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Interaction of *Mycoplasma ovipneumoniae* with sheep alveolar macrophages

by

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GENERAL INTRODUCTION

Ovine pneumonia is one of the most common infectious diseases of sheep regardless of whether the animals are raised intensively indoors or under nomadic conditions (42). The disease, characterized clinically by signs of fever, depression, nasal discharge, paroxysmal coughing and variable mortality, probably ranks as the number one disease affecting the world's sheep population. The obvious economic impact of this disease results from deaths, reduced growth rate, delayed marketing and cost of treatment. An indication of the importance of pneumonia in sheep in North America is provided by the results of a study by Elazhary et al. (49) which showed that nearly 30% of sheep and goats in Quebec, Canada suffered from respiratory disease. A more recent survey conducted in Michigan, USA by Rook (125) found that 17% of pre-weaning mortality of lambs was due to pneumonia. In Africa, studies conducted in Sudan (1) and in Mali (103) indicated that respiratory diseases were common problems in sheep and goats. In Iowa, economic losses to sheep producers from respiratory diseases are also significant. A survey of Iowa sheep producers conducted by Hartwig and Eness (67) indicated that pneumonia was one of their most important disease problems.

Pneumonia in sheep is caused by the complex interaction of environmental factors producing stress and a variety of

microorganisms acting synergistically (42,143). Among these microorganisms, Pasteurella sp. are considered the most commonly isolated from both pneumonic and normal sheep. However, challenge experiments have shown that Pasteurella sp. alone have limited ability to produce pneumonia and probably require a predisposing infection by another infectious agent (42,86,143). A number of microorganisms, including viruses, mycoplasmas and chlamydia have been suggested to play this role.

Mycoplasma ovipneumoniae has been isolated from the lungs of pneumonic as well as normal sheep in many countries (5,6,15, 42,96,111). During outbreaks of respiratory disease in Iowa sheep, a serological response to M. ovipneumoniae and P. hemolytica was demonstrated (90,50). These results provided evidence for the involvement of M. ovipneumoniae and P. hemolytica in the disease process. At the same time, no significant antibody titers for parainfluenza-type 3 (PI3), infectious bovine rhinotracheitis (IBR) respiratory syncytial (RS), or bovine viral diarrhea (BVD) viruses were noted.

Mycoplasmas possess various virulence factors including suppression of the host pulmonary defence mechanisms that may facilitate colonization of the respiratory tract by secondary infectious agents. Many in vitro studies have shown that in the absence of specific antibodies mycoplasmas not only can survive in culture with phagocytic cells but also interfere

with ingestion and killing of a second bacterial target (11,75,115,137,149). Theoretically, mycoplasma-mediated alterations in phagocytic cell function could have major adverse effects on disease processes in the respiratory tract. However, as far as M. ovipneumoniae is concerned little is known of its inter-relationship with the defense mechanisms of ovine lung (3). In the following study, the ability of M. ovipneumoniae (field strain) to alter sheep alveolar macrophage functions was examined in vitro. In addition, attempts were made to determine surface receptors for Fc component of globulin and complement on sheep alveolar macrophages and the effect of M. ovipneumoniae on their expression.

Explanation of Thesis Format

This thesis consists of a general introduction, review of the literature, one paper suitable for publication (composed of an abstract followed by introduction, materials and methods, results, discussion and references sections), general summary and conclusions, literature cited in the general introduction, literature review, and summary and conclusions, and acknowledgments.

LITERATURE REVIEW

Many of the respiratory diseases associated with mycoplasmas are complex and may have a multifactorial etiology. While the etiologic role of mycoplasmas has clearly been demonstrated with certain mycoplasmal organisms in specific hosts, their role in other instances has not been confirmed. Isolation of mycoplasmas from apparently healthy animals is not an unusual occurrence. Even though extensive experimentation has been reported on the mechanisms of pathogenesis of mycoplasmas, it is clear that the subject is extremely complex and there is a need for additional information. Establishment of cause and effect or fulfillment of Koch's postulates has been difficult. The virulence factors of mycoplasmas can be divided into four main functional components: (1) adherence to host tissues, (2) toxin production, (3) nonspecific activation of host immune responses, and (4) suppression of the host defence mechanisms leading to secondary bacterial infection. This last component may be an important mechanism by which mycoplasmas can contribute to respiratory diseases in humans and animals. Immunosuppression has been studied with several respiratory mycoplasmas but, little information is available on M. ovipneumoniae. Therefore, this literature review will address interactions of mycoplasmas in general with host immune system

cells. Prior to that, a brief review of the etiology of respiratory disease of sheep is presented. A review of surface receptors on phagocytic cells is also included.

