmas for mouse lymphocytes. *Infect. Immun.* 18:393-399.
5. Biberfeld, G., and E. Nilsson. 1978. Mitogenicity of Mycoplasma fermentans for human lymphocytes. *Infect. Immun.* 21:48-54.
6. Cole, B. C., L. R. Washburn, G. J. Sullivan, and J. R. Ward. 1982. Specificity of a mycoplasma mitogen for lymphocytes from human and various animal hosts. *Infect. Immun.* 36:662-666.
7. Naot, Y., R. Siman-Tov, and H. Ginsburg. 1979. Mitogenic activity of Mycoplasma pulmonis. II. Studies on the biochemical nature of the mitogenic factor. *Eur. J. Immunol.* 9:149-154.
8. Katz, R., R. Siman-Tov, and Y. Naot. 1983. Comparison of mitogens from Mycoplasma pulmonis and Mycoplasma neurolyticum. *Yale J. Biol. Med.* 56:613-621.
9. Adegboye, D. 1978. Attempts to demonstrate cell-mediated immune response during Mycoplasma suis pneumoniae infection of pigs. *Res. Vet. Sci.* 25:323-330.
10. Kristensen, B., Ph. Paroz, J. Nicolet, M. Wanner, and A. L. de Weck. 1981. Cell-mediated and humoral immune response in swine after vaccination and natural infection with Mycoplasma hyopneumoniae. *Am. J. Vet. Res.* 42:784-788.
11. Naot, Y., S. Davidson, and E. S. Lindenbaum. 1981. Mitogenicity and pathogenicity of Mycoplasma pulmonis in rats. I. Atypical interstitial pneumonia induced by mitogenic mycoplasmal membranes. *J. Infect. Dis.* 143:55-62.
12. Naot, Y., S. Davidson, and E. S. Lindenbaum. 1984. Role of mitogenicity in pathogenicity of mycoplasmas for murine hosts. *Ann. Microbiol. (Paris)* 135A:95-101.

13. Roberts, D. H. 1973. Preliminary studies on the cell-mediated immune response in pigs to Mycoplasma hyopneumoniae. Br. Vet. J. 129: 427-438.
14. Kishima, M., R. F. Ross, and C. Kuniyasu. 1985. Cell-mediated and humoral immune response to Mycoplasma hyopneumoniae in pigs enhanced by dextran sulfate. Am. J. Vet. Res. 46:456-462.
15. Nicolet, J., and E. Rivera. 1976. Preliminary observations with the leucocyte migration inhibition test in the enzootic pneumonia of pig. Proc. 4th Int. Pig Vet. Soc. 1976:PP8.
16. Slavik, M. F., and W. P. Switzer. 1972. Development of a micro-titration complement-fixation test for diagnosis of mycoplasmal swine pneumonia. Iowa State J. Res. 47:117-128.
17. Holmgren, N. 1974. An indirect haemagglutination test for detection of antibodies against Mycoplasma hyopneumoniae using formalinized tanned swine erythrocytes. Res. Vet. Sci. 16:341-346.
18. Bruggmann, S., H. Keller, H. U. Bertschinger, and B. Engberg. 1977. Quantitative detection of antibodies to Mycoplasma suis pneumoniae in pigs' sera by an enzyme-linked immunosorbent assay. Vet. Rec. 101: 109-111.
19. Friis, N. F. 1975. Some recommendations concerning primary isolation of Mycoplasma suis pneumoniae and Mycoplasma flocculare. A survey. Nord. Vet. Med. 27:337-339.
20. Friis, N. F. 1979. Selective isolation of slowly growing acidifying mycoplasmas from swine and cattle. Acta Vet. Scand. 20:607-609.
21. Switzer, W. P. 1972. Mycoplasmal pneumonia of swine. J. Am. Vet. Med. Assoc. 160:651-653.
22. Razin, S. 1983. Cell lysis and isolation of membranes. Pages 225-233 in S. Razin and J. G. Tully, eds. Methods in Mycoplasmaology. Vol. 1. Academic Press, New York, New York.
23. Lowry, H., A. L. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement and the Folin phenol reagent. J. Biol. Chem. 193:265-275.
24. Bentley, O. E., and D. O. Farrington. 1980. Evaluation of an induced Pasteurella multocida swine pneumonia model. Am. J. Vet. Res. 41: 1870-1873.

25. Whittlestone, P. 1972. The role of mycoplasmas in the production of pneumonia in the pig. Pages 263-283 in Pathogenic Mycoplasmas. A Ciba Foundation Symposium. Associated Scientific Publishers, Amsterdam, The Netherlands.
26. Ross, R. F., B. J. Zimmermann-Erickson, and T. F. Young. 1984. Characteristics of protective activity of Mycoplasma hyopneumoniae vaccine. Am. J. Vet. Res. 45:1899-1905.
27. Laboratory Branch of the Communicable Disease Center. 1965. Standardized diagnostic complement fixation methods and adaptation to microtest. U.S. Department of Health, Education, and Welfare, Public Health Monogr. 74.
28. Amanfu, W., C. N. Weng, R. F. Ross, and H. J. Barnes. 1984. Diagnosis of mycoplasmal pneumonia of swine: sequential study by direct immunofluorescence. Am. J. Vet. Res. 45:1349-1352.
29. Friis, N. F. 1974. Mycoplasma suis pneumoniae and Mycoplasma flocculare in comparative pathogenicity studies. Acta Vet. Scand. 15:507-518.
30. Tajima, M., T. Yagihashi, T. Nunoya, A. Takeuchi, and F. Ohashi. 1984. Mycoplasma hyopneumoniae infection in pigs immunosuppressed by thymectomy and treatment with antithymocyte serum. Am. J. Vet. Res. 45:1928-1932.
31. Denny, F. W., D. Taylor-Robinson, and A. C. Allison. 1972. The role of thymus-dependent immunity in Mycoplasma pulmonis infections of mice. J. Med. Microbiol. 5:327-336.
32. Singer, S. H., M. Ford, and R. L. Kirschstein. 1972. Respiratory diseases in cyclophosphamide-treated mice. I. Increased virulence of Mycoplasma pulmonis. Infect. Immun. 5:953-956.
33. Taylor, G., D. Taylor-Robinson, and G. W. Fernald. 1973. Reduction in the severity of Mycoplasma pneumoniae-induced pneumonia in hamsters by immunosuppressive treatment with anti-thymocyte sera. J. Gen. Microbiol. 7:343-348.

SECTION II. STIMULATION OF LYMPHOCYTES FROM, AND INOCULATION OF
CESAREAN-DERIVED, COLOSTRUM-DEPRIVED PIGS WITH
MYCOPLASMA HYOPNEUMONIAE MEMBRANES

Stimulation of lymphocytes from, and inoculation of
Cesarean-derived, colostrum-deprived pigs with
Mycoplasma hyopneumoniae membranes

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SUMMARY

Nonspecific mitogenicity of Mycoplasma hyopneumoniae membranes for swine lymphocytes and potential for these membranes to induce lesions seen in mycoplasmal pneumonia of swine (MPS) were investigated. Membranes were prepared by osmotic lysis of mycoplasmal cells and administered intratracheally (IT) to 5-week-old, Cesarean-derived, colostrum-deprived pigs. Seven days post-inoculation pigs were necropsied and lungs examined for presence of gross and microscopic lesions. Nonspecific and specific lymphocyte stimulation responses were determined using peripheral blood lymphocytes (PBL) collected before and after IT inoculation and bronchial lymph node lymphocytes (BLNL) collected at necropsy.

Examination of lungs from membrane-inoculated pigs revealed no gross lesions; however, microscopic lesions consisting of mild perivascular and peribronchiolar lymphoid hyperplasia with occasional penetration of the muscular layer by lymphocytes were seen in the submucosa of bronchioles. The distribution of microscopic lesions in the different regions of the lungs did not have any specific pattern and were seen in every lobe at one time or another in these experiments. Although substantially less severe the lesions resembled those found in lungs of pigs infected with virulent M. hyopneumoniae.

Positive stimulation of PBL from control and membrane-inoculated pigs by M. hyopneumoniae membranes was detected on all but one occasion. Mean stimulation indices (SI) ranged from 2.8 to 17.4 for control pigs and from 3.7 to 9.9 for membrane-inoculated pigs. The response appeared to be

nonspecific in nature. BLNL from control and membrane-inoculated pigs were also stimulated by membrane preparations. Contrary to PBL, the response of BLNL appeared to be influenced by previous exposure to the membranes, control pigs having SI ranging from 3.0 to 16.5 whereas SI for membrane-inoculated pig ranged from 18.6 to 25.5.

The findings indicate that M. hyopneumoniae has some degree of non-specific mitogenicity for swine lymphocytes and that this mitogenic activity might have a role in development of MPS lesions.

INTRODUCTION

The nonsepcific mitogenicity of various mycoplasmas for lymphocytes from different animal species has been recognized for several years (1,2, 3,4). This mitogenic activity has been associated with whole cell (3,4, 5), membrane (2,3,4,6,7,8,9), and supernate (10,11,12) preparations from mycoplasma cultures. In the case of Mycoplasma pulmonis, a link has been established between mitogenic activity for rat lymphocytes and the development of lesions of pneumonia following intranasal inoculation of rats with membranes prepared from that organism (13,14).

Mitogenicity of M. hyopneumoniae for swine lymphocytes has been reported as a sporadic event (15,16); however we recently obtained more convincing evidence that the organism is mitogenic for swine lymphocytes (17). Furthermore, microscopic lesions of pneumonia resembling those seen in MPS developed in the same swine following IT inoculation with M. hyopneumoniae membranes. Unfortunately, the basis for development of the lesions was not clearly determined because Mycoplasma flocculare, an organism closely related to M. hyopneumoniae, was isolated from a few of the swine.

In the present report, we provide further evidence of the pathogenic potential of M. hyopneumoniae membranes by demonstrating that they are mitogenic for unsensitized lymphocytes from Cesarean-derived, colostrum-deprived swine and that they induce lesions in such swine when given IT.

MATERIALS AND METHODS

Experimental Animals

Cesarean-derived, colostrum-deprived (CDCD), 5-week-old Yorkshire pigs were purchased from a commercial supplier (Merrick-Struve Laboratories, Manilla, IA). Animals were housed in groups of three, in plexiglass isolation chambers ventilated with air filtered through fiberglass filter medium.^a They were fed a nonpelleted, 18% protein swine starter ration without any growth promoters or other antibacterial substances.

Experimental Design

The same experimental design was followed in each of the two experiments comprising this study except that the number of pigs in each experiment varied. In the first experiment, 2 groups of 3 pigs each were used whereas 4 groups of 3 pigs each were used in the second experiment. In each experiment, half of the pigs were inoculated IT (18) 3 days in a row with 5 ml of M. hyopneumoniae membrane suspension and the other half inoculated with 5 ml of buffer with formaldehyde but without membranes.

Blood samples for PBL isolation and serum collection were taken once before and at 2 and 6 days post-inoculation (DPI). All pigs were killed and necropsied 7 DPI.

^aAmerican Air Filter Company, Inc., Zion, Ill.

Mycoplasma hyopneumoniae Membranes

The 1262 strain of M. hyopneumoniae, isolated originally from a field case of MPS, triple-cloned and in the 40-42 passage^b, was used to prepare membrane suspensions for IT inoculations and antigen for the lymphocyte stimulation test. Mycoplasmas were grown in Friis mycoplasma broth (19,20) with acid adjusted serum (21) for 3 days at 37°C in a shaking waterbath.^c Membranes were obtained by an osmotic shock technique described previously (17).

For use in the lymphocyte stimulation test, M. hyopneumoniae membranes were diluted to 200 µg of protein/ml in a 1/20 dilution of 0.15M NaCl-0.05M tris (hydroxymethyl) aminomethane-0.01M 2-mercaptoethanol buffer and stored at -70°C in 1-ml aliquots. Protein concentration was determined according to Lowry et al. (22) with bovine plasma proteins^d as the standard. For the IT inoculation, the membranes were resuspended in the same buffer as above to give a suspension equivalent to 1×10^{11} color changing units per ml of viable whole cells. Nonviability and sterility of the membrane preparations were checked by inoculating samples

^bB. Zimmermann-Erickson, Veterinary Medical Research Institute, Ames, Ia.

^cAquatherm Waterbath Shaker, New Brunswick Sci. Comp., Inc., Edison, N.J.

^dLab-Trol Chemistry Control, Dade Diagnostics Inc., Aguada, Puerto Rico.

on both Friis mycoplasma agar and broth. Furthermore, to ensure complete nonviability of membrane preparations administered to pigs formaldehyde was added to give a final concentration of 0.25%.

Isolation of Lymphocytes

Heparinized blood samples (20 U heparin/ml of blood) were diluted 1:2 with phosphate buffered saline solution containing 1% glucose and PBL isolated by density centrifugation on Ficoll-sodium diatrizoate solution (Histopaque-1077^e) as described previously (17). On days of necropsy, bronchial lymph nodes were aseptically collected and placed in Hanks' balanced salt solution without Ca^{++} or Mg^{++f} (HBSS). Bronchial lymph node lymphocyte suspensions were prepared as described previously (17).

Lymphocyte Stimulation Test

Lymphocytes were dispensed in 100 μl volumes in the wells of micro-titer plates^f with equal volumes of concanavalin A type IV^e (Con A, 4 $\mu\text{g}/\text{ml}$), phytohemagglutinin A type V^e (PHA 10 $\mu\text{g}/\text{ml}$), pokeweed mitogen^e (PWM 5 $\mu\text{g}/\text{ml}$), heated (60 min. at 56°C) or nonheated M. hyopneumoniae cell membranes (4 $\mu\text{g}/\text{ml}$) or control medium (HBSS). All cultures were set up in triplicate and incubated for 3 days (Con A, PHA, PWM, and HBSS),

^eSigma Chemical Co., St. Louis, Mo.

^fFlow Laboratories Inc., McLean, Va.

or 5 days (Con A, M. hyopneumoniae, and HBSS) at 37°C in an atmosphere of 5% CO₂ in air and high humidity. Labeling and counting of incorporated [³H]-thymidine^g (specific activity 6.7 Ci/mM) was done as described previously (17).

Necropsy

Anesthesia was induced by intravenous injection of pentobarbital sodium and pigs were exsanguinated. After a piece of the right cardiac lobe was taken for microbiologic examination, lungs were removed and examined for gross lesions.

Histopathology

Five samples of lung tissue were taken from each of the 7 lobes of every pig and fixed in 10% formalin. Tissues were cut at 5 µm, processed according to standard procedures and sections stained with hematoxylin and eosin. Using light microscopy, tissues were examined for presence of lesions characteristic of MPS (23). For each section, individual scores on a scale of 0 to 3 (17) were given to quantify the extent of perivascular and peribronchiolar lymphoid hyperplasia, the extent of lymphocyte accumulation in the submucosa of bronchioles, and the amount of exudate in the airways and alveoli. A cumulative score was then derived for each group of pigs (17).

^gNew England Nuclear, Boston, Mass.

Bacteriologic and Mycoplasmal Cultural Procedures

A piece of right cardiac lobe was ground in Friis liquid media (without added antibiotics) using a TenBroeck tissue grinder. Serial tenfold dilutions of the lung homogenate were made in Friis broth with methicillin and bacitracin. Samples of secretions from the nasal cavity, trachea, and left bronchus were collected with sterile cotton-tipped applicators. All bacteriologic and mycoplasmal cultural procedures used were similar to those described by Ross et al. (24).

Complement-Fixation Test

All serum samples collected were assayed for presence of antibodies to M. hyopneumoniae using a modified (25) direct microtiter complement-fixation test (CF) (26).

Statistical Analysis

Analysis of variance was used to compare the SI of control and membrane-inoculated groups in each of the 2 experiments.

Double-sided Student t-test was used to compare the mean lesion score of control and membrane-inoculated pigs in each of the 2 experiments.

RESULTS

Pathology

Examination at necropsy revealed no evidence of gross lesions of pneumonia in lungs of control or membrane-inoculated pigs in either experiment. Upon microscopic examination of lung sections from control pigs in both experiments, no lymphocytes were seen around blood vessels but variable numbers of lymphocytes were present around bronchioles. Mild (Figure 1) to moderate (Figure 2) focal accumulations of lymphocytes were seen in lungs of all membrane-inoculated pigs. The lesions did not have any specific pattern of distribution throughout the different regions of the lungs and were seen in every lobe at one time or another during the course of these experiments. Accumulation of lymphocytes was seen around both blood vessels and bronchioles but was most frequent around bronchioles. Occasionally, lymphocytes infiltrated the submucosa of bronchioles and collected between the bronchial epithelium and the muscular layer surrounding the bronchioles (Figure 3). The lymphoid hyperplasia in the submucosa of airways or in the peribronchiolar and perivascular areas was occasionally organized into nodule formations. These nodules had mild to moderate development and on rare occasions protruded into the lumina of airways (Figure 4).

In the first experiment, although lesions were seen in lungs of membrane-inoculated pigs the mean lesion score was not significantly different from the score of control pigs because of the mildness of the lesions observed in the samples (Table 1). However, in the second experiment

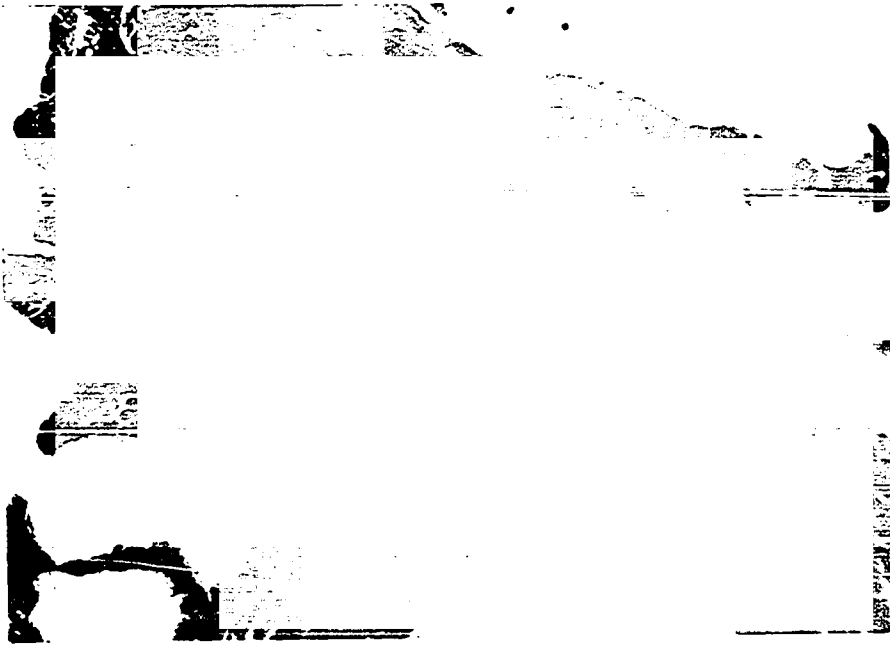


Figure 1. Mild peribronchiolar lymphoid hyperplasia in lungs from membrane-inoculated pig, 7 DPI. 80X

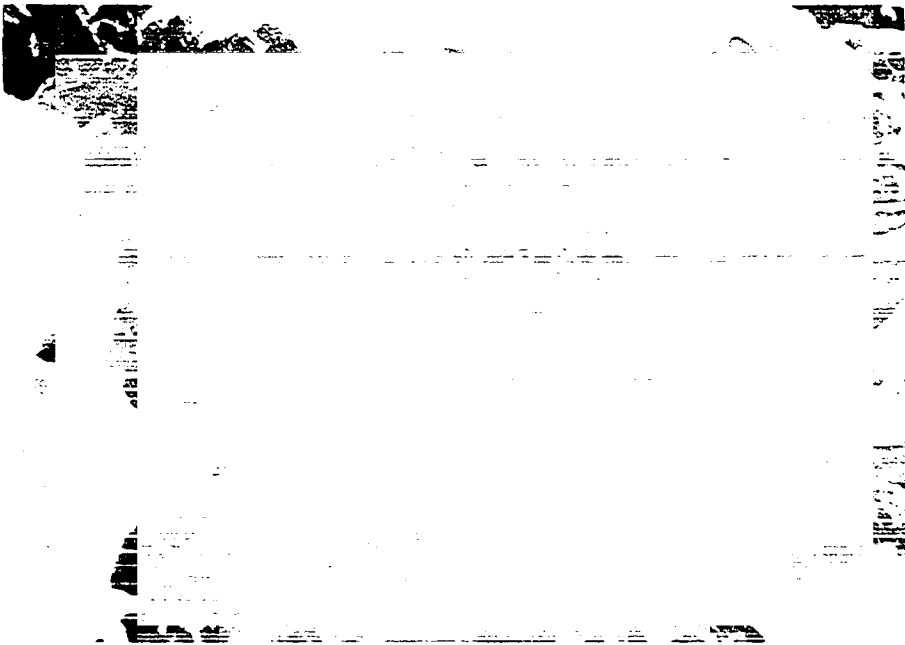


Figure 2. Moderate peribronchiolar lymphoid hyperplasia in lungs from membrane-inoculated pig, 7 DPI. 128X

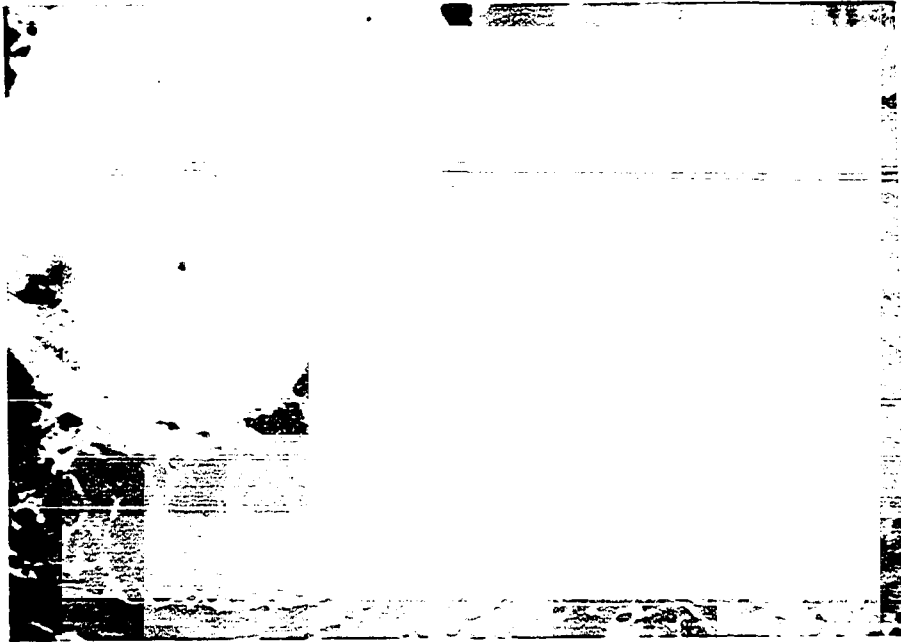


Figure 3. Moderate peribronchiolar lymphoid hyperplasia with penetration of the muscular layer by lymphocytes. Membrane-inoculated pig, 7 DPI. 100X



Figure 4. Lymphocytes in submucosa of bronchiole organized into nodule formation protruding into the lumen. Membrane-inoculated pig, 7 DPI. 96X

Table 1. Individual and mean microscopic lesion score of pneumonia of pigs inoculated intratracheally with M. hyopneumoniae membranes

Experiment	Group	Microscopic lesion score							Mean ^{**}
		Individual pigs							
1	Control	0.00	0.00	0.00					0.00 ± 0.00 ⁺
	Membrane-Inoculated	0.29	0.71	1.29					0.76 ± 0.50
2	Control	1.00	0.00	0.71	0.29	0.57	0.29		0.47 ± 0.36 ^a
	Membrane-Inoculated	1.29	0.71	1.43	0.86	1.59	1.43		1.22 ± 0.35 ^b

** Groups with different superscript differ (p<0.01).

⁺ $\bar{X} \pm SD$.

membrane-inoculated pigs had a significantly higher ($p < .01$) mean lesion score than control pigs (Table 1). Differences in individual score were seen in each group in both experiments. Microscopic lesions were slightly more pronounced in membrane-inoculated pigs of the second experiment (Table 1). The development of peribronchiolar lymphoid tissue in control pigs was highly variable and was found more abundant in pigs of the second experiment (Table 1). The 3 control pigs in experiment 1 received a score of 0 due to the very limited development of peribronchiolar lymphoid tissue.

Lymphocyte Stimulation Test

Lymphocytes collected from blood of pigs in all groups prior to IT inoculation were stimulated by M. hyopneumoniae membranes (Table 2). In general, stimulation of pre-inoculation PBL was obtained with heated and nonheated membranes except that no significant response was detected with PBL from control pigs using nonheated membranes in experiment 2 (Table 2). Stimulation indices obtained pre-inoculation and 2 DPI with PBL from membrane-inoculated pigs were slightly, but not significantly, higher than those from control pigs regardless of whether or not the membrane suspensions were heated. At 6 DPI positive SI were again found following stimulation of PBL by M. hyopneumoniae membranes (Table 2). In the second experiment, the PBL from control pigs had higher SI than membrane-inoculated pigs when heated or nonheated M. hyopneumoniae were used.

When BLNL were exposed to M. hyopneumoniae membranes positive SI were obtained with lymphocytes from all groups (Table 3). In the first experiment, higher but not significantly different indices were obtained

Table 2. Effect of intratracheal inoculation of M. hyopneumoniae membranes on responsiveness of peripheral blood lymphocytes to the organism

Group	<u>M. hyopneumoniae</u> membrane	Days post-inoculation					
		Experiment 1 (n=3)			Experiment 2 (n=6)		
		-1	2	6	-1	2	6
Control	Heated [@]	4.2±2.5	3.9±3.8	8.3±2.6	3.7±2.7	6.5±3.1	17.4±12.2
	Nonheated	3.6±0.6	3.0±1.7	5.2±0.2	2.8±1.1	6.4±2.5	10.4±6.1
Membrane- Inoculated	Heated	4.8±2.4	7.6±6.0	8.2±3.8	7.1±2.4	9.9±6.3	8.3±4.0
	Nonheated	3.7±2.8	5.6±2.3	7.3±3.4	4.3±3.3	8.5±5.3	8.1±2.9

[@]56°C, 60 minutes.

⁺Stimulation index: $\bar{X} \pm SD$.

Table 3. Effect of intratracheal inoculation of M. hyopneumoniae membranes on responsiveness of bronchial lymph node lymphocytes to the organism

Experiment (n=No. of pigs per group)	Group	<u>M. hyopneumoniae</u> membranes	
		Heated [@]	Nonheated
1 (n=3)	Control	3.0±1.2 ⁺	3.2±1.5
	Membrane- Inoculated	18.6±10.6	21.4±15.3
2 (n=6)	Control	16.5±10.6	11.1±8.9
	Membrane- Inoculated	25.5±15.0	19.8±10.9

[@]56°C, 60 minutes.

⁺Stimulation index: $\bar{X} \pm \text{SD}$.

with lymphocytes from membrane-inoculated pigs than with lymphocytes from control pigs. Comparison of SI obtained with BLNL from control pigs in the two experiments revealed much higher responses in the second experiment. Even though the SI of BLNL from control pigs in the second experiment were large, the SI of BLNL from membrane-inoculated pigs were larger when both heated and nonheated membranes were used (Table 3).

The response of PBL to stimulation by ConA, PHA, and PWM was highly variable from day to day in both groups and in each experiment but no consistent difference between control and inoculated pigs could be detected (Table 4). Stimulation of BLNL by ConA, PHA, and PWM also gave highly variable SI and again no difference between groups seemed to exist (Table 4).

Bacteriology

Bordetella bronchiseptica, Pasteurella multocida, or Haemophilus spp. were not isolated from samples from the nasal cavity, trachea, bronchus or lung of control or membrane-inoculated pigs in either experiment. Similarly, mycoplasma were not isolated from lung homogenates from any of the pigs.

Table 4. Response of lymphocytes to stimulation by various mitogens

Experiment (n=No. of pigs per group)	Group	Mitogen ^b	Days post-inoculation (origin of cells) ^a	
			-1 (PB)	2 (PB)
1 (n=3)	Control	ConA	47.3±44.3	35.1±29.5
		PHA	33.1±21.0	71.0±61.9
		PWM	25.8±14.0	119.7±50.5
	Membrane- inoculated	ConA	29.4±24.8	24.6±24.8
		PHA	25.8±10.5	71.4±103.3
		PWM	13.1±1.6	56.3±73.5
2 (n=6)	Control	ConA	32.1±30.8	50.4±49.9
		PHA	92.1±78.2	157.3±150.5
		PWM	40.8±35.0	87.0±82.2
	Membrane- inoculated	ConA	34.8±20.1	30.7±24.1
		PHA	128.1±78.6	93.2±63.6
		PWM	114.5±77.0	80.6±75.9

^aPB: peripheral blood; BLN: bronchial lymph node.

^bConA: concanavalin A (4 µg/ml); PHA: phytohemagglutinin (10 µg/ml); PWM: pokeweed mitogen (5 µg/ml).

^cStimulation index: $\bar{X} \pm SD$.

6 (PB)	7 (BLN)
36.7±19.2	457.4±106.3
45.8±26.2	325.9±260.8
46.3±27.4	408.0±225.9
35.3±9.48	815.0±275.0
37.5±4.65	907.0±442.0
23.5±5.8	806.0±234.0
44.1±33.4	195.1±133.1
104.2±78.4	230.7±164.9
66.7±34.6	180.8±128.1
22.6±16.1	151.6±90.7
30.9±23.5	259.7±202.9
25.6±20.6	203.8±161.4

DISCUSSION

In the present study, IT inoculation of CDCD pigs with membrane preparations of M. hyopneumoniae induced microscopic lesions of perivascular and peribronchiolar lymphocytic hyperplasia resembling those seen in MPS. Previous experiments had indicated that similar membrane preparations could also induce this type of lesions in conventionally-reared pigs (17).

The microscopic lesions observed were far less severe than what is seen in natural or experimental infection with viable organisms. Microscopic lesions are generally detectable in lungs of pigs 7 days following exposure to M. hyopneumoniae (22). Presence of viable replicating M. hyopneumoniae in lungs of infected pigs could cause continued stimulation of lymphocytes by mitogen associated with this organism and might explain the increased severity of lesions in natural and experimental infection. This sustained stimulation of lymphocytes could also account for the long duration of MPS.

The potential for nonspecific mitogen to initiate or exacerbate lung disease has to be considered especially in light of the fact that concanavalin A, a nonspecific mitogen, induced lesions of interstitial pneumonia when administered intranasally to rats (13). The highly contaminated air present in swine confinement housing (27,28) could be a source of nonspecific mitogen such as endotoxin of gram-negative bacteria (28) and possibly mycoplasma in infected herds.

Although the experiments were not done at the same time, the response of conventionally-reared and CDCD pigs to the membranes appeared to be

similar. However, severity of microscopic lesions was less pronounced in CDCD pigs than in conventionally-reared pigs. A possible explanation for the difference in severity of microscopic lesions could be that cell populations or cell numbers in the lungs or regional lymph nodes of the two groups of pigs differed. CDCD pigs were less exposed to stimulation by the environment and thus their local immune system would be less prepared to react to membrane exposure. In studies on lung lavage fluid nonspecific antigenic stimulation was required in order to elicit a population of resident macrophages in airways of CDCD piglets (29). Additionally, a significantly lower proportion of T-helper lymphocytes has been observed in spleens of germ-free mice compared to conventional mice (30). The percentage of antibody producing cells in spleens of each type of mice were not different.

Thymectomy has been shown to reduce the severity of microscopic lesions developing following infection with M. pulmonis in mice (31), Mycoplasma pneumoniae in hamsters (32), and M. hyopneumoniae in pigs (33). Although these findings provided evidence for the role of T-lymphocytes in lesion development it is highly probable that their presence in the lungs is necessary to help control the infection because mycoplasmas were able to multiply to much higher numbers in the lungs (31,32,33) and to invade other organs in thymectomyzed animals (31). The T-lymphocytes could control the infection by interacting directly with mycoplasmas but they are more likely to have a helper role since local humoral responses are generally thought to be of major importance in controlling respiratory mycoplasmoses (34,35,36).

As noted previously (17) the response of PBL to membrane preparations did not appear to be influenced by previous exposure of the pigs to membranes. However, positive stimulation of PBL was found in all but one instance in the present experiments and was sporadically encountered in previous ones (17). Mitogenic factors associated with various mycoplasmas have been found to stimulate T (11,12), B (4,5,8,9), or both (2,6,7) populations of lymphocytes from various animal species. Possible differences in ratio of lymphocytes subpopulations between CDCD and conventionally-reared pigs combined with preferential stimulation of one of these subpopulations could explain the differences noted in stimulation of PBL.

Positive SI observed with BLNL from control pigs were fairly large in the second experiment but still the response of BLNL appeared to be influenced by previous exposure to the membranes since BLNL from membrane-inoculated pigs had higher SI than control pigs in each experiment. This probably results from sensitization of lymphocytes in the lungs to M. hyopneumoniae followed by local traffic of lymphocytes to the regional lymph nodes. Earlier studies (17,36) indicated that a substance containing protein was a likely candidate responsible for sensitizing lymphocytes due to its susceptibility to heat treatment. In the first experiment of this study, heat treatment of the membranes reduced the response of BLNL to stimulation. However, in the second experiment, higher SI were observed with BLNL following heat treatment of the membranes. This was observed with BLNL from both control and membrane-inoculated pigs which might indicate that although specific stimulation of BLNL from membrane-

inoculated pigs could be taking place, nonspecific stimulation by membranes would be prevalent in this case.

In previous experiments (17), detection of positive SI concurrently with isolation of M. flocculare from the lungs of some pigs raised the question as to whether or not this mycoplasma influenced the response of BLNL to stimulation by M. hyopneumoniae membranes. This point was brought about because of the antigenic relatedness found between these two mycoplasmas by agar gel diffusion, CF test, immunofluorescence, and growth precipitation test (37) as well as by two-dimensional immunoelectrophoresis (38). In the current experiments, obtaining positive SI with lymphocytes from CDCD pigs from which no porcine mycoplasmas were isolated confirms the mitogenic and lesion inducing potential of membranes from M. hyopneumoniae for swine lymphocytes.

REFERENCES

1. Ginsburg, H., and J. Nicolet. 1973. Extensive transformation of lymphocytes by a mycoplasma organism. *Nature New Biol.* 246:143-146.
2. Biberfeld, G., and E. Gronowicz. 1976. Mycoplasma pneumoniae is a polyclonal B-cell activator. *Nature* 261:238-239.
3. Naot, Y., J. G. Tully, and H. Ginsburg. 1977. Lymphocyte activation by various Mycoplasma strains and species. *Infect. Immun.* 18:310-317.
4. Cole, B. C., K. E. Aldridge, and J. R. Ward. 1977. Mycoplasma-dependent activation of normal lymphocytes: mitogenic potential of mycoplasmas for mouse lymphocytes. *Infect. Immun.* 18:393-399.
5. Kirchner, H., H. Brunner, and H. Ruhl. 1977. Effect of A. laidlawii on murine and human lymphocyte cultures. *Clin. Exp. Immunol.* 29:176-180.
6. Biberfeld, G., and E. Nilsson. 1978. Mitogenicity of Mycoplasma fermentans for human lymphocytes. *Infect. Immun.* 21:48-54.
7. Naot, Y., S. Merchav, E. Ben-David, and H. Ginsburg. 1979. Mitogenic activity of Mycoplasma pulmonis. I. Stimulation of rat B and T lymphocytes. *Immunology* 36:399-406.
8. Naot, Y., S. Merchav, and H. Ginsburg. 1979. Mycoplasma neurolyticum: a potent mitogen for rat B lymphocytes. *Eur. J. Immunol.* 9:185-189.
9. Naot, Y., and H. Ginsburg. 1978. Activation of B lymphocytes by mycoplasma mitogen(s). *Immunology* 34:715-720.
10. Cole, B. C., L. R. Washburn, G. J. Sullivan, and J. R. Ward. 1982. Specificity of a mycoplasma mitogen for lymphocytes from human and various animal hosts. *Infect. Immun.* 36:662-666.
11. Cole, B. C., G. J. Sullivan, R. A. Daynes, I. A. Sayed, and J. R. Ward. 1982. Stimulation of mouse lymphocytes by a mitogen derived from Mycoplasma arthritidis. II. Cellular requirements for T cell transformation mediated by a soluble mycoplasma mitogen. *J. Immunol.* 128:936-938.
12. Daynes, R. A., J. M. Novak, and B. C. Cole. 1982. Comparison of the cellular requirements for human T cell transformation by a soluble mitogen derived from Mycoplasma arthritidis and concanavalin A. *J. Immunol.* 129:936-938.