Etiology of Sheep Pneumonia

Ovine pneumonia is a very common infectious disease of sheep worldwide regardless of the management system (42). Points which emerge from the literature are the confusion in the terminology and the classification of pneumonias of sheep since, in the past, this has been based primarily upon the morphology of the lesions. Traditionally, most pneumonias of sheep have been classified as either an acute exudative type resembling acute pneumonic pasteurellosis or a chronic proliferative type resembling atypical pneumonia (42).

The etiology of sheep pneumonia is very complex and has not been clearly defined. The available information has been derived from isolation of microorganisms from pneumonic lungs and attempts to reproduce the disease with an individual agent or a combination of agents. Establishment of cause and effect or fulfillment of Koch's postulates has been difficult due to the complex etiology and the poorly defined lesions.

A wide variety of microorganisms have been isolated from the respiratory tract of pneumonic sheep including Pasteurella sp., Mycoplasma sp., chlamydia, viruses, nematode parasites, fungal agents, and occasionally other bacteria such as

Escherichia coli, Corynebacterium sp., Staphylococcus sp., Neisseria sp. (7,8,42,143), and nonidentified Gram negative bacteria (21). Among these microorganisms, Pasteurella sp. are reported to be the most commonly isolated from both pneumonic and normal sheep. It is often found in pure culture in lungs with acute exudative pneumonia but also may be present in cases of chronic proliferative pneumonia. However, in all these lesions, it is not unusual to find Pasteurella sp. in association with Mycoplasma sp. and many other bacteria.

There have been many attempts to reproduce ovine pneumonia with a single agent or combinations of microorganisms. A number of viral agents including PI3, several types of adenoviruses and reoviruses were reported to induce clinical disease which was usually self limiting (42,143). The majority of lambs inoculated with Chlamydia psittaci developed pneumonia with lesions resembling atypical pneumonia but the infection did not persist (42). Attempts to induce ovine pneumonia with pure cultures of Pasteurella hemolytica have given inconsistent results. In most cases, the bacteria were rapidly cleared from the respiratory tract although sometimes they persisted and multiplied to induce exudative pneumonia or, occasionally, a proliferative type of lesions (86).

Historically, the isolation of Mycoplasma sp. from the respiratory tract of sheep was first reported in 1955 by Greig

(64). However, a potential role for mycoplasmas in ovine pneumonia was initially suggested by Hamdy et al. (65) who consistently were able to reproduce the disease by stressing lambs inoculated intratracheally with a mycoplasma agent and Pasteurella multocida. Meanwhile, there were reports of isolation of mycoplasma from the respiratory tract of pneumonic sheep in many countries (88). Also, some investigations conducted in Australia associated Mycoplasma sp. with many cases of pneumonia appearing in lambs 5 to 10 weeks of age (142,145). Clinical signs reported were coughing, sneezing and a mucoid nasal discharge. While the morbidity was high the mortality was low. The lungs from all animals that died or were sacrificed had pneumonic lesions that were characterized as a proliferative interstial pneumonia. At the same time, a cytopathogenic agent was cultivated in bovine testicular cell culture and pneumonia similar to the natural disease was produced in lambs by intratracheal inoculation of the cell culture grown organisms. Further studies by Carmichael et al. (24) confirmed the nature of the agent and it was named M. ovipneumoniae.