13. Naot, Y., S. Davidson, and E. S. Lindenbaum. 1981. Mitogenicity and pathogenicity of Mycoplasma pulmonis in rats. I. Atypical interstitial pneumonia induced by mitogenic mycoplasmal membranes. *J. Infect. Dis.* 143:55-62.
14. Naot, Y., S. Davidson, and E. S. Lindenbaum. 1984. Role of mitogenicity in pathogenicity of mycoplasmas for murine hosts. *Ann. Microbiol. (Paris)* 135A:95-101.
15. Adegboye, D. 1978. Attempts to demonstrate cell-mediated immune response during Mycoplasma suis pneumonia infection of pigs. *Res. Vet. Sci.* 25:323-330.
16. Kristensen, B., Ph. Paroz, J. Nicolet, M. Wanner, and A. L. de Weck. 1981. Cell-mediated and humoral immune response in swine after vaccination and natural infection with Mycoplasma hyopneumoniae. *Am. J. Vet. Res.* 42:784-788.
17. Messier, S., R. F. Ross, and B. Zimmermann-Erickson. 1986. Role of Mycoplasma hyopneumoniae membranes in the pathogenesis of mycoplasmal pneumonia of swine. (Manuscript in preparation).
18. Bentley, O. E., and D. O. Farrington. 1980. Evaluation of an induced Pasteurella multocida swine pneumonia model. *Am. J. Vet. Res.* 41:1870-1873.
19. Friis, N. F. 1975. Some recommendations concerning primary isolation of Mycoplasma suis and Mycoplasma flocculare: A survey. *Nord. Vet. Med.* 27:337-339.
20. Friis, N. F. 1979. Selective isolation of slowly growing acidifying mycoplasmas from swine and cattle. *Acta Vet. Scand.* 20:607-609.
21. Switzer, W. P. 1972. Mycoplasmal pneumonia of swine. *J. Am. Vet. Med. Assoc.* 160:651-653.
22. Lowry, H., A. L. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement and the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
23. Whittlestone, P. 1972. The role of mycoplasmas in the production of pneumonia in the pig. Pages 263-283 in Pathogenic Mycoplasmas. A Ciba Foundation Symposium. Associated Scientific Publishers, Amsterdam, The Netherlands.
24. Ross, R. F., B. J. Zimmermann-Erickson, and T. F. Young. 1984. Characteristics of protective activity of Mycoplasma hyopneumoniae vaccine. *Am. J. Vet. Res.* 45:1899-1905.

25. Slavik, M. F., and W. P. Switzer. 1972. Development of a microtitration complement-fixation test for diagnosis of mycoplasmal swine pneumonia. *Iowa State J. Res.* 47:117-128.
26. Laboratory Branch of the Communicable Disease Center. 1965. Standardized diagnostic complement fixation methods and adaptation to microtest. US Department of Health, Education, and Welfare, Public Health Monogr. 74.
27. Elliot, L. F., M. McCalla, and J. A. Deshazer. 1980. Bacteria in the air of housed swine units. *Appl. Environ. Microbiol.* 32:270-273.
28. Clark, S., R. Rylander, and L. Larsson. 1983. Airborne bacteria, endotoxin and fungi in dust in poultry and swine confinement buildings. *Am. Ind. Hyg. Assoc. J.* 44:537-541.
29. Rothlein, R., R. Gallilu, and Y. B. Kim. 1981. Development of alveolar macrophages in specific pathogen-free and germ-free Minnesota miniature swine. *J. Reticuloendothel. Soc.* 30:384-395.
30. Ohwaki, M., N. Yasutake, H. Yasui, and R. Ogura. 1977. A comparative study of the humoral immune responses in germfree and conventional mice. *Immunology* 32:43-50.
31. Denny, F. W., D. Taylor-Robinson, and A. C. Allison. 1972. The role of thymus-dependent immunity in Mycoplasma pulmonis infections of mice. *J. Med. Microbiol.* 5:327-336.
32. Tajima, M., T. Yagihashi, T. Nunoya, A. Takeuchi, and F. Ohashi. 1984. Mycoplasma hyopneumoniae infection in pigs immunosuppressed by thymectomy and treatment with antithymocyte serum. *Am. J. Vet. Res.* 45:1928-1932.
33. Taylor, G., D. Taylor-Robinson, and G. W. Fernald. 1974. Reduction in the severity of Mycoplasma pneumoniae-induced pneumonia in hamsters by immunosuppressive treatment with anti-thymocyte sera. *J. Med. Microbiol.* 7:343-348.
34. Brunner, H., H. B. Greenberg, W. D. James, R. L. Horswood, R. B. Couch, and R. M. Chanock. 1973. Antibody to Mycoplasma pneumoniae in nasal secretions and sputa of experimentally infected human volunteers. *Infect. Immun.* 8:612-620.
35. Howard, C. J., R. N. Gourlay, and G. Taylor. 1980. Immunity to Mycoplasma bovis infections of the respiratory tract of calves. *Res. Vet. Sci.* 28:242-249.

36. Messier, S., R. F. Ross, and P. S. Paul. 1986. Humoral and cell-mediated immune responses of pigs following experimental Mycoplasma hyopneumoniae infection. (Manuscript in preparation).
37. Friis, N. F. 1977. Mycoplasma suipneumoniae and Mycoplasma flocculare in the growth precipitation test. Acta Vet. Scand. 18:168-175.
38. Ro, L. H., and R. F. Ross. 1983. Comparison of Mycoplasma hyopneumoniae strains by serologic methods. Am. J. Vet. Res. 44:2087-2094.

SECTION III. HUMORAL AND CELL-MEDIATED IMMUNE RESPONSES OF
PIGS FOLLOWING EXPERIMENTAL MYCOPLASMA
HYOPNEUMONIAE INFECTION

Humoral and cell-mediated immune responses of pigs
following experimental Mycoplasma hyopneumoniae infection

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SUMMARY

The cell-mediated and humoral immune responses of pigs following experimental Mycoplasma hyopneumoniae infection were investigated. The response of peripheral blood (PBL) and bronchial lymph node (BLNL) lymphocytes to stimulation by M. hyopneumoniae antigens was evaluated by lymphocyte stimulation test and presence of specific antibodies in serum and lung washing (LW) samples assayed by complement-fixation test (CF) and ELISA. Additionally, immunoglobulin-positive cells in lung and bronchial lymph node (BLN) were identified by indirect fluorescent antibody test (IFA). Positive stimulation of PBL from infected and noninfected pigs occurred on every occasion PBL were tested and lymphocytes from infected pigs generally had higher stimulation indices (SI). The response of BLNL appeared more antigen-specific and BLNL from infected pigs had significantly higher SI at 2 and 6 weeks post-inoculation. CF antibodies to M. hyopneumoniae were detected in sera and LW from infected pigs. The ELISA indicated that IgA antibodies predominated in LW from most infected pigs and the intensity of the reaction exhibited by the IgG antibodies increased over the course of the infection. Cells producing IgM and IgA isotypes were found in lungs of all pigs but IgG-positive cells were present only in lungs of infected pigs. Of all the immune responses developing in mycoplasmal pneumonia of swine, the local humoral response seems to better parallel recovery from the disease.

INTRODUCTION

Both cell-mediated and humoral immune responses to Mycoplasma hyopneumoniae have been detected following experimental infection (1) or vaccination (2,3) but their exact role in protection against, or in pathogenesis of mycoplasmal pneumonia of swine (MPS) have not been fully resolved. The local humoral response was found to be important in providing some degree of protection against mycoplasmal pneumonias similar to MPS occurring in other animal species (4,5,6,7). The importance of this immune response in MPS was suggested when the percentage and total number of B-lymphocytes in lungs increased following infection with M. hyopneumoniae (8). The number of immunoglobulin-producing cells in lung tissue, as well as antibody titers to M. hyopneumoniae in tracheobronchial secretions increased during the course of experimental MPS (9). Suppression of T-cell response, achieved by thymectomy, injection of antithymocyte serum, or treatment with cyclophosphamide, and subsequent infection of animals with mycoplasma species known to cause pneumonia resulted in development of less severe microscopic lesions of pneumonia than in immunocompetent infected animals (10,11,12,13). But at the same time, suppression of this immune response allowed mycoplasmas to grow and reach numbers 10 to 1000 fold higher than in immunocompetent infected animals (10,12,13, 14). Also, spread of mycoplasmas to other organs occurred more frequently in immunosuppressed infected animals (10).

In an attempt to help elucidate the role of the immune response in the pathogenesis of MPS, the local and systemic humoral and cell-mediated

immune responses of pigs experimentally infected with M. hyopneumoniae were investigated.

MATERIALS AND METHODS

Experimental Animals

Yorkshire and Hampshire pigs, 7 to 10 weeks old, of both sexes were obtained from a barrier-maintained, closed herd at the animal resource station at Iowa State University. The herd was originally established from Cesarean-derived, isolation-reared animals. The pigs were fed a 16% protein swine grower ration without any growth promoters or other antibacterial substances. Pigs were housed in four groups of seven animals in separate isolation rooms.

Experimental Design

Twenty-eight pigs were randomly distributed on the basis of litter and sex into two groups of equal size. Pigs in one group were each inoculated once intratracheally (IT) (15) with 5 ml of 10% pneumonic lung suspension containing M. hyopneumoniae. This pig-passaged lung inoculum, designated M. hyopneumoniae strain 194, was derived from a field case of MPS and has been shown to be free of detectable bacteria, mycoplasmas other than M. hyopneumoniae and viruses. The other group received a placebo inoculation IT consisting of mycoplasma culture broth.

Blood samples were collected for peripheral blood lymphocytes (PBL) isolation and serum collection once before and at weekly intervals following inoculation. Pigs were killed and necropsies done on five pigs from each group at 2 and 4 weeks post-inoculation (WPI) and on the remaining pigs at 6 WPI.

Mycoplasma hyopneumoniae Membranes

The 194 strain (field isolate, passage 7) of M. hyopneumoniae was used to prepare the antigen for the lymphocyte stimulation test. The organisms were grown in Friis mycoplasma broth (16,17) with acid adjusted swine serum instead of whole serum (18). Mycoplasmas were grown in flasks by shaking (50 rpm) at 37°C for 3 days in a waterbath.^a

Membranes of M. hyopneumoniae were obtained by slight modification of the osmotic lysis technique of Razin (19). Briefly, cells were pelleted by centrifugation at 12,000 X g for 15 minutes, washed once with a large volume of 0.02M Tris-HCl buffer, pH 7.0. The cells were then resuspended in prewarmed (37°C) 2M glycerol (in 0.25M NaCl) solution and incubated at 37°C for 15 minutes. The cell suspension was then transferred into 50 volumes of warm (37°C) deionized water and incubated at 37°C for 15 minutes. The membranes were collected by centrifugation at 27,000 X g for 40 minutes and washed once with 0.05M NaCl-0.01M phosphate buffer, pH 7.0. The cells were resuspended in diluted buffer [1/20 dilution of 0.15M NaCl-0.05M tris (hydroxymethyl) aminomethane-0.01M 2-mercaptoethanol, pH 7.4] and the protein concentration determined according to Lowry et al. (20) with bovine plasma proteins as the standard.^b For use in the lymphocyte stimulation test, M. hyopneumoniae membranes were diluted to 200 µg of protein/ml and stored at -70°C in 1-ml aliquots. Nonviability and

^a Aquatherm Waterbath Shaker, New Brunswick Sci. Comp., Edison, N.J.

^b Lab-Trol Chemistry Control, Dade Diagnostic Inc., Aquada, Puerto Rico.

sterility of the membrane preparations were checked by inoculating samples on both Friis mycoplasma agar and broth.

Isolation of Lymphocytes

Heparinized blood samples (20 U heparin/ml of blood) were diluted 1:2 with phosphate buffered saline solution containing 1% glucose (PBSS-glucose). The diluted blood was layered on a Ficoll-sodium diatrizoate solution (Histopaque-1077^c) and centrifuged at 400 X g for 30 minutes at room temperature. The PBL rich band was collected and washed once with PBSS-glucose by centrifugation at 150 X g for 10 minutes. Contaminating erythrocytes were lysed by incubating the PBL in 0.83% NH₄Cl-0.0M phosphate buffer (pH 7.2) for 5 minutes at room temperature. The PBL were then washed twice with PBSS-glucose. Following the final wash the PBL were resuspended in RPMI-1640^d medium supplemented with fetal bovine serum^d (15%), penicillin^d (50 IU/ml), streptomycin^d (50 µg/ml), kanamycin^d (50 µg/ml), L-glutamine (2mM) and HEPES (10mM). The PBL were counted and the concentration adjusted to 2×10^6 lymphocytes/ml. Viability as determined by trypan blue dye exclusion test was >98%.

On days of necropsy, bronchial lymph nodes were aseptically collected and placed in Hanks' balanced salt solution without Ca++ or Mg++^d (HBSS). The lymph nodes were minced using a pair of forceps and a scalpel. The

^cSigma Chemical Co., St. Louis, Mo.

^dFlow Laboratories Inc., McLean, Va.

cell suspension was transferred to a tube and the large pieces allowed to sediment. The supernate was removed and centrifuged at 150 X g for 10 minutes at room temperature. Cells were resuspended in NH_4Cl -phosphate buffer and incubated as above. Bronchial lymph node lymphocytes (BLNL) were washed twice with PBSS-glucose and resuspended in supplemented RPMI-1640. Cell concentration was adjusted to 2×10^6 lymphocytes/ml. Viability of BLNL was >90%.

Lymphocyte Stimulation Test

Lymphocytes were dispensed in 100- μl volumes in the wells of micro-titer plates^d with equal volumes of concanavalin A type IV^c (ConA 4 $\mu\text{g/ml}$), phytohemagglutinin A type V^c (PHA 10 $\mu\text{g/ml}$), pokeweed mitogen^c (PWM 5 $\mu\text{g/ml}$), heated (60 min. at 56°C) or nonheated M. hyopneumoniae cell membranes (4 $\mu\text{g/ml}$) or control medium (HBSS). All cultures were set up in triplicate and incubated for 3 days (ConA, PHA, PWM and HBSS), or 5 days (ConA, M. hyopneumoniae and HBSS), at 37°C in an atmosphere of 5% CO_2 in air and high humidity. Eighteen hours before harvesting the respective plates, 1 μCi of [³H]-thymidine^e (specific activity 6.7 Ci/mmol) was added to each well. The cells were collected on glass fiber paper strips^f using a cell harvester (Microharvester^f). After air-drying, glass fiber disks were placed in 4 ml of scintillation cocktail [42 ml PPO-POPOP concentrate

^eNew England Nuclear, Boston, Mass.

^fBellco Glass Inc., Vineland, N.J.

(Liquifluor^e) plus 1000 ml toluene] and [³H]-thymidine incorporation counted using a liquid scintillation counter.^g Results were expressed as a stimulation index calculated as follows: SI=cpm in stimulated cultures/cpm in control cultures. Because great variations were seen in cpm of control cultures, a SI of 3 or greater was considered positive.

Necropsy

Following electrocution, pigs were exsanguinated. After a piece of the right cardiac lobe was taken aseptically for microbiologic examination, lungs were removed and examined for gross lesions of MPS. The number of lobes with lesions was recorded and the lesions were sketched. Mean percentage of area of lungs with lesions was measured with an image analyzer.^h

Lung Washings

After the gross lesions of MPS were sketched, the left bronchus was clamped and the left lung removed; 30 ml of phosphate buffered saline (PBS) were injected into the bronchus and the lung massaged for about 1 minute. The lung washings (LW) (5 to 10 ml) were centrifuged at 1000 X g for 10 minutes and the supernate stored at -70°C. Before performing assays to detect antibodies to M. hyopneumoniae, the LW were concentrated by freeze-drying and reconstituted to a 1-ml volume.

^gLS-230, Beckman Instrument Inc., Fullerton, Ca.

^hModel MOP-3, Carl Zeiss Inc., Thornwood, N.Y.

Histopathology

Samples of lung tissue were taken from 4 lobes with lesions, when possible, and fixed in 10% buffered formalin. Tissues were cut at 5 μ m, processed according to standard procedures and sections stained with hematoxylin and eosin. Using light microscopy, the tissues were examined for presence of lesions characteristic of MPS (21).

Bacteriologic and Mycoplasmal Cultural Procedures

All bacteriologic and mycoplasmal cultural procedures used were similar to those described by Ross et al. (22). A piece of right cardiac lobe was ground in Friis liquid medium (without added antibiotics) using a TenBroeck tissue grinder. Serial tenfold dilutions of the lung homogenate in Friis broth with methicillin and bacitracin were carried out to 10^{-7} . Samples of secretions from the nasal cavity, trachea, and right bronchus were collected with sterile cotton-tipped applicators.

Complement-Fixation Test

All serum samples collected and reconstituted LW were assayed for presence of antibodies to M. hyopneumoniae by a modified (23) direct microtiter complement-fixation-test (CF) (24).