Surveys conducted in many countries have shown that M. ovipneumoniae is the most commonly isolated mycoplasma from ovine lungs (15,111) as well as from the ovine nasal cavity (23). The agent is also commonly isolated from proliferative types of pneumonia as well as from lungs with typical

exudative pneumonia, from chronic pneumonias that followed a viral infection, and from normal lungs (42). Other Mycoplasma sp. including M. arginini, Acholeplasma laidlawii and ureaplasmas have also been recovered from the respiratory tract of both pneumonic and normal sheep (23,42). However, like M. ovipneumoniae, their role in the causation of the disease is not well proven.

Attempts to produce pneumonia in sheep with pure cultures of M. ovipneumoniae have yielded variable results. In some early studies conducted in Australia, nearly all animals developed severe pneumonia (142,145). On the other hand, only 25 to 50% of the animals developed mild pneumonia in studies conducted in Scotland (53,61), and less than 20% showed lesions in experiments conducted in New Zealand (5,43). Attempts to recover M. ovipneumoniae from the pneumonic lungs in all these experiments usually were unsuccessful and, even where M. ovipneumoniae was present, bacterial numbers in both healthy and pneumonic lungs were similar. Because of the inconsistent results in producing the experimental disease and the failure to recover the agent from the lesions, the results of these experiments did not establish the etiological significance of M. ovipneumoniae beyond doubt. However, attempts to produce the disease in lambs with a homogenate of pneumonic lung containing M. ovipneumoniae alone or in combination with P. hemolytica have yielded proliferative

pneumonia in most animals (86). This has led to the hypothesis that the challenge organisms in the early studies might have been attenuated during isolation and cloning on artificial medium or that strains varied in virulence (83,86,87). In this regard, it is interesting to note that the early studies were conducted utilizing tissue culture grown organisms. Another factor of variability in the challenge studies could be the nature of the experimental animal. Naturally-reared lambs were more likely to develop pneumonia than were specific pathogen-free animals (86).

Host Phagocytic Cell Defence Mechanisms

The major phagocytic cells of mammals belong to two complementary systems. The mononuclear-phagocyte system, consists of circulating monocytes and tissue macrophages. The second is represented by the polymorphonuclear neutrophil granulocytes also known as neutrophils. Their major function is the destruction of invading microorganisms through the process of phagocytosis. A successful phagocytic action by phagocytic cells requires directed migration of phagocytes, attachment, ingestion, phagosome-lysosome fusion, stimulation of oxygen-independent and oxygen-dependent killing mechanisms, and activation of specific immune responses and cytokine production (41,59,95).

Phagocytic cells are attracted by many bacterial products such as N-formylated tripeptides, by factors released by damaged cells, or by activation of components of the complement system such as C5a and C3a (41).

Once the phagocytic cells encounter the invading microorganism, they must bind it firmly. This attachment is usually mediated through antibody molecules and the third component of complement (C3), known as opsonins. Neutrophils and macrophages carry specific receptors for the Fc portion of antibody molecules and C3 on their surface (59). Therefore, particles coated with these molecules facilitate their binding to the phagocytic cells. After attachment is complete, the phagocytes will attempt to internalize the particles into a vacuole called a phagosome. Antibodies and C3 again play a major role in this process by rendering the surface of the particles more hydrophobic. However, it is reported that receptors for C3 promote only binding but not ingestion of some particles (18,66,94).

Destruction of the ingested particles occurs mainly through two distinct mechanisms, oxygen-independent and oxygen-dependent. Within the cytoplasm of phagocytic cells are several granules (lysosomes) that contain microbicidal enzymes such as lysozyme, proteases and peroxidase. Fusion of phagosome to these lysosomes (phagolysosomes) triggers the release of the lysosomal enzymes that can kill most

microorganisms. Of more importance in destroying infectious agents is the oxygen-dependent mechanism known as the respiratory burst. Changes in the plasma membrane initiate an intracellular activation of cytochrome oxidase that result in a burst of oxygen consumption. This leads to the production of highly toxic oxygen metabolites such as superoxide anion, hydrogen peroxide, hydroxyl radicals and singlet oxygen (41,59). In most instances, the infectious agents are killed by this oxidative burst. However, in some cases, the organisms escape and the animal must initiate an appropriate immune response to augment its defence mechanisms. Even more, elimination of some intracellular pathogens requires induction of specific T cell products known as lymphokines that activate phagocytic cells and other T cells to kill these pathogens (39).