Direct Immunofluorescence

Lung samples collected contiguous to the 4 samples collected for histopathology were embedded immediately in an ethylene glycol-based

medium (OCTⁱ) and frozen rapidly over dry ice. Evaluation for presence of M. hyopneumoniae or its antigens was done by a direct immunofluorescence procedure (25).

Indirect Immunofluorescence

Three sections per pig of frozen lung tissue and bronchial lymph node (BLN) collected at necropsy were cut at 6 μ m with a refrigerated microtome.^j Tissue sections were fixed in acetone for 10 minutes and air-dried. For each pig individual lung and BLN, tissue sections were overlaid with 50 μ l of murine monoclonal antibodies raised against either swine IgM, IgG, or IgA and incubated for 30 minutes at 37°C in a humid chamber. Slides were washed twice in cold PBS for 5 minutes with agitation. Tissue sections were overlaid with 50 μ l of a fluorescein-conjugated goat anti-mouse IgG^k (H&L chain specific) and incubated for 30 minutes at 37°C in a humid chamber. Slides were rinsed twice in cold PBS for 5 minutes with agitation. Tissue sections were air-dried, mounted with glycerol-PBS (9:1) and kept at 4°C until examined. Sections of lung and BLN were examined with a binocular microscope^l equipped with a dark field condenser and an Osram HBO 200 mercury vapor lamp. A blue excitatory filter (BG112) and a yellow barrier

ⁱLab-Tek Products, Division of Miles Laboratories Inc., Naperville, Ill.

^jAmerican Optical, Buffalo, N.Y.

^kPel-Freez Biologicals, Rogers, Ark.

^lErnst Leitz, Wetzlar, West Germany

filter (K530) were used. The intensity of fluorescence was scored on a 0 to 3+ scale (26).

Enzyme-Linked Immunosorbent Assay

All reconstituted LW and the corresponding serum samples collected the day before necropsy were assayed for identification of the immunoglobulin classes of antibodies to M. hyopneumoniae by an enzyme-linked immunosorbent assay (ELISA). Preparation of the antigen and all solutions used were the same as those described by Piffer et al. (27). Following adsorption of the antigen to the plates, these were stored at -20°C until used. On day of use, plates were warmed at room temperature and washed 4 times. LW or serum to be tested (100 μl) were added to the wells and the plates incubated at 37°C for one hour. The plates were then washed 4 times and 100 μl of murine monoclonal anti-swine IgM, IgG, or IgA added to the appropriate wells. Following incubation for one hour at 37°C , plates were washed 4 times and 100 μl of peroxidase-labeled goat anti-mouse IgG^k (H&L chain specific) added to each well. Plates were incubated for one hour at 37°C . The plates were washed 4 times and 100 μl of substrate (5-aminosalicylic acid and H_2O_2) added. After 30 minutes, the enzymatic reaction was stopped by adding 20 μl of 1N NaOH. Absorbance at 488 nm was measured directly in the microtiter plate wells.

Statistical Analysis

Analysis of variance was used to compare the mean stimulation indices of the two groups. The two-sided Student t-test was used to compare mean areas of lung with lesions of pneumonia.

RESULTS

Pathology

Gross lesions of pneumonia were found in all M. hyopneumoniae inoculated pigs. Lungs of infected pigs killed at 2 and 4 WPI had lesions in every lobe while lungs of infected pigs killed at 6 WPI had lesions in all but 4 lobes (Table 1). No significant difference was found in the percentage of lung with pneumonia in pigs killed at 2 and 4 WPI, but a significant decrease in the amount of gross lesions had occurred at 6 WPI (Table 1).

Upon microscopic examination, typical lesions of MPS consisting of peribronchial, peribronchiolar, and perivascular lymphoid hyperplasia were seen in every lobe obtained from infected pigs. At 6 WPI, the lymphoid hyperplasia appeared to be more severe around or close to bronchioles, and frequently lymphoid nodule formations could be seen scattered throughout sections of lung.

Bacteriologic Examination

Lung homogenates from all noninfected pigs were culture-negative for Bordetella bronchiseptica, Pasteurella multocida, Haemophilus spp., or mycoplasmas. Cultures of lung homogenates from infected pigs resulted in isolation of M. hyopneumoniae from all samples; lung homogenates from all infected pigs were culture-negative for bacteria.

No B. bronchiseptica, P. multocida, Haemophilus spp., or other porcine mycoplasmas were isolated from nasal secretions, tracheal secretions or

Table 1. Pathological and microbiological findings following experimental M. hyopneumoniae infection

WPI	(n=No. of pigs)	Gross lesions		Isolation of <u>M. hyopneumoniae</u>	Direct immunofluorescence	
		No. of lobes positive/No. examined	Percentage of lung pneumonic ^{*,+}	No. of pigs positive	No. of pigs positive	No. of lobes positive/No. examined
2	Noninfected (n=5)	0/35	0	0	0	0/20
	Infected (n=5)	35/35	7.31±3.47 ^a	5	5	14/20
4	Noninfected (n=5)	0/35	0	0	0	0/20
	Infected (n=5)	35/35	11.18±6.94 ^a	5	5	16/20
6	Noninfected (n=4)	0/28	0	0	0	1/16
	Infected (n=4)	24/28	2.8±2.7 ^b	4	4	7/16

* Groups with different superscripts differ (p<.05).

⁺ $\bar{X} \pm \text{SD}$.

bronchial secretions of either infected or noninfected pigs.

Direct Fluorescent Antibody Test

Presence of M. hyopneumoniae or its antigens was detected by direct fluorescent antibody (DFA) technique in one lobe from one noninfected pig and the intensity of fluorescence was low. In lung sections from infected pigs, the DFA test was positive for 70% of examined lobes from pigs killed 2 WPI; 80% of the examined lobes from pigs killed 4 WPI; and 43% of the examined lobes from pigs killed 6 WPI (Table 1).

Complement-Fixation Test

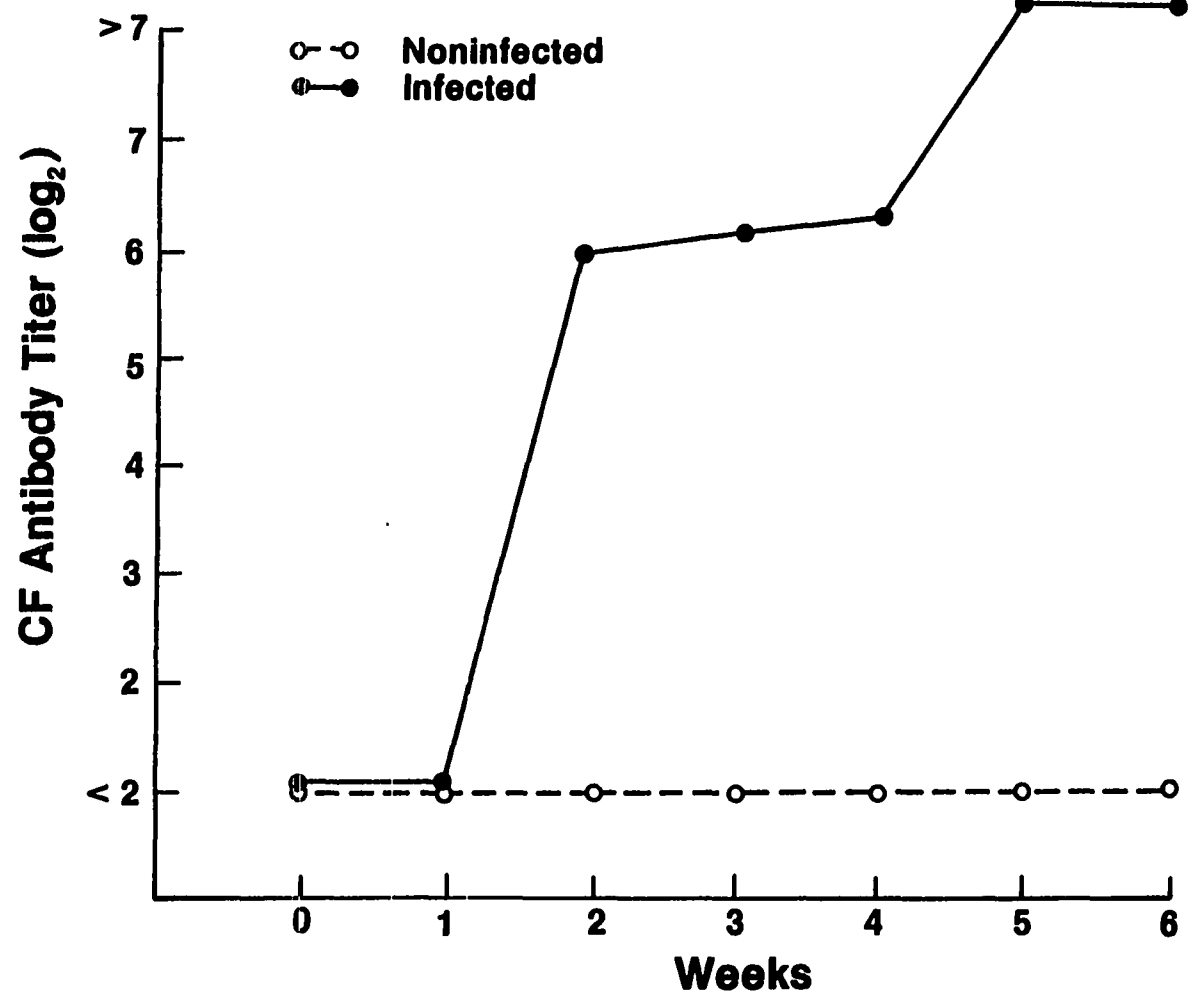
All infected pigs had detectable CF antibody titers in their serum by the second week post-inoculation. The mean antibody titer increased over the next few weeks and remained high until the end of the experiment (Figure 1).

Complement-fixing antibodies to M. hyopneumoniae were present in samples of lung washings collected from every infected pig killed at 2 and 6 WPI and in 4 out of 5 pigs killed 4 WPI.

Lymphocyte Stimulation Test

In all instances when PBL from infected and noninfected pigs were stimulated with M. hyopneumoniae membranes, positive SI were obtained. Considerable weekly variations were noted but generally the response of PBL from infected pigs was higher than the one obtained with PBL from non-infected pigs. Significant differences ($p < .05$) between infected and

Figure 1. Complement-fixing antibody titer (geometric mean titers) following exposure to M. hyopneumoniae



noninfected pigs were noted at 4 and 6 WPI when nonheated membrane preparations were used (Figure 2), and also at 6 WPI when heated membrane preparations were used, PBL from infected pigs having the higher SI (Figure 3). Heat-treatment of the membranes did not have a consistent effect on PBL from infected and noninfected pigs. On some occasions increased SI were noted while on other occasions a decrease in SI had occurred (Figures 2 and 3).

Nonspecific stimulation of BLNL from noninfected animals was seen at 2 and 4 WPI but SI were low (Table 2). High SI with BLNL from infected pigs were obtained 2 WPI and a smaller, but still, significantly increased SI ($p < .01$) was detected at 6 WPI (Table 2). At 4 WPI, no significant difference between the SI obtained with BLNL from infected and noninfected pigs was detected. Heat-treatment of the membranes significantly ($p < .05$) reduced the response of BLNL from infected pigs at 2 and 6 WPI from 42.4 to 7.2, and from 7.3 to 2.8 respectively (Table 2).

Weekly responses of PBL to ConA, PHA, and PWM gave mean SI ranging from 20 to 140. Responses of BLNL to these mitogens were stronger, SI ranging almost always between 100 and 400. No significant difference was found between responses of infected and noninfected pigs.

Indirect Fluorescent Antibody Test

Small amounts of IgM- and IgA-positive cells were seen by indirect fluorescent antibody (IFA) technique in most lung sections from noninfected pigs killed at 2, 4, and 6 WPI. In sections of lungs from infected pigs killed 2 WPI, the number of IgM-positive cells was only slightly

Figure 2. Mean stimulation index of peripheral blood lymphocytes following exposure to nonheated membranes of M. hyopneumoniae

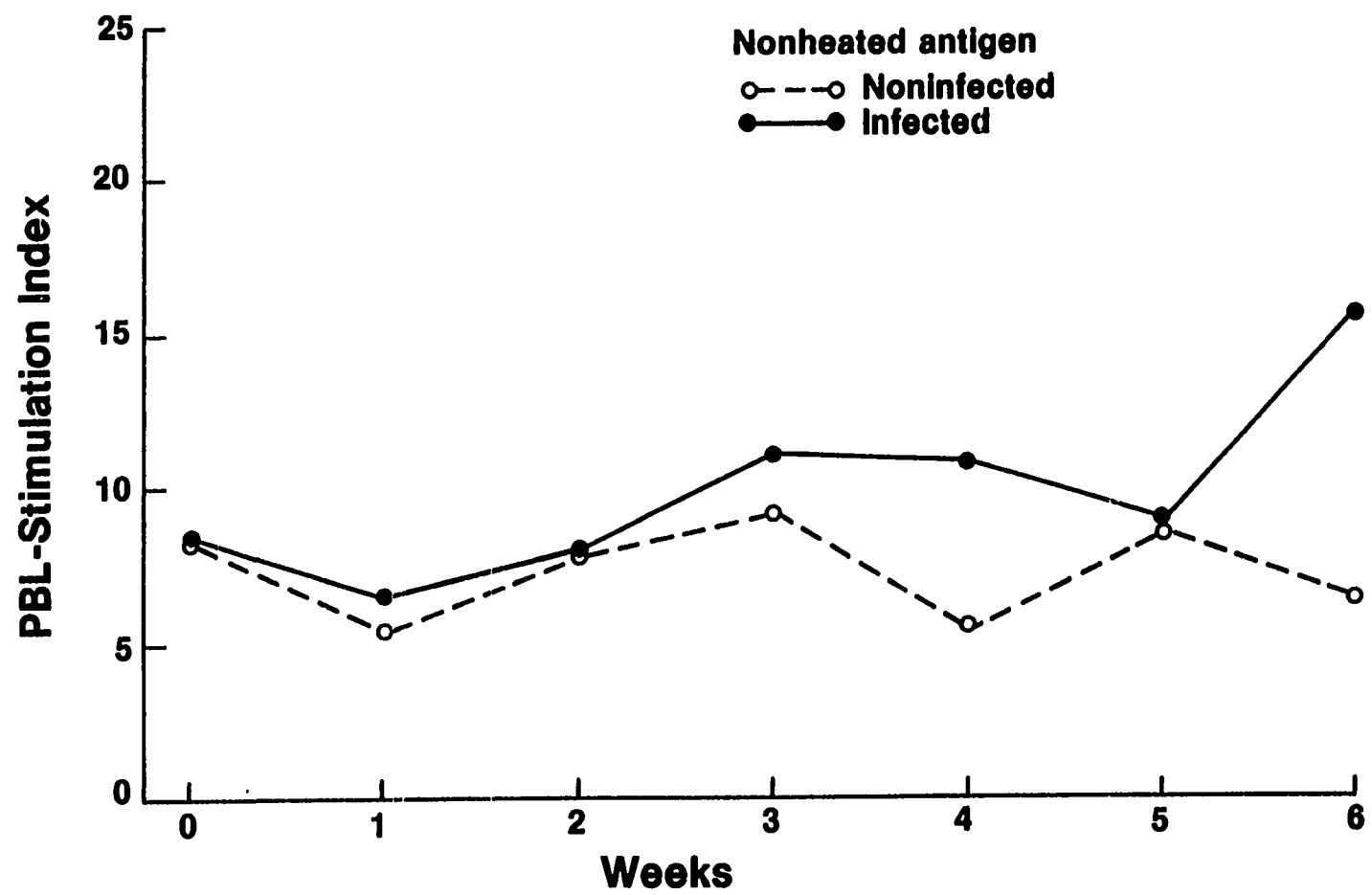


Figure 3. Mean stimulation index of peripheral blood lymphocytes following exposure to heated membranes of M. hyopneumoniae

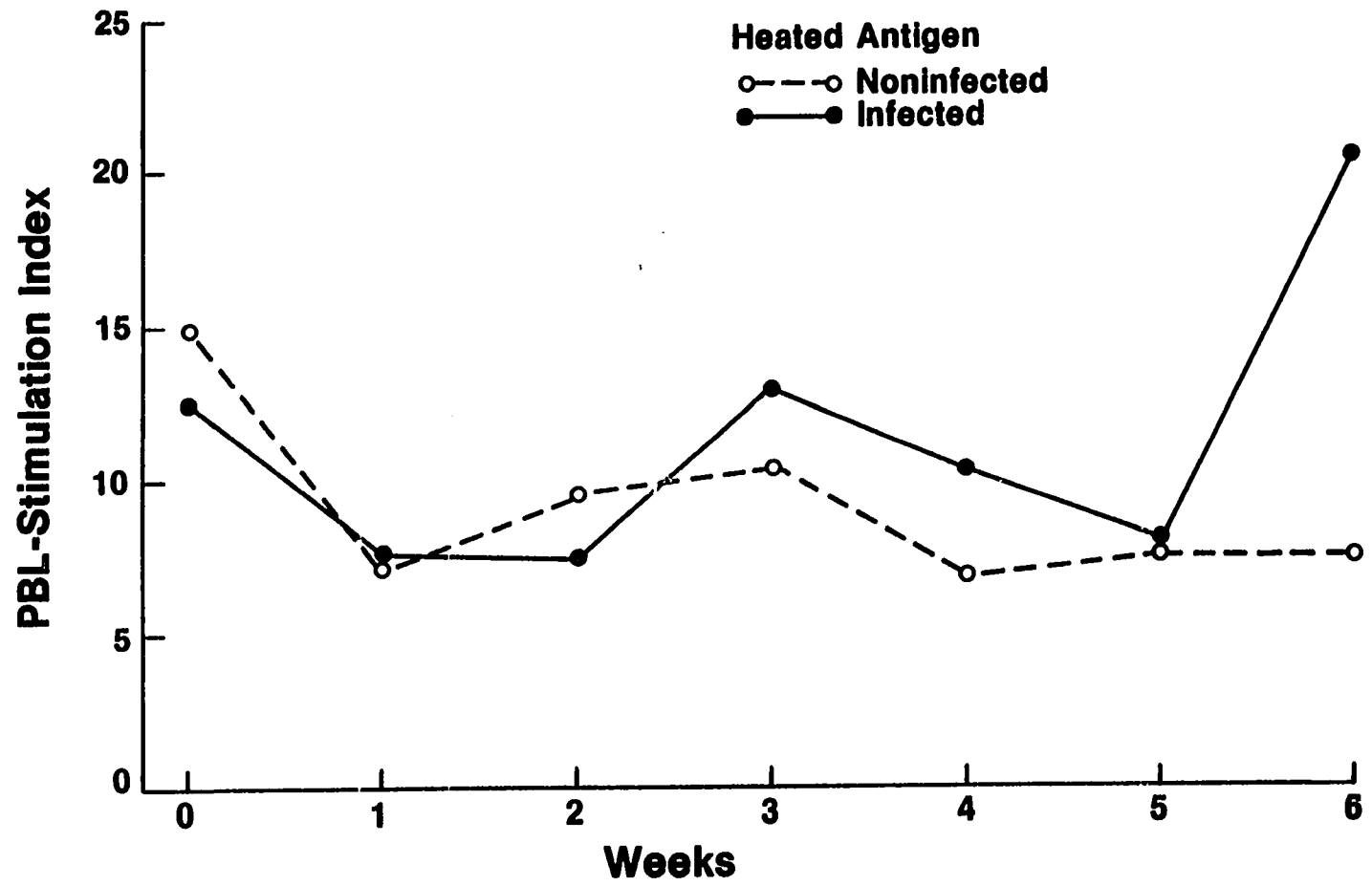


Table 2. Effect of experimental M. hyopneumoniae infection on responsiveness of bronchial lymph node lymphocytes to the organism

<u>M. hyopneumoniae</u> membranes	Group	Weeks post-inoculation		
		2 ^{**} (n=5) [#]	4 (n=5)	6 ^{**} (n=4)
Nonheated	Noninfected	4.6±2.4 ^{a,+}	3.0±2.3	1.4±0.5 ^a
	Infected	42.4±21.5 ^b	3.9±5.5	7.3±3.4 ^b
Heated [@]	Noninfected	1.4±0.7 ^{a,b,c}	2.2±1.8	1.6±0.5 ^{a,b}
	Infected	7.2±3.6 ^{a,b,d}	1.3±1.1	2.8±1.8 ^{a,b}

^{**} Groups with different superscripts in each column differ (p<.01).