Another mechanism by which phagocytic cells contribute to host defence against infectious agents is their ability to lyse these infectious agents through a process termed antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cellular cytotoxicity. Cells that possess receptors for the Fc region of immunoglobulins and C3b may bind to foreign target cells in the presence of specific antibody and complement. Cells that can participate in this process include macrophages, neutrophils, eosinophils, killer (K) cells, and some subsets of T-lymphocytes (68,135). The mechanism of this

cytotoxicity is unknown, although, the concept of a granule exocytosis model as proposed by Henkart (68) is generally accepted at least for cytotoxic T cells and K cells. According to this concept, cytoplasmic granules fuse with the plasma membrane of the target cells and at the cytotoxic cell-target cell interface a number of substances including a potent cytotoxic pore-forming material known as perforin are released.

Despite these antimicrobial defences of the host, some pathogenic organisms have developed methods to circumvent the killing process of phagocytic cells, and in some cases they used host defence cells for their own advantage. The common strategy utilized by many pathogenic organisms to avoid destruction by host cells is to circumvent one or more of the events outlined above.

Bacteria-Phagocytic Cell Interactions

Some pathogenic organisms including mycoplasma species and gonococci readily attach to phagocytic cells without being ingested and proliferate on the surface of these cells. This may compromise the ability of these phagocytes to ingest and kill a second bacterial agent. However, addition of specific antibodies against these agents promotes ingestion and killing by the phagocytic cells (41).

Another very effective mechanism used by pathogenic microorganisms to minimize their interaction with the phagocytic cells is to inhibit phagosome-lysosome fusion. Legionella pneumophila, a facultative intracellular Gram-negative bacterium which causes Legionnaires' disease, is capable of multiplying in human alveolar macrophages by inhibiting phagosome-lysosome fusion, thus evading the killing mechanism of macrophages (41,71). Inhibition of phagosome-lysosome fusion also has been documented for Brucella abortus, mycobacterium species and chlamydia (41).

Pathogenic microorganisms also have developed various tactics to avoid the oxidative killing mechanism of phagocytic cells. Haemophilus somnus has been shown to limit the respiratory burst of bovine neutrophils as measured by the iodination test, and a low molecular weight nucleotide has been incriminated (29,79). Similarly, Toxoplasma gondii has been shown to fail to induce the release of the macrophage's reactive oxygen metabolites (27). Failure of other bacteria including B. abortus and Salmonella typhi to stimulate an oxidative burst when they are ingested also has been reported (41). Other bacteria such as Listeria monocytogenes produce a large amount of superoxide dismutase that converts oxygen radicals to hydrogen peroxide which can be inactivated by catalase enzyme (41).

An avoidance mechanism of certain microorganisms is through the production of specific cytotoxins. Respiratory bacterial pathogens including P. haemolytica and H. somnus produce cytotoxins, known as leukotoxin that are highly toxic for alveolar macrophages (40). In the same way, H. pleuropneumoniae extracts were cytocidal for swine alveolar macrophages (112).

In addition, the production of antiphagocytic surface components such as capsules help some pathogenic microorganisms to avoid phagocytosis. For instance, P. haemolytica avoids phagocytosis by means of its capsular material that can adversely affect bovine neutrophil phagocytic function (40). Numerous other examples include S. aureus, Corynebacterium equi, and Klebsiella pneumoniae (41). The mechanisms by which the capsular material protects bacteria from phagocytosis include decreased binding of serum opsonins, inaccessibility of ligands, and decreased hydrophobicity of the bacterial surface (41).

Surface Receptors on Phagocytic Cells

Phagocytic activity of phagocytic cells depends heavily on their membrane receptors including Fc and complement (C) receptors which mediate both particle attachment and endocytosis. The importance of these surface receptors in host defence mechanisms relies also on their ability to

mediate cytotoxicity through antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity. Even though the expression of these surface markers appears to correlate with cell differentiation, a variable number of mature cells can usually be demonstrated to not express either class of receptor markers (117). In addition to macrophages and neutrophils, basophils, eosinophils and subsets of lymphocytes are also reported to possess similar surface receptors.

Studies on Fc receptors (FcR) have shown that all immunoglobulin (Ig) classes are capable of binding to immune cells, although some cells are found to have more affinity for certain Ig classes than others (63,138,141,144,156). It seems likely that IgG molecules bind to macrophages and neutrophils with high avidity, while IgE molecules bind more to eosinophils and basophils. However, due to the complexity of the subject, only the main points concerning FcR for IgG will be cited here.

Heterogeneity and binding specificity of FcR for IgG on murine macrophages are probably the best studied, and at least two different types of FcR for IgG have been identified (151,123). This heterogeneity of Ig FcR was first observed by Kossard and Nelson (97) who demonstrated that erythrocytes sensitized with early antisera failed to form rosettes with trypsinized macrophages, whereas erythrocytes sensitized with hyperimmune serum formed rosettes with both trypsinized and

nontrypsinized macrophages. This suggested the presence of more than one class of receptor for IgG molecules on murine macrophages. Furthermore, studies conducted by Askenase and Hayden (14), Unkeless and Eisen (152), Heusser et al. (69), and Diamond et al. (46) provided evidence that the IgG subclass that bound to a trypsin-sensitive site was IgG2a. Although the presence of more than one class of receptors for IgG on mouse macrophages is recognized by almost all these investigators, their exact number still remains unclear. Binding and competitive inhibition studies have yielded conflicting results. Walker (158) used normal macrophages and macrophage cell lines to demonstrate that binding of monomeric or aggregated IgG2a was not inhibited by aggregated IgG2b, suggesting the existence of two binding sites. Diamond et al. (46), using two different macrophage cell lines as well as normal macrophages, also found some evidence for two binding sites, one for IgG2a and other for IgG2b. In contrast, Heusser et al. (69) and Anderson et al. (12) found three binding sites on mouse macrophages as well as macrophage cell lines.

The human FcR, as in the mouse, are also heterogenous and show different affinities for IgG subclasses. Three distinct types of FcR have been determined. FcRI binds to monomeric IgG with high affinity and in rank order of affinity to IgG1=IgG3>IgG4. It is expressed mainly on macrophages. The two other receptors termed FcRII and FcRIII have lower affinity

for IgG. FcRII binds to all subclasses of IgG and is expressed on phagocytic cells, B cells and platelets. On the other hand, FcRIII preferentially binds to IgG1 and IgG3, and is expressed on all types of phagocytes and lymphocytes (95,153)

In contrast to murine species and man, the domestic animal IgG subclass specificity for FcR has not been well documented. Reports in the literature have been confined mainly to evaluations of the presence of receptors for IgG complexes. Therefore, the existence of functionally and antigenically distinct FcR for IgG among domestic animals leukocytes remains to be further investigated. However, studies of FcR for IgG isotypes on bovine phagocytic cells by the rosette formation technique have shown that neutrophils produced more rosettes with IgG2-coated erythrocytes than with IgG1-sensitized cells. When bovine macrophages were examined by the same method, it was found that receptors for IgG1 were more frequent than those for IgG2 (77). Other studies showed no difference in numbers of IgG1 and IgG2 receptors on bovine monocytes (132). Howard (74) compared the ability of bovine IgG1 and IgG2 to promote killing of M. bovis by bovine alveolar macrophages and neutrophils and found that both IgG1 and IgG2 promote mycoplasma killing by macrophages, while only IgG2 promoted killing by neutrophils. These results provided some evidence for the existence of different types of receptors for IgG subclasses on bovine phagocytes. Similarly,

binding of subclasses of sheep IgG on sheep phagocytes was examined by Fleit et al. (52) using an immunofluorescence technique. It was found that alveolar macrophages bound IgG1 but not IgG2, while peripheral neutrophils bound IgG2 but not IgG1. This is similar to the results obtained from cattle. However, the existence of different types of FcR for IgG subclasses in both bovine and ovine phagocytic cells needs to be further investigated.