[#] Number of pigs per group.

⁺ Stimulation index; $\bar{X} \pm \text{SD}$.

[@] 56°C, 60 minutes.

greater than in noninfected pigs, while greater numbers of IgA-positive cells were present, and IgG-positive cells were found in sections from all 5 infected pigs (Table 3). At 4 WPI, IgM-positive cells were detected in lung sections from infected pigs in amounts almost similar to what was found in noninfected pigs. Greater numbers of IgA-positive cells were found in lung sections from infected pigs than in noninfected pigs (Table 3). Almost identical quantities of IgA-positive cells were detected by IFA in lung sections from infected pigs killed at 2 and 4 WPI. Presence of IgG-positive cells noted in sections of lungs from infected pigs killed 4 WPI generally appeared to be more numerous than in sections from pigs killed 2 WPI (Figures 4 and 5). At 6 WPI, lung sections from infected pigs contained small numbers of IgM-positive cells, while IgG- and IgA-positive cells were fairly abundant (Table 3).

At 2 and 4 WPI, examination of bronchial lymph node (BLN) by IFA did not reveal great differences between infected and noninfected pigs in regard to total number of cells found or class of immunoglobulin-positive cells present, but germinal centers were seen more frequently in BLN from infected pigs (Figures 6 and 7). At 6 WPI, again little difference between infected and noninfected pigs was seen in regard to IgM- and IgA-positive cells, but IgG-positive cells appeared more numerous in BLN from infected pigs (Table 3). Germinal centers were also found frequently in these pigs.

ELISA

Initial testing of the LW by ELISA was done with a 1:10 dilution of the concentrated lungs washings. Two weeks post-inoculation specific

Table 3. Immunoglobulin-positive cells in lung and bronchial lymph node after experimental M. hyopneumoniae infection

WPI	Group (n=No. of pigs)	Tissue					
		Lung			Bronchial lymph node		
		IgM	IgG	IgA	IgM	IgG	IgA
2	Noninfected (n=5)	+ [@]	0	+	++	+	++
	Infected (n=5)	+	+	++	++	+	++
4	Noninfected (n=5)	±	0	+	+++	++	++
	Infected (n=5)	+	++	++	+++	++	++
6	Noninfected (n=4)	±	0	+	++	±	++
	Infected (n=4)	+	++	++	+++	+++	+++

[@]Fluorescence in sections of lung and lymph node was graded as follows:
 0 = no positive cell, ±: rare single cell, +: single positive cell,
 ++: scattered areas of positive cells, +++: many areas of numerous positive
 cells. One section of tissue per pig was examined for each isotype and
 grades were "averaged" for the number of pigs in each group.

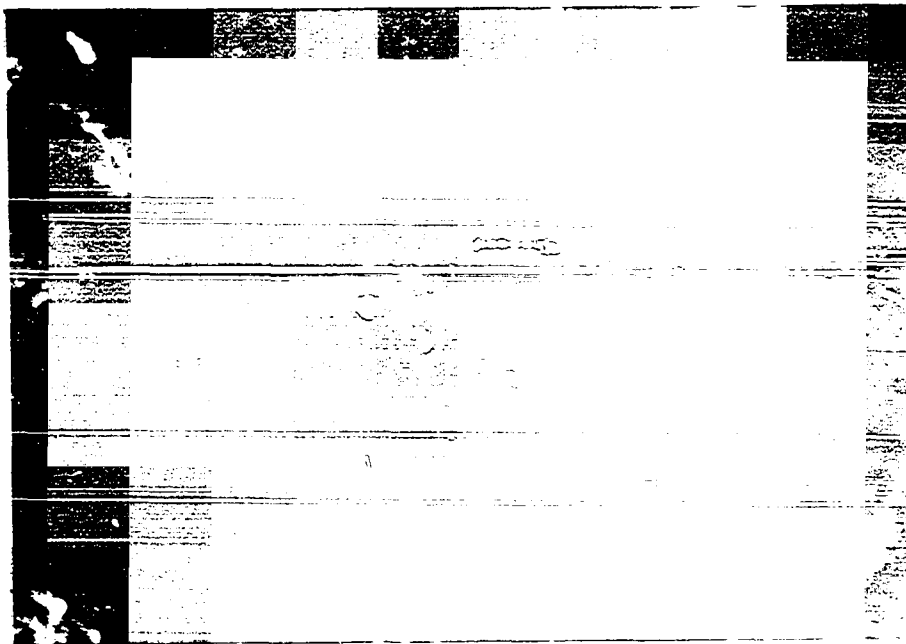


Figure 4. IgG-positive cells in lung from M. hyopneumoniae infected pig 2 WPI. 80X

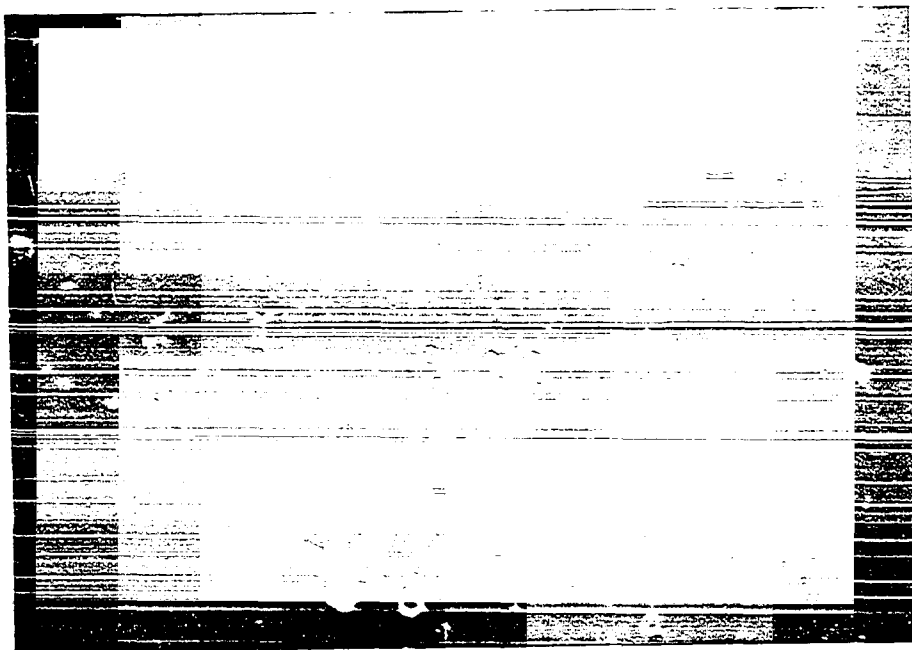


Figure 5. IgG-positive cells in lung from M. hyopneumoniae infected pig 4 WPI. 80X

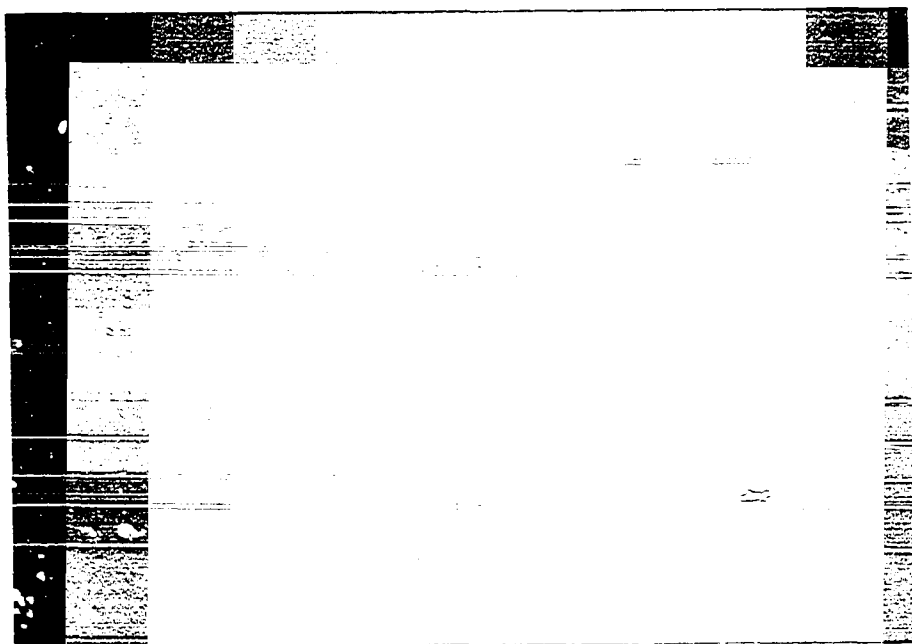


Figure 6. IgM-positive cells in bronchial lymph node from noninfected pig 2 WPI. 40X

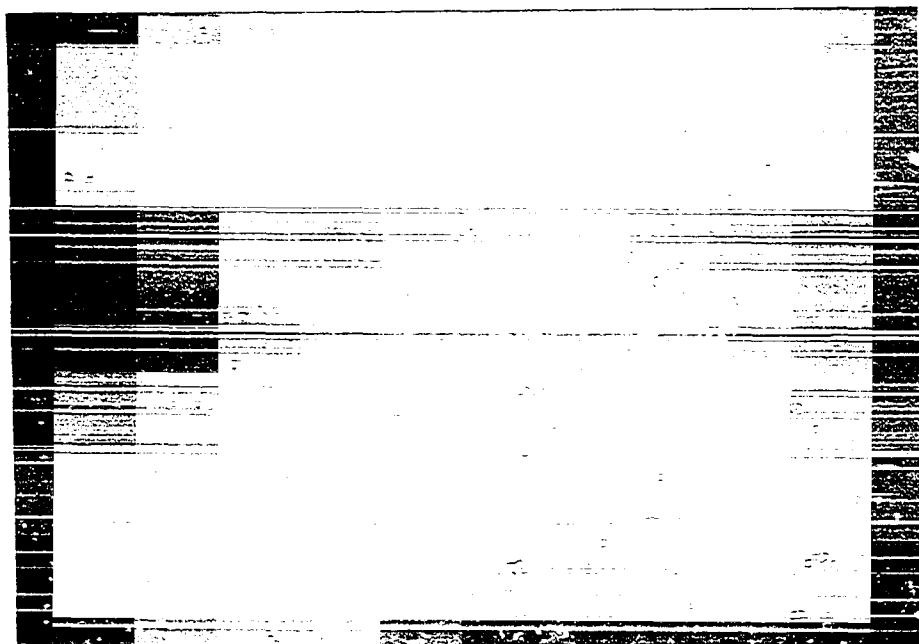


Figure 7. IgM-positive cells in bronchial lymph node from M. hyopneumoniae infected pig 2 WPI. 40X

antibodies to M. hyopneumoniae could be detected in all LW collected from infected pigs. All three isotypes of immunoglobulin were found, IgG being the most frequent (Table 4). At 4 WPI, specific antibodies were again detected in LW from all infected pigs and antibodies of the three isotypes were found; IgG and IgA antibodies were both present in all samples. Six weeks post-inoculation strong reactions were observed with all three isotypes when using LW from the four infected pigs.

Additional dilutions of the LW from infected pigs had to be done and the ELISA repeated because it was difficult to tell, especially with pigs killed at 4 and 6 WPI, which of the three isotypes was predominant. Two weeks post-inoculation, IgG antibodies predominated in LW of 3 pigs as detected in the 1:10 dilution of LW. In the two other pigs, one had slightly higher levels of IgM antibodies while the other one had slightly higher levels of IgA. At 4 WPI, 4 out of 5 infected pigs had higher levels of IgA relative to the other two isotypes. Two of these 4 pigs also had moderately higher levels of IgG antibodies. The last pig had moderate levels of IgA compared to the other isotypes. Six weeks post-inoculation, IgA antibodies were predominant in 2 of the 4 infected pigs. They were also found in high levels in association with high levels of IgG in one pig, and in moderate amount in one pig that had IgG as the predominant isotype. IgG antibodies were found in moderate amount in two pigs, while IgM antibodies were in moderate amount in one pig with high levels of IgG and IgA antibodies.

Specific serum antibodies to M. hyopneumoniae were found in serum samples from 2 of the 5 infected pigs killed 2 WPI, one having only IgM

Table 4. Isotype of antibodies to M. hyopneumoniae in lung washings from infected pigs

WPI	Immunoglobulin														
	IgM [#]					IgG					IgA				
2	++ [@]	-	+	+	++	++	++	++	++	++	+	-	+	++	+
4	-	-	++	-	++	++	++	++	++	++	++	++	++	++	++
6	++	++	++	++		++	++	++	++		++	++	++	++	

[#] For each immunoglobulin isotype, each column represents results from individual pig.

[@] -: negative, +: OD₄₈₈ within 2 and 3 SD above mean OD₄₈₈ reading of lung washing from noninfected pigs, ++: OD₄₈₈ value greater than 3 SD above mean OD₄₈₈ reading of lung washing from noninfected pigs.

and the other IgM and IgG antibodies. At 4 WPI, antibodies to M. hyo-
pneumoniae were detected in serum samples from all infected pigs and all
3 isotypes were found. Strong reactions to all isotypes tested were also
obtained with serum samples from the four infected pigs killed 6 WPI.

DISCUSSION

Although weekly variations were noted, the M. hyopneumoniae membrane preparations used in this experiment had a consistent nonspecific mitogenic effect for swine PBL as indicated by detection of positive SI with lymphocytes from noninfected pigs. Earlier reports had mentioned possible nonspecific mitogenicity associated with M. hyopneumoniae (1,3). In previous experiments (28), we inconsistently detected nonspecific mitogenicity for swine PBL when using membranes prepared with a highly passaged field isolate of M. hyopneumoniae (strain 1262, passage 40-42). In the present experiment, membrane suspensions were prepared using a low passage of a field isolate (M. hyopneumoniae strain 194, passage 7) and it is possible that differences in concentration of mitogenic factor exist between these two strains. Strain differences in mitogenic activity of Mycoplasma pulmonis for rat lymphocytes have been documented (29).

Specific activation of PBL seems to take place in advancing stages of MPS since significant differences in SI of infected and noninfected pigs were noted at 4 and 6 WPI when using nonheated membranes, and at 6 WPI when using heated membrane preparations of M. hyopneumoniae. Highly variable results have been obtained with lymphocyte stimulation tests using PBL from pigs infected with M. hyopneumoniae. Kristensen et al. (3) reported finding positive stimulation of PBL one week following contact-exposure, whereas Adegboye (1) first detected positive SI 15 weeks after infection. After the initial detection, results were inconsistent in the following weeks. In human pneumonia caused by Mycoplasma pneumoniae sensitized PBL

were generally detected within a month following onset of disease and persisted for several years (30,31,32,33).

The response of BLNL to stimulation by M. hyopneumoniae membranes appeared to be more antigen-specific than the response of PBL. Positive stimulation of BLNL from noninfected pigs occurred only twice when using heated and no-heated membrane preparations, whereas positive SI with BLNL from infected pigs were consistently obtained when nonheated membrane preparations were used and on one occasion when heated membrane preparations were used. In children with recent or active infection with M. pneumoniae, the response of lymphocytes from adenoid vegetations to stimulation by M. pneumoniae antigens was a better indicator of infection than the response of PBL from the same patients (34). Kristensen et al. (3) on the other hand found that PBL from M. hyopneumoniae infected pigs were responsive to stimulation by the homologous antigen whereas lymphocytes from bronchial lymph node were not stimulated. Significant reduction in the response of BLNL from infected pigs after heat-treatment of the membrane preparations added support to the observation made previously (29) that the principal antigen(s) recognized by the lymphocytes in the lymphocyte stimulation test is or contains protein as its major component.

Depression of cell-mediated immunity, as indicated by loss of responsiveness of lymphocytes to stimulation by PHA, has been reported following M. hyopneumoniae infection (35) and in other mycoplasmal pneumonias (36,37, 38,39). In the present experiment, no significant difference in responses of PBL and BLNL from infected and noninfected pigs to stimulation by ConA, PHA, and PWM was detected.

Serum samples from all 5 infected pigs had detectable CF antibody titers by 2 WPI but the ELISA detected specific antibodies to M. hyopneumoniae in only two of these pigs. The reason for this discrepancy is difficult to understand because the ELISA has been found more sensitive than the CF for detection of antibodies in the early stages of MPS (27). At 4 and 6 WPI, antibodies to M. hyopneumoniae were detected by both CF and ELISA in serum samples from every infected pig. By ELISA, we found that antibodies to M. hyopneumoniae of all isotypes tested were present in serum from infected pigs and IgG antibodies generally gave the strongest reaction. Suter et al. (9) recently reported that the antibodies to M. hyopneumoniae detected by ELISA in sera of infected pigs were of the IgG isotype only. The specificity of the monoclonal antibodies used to detect the various isotypes of antibodies coupled to the sensitivity of the ELISA would explain the differences noted between our results and those of Suter et al. (9). Holmgren (40) found IgG and IgA antibodies to M. hyopneumoniae by indirect haemagglutination. IgA antibodies have also been found in human sera following M. pneumoniae infection (41,42).

A local humoral immune response to M. hyopneumoniae was detected in all infected pigs either by CF, ELISA, or both. Early following inoculation (2 WPI), IgG antibodies were the most frequently identified in LW. As the disease progressed (4 WPI) IgA gave stronger reactions in ELISA and IgG antibodies could still be found in LW from all infected pigs. By 6 WPI, reactions seen by ELISA with specific IgG antibodies in LW from infected pigs were stronger when compared to results at 2 and 4 WPI. Antibodies of the IgM isotype were also found in LW from most infected pigs.

Suter et al. (9) reported steady increases of IgG and IgA antibodies to M. hyopneumoniae in tracheobronchial secretions up to 5 weeks post-infection with IgG antibodies generally giving the strongest reaction. By 8 weeks post-infection IgA antibodies had kept increasing whereas IgG antibodies had decreased. Holmgren (40) reported presence of IgA and IgG antibodies in tracheobronchial secretions of M. hyopneumoniae infected pigs but questioned the production of IgG antibodies in lung tissue and suggested that they were transuded from the serum. Following swine influenza, Charley and Corthier (43) could not find evidence of antibody transfer from serum to pulmonary secretions and concluded that antibodies found in lungs were locally produced.

No difference in quantity of IgM-positive cells between infected and noninfected pigs could be detected by IFA during the experiment. On the other hand, IgA-positive cells were more numerous in lung sections from infected pigs and IgG-positive cells were detected only in lungs from infected pigs. The number of IgA-positive cells in lungs of infected pigs did not change much between 2 and 4 WPI whereas the number of IgG-positive cells appeared to have increased during the same time period. Six weeks after inoculation the number of IgA- and IgG-positive cells were still high. Suter et al. (9) reported that increase of IgA- and IgG-positive cells had occurred 3 weeks after M. hyopneumoniae infection but by 5 WPI, a rapid decrease occurred. In M. pneumoniae-infected hamsters, the peribronchiolar and perivascular lymphoid hyperplasia consisted mainly of IgM-positive cells with variable amounts of IgG-positive cells and minimal numbers of IgA-positive cells (26). Mice infected with M. pulmonis were

found to have IgG as the predominant isotype of immunoglobulin-positive cells in perivascular and peribronchiolar lymphoid hyperplasia (44). Genetic differences in rat strains probably accounted for the fact that following M. pulmonis infection only IgA-positive cells increased in lungs of F344 rats (45,46), and IgM-, IgG-, and IgA-positive cells increased in LEW rat lungs (46).

Although no great difference was found in number or class of immunoglobulin-positive cells in BLN from infected and noninfected pigs in early stages of MPS, detection of more germinal centers in BLN from infected pigs suggests involvement of the local immune response. In later stages of the disease (6 WPI), IgG-positive cells were more numerous in lymph nodes from infected pigs than noninfected pigs. These results are consistent with those of Suter et al. (9). In addition, the results support the observation that, following M. hyopneumoniae infection, cell proliferation occurred in germinal centers (47), as well as in paracortical areas (12,47).

When evaluating the different immune responses developing in experimentally induced MPS it seems that the local humoral immune response better parallels recovery from the disease. This is based on the findings that significant reduction in lesions of pneumonia and reduced detection of M. hyopneumoniae by IFA was observed when large amounts of immunoglobulin-positive cells in lungs and strong positive reactions to M. hyopneumoniae in ELISA with LW were detected. Increasing intensity of IgG antibodies in ELISA and greater numbers of IgG-positive cells would suggest a more active role for this immunoglobulin class, even though IgA antibodies are also fairly abundant. In the porcine respiratory tract, IgG antibodies and

antibody-producing cells have generally been associated with the lower respiratory tract, while IgA are predominant in the upper respiratory tract (48,49). Studies on M. pulmonis infection in mice have indicated that IgM, IgG, or IgA could mediate resistance to infection with this mycoplasma (50).

Cell-mediated immunity, as detected by the lymphocyte stimulation test, was noticed early post-inoculation with BLNL and later on with PBL. It is possible that sensitization of local lymphocytes occurs soon after inoculation and that sensitized lymphocytes will reach the systemic circulation after a certain time. Detection of stronger stimulation to M. pneumoniae with PBL from older patients suggested that repeated asymptomatic infection resulted in increasing numbers of sensitized lymphocytes reaching the systemic circulation (51).

REFERENCES

1. Adegboye, D. S. 1978. Attempts to demonstrate cell-mediated immune response during Mycoplasma suis pneumoniae infection of pigs. Res. Vet. Sci. 25:323-330.
2. Kishima, M., R. F. Ross, and C. Kuniyasu. 1985. Cell-mediated and humoral immune response to Mycoplasma hyopneumoniae in pigs enhanced by dextran sulfate. Am. J. Vet. Res. 46:456-462.
3. Kristensen, B., Ph. Paroz, J. Nicolet, M. Wanner, and A. L. de Weck. 1981. Cell-mediated and humoral response in swine after vaccination and natural infection with Mycoplasma hyopneumoniae. Am. J. Vet. Res. 42:784-788.
4. Brunner, H., H. B. Greenberg, W. D. James, R. L. Horswood, R. B. Couch, and R. M. Chanock. 1973. Antibody to Mycoplasma pneumoniae in nasal secretions and sputa of experimentally infected human volunteers. Infect. Immun. 8:612-620.
5. Fernald, G. W., and W. A. Clyde, Jr. 1970. Protective effect of vaccines in experimental Mycoplasma pneumoniae disease. Infect. Immun. 1:559-565.
6. Howard, C. J., R. N. Gourlay, and G. Taylor. 1980. Immunity to Mycoplasma bovis infections of the respiratory tract of calves. Res. Vet. Sci. 29:242-249.
7. Taylor, G., and C. J. Howard. 1980. Class-specific antibody responses to Mycoplasma pulmonis in sera and lungs of infected and vaccinated mice. Infect. Immun. 29:1160-1168.
8. Charley, B., and H. Salmon. 1979. Influence of a mycoplasma respiratory tract infection on sheep erythrocyte and complement receptor-bearing cell composition in the respiratory tract of swine. Am. J. Vet. Res. 40:1257-1259.
9. Suter, M., M. Kobisch, and J. Nicolet. 1985. Stimulation of immunoglobulin-containing cells and isotype-specific antibody response in experimental Mycoplasma hyopneumoniae infection in specific-pathogen-free pigs. Infect. Immun. 49:615-620.
10. Denny, F. W., D. Taylor-Robinson, and A. C. Allison. 1972. The role of thymus-dependent immunity in Mycoplasma pulmonis infections of mice. J. Med. Microbiol. 5:327-336.

11. Fernald, G. W., and W. A. Clyde, Jr. 1974. Cell-mediated immunity in M. pneumoniae infections. Pages 421-427 in J. M. Bove and J. F. Duplan, eds. Les Mycoplasmes de l'Homme, des Animaux, des Vegetaux et des Insectes. Colloques INSERM vol. 33. INSERM, Paris, France.
12. Tajima, M., T. Yagihashi, T. Nunoya, A. Takeuchi, and F. Ohashi. 1984. Mycoplasma hyopneumoniae infection in pigs immunosuppressed by thymectomy and treatment with antithymocyte serum. Am. J. Vet. Res. 45:1928-1932.
13. Taylor, G., D. Taylor-Robinson, and G. W. Fernald. 1974. Reduction in the severity of Mycoplasma pneumoniae-induced pneumonia in hamsters by immunosuppressive treatment with anti-thymocyte sera. J. Med. Microbiol. 7:343-348.
14. Singer, S. H., M. Ford, and R. L. Kirschstein. 1972. Respiratory diseases in cyclophosphamide-treated mice. I. Increased virulence of Mycoplasma pulmonis. Infect. Immun. 5:953-956.
15. Bentley, O. E., and D. O. Farrington. 1980. Evaluation of an induced Pasteurella multocida swine pneumonia model. Am. J. Vet. Res. 41:1870-1873.
16. Friis, N. F. 1975. Some recommendations concerning primary isolation of Mycoplasma suis pneumoniae and Mycoplasma flocculare. A Survey. Nord. Vet. Med. 27:337-339.
17. Friis, N. F. 1979. Selective isolation of slowly growing acidifying mycoplasmas from swine and cattle. Acta Vet. Scand. 20:607-609.
18. Switzer, W. P. 1972. Mycoplasmal pneumonia of swine. J. Am. Vet. Med. Assoc. 160:651-653.
19. Razin, S. 1983. Cell lysis and isolation of membranes. Pages 225-233 in S. Razin and J. G. Tully, eds. Methods in Mycoplasmaology. Vol. 1. Academic Press, New York, New York.
20. Lowry, H., A. L. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement and the Folin phenol reagent. J. Biol. Chem. 193:265-275.
21. Whittlestone, P. 1972. The role of mycoplasmas in the production of pneumonia in the pig. Pages 263-283 in Pathogenic Mycoplasmas. A Ciba Foundation Symposium. Associated Scientific Publishers, Amsterdam, The Netherlands.

22. Ross, R. F., B. J. Zimmermann-Erickson, and T. F. Young. 1984. Characteristics of protective activity of Mycoplasma hyopneumoniae vaccine. *Am. J. Vet. Res.* 45:1899-1905.
23. Slavik, M. F., and W. P. Switzer. 1972. Development of a micro-titration complement-fixation test for diagnosis of mycoplasmal swine pneumonia. *Iowa State J. Res.* 47:117-128.
24. Laboratory Branch of the Communicable Disease Center. 1965. Standardized diagnostic complement fixation methods and adaptation to microtest. U.S. Department of Health, Education, and Welfare, Public Health Monogr. 74.
25. Amanfu, W., C. N. Weng, R. F. Ross, and H. J. Barnes. 1984. Diagnosis of mycoplasmal pneumonia of swine: Sequential study by direct immunofluorescence. *Am. J. Vet. Res.* 45:1349-1352.
26. Fernald, G. W., W. A. Clyde, Jr., and J. Bienenstock. 1972. Immuno-globulin-containing cells in lungs of hamsters infected with Mycoplasma pneumoniae. *J. Immunol.* 108:1400-1408.
27. Piffer, I. A., T. F. Young, A. Petenate, and R. F. Ross. 1984. Comparison of complement fixation test and enzyme-linked immuno-sorbent assay for detection of early infection with Mycoplasma hyo-pneumoniae. *Am. J. Vet. Res.* 45:1122-1126.
28. Messier, S., R. F. Ross, and B. Zimmermann-Erickson. 1986. Role of Mycoplasma hyopneumoniae membranes in the pathogenesis of mycoplasmal pneumonia of swine. (Manuscript in preparation).
29. Naot, Y., J. G. Tully, and H. Ginsburg. 1977. Lymphocyte activation by various Mycoplasma strains and species. *Infect. Immun.* 18:310-317.
30. Biberfeld, G., P. Biberfeld, and G. Sterner. 1974. Cell-mediated immune response following Mycoplasma pneumoniae infection in man. I. Lymphocyte stimulation. *Clin. Exp. Immunol.* 17:29-41.
31. Fernald, G. W. 1972. In vitro response of human lymphocytes to Mycoplasma pneumoniae. *Infect. Immun.* 5:552-558.
32. Fernald, G. W. 1973. Role of host response in Mycoplasma pneumoniae disease. *J. Infect. Dis.* 127 (Suppl.):S55-S58.
33. Mogensen, H. H., V. Andersen, and K. Lind. 1976. Lymphocyte transformation studies in Mycoplasma pneumoniae infections. *Infection* 4 (Suppl. 1):S21-S24.

34. Mogensen, H. H., K. I. Meistrup-Larsen, L. P. Ryder, and K. Lind. 1984. Mycoplasma pneumoniae-stimulation of lymphocytes obtained from adenoid vegetations and blood in children with and without serological evidence of Mycoplasma pneumoniae infection. Acta Pathol. Microbiol. Immunol. Scand. (C) 92:313-317.
35. Adegboye, D. S. 1978. A review of mycoplasma-induced immuno-suppression. Br. Vet. J. 134:556-560.
36. Bennett, R. H., and D. E. Jasper. 1977. Immunosuppression of humoral and cell-mediated responses in calves associated with inoculation of Mycoplasma bovis. Am. J. Vet. Res. 38:1731-1738.
37. Kaklamanis, E., and M. Pavlatos. 1972. The immunosuppressive effect of mycoplasma infection. I. Effect on the humoral and cellular response. Immunology 22:695-702.
38. Roberts, D. H., R. S. Windsor, W. N. Masiga, and C. G. Kariavu. 1973. Cell-mediated immune response in cattle to Mycoplasma mycoides var. mycoides. Infect. Immun. 8:349-354.
39. Sabato, A. R., D. M. Cooper, and Y. H. Thong. 1981. Transitory depression of immune function following Mycoplasma pneumoniae infection in children. Pediatr. Res. 15:813-816.
40. Holmgren, N. 1974. On the immune response in porcine serum and tracheobronchial secretions following experimental infection with Mycoplasma hyopneumoniae. Zentralbl. Veterinarmed. (B) 21:188-201.
41. Biberfeld, G., and G. Sterner. 1971. Antibodies in bronchial secretions following natural infection with Mycoplasma pneumoniae. Acta Pathol. Microbiol. Immunol. Scand. (B) 79:599-605.
42. Fernald, G. W., W. A. Clyde, Jr., and F. W. Denny. 1967. Nature of the immune response to Mycoplasma pneumoniae. J. Immunol. 98:1028-1038.
43. Charley, B., and G. Corthier. 1977. Local immunity in the pig respiratory tract. II. Relationship of serum and local antibodies. Ann. Microbiol. (Inst. Pasteur) 128B:109-119.
44. Cassell, G. H., J. R. Lindsey, and H. J. Baker. 1974. Immune response of pathogen-free mice inoculated intranasally with Mycoplasma pulmonis. J. Immunol. 112:124-136.
45. Davis, J. K., P. A. Maddox, R. B. Thorp, and G. H. Cassell. 1980. Immunofluorescent characterization of lymphocytes in lungs of rats infected with Mycoplasma pulmonis. Infect. Immun. 27:255-259.

46. Davis, J. K., R. B. Thorp, P. A. Maddox, M. B. Brown, and G. H. Cassell. 1982. Murine respiratory mycoplasmosis in F344 and LEW rats: evolution of lesions and lung lymphoid cell populations. *Infect. Immun.* 36:720-729.
47. Adegboye, D. S. 1978. The immunological response of pig bronchial lymph node to Mycoplasma suis. *J. Comp. Pathol.* 88:97-104.
48. Bradley, P. A., F. J. Bourne, and P. J. Brown. 1976. The respiratory tract immune system in the pig. I. Distribution of immunoglobulin-containing cells in the respiratory tract mucosa. *Vet. Pathol.* 13: 81-89.
49. Corthier, G., B. Charley, and J. Franz. 1980. Local immunity in the pig respiratory tract. III. Immunoenzymatic determination of local immunoglobulin classes sharing anti-influenza activity. *Ann. Virol. (Inst. Pasteur)* 131E:355-363.
50. Taylor, G., and C. J. Howard. 1981. Protection of mice against Mycoplasma pulmonis infection using purified mouse immunoglobulins: comparison between protective effect and biological properties of immunoglobulin classes. *Immunology* 43:519-525.
51. Fernald, G. W., A. M. Collier, and W. A. Clyde, Jr. 1975. Respiratory infections due to Mycoplasma pneumoniae in infants and children. *Pediatrics* 55:327-335.

GENERAL DISCUSSION

A characteristic common to various mycoplasmal pneumonias is the nature of the microscopic lesions observed in lungs from infected animals. Peribronchiolar and perivascular lymphoid hyperplasia are typical lesions found following infection with M. hyopneumoniae in pigs, M. pulmonis in rats and mice, and M. pneumoniae in humans (36). Perivascular and peribronchiolar cuffing has also been observed in gnotobiotic calves inoculated with M. bovis (139) and Ureaplasma spp. (140), as well as in colostrum-deprived, specific-pathogen-free lambs inoculated with M. ovis-pneumoniae (141).

Because of the major involvement of lymphocytes in the microscopic lesions of MPS investigations were undertaken to evaluate various responses of lymphocytes from swine exposed to M. hyopneumoniae. Some of the responses, such as antibody production and cell proliferation following stimulation by mitogen or antigen, can give information as to the functional activity of the lymphocytes. Additionally, visualization of lymphocytes in lung sections of pigs inoculated with M. hyopneumoniae membranes and identification of some of the lymphocytes by immunofluorescence provide further evidences as to the interactions between M. hyopneumoniae and the porcine immune system.