In addition to macrophages, other phagocytic cells and B lymphocytes also have on their surface a variety of receptors specific for the third component of complement (C3) that are detected by red blood cells sensitized with IgM and reacted with serum complement. At the present time, at least nine different types of complement receptors (CR) are believed to exist. However, only four of these have been fully characterized. These are designated as: CR1 which binds to C3b, C4b, iC4b and with much lower affinity to iC3b, CR2 which is specific for C3d, C3dg (region of the C3b fragment that becomes exposed after cleavage of C3b into iC3b) and iC3b, CR3 which binds to iC3b and with much lower affinity to C3dg and C3d, and CR4 which is structurally distinct from CR2 but has similar ligand specificity (95,130). Macrophages and neutrophils have been shown to express CR1, CR3, and CR4 type receptors but not CR2. On the other hand, B lymphocytes have

on their surface membrane CR1, CR2, and CR3 type receptors but not CR4 (95,131).

The function of the various CR appears to vary depending on the type of cells that carry them. For example, CR1 that is expressed also on erythrocytes is involved in the clearance and neutralization of circulating immune complexes by binding and carrying them into the liver and spleen, where they are destroyed by macrophages. On the other hand, its function on macrophages and neutrophils is to opsonize particles for phagocytosis and cytotoxicity (95).

Although FcR for IgG and C receptors have been described on bovine (77,105), caprine (100), swine (28,66), equine (48), human (119), guinea pigs (82), and to a limited extent, on ovine (52) phagocytic cells, the effects of microorganisms on their expression is not well documented. However, Caruso and Ross (25) reported that alveolar macrophages from pigs experimently infected with M. hyopneumoniae and Actinobacillus pleuropneumoniae had greater phagocytic activity towards sensitized and nonsensitized sheep red blood cells than noninfected control pigs. Similarly, Harmsen and Jeska (66) found that infection of pigs with Toxoplasma gondii resulted in an increased number of pig AM exhibiting C receptors, while the number of FcR for IgG remained unchanged compared with the noninfected control pigs. However, they concluded that this augmentation of C receptors did not independently enhance the

ability of these pig AM to endocytose SRBC. It is interesting to note that both studies were performed under in vivo conditions. It has also been shown that intraperitoneal injection of some substances such as thioglycollate medium or bacillus Calmette-Guerin into mice resulted in the activation of peritoneal macrophages by increasing their surface receptors (18). In contrast, administration of glucocorticoids in guinea pigs (110) and human (55) decreased the expression of FcR on phagocytic cells. Similarly, prolonged exposure of guinea pigs to corticosteroids in vivo can cause alterations in membrane Fc receptor function of AM (81) and the loss of these Fc receptors caused a marked decrease in cytotoxic effector function of these macrophages (80).

Pathogenesis of Mycoplasmal Infection

The ability of an infectious agent including mycoplasmas to invade and initiate disease is dependent on a complex interaction with host defence mechanisms. Mycoplasmas are, in general, pathogens of the mucosal surfaces and therefore resistance at this site will determine the outcome of the infection. At this level, the role of host phagocytic cells in containing the invaders is of major importance. Both neutrophils and macrophages are professional phagocytes capable of trapping and destroying particulate materials through the action of various mechanisms. However, many

infectious agents, including Mycoplasma sp., have evolved several virulence factors that allow them to resist and colonize the host tissues.

Virulence Factors

Adherence to Host Tissues Attachment of mycoplasmas to host cells is crucial for their survival within a host. The general ability of mycoplasmas to attach to host cells has been studied most extensively with the respiratory pathogens, in particular M. pneumoniae, the causative agent of atypical pneumonia in man. The consequences of attachment of mycoplasmas are often injury to the host cell including biochemical alterations such as decreases in ATP, cyclic AMP, carbohydrate utilization and oxidative metabolism (26).

Even though hemadsorption of red cells by M. pneumoniae was described first by Del Guidice and Parvia (45), early information available on the importance of the attachment process in the pathogenicity of mycoplasmas came from Sobeslavsky et al. (139) who reported the utilization of neuraminic acid receptors of host cells by M. pneumoniae. Shortly thereafter, Manchee and Taylor-Robinson (104) confirmed these findings, denoted differences in the avidity for neuraminic acid receptors among strains of M. gallisepticum, and concluded the existence of a receptor gradient that might reflect differences in pathogenicity.