Investigations into nonspecific mitogenic factors associated with M. hyopneumoniae indicated that membrane preparations of this organism stimulated PBL from conventionally-reared pigs sporadically (142), whereas PBL from CDCD pigs exhibited positive stimulation in all but one instance

when tested (143). Prior to these reports, nonspecific stimulation of swine lymphocytes by M. hyopneumoniae was suspected on only two occasions (14,15).

The relevance of the mitogenic activity of membranes for swine lymphocytes in the pathogenesis of MPS can probably be better appreciated if we consider the fact that membrane preparations induced microscopic lesions of pneumonia resembling those of MPS when given IT to pigs (142, 143). Although an association has been established between mitogenicity and lesion inducing potential for M. pulmonis in rats (63), the exact significance of this association in the pathogenesis of the disease is still not completely resolved.

Antigen-specific interactions between antigens and the immune system of exposed animals can involve a humoral response, a cell-mediated response or sometimes both. In most mycoplasmal infections, antigen-specific interactions are generally evaluated on the basis of antibody levels in serum. Also, antigen-specific CMI following respiratory mycoplasmal infection is reported for M. pneumoniae (113,114,115,116,117, 118) and M. hyopneumoniae (13,14,15). These specific responses were generally observed with PBL and on one occasion with adenoid vegetations following M. pneumoniae infection (117). In the present studies, specific interactions between the porcine immune system and M. hyopneumoniae involved mainly the lymphocytes from bronchial lymph nodes (142,143,144) and antibodies present in lung secretions as well as in serum (144).

At this point, a hypothesis as to the possible role of M. hyopneumoniae in the pathogenesis of MPS can be put forward. It is possible

that membranes, and most probably live organisms, nonspecifically stimulate lymphocytes present in the lungs. Stimulated lymphocytes could start multiplying and/or releasing lymphokines that would attract other lymphocytes to the site. With lymphocytes accumulating in the lungs, microscopic lesions of, or resembling those of MPS should be detectable after 5 to 7 days based on the results of the lymphocyte stimulation test (142,143). This interval is within the time frame of detection of microscopic lesions in lungs of pigs receiving M. hyopneumoniae membranes (142,143) and also of lesion development in natural or experimental infection (132). If the stimulation of lymphocytes is allowed to persist, as in the case of experimental infection (144), appropriate clone(s) of B-lymphocytes could be triggered to produce specific antibodies that would eventually result in control of the infection. This scheme would be consistent with our observations that severity of microscopic lesions of MPS was reduced by previous exposure to M. hyopneumoniae membranes (142), and that recovering stages of MPS are associated with increasing development of the local humoral response (144).

The results additionally suggest that specific antigen recognized by BLNL of sensitized animals would be different from the nonspecific mitogenic factor stimulating lymphocytes. This is based on the observations that heat treatment of membranes used in lymphocyte stimulation test generally reduced the SI of BLNL from membrane-inoculated or M. hyopneumoniae-infected pigs whereas the response of BLNL from control pigs was not affected by heating the membranes. Heat resistance would suggest that the mitogenic factor associated with M. hyopneumoniae is

not constituted primarily of protein, such as those associated with M. pulmonis (56) and probably M. arthritidis (53,54). This would leave carbohydrates and/or lipids as possible constituent(s) of the mitogenic factor. The mitogenic factor of M. neurolyticum is composed mainly of carbohydrates with possible involvement also of lipids and proteins (47).

Additional information on the interactions between M. hyopneumoniae and the porcine immune system is needed to fully understand the pathogenesis of MPS. Difference observed in response of lymphocytes to stimulation by membranes from high passage field strain (142) and low passage field strain (144) might indicate variations in concentration or potency of mitogenic factor between strains. Also, difference noted in susceptibility of lymphocytes from conventionally-reared and CDCD pigs to stimulation by M. hyopneumoniae membranes would warrant further investigations into determining which lymphocyte subpopulations are primarily stimulated by the membranes.

Although numerous areas concerning the pathogenesis of MPS still need clarification it seems that based on the information gathered in the different experiments of this study, induction of an efficient local immune response as a method of control of MPS has to be attempted.

REFERENCES

1. Maré, C. J., and W. P. Switzer. 1965. Mycoplasma hyopneumoniae a causative agent of virus pig pneumonia. Vet. Med. 60:841-846.
2. Goodwin, R. F. W., A. P. Pomeroy, and P. Whittlestone. 1965. Production of enzootic pneumonia in pigs with a mycoplasma. Vet. Rec. 77:1247-1249.
3. Young, T. F., R. F. Ross, and J. Drisko. 1983. Prevalence of antibodies to Mycoplasma hyopneumoniae in Iowa swine. Am. J. Vet. Res. 44:1946-1948.
4. Switzer, W. P., and R. F. Ross. 1975. Mycoplasmal diseases. Pages 741-764 in Diseases of Swine. 4th ed. Iowa State University Press, Ames, Iowa.
5. Whittlestone, P. 1979. Porcine mycoplasmas. Pages 134-171 in J. G. Tully and R. F. Whitcomb, eds. The Mycoplasmas. Vol. 2. Academic Press, New York.
6. Lam, K. M., and W. P. Switzer. 1971. Mycoplasmal pneumonia of swine: active and passive immunizations. Am. J. Vet. Res. 32:1737-1741.
7. Goodwin, R. F. W., and P. Whittlestone. 1973. Enzootic pneumonia of pigs: immunization attempts inoculating Mycoplasma suis pneumoniae antigen by various routes and with different adjuvants. Br. Vet. J. 129:456-462.
8. Slavik, M., W. Schuller, and W. P. Switzer. 1979. Immunization of swine with Mycoplasma hyopneumoniae vaccines. Iowa State J. Res. 53:297-300.
9. Farrington, D. O. 1976. Immunization of swine against mycoplasmal pneumonia. Proc. 4th Int. Pig Vet. Soc. 1976:PP4.
10. Etheridge, J. R., and L. C. Lloyd. 1982. A method for assessing induced resistance to enzootic pneumonia of pigs. Res. Vet. Sci. 33:188-191.
11. Ross, R. F., B. J. Zimmermann-Erickson, and T. F. Young. 1984. Characteristics of protective activity of Mycoplasma hyopneumoniae vaccine. Am. J. Vet. Res. 45:1899-1905.
12. Goodwin, R. F. W. 1973. Field trials with a formalinized vaccine against enzootic pneumonia of pigs. Br. Vet. J. 129:465-470.

13. Kishima, M., R. F. Ross, and C. Kuniyasu. 1985. Cell-mediated and humoral immune response to Mycoplasma hyopneumoniae in pigs enhanced by dextran sulfate. *Am. J. Vet. Res.* 46:456-462.
14. Kristensen, B., Ph. Paroz, J. Nicolet, M. Wanner, and A. L. de Weck. 1981. Cell-mediated and humoral response in swine after vaccination and natural infection with Mycoplasma hyopneumoniae. *Am. J. Vet. Res.* 42:784-788.
15. Adegboye, D. S. 1978. Attempts to demonstrate cell-mediated immune response during Mycoplasma suis pneumoniae infection of pigs. *Res. Vet. Sci.* 25:323-330.
16. Brunner, H., H. B. Greenberg, W. D. James, R. L. Horswood, R. B. Couch, and R. M. Chanock. 1973. Antibody to Mycoplasma pneumoniae in nasal secretions and sputa of experimentally infected human volunteers. *Infect. Immun.* 8:612-620.
17. Fernald, G. W., and W. A. Clyde, Jr. 1970. Protective effect of vaccines in experimental Mycoplasma pneumoniae disease. *Infect. Immun.* 1:559-565.
18. Howard, C. J., R. N. Gourlay, and G. Taylor. 1980. Immunity to Mycoplasma bovis infections of the respiratory tract of calves. *Res. Vet. Sci.* 28:242-249.
19. Taylor, G., and C. J. Howard. 1980. Class-specific antibody responses to Mycoplasma pulmonis in sera and lungs of infected and vaccinated mice. *Infect. Immun.* 29:1160-1168.
20. Tajima, M., and T. Yagihashi. 1982. Interaction of Mycoplasma hyopneumoniae with the porcine respiratory epithelium as observed by electron microscopy. *Infect. Immun.* 37:1162-1169.
21. Geary, S. J., and E. M. Walczak. 1983. Cytopathic effect of whole cells and purified membranes of Mycoplasma hyopneumoniae. *Infect. Immun.* 41:132-136.
22. Ginsburg, H., and J. Nicolet. 1973. Extensive transformation of lymphocytes by a mycoplasma organism. *Nature New Biol.* 246:143-146.
23. Wise, K. S., G. H. Cassell, and R. T. Acton. 1978. Selective association of murine T lymphoblastoid cell surface alloantigens with Mycoplasma hyorhinis. *Proc. Natl. Acad. Sci. USA* 75:4479-4483.
24. Biberfeld, G., and E. Gronowicz. 1976. Mycoplasma pneumoniae is a polyclonal B-cell activator. *Nature* 261:238-239.

25. Rinaldo, C. R., Jr., J. C. Overall, Jr., B. C. Cole, and L. A. Glasgow. 1973. Mycoplasma-associated induction of interferon in ovine leukocytes. *Infect. Immun.* 8:796-803.
26. Aldridge, K. E., B. C. Cole, and J. R. Ward. 1977. Mycoplasma-dependent activation of normal lymphocytes: induction of a lymphocyte-mediated cytotoxicity for allogeneic and syngeneic mouse target cells. *Infect. Immun.* 18:377-385.
27. Levin, D., H. Gershon, and Y. Naot. 1985. Production of interleukin-2 by rat lymph node cells stimulated by Mycoplasma pulmonis membranes. *J. Infect. Dis.* 151:541-544.
28. Bredt, W. 1979. Motility, Pages 141-155 in M. F. Barile and S. Razin, eds. *The Mycoplasmas*. Vol. 1. Academic Press, New York.
29. Biberfeld, G., and P. Biberfeld. 1970. Ultrastructural features of Mycoplasma pneumoniae. *J. Bacteriol.* 102:855-861.
30. Cassell, G. H., and A. Hill. 1979. Murine and other small-animal mycoplasmas. Pages 235-273 in J. G. Tully and R. F. Whitcomb, eds. *The Mycoplasmas*. Vol. 2. Academic Press, New York.
31. Zucker-Franklin, D., M. Davidson, and L. Thomas. 1966. The interaction of mycoplasmas with mammalian cells. I. HeLa cells, neutrophils, and eosinophils. *J. Exp. Med.* 124:521-532.
32. Collier, A. M., and W. A. Clyde, Jr. 1971. Relationship between Mycoplasma pneumoniae and human respiratory epithelium. *Infect. Immun.* 3:694-701.
33. Howard, C. J., and L. H. Thomas. 1974. Inhibition by Mycoplasma dispar of ciliary activity in tracheal organ cultures. *Infect. Immun.* 10:405-408.
34. Westerberg, S. C., C. B. Smith, B. B. Wiley, and C. Jensen. 1972. Mycoplasma-virus interrelationships in mouse tracheal organ cultures. *Infect. Immun.* 5:840-846.
35. Hirth, R. S., M. E. Tourtelotte, and S. W. Nielsen. 1970. Cytopathic effects and ultrastructure of Mycoplasma agalactiae var. bovis (Donetta strain). *Infect. Immun.* 2:105-111.
36. Gourlay, R. N., and C. J. Howard. 1982. Respiratory mycoplasmosis. *The Respiratory Systems*. Adv. Vet. Sci. Comp. Med. 26:289-332. Academic Press, New York.

37. Howard, C. J., and R. N. Gourlay. 1974. An electron-microscopic examination of certain bovine mycoplasmas stained with ruthenium red and the demonstration of a capsule on Mycoplasma dispar. J. Gen. Microbiol. 83:393-398.
38. Feizi, T., and D. Taylor-Robinson. 1967. Cold agglutinin anti-I and Mycoplasma pneumoniae. Immunology 13:405-409.
39. Biberfeld, G. 1979. Autoimmune reactions associated with Mycoplasma pneumoniae infection. Zentralbl. Bakteriol. Mikrobiol. Hyg. (A) 245:144-149.
40. Powell, D. A., and W. A. Clyde, Jr. 1975. Opsonin-reversible resistance of Mycoplasma pneumoniae to in vitro phagocytosis by alveolar macrophages. Infect. Immun. 11:540-550.
41. Howard, C. J., G. Taylor, J. Collins, and R. N. Gourlay. 1976. Interaction of Mycoplasma dispar and Mycoplasma agalactiae subsp. bovis with bovine alveolar macrophages and bovine lacteal polymorphonuclear leukocytes. Infect. Immun. 14:11-17.
42. Davis, J. K., K. M. Delozier, D. K. Asa, F. C. Minion, and G. H. Cassell. 1980. Interactions between murine alveolar macrophages and Mycoplasma pneumoniae in vitro. Infect. Immun. 29:590-599.
43. Al-Kaissi, A., and M. R. Alley. 1983. Electron microscopic studies of the interaction between ovine alveolar macrophages and Mycoplasma ovipneumoniae in vitro. Vet. Microbiol. 8:571-584.
44. Taylor, G., and C. J. Howard. 1980. Interaction of Mycoplasma pulmonis with mouse peritoneal macrophages and polymorphonuclear leucocytes. J. Med. Microbiol. 13:19-30.
45. Cole, B. C., K. E. Aldridge, and J. R. Ward. 1977. Mycoplasma-dependent activation of normal lymphocytes: mitogenic potential of mycoplasmas for mouse lymphocytes. Infect. Immun. 18:393-399.
46. Biberfeld, G., and E. Nilsson. 1978. Mitogenicity of Mycoplasma fermentans for human lymphocytes. Infect. Immun. 21:48-54.
47. Katz, R., R. Siman-Tov, and Y. Naot. 1983. Comparison of mitogens from Mycoplasma pulmonis and Mycoplasma neurolyticum. Yale J. Biol. Med. 56:613-621.
48. Naot, Y., J. G. Tully, and H. Ginsburg. 1977. Lymphocyte activation by various Mycoplasma strains and species. Infect. Immun. 18:310-317.

49. Naot, Y., S. Merchav, E. Ben-David, and H. Ginsburg. 1979. Mitogenic activity of Mycoplasma pulmonis. I. Stimulation of rat B and T lymphocytes. *Immunology* 36:399-406.
50. Naot, Y., S. Merchav, and H. Ginsburg. 1979. Mycoplasma neurolyticum: a potent mitogen for rat B lymphocytes. *Eur. J. Immunol.* 9:185-189.
51. Naot, Y., and H. Ginsburg. 1978. Activation of B lymphocytes by mycoplasma mitogen(s). *Immunology* 34:715-720.
52. Cole, B. C., L. R. Washburn, G. J. Sullivan, and J. R. Ward. 1982. Specificity of a mycoplasma mitogen for lymphocytes from human and various animal hosts. *Infect. Immun.* 36:662-666.
53. Cole, B. C., G. J. Sullivan, R. A. Daynes, I. A. Sayed, and J. R. Ward. 1982. Stimulation of mouse lymphocytes by a mitogen derived from Mycoplasma arthritidis. II. Cellular requirements for T cell transformation mediated by a soluble mycoplasma mitogen. *J. Immunol.* 128:2013-2018.
54. Daynes, R. A., J. M. Novak, and B. C. Cole. 1982. Comparison of cellular requirements for human T cell transformation by a soluble mitogen derived from Mycoplasma arthritidis and concanavalin A. *J. Immunol.* 129:936-938.
55. Kirchner, H., H. Brunner, and H. Rühl. 1977. Effect of A. laidlawii on murine and human lymphocyte cultures. *Clin. Exp. Immunol.* 29:176-180.
56. Naot, Y., R. Siman-Tov, and H. Ginsburg. 1979. Mitogenic activity of Mycoplasma pulmonis. II. Studies on the biochemical nature of the mitogenic factor. *Eur. J. Immunol.* 9:149-154.
57. McConnell, I., A. Munro, and H. Waldmann. 1981. The biology of the major histocompatibility system. MHS immune response genes. Pages 191-203 in The immune system: A course. Blackwell Scientific Publications, Oxford, England.
58. Stanbridge, E. J., K. A. Bretzius, and R. F. Good. 1981. Mycoplasma-lymphocyte interactions: Ir gene control of mitogenesis and a paradoxical interaction with Thy-1 bearing cells. *Isr. J. Med. Sci.* 17:628-632.
59. Davis, J. K., and G. H. Cassell. 1982. Murine respiratory mycoplasmosis in LEW and F344 rats: strain differences in lesion severity. *Vet. Pathol.* 19:280-293.

60. Davis, J. K., R. B. Thorp, J. W. Simecka, and G. H. Cassell. 1983. Non-specific mitogenic responses of LEW and F344 rat lymphocytes toward Mycoplasma pulmonis. Yale J. Biol. Med. 56:914-915.
61. Naot, Y., S. Davidson, and E. S. Lindenbaum. 1984. Role of mitogenicity in pathogenicity of mycoplasmas for murine hosts. Ann. Microbiol. (Paris) 135A:95-101.
62. Davis, J. K., J. W. Simecka, J. S. P. Williamson, S. E. Ross, M. M. Juliana, R. B. Thorp, and G. H. Cassell. 1985. Nonspecific lymphocyte responses in F344 and LEW rats: susceptibility to murine respiratory mycoplasmosis and examination of cellular basis for strain differences. Infect. Immun. 49:152-158.
63. Naot, Y., S. Davidson, and E. S. Lindenbaum. 1981. Mitogenicity and pathogenicity of Mycoplasma pulmonis in rats. I. Atypical interstitial pneumonia induced by mitogenic mycoplasmal membranes. J. Infect. Dis. 143:55-62.
64. Biberfeld, G. 1977. Activation of human lymphocyte subpopulations by Mycoplasma pneumoniae. Scand. J. Immunol. 6:1145-1150.
65. Biberfeld, G., P. Arneborn, M. Forsgren, L. V. von Stedingk, and S. Blomqvist. 1983. Non-specific polyclonal antibody response induced by Mycoplasma pneumoniae. Yale J. Biol. Med. 56:639-642.
66. Stanbridge, E. J., and R. L. Weiss. 1978. Mycoplasma capping on lymphocytes. Nature 276:583-587.
67. Butler, G. H., and E. J. Stanbridge. 1983. Infection of mouse lymphoblastoid cell lines with Mycoplasma hyorhinis: complex nature of mycoplasma-host cell interactions. Infect. Immun. 42:1136-1143.
68. Stanbridge, E. J. 1982. Mycoplasma-lymphocyte interactions and their possible role in immunopathologic manifestations of mycoplasmal disease. Rev. Infect. Dis. 4 (Suppl. 1):S219-S226.
69. Wise, K. S., F. C. Minion, and H. C. Cheung. 1982. Translocation of Thy-1 antigen and a fluorescent lipid probe during lymphoblastoid cell interaction with Mycoplasma hyorhinis. Rev. Infect. Dis. 4 (Suppl. 1):S210-S218.
70. Rinaldo, C. R., Jr., B. C. Cole, J. C. Overall, Jr., J. R. Ward, and L. A. Glasgow. 1974. Induction of interferon in ovine leukocytes by species of mycoplasma and acholeplasma. Proc. Soc. Exp. Biol. Med. 146:613-618.