Collier and Baseman (38) suggested that neuraminic acid was a component of receptors of host cells since treatment of tracheal rings with neuraminidase could partially inhibit adherence. They also noted that avirulent mycoplasmas were not capable of adhering to the tracheal rings, and suggested that a tip-like structure seemed to be necessary for attachment. In 1976, Powell et al. (114) also demonstrated the ability of neuraminidase to impair attachment of M. pneumoniae to tracheal rings. Further studies on cell receptors for M. pneumoniae confirmed that adherence occurs through the interaction of membrane proteins of the microorganisms to a receptor moiety on the surface of host cells. The adhesin is the P1 protein concentrated in a tip structure of the microorganism but also present on other areas of the membrane. However, the importance of the P1 in attachment in vivo is not completely understood since avirulent strains of M. pneumoniae are unable to attach to host cells but still they possess the P1 protein (37).

A considerable number of Mycoplasma sp. other than M. pneumoniae have been shown to possess the ability to adhere to host cells. M. gallisepticum attaches to the tracheal epithelium of chickens by means of its organelles (154), and a glycophorin (glycoprotein) was reported to be its specific receptor (16). The existence of cross reactions between epitopes of P1 protein of M. pneumoniae and a similar size

protein in M. gallisepticum was noted (30), although the binding protein has not been fully characterized. On the other hand, adherence of M. pulmonis to host cells was shown to be a different process than that of M. pneumoniae and M. gallisepticum. It was shown that neuraminic acid did not act as a receptor in host cells and that the organism lacked an attachment organelle (108,146). Consequently, it was speculated that adhesion of M. pulmonis to host cells was mediated by a general interaction of a capsule-like surface material on its membrane with the host cells. Cytadsorptive virulent strains of M. pulmonis have more capsular material than substrains that have been passaged in vitro until they are no longer virulent (146). It has also been proposed that the capsular material of M. dispar, a causative agent of pneumonia in calves and M. hyopneumoniae, the etiologic agent of enzootic pneumonia in swine, plays an important role in the colonization of respiratory tract through the attachment process (128). Recently, Rosenbusch (126) proposed a role for superoxide dismutase (SOD) in the mucosal-colonizing mycoplasmas since several species of mycoplasma that are mucosal colonizers including M. bovoculi, M. bovirhinis, M. arginini, M. dispar and M. hyopneumoniae were found to produce the enzyme. On the other hand, M. bovis, M. gallisepticum, M. pulmonis and M. pneumoniae, which are known to be capable of causing systemic infections, were found not to produce SOD.

Concerning M. ovipneumoniae, even though experimental studies revealed its ability to induce ciliostasis and loss of cilia in ovine cells (85), no information on its mechanisms of adherence to host cells has been reported.

Toxin Production The neurotoxicity of M. neurolyticum, the causative agent of rolling disease in mice and rats, has been recognized since the work of Sabin (133). Neurotoxic properties of 3 other species, M. arthritidis, M. pulmonis and M. gallisepticum, were described by Thomas (148) who showed that the organisms must be alive to produce toxin and larger doses must be used to produce toxicity.

The spiroplasmas from plants can also affect neurologic tissue when inoculated into appropriate hosts, although the role of toxins has not been clearly demonstrated (57). There are reports that the corn stunt spiroplasma can colonize the neural tissue of its natural insect vectors resulting in behavioral abnormalities such as irritability (57). Spiroplasma mirum, a causative agent of suckling mouse cataract is also reported to affect ocular and neural tissues of rodents (57).

Many of the mycoplasmas causing pneumonia induce ciliostasis and loss of cilia. It is believed that the basis for these cytopathic changes might be due to oxidative attack by hydrogen peroxide on host cell membranes. M. pneumoniae

actively produces superoxide anions as well as hydrogen peroxide. These can be introduced into the host cell through the beneficial effects of intimate contact with the cell membrane. Once within the cell, the superoxide anions inactivate the host epithelial catalase enzyme, thus permitting the oxidative action of hydrogen peroxide produced by both the organism and the host cells (9,91). Hydrogen peroxide is also reportedly produced by M. hyorhinis (113), and Acholeplasma laidlawii (118). However, this role of hydrogen peroxide in the pathogenicity of mycoplasmas needs to be further investigated since some known highly pathogenic mycoplasma species such as M. hyopneumoniae (113) and M. hyosynoviae (129) do not produce hydrogen peroxide. Gabridge and Barden-Stahl (58) proposed a role of adenine in the ciliostasis induced by M. pneumoniae. The transfer of adenine from the host cell membranes to the microorganisms decreases the quantity of ATP available to the cell and results in loss of energy for ciliary motion.

Other potential mediators of toxicity in mycoplasma infections are galactan, a phenol-water extract of M. mycoides subsp. mycoides and lipoglycan extracted from several Acholeplasma species. The evidence that these two products may play a pathologic role similar to the endotoxin of gram negative bacteria has been suggested by several authors over

the past years, although biochemically they are different from classical lipopolysaccharide (LPS) (101,136,155,157).

The possibility that mycoplasma pathogenicity might be linked to certain types of enzymes (proteases, collagenases) has also been investigated. Liquefaction of formalin-denatured gelatin by cultures of M. mycoides subsp. mycoides and subsp. capri was described by Freundt (54). Liquefaction of coagulated serum by M. capricolum and M. mycoides subsp. mycoides has also been described (57). Proteases specific for IgA have been found in Ureaplasma urealyticum (93,122).

Several other factors which may play a role in the pathogenesis of mycoplasmal disease have been reported. A polysaccharide capable of eliciting an inflammatory response in the skin of guinea pigs and the mammary gland of cows was extracted from the membrane of M. bovis (60). Two membrane proteins of M. hyopneumoniae have been shown to be responsible for the cytotoxicity for porcine pulmonary fibroblasts (128).

Interactions with the Immune System

Mycoplasma-Phagocytic Cell Interactions An important feature of several species of mycoplasma including those that cause respiratory infections is their ability to survive quite well in cultures of macrophages and neutrophils in the absence of specific antibodies. This phenomenon has been observed with M. pneumoniae (22,115), M. pulmonis (89), M. bovis (78) and M.

dispar (11,78). The mechanism by which mycoplasmas inhibit the phagocytic capacity of phagocytes is unknown. In addition to anti-phagocytic capsular material found in some mycoplasma species (11,73), it can be speculated that this survival may be due to a passive mechanism. The intimate contact of mycoplasmas with the cell membranes of the phagocytes may prevent the stimulation of appropriate receptors, or even when stimulated, the density is insufficient for engulfment (72). Thus, this may also compromise the ability of phagocytic cells to ingest and kill a second bacterial target. In this regard, M. hominis, M. arthritidis (137,149), M. bovis (75), and M. dispar (11,75) have already been reported to interfere with the ability of phagocytic cells to phagocytose Escherichia coli or Staphylococcus aureus in vitro. However, there are reported findings that M. arthritidis did not attach to cells but still inhibited phagocytosis (73). Furthermore, Thomsen and Heron (149) demonstrated that in vivo exposure of rat neutrophils to M. arthritidis and M. hominis also depressed their ability to cause phagocytosis of E. coli. Caruso and Ross (25) also reported that the percentage of AM phagocytosing opsonized sheep RBC was not affected by in vivo infection of pigs with M. hyopneumoniae alone but, was suppressed by a combined infection with M. hyopneumoniae and Actinobacillus pleuropneumoniae.

In contrast, certain investigators have reported that mycoplasmas were found to activate macrophage phagocytic activity. Normal mouse peritoneal macrophages in the presence of M. arginini or M. pulmonis, enhanced their cytotoxicity for target cells and their microbicidal capacities for intracellular organisms (70). This is difficult to reconcile with the previously mentioned in vitro and in vivo studies. It is interesting to note that certain microorganisms other than mycoplasmas have been also observed to interact with normal macrophages and neutrophils in vitro, resulting in an impaired ability