71. Cole, B. C., J. C. Overall, Jr., P. S. Lombardi, and L. A. Glasgow. 1976. Induction of interferon in ovine and human lymphocyte cultures by mycoplasmas. *Infect. Immun.* 14:88-94.
72. Sokhey, J., A. I. Solovieva, and V. I. Vasilieva. 1977. Induction of interferon in mice and cell cultures by Mycoplasma pneumoniae. *Acta Virol.* 21:485-489.
73. Rinaldo, C. R., Jr., B. C. Cole, J. C. Overall, Jr., and L. A. Glasgow. 1974. Induction of interferon in mice by mycoplasmas. *Infect. Immun.* 10:1296-1301.
74. Beck, J., H. Brunner, F. Marcucci, H. Kirchner, and J. Wietzerbin. 1982. Induction of interferon by mycoplasmas in mouse spleen cell cultures. *J. Interferon Res.* 2:31-36.
75. Birke, C., H. H. Peter, U. Langenberg, W. J. P. Muller-Hermes, J. H. Peters, J. Heitmann, W. Leibold, H. Dallugge, E. Krapf, and H. Kirchner. 1981. Mycoplasma contamination in human tumor cell lines: effect on interferon induction and susceptibility to natural killing. *J. Immunol.* 127:94-98.
76. Cole, B. C., and R. N. Thorpe. 1983. Induction of human γ interferons by a mitogen derived from Mycoplasma arthritidis and by phytohemagglutinin: differential inhibition with monoclonal anti-HLA.DR antibodies. *J. Immunol.* 131:2392-2396.
77. Arai, S., T. Munakata, and K. Kuwano. 1983. Mycoplasma interaction with lymphocytes and phagocytes: role of hydrogen peroxide released from M. pneumoniae. *Yale J. Biol. Med.* 56:631-638.
78. Farrar, J. J., W. R. Benjamin, M. L. Hilfiker, M. Howard, W. L. Farrar, and J. Fuller-Farrar. 1982. The biochemistry, biology, and role of interleukin 2 in the induction of cytotoxic T cell and antibody-forming B cell responses. *Immunol. Rev.* 63:129-166.
79. Gillis, S., and K. A. Smith. 1977. Long term culture of tumour-specific cytotoxic T cells. *Nature* 268:154-156.
80. Ruscetti, F. W., D. A. Morgan, and R. C. Gallo. 1977. Functional and morphologic characterization of human T cells continuously grown in vitro. *J. Immunol.* 119:131-138.
81. Biberfeld, G., and G. Sterner. 1971. Antibodies in bronchial secretions following natural infection with Mycoplasma pneumoniae. *Acta Pathol. Microbiol. Immunol. Scand. (B)* 79:599-605.

82. Cassell, G. H., J. R. Lindsey, and H. J. Baker. 1974. Immune response of pathogen-free mice inoculated intranasally with Mycoplasma pulmonis. J. Immunol. 112:124-136.
83. Goodwin, R. F. W., and R. G. Hodgson. 1970. The passive haemagglutination test for the detection of Mycoplasma suis pneumoniae and the possible diagnosis of enzootic pneumonia in pigs. J. Hyg. (Camb.) 68:327-336.
84. Holmgren, N. 1974. On the immune response in porcine serum and tracheobronchial secretions following experimental infection with Mycoplasma hyopneumoniae. Zentralbl. Veterinarmed. (B) 21:188-201.
85. Suter, M., M. Kobisch, and J. Nicolet. 1985. Stimulation of immunoglobulin-containing cells and isotype-specific antibody response in experimental Mycoplasma hyopneumoniae infection in specific-pathogen-free pigs. Infect. Immun. 49:615-620.
86. Thomas, L. H., R. N. Gourlay, E. J. Stott, C. J. Howard, and J. C. Bridger. 1982. A search for new microorganisms in calf pneumonia by the inoculation of gnotobiotic calves. Res. Vet. Sci. 33:170-182.
87. Boulanger, P., and C. L'Ecuyer. 1968. Enzootic pneumonia of pigs: complement-fixation tests for the detection of mycoplasma antibodies in the serum of immunized rabbits and infected swine. Can. J. Comp. Med. 32:547-554.
88. Slavik, M. F., and W. P. Switzer. 1972. Development of a micro-titration complement-fixation test for diagnosis of mycoplasmal swine pneumonia. Iowa State J. Res. 47:117-128.
89. Holmgren, N. 1974. An indirect haemagglutination test for detection of antibodies against Mycoplasma hyopneumoniae using formalinized tanned swine erythrocytes. Res. Vet. Sci. 16:341-346.
90. Lam, K. M., and W. P. Switzer. 1972. Mycoplasmal pneumonia of swine: Serologic response in pigs. Am. J. Vet. Res. 33:1329-1332.
91. Fujikara, T., S. Namioka, and S. Shibata. 1970. Tube agglutination test of Mycoplasma hyopneumoniae infection in swine. Natl. Inst. Anim. Health 10:42-43.
92. Pijoan, C., and E. Boughton. 1974. Tube agglutination tests with Mycoplasma hyopneumoniae and M. hyorhinis. Br. Vet. J. 130:593-598.
93. Bruggmann, S., H. Keller, H. U. Bertschinger, and B. Engberg. 1977. Quantitative detection of antibodies to Mycoplasma suis pneumoniae in pigs' sera by an enzyme-linked immunosorbent assay. Vet. Rec. 101: 109-111.

94. Piffer, I. A., T. F. Young, A. Petenate, and R. F. Ross. 1984. Comparison of complement fixation test and enzyme-linked immunosorbent assay for detection of early infection with Mycoplasma hyopneumoniae. Am. J. Vet. Res. 45:1122-1126.
95. Armstrong, C. H., M. J. Freeman, L. Sands-Freeman, M. Lopez-Osuna, T. Young, and L. J. Runnels. 1983. Comparison of the enzyme-linked immunosorbent assay, the indirect hemagglutination test and the complement fixation test for detecting antibodies to Mycoplasma hyopneumoniae. Can. J. Comp. Med. 47:464-470.
96. Freeman, M. J., M. Lopez-Osuna, C. H. Armstrong, and L. Sands-Freeman. 1984. Evaluation of the indirect hemagglutination assay as a practical serodiagnostic test for mycoplasmal pneumonia of swine. Vet. Microbiol. 9:259-270.
97. Brunner, H. 1974. Studies on the pathogenesis of experimental Mycoplasma pneumoniae infection in the guinea pig. Pages 411-419 in J. M. Bove and J. F. Duplan, eds. Les Mycoplasmes de l'Homme, des Animaux, des Vegetaux et des Insectes. Colloques INSERM vol. 33. INSERM, Paris, France.
98. Brunner, H., U. Bruck, D. Lambert, D. Sziegoleit, and H. Schiefer. 1976. Response of guinea pigs and hamsters to experimental infection with Mycoplasma pneumoniae. Infection 4 (Suppl. 1):S13-S16.
99. Davis, J. K., R. B. Thorp, P. A. Maddox, M. E. Brown, and G. H. Cassell. 1982. Murine respiratory mycoplasmosis in F344 and LEW rats: evolution of lesions and lung lymphoid cell populations. Infect. Immun. 36:720-729.
100. Howard, C. J., R. N. Gourlay, and G. Taylor. 1977. Induction of immunity in calves to Mycoplasma bovis infection of the respiratory tract. Vet. Microbiol. 2:29-37.
101. Taylor, G., and C. J. Howard. 1981. Protection of mice against Mycoplasma pulmonis infection using purified mouse immunoglobulins: comparison between protective effect and biological properties of immunoglobulin classes. Immunology 43:519-525.
102. Biberfeld, G., and G. Sterner. 1976. Tuberculin anergy in patients with Mycoplasma pneumoniae infection. Scand. J. Infect. Dis. 8: 71-73.
103. Charley, B., and H. Salmon. 1979. Influence of a mycoplasma respiratory tract infection on sheep erythrocyte and complement receptor-bearing cell composition in the respiratory tract of swine. Am. J. Vet. Res. 40:1257-1259.

104. Denny, F. W., D. Taylor-Robinson, and A. C. Allison. 1972. The role of thymus-dependent immunity in Mycoplasma pulmonis infections of mice. *J. Med. Microbiol.* 5:327-336.
105. Fernald, G. W., W. A. Clyde, Jr., and J. Bienenstock. 1972. Immunoglobulin-containing cells in lungs of hamsters infected with Mycoplasma pneumoniae. *J. Immunol.* 108:1400-1408.
106. Davis, J. K., P. A. Maddox, R. B. Thorp, and G. H. Cassell. 1980. Immunofluorescent characterization of lymphocytes in lungs of rats infected with Mycoplasma pulmonis. *Infect. Immun.* 27:255-259.
107. Tajima, M., T. Yagihashi, T. Nunoya, A. Takeuchi, and F. Ohashi. 1984. Mycoplasma hyopneumoniae infection in pigs immunosuppressed by thymectomy and treatment with antithymocyte serum. *Am. J. Vet. Res.* 45:1928-1932.
108. Singer, S. H., M. Ford, and R. L. Kirschstein. 1972. Respiratory diseases in cyclophosphamide-treated mice. I. Increased virulence of Mycoplasma pulmonis. *Infect. Immun.* 5:953-956.
109. Fernald, G. W., and W. A. Clyde, Jr. 1974. Cell-mediated immunity in M. pneumoniae infections. Pages 421-427 in J. M. Bove and J. F. Duplan, eds. *Les Mycoplasmes de l'Homme, des Animaux, des Vegetaux et des Insectes. Colloques INSERM vol. 33.* INSERM, Paris, France.
110. Taylor, G., D. Taylor-Robinson, and G. W. Fernald. 1974. Reduction in the severity of Mycoplasma pneumoniae-induced pneumonia in hamsters by immunosuppressive treatment with anti-thymocyte sera. *J. Med. Microbiol.* 7:343-348.
111. Taylor, G., and D. Taylor-Robinson. 1976. Effects of active and passive immunization on Mycoplasma pulmonis-induced pneumonia in mice. *Immunology* 30:611-618.
112. Tomasi, T. B., Jr. 1983. Mechanisms of immune regulation at mucosal surfaces. *Rev. Infect. Dis.* 5 (Suppl. 4):S784-S792.
113. Biberfeld, G., P. Biberfeld, and G. Sterner. 1974. Cell-mediated immune response following Mycoplasma pneumoniae infection in man I. Lymphocyte stimulation. *Clin. Exp. Immunol.* 17:29-41.
114. Fernald, G. W. 1972. In vitro response of human lymphocytes to Mycoplasma pneumoniae. *Infect. Immun.* 5:552-558.
115. Fernald, G. W. 1973. Role of host response in Mycoplasma pneumoniae disease. *J. Infect. Dis.* 127 (Suppl.):S55-S58.

116. Mogensen, H. H., V. Andersen, and K. Lind. 1976. Lymphocyte transformation studies in Mycoplasma pneumoniae infections. *Infection* 4 (Suppl. 1):S21-S24.
117. Mogensen, H. H., K. I. Meistrup-Larsen, L. P. Ryder, and K. Lind. 1984. Mycoplasma pneumoniae-stimulation of lymphocytes obtained from adenoid vegetations and blood in children with and without serological evidence of Mycoplasma pneumoniae infection. *Acta Pathol. Microbiol. Immunol. Scand. (C)* 92:313-317.
118. Fernald, G. W., A. M. Collier, and W. A. Clyde, Jr. 1975. Respiratory infections due to Mycoplasma pneumoniae in infants and children. *Pediatrics* 55:327-335.
119. Adegboye, D. S. 1978. The immunological response of pig bronchial lymph node to Mycoplasma suis. *J. Comp. Pathol.* 88:97-104.
120. Biberfeld, G. 1974. Cell-mediated immune response following Mycoplasma pneumoniae infection in man. II. Leucocyte migration inhibition. *Clin. Exp. Immunol.* 17:43-49.
121. Roberts, D. H., R. S. Windsor, W. N. Masiga, and C. G. Kariavu. 1973. Cell-mediated immune response in cattle to Mycoplasma mycoides var. mycoides. *Infect. Immun.* 8:349-354.
122. Nicolet, J. and E. Rivera. 1976. Preliminary observations with the leucocyte migration inhibition test in the enzootic pneumonia of pig. *Proc. 4th Int. Pig Vet. Soc.* 1976:PP8.
123. Roberts, D. H. 1973. Preliminary studies on the cell-mediated immune response in pigs to Mycoplasma hyopneumoniae. *Br. Vet. J.* 129:427-438.
124. Bennett, R. H., and D. E. Jasper. 1977. Immunosuppression of humoral and cell-mediated responses in calves associated with inoculation of Mycoplasma bovis. *Am. J. Vet. Res.* 38:1731-1738.
125. Kaklamanis, E., and M. Pavlatos. 1972. The immunosuppressive effect of mycoplasma infection. I. Effect on the humoral and cellular response. *Immunology* 22:695-702.
126. Bergquist, L. M., B. H. S. Lau, and C. E. Winter. 1974. Mycoplasma-associated immunosuppression: effect on hemagglutinin response to common antigens in rabbits. *Infect. Immun.* 9:410-415.
127. Sabato, A. R., D. M. Cooper, and Y. H. Thong. 1981. Transitory depression of immune function following Mycoplasma pneumoniae infection in children. *Pediatr. Res.* 15:813-816.

128. Adegbeye, D. S. 1978. A review of mycoplasma-induced immuno-suppression. Br. Vet. J. 134:556-560.
129. Biberfeld, G., and G. Sterner. 1976. Effect of Mycoplasma pneumoniae infection on cell-mediated immunity. Infection 4 (Suppl. 1):S17-S20.
130. Filia, M., B. A. Myhre, L. T. Chinh, M. Territo, T. S. Edgington, and H. Kattlove. 1974. Pathogenesis of anemia associated with Mycoplasma pneumoniae. Acta Haematol. 51:297-301.
131. Niklasson, P. M., and R. C. Williams, Jr. 1974. Studies of peripheral blood T- and B-lymphocytes in acute infections. Infect. Immun. 9:1-7.
132. Whittlestone, P. 1972. The role of mycoplasmas in the production of pneumonia in the pig. Pages 263-283 in Pathogenic Mycoplasmas. A Ciba Foundation Symposium. Associated Scientific Publishers, Amsterdam, The Netherlands.
133. Livingston, C. W., Jr., E. L. Stair, N. R. Underdahl, and C. A. Mebus. 1972. Pathogenesis of mycoplasmal pneumonia in swine. Am. J. Vet. Res. 33:2249-2258.
134. Baskerville, A. 1972. Development of the early lesions in experimental enzootic pneumonia of pigs: an ultrastructural and histologic study. Res. Vet. Sci. 13:570-578.
135. Mebus, C. A., and N. R. Underdahl. 1977. Scanning electron microscopy of trachea and bronchi from gnotobiotic pigs inoculated with Mycoplasma hyopneumoniae. Am. J. Vet. Res. 38:1249-1254.
136. Baskerville, A., and C. L. Wright. 1973. Ultrastructural changes in experimental enzootic pneumonia of pigs. Res. Vet. Sci. 14:155-160.
137. Ross, R. F. 1981. Mycoplasmal diseases. Pages 535-549 in Diseases of Swine. 5th ed. Iowa State University Press, Ames, Iowa.
138. Williams, P. P., and J. E. Gallagher. 1978. Cytopathogenicity of Mycoplasma hyopneumoniae in porcine tracheal ring and lung explant organ cultures alone and in combination with monolayer cultures of fetal lung fibroblasts. Infect. Immun. 20:495-502.
139. Gourlay, R. N., and C. J. Howard. 1978. Isolation and pathogenicity of mycoplasmas from the respiratory tract of calves. Pages 295-304 in W. B. Martin, ed. Respiratory Diseases in Cattle. Current Topics in Veterinary Medicine. Vol. 3. Martinus Nijhoff, The Hague, The Netherlands.

140. Howard, C. J., R. N. Gourlay, L. H. Thomas, and E. J. Stott. 1976. Induction of pneumonia in gnotobiotic calves following inoculation of Mycoplasma dispar and ureaplasmas (T-mycoplasmas). Res. Vet. Sci. 21:227-231.
141. Foggie, A., G. E. Jones, and D. Buxton. 1976. The experimental infection of specific pathogen free lambs with Mycoplasma ovipneumoniae. Res. Vet. Sci. 21:28-35.
142. Messier, S., R. F. Ross, and B. Zimmermann-Erickson. 1985. Role of Mycoplasma hyopneumoniae membranes in the pathogenesis of mycoplasmal pneumonia of swine. (Manuscript in preparation).
143. Messier, S., and R. F. Ross. 1986. Stimulation of lymphocytes from, and inoculation of cesarean-derived, colostrum-deprived pigs with Mycoplasma hyopneumoniae membranes. (Manuscript in preparation).
144. Messier, S., R. F. Ross, and P. S. Paul. 1986. Humoral and cell-mediated immune responses of pigs following experimental Mycoplasma hyopneumoniae infection. (Manuscript in preparation).

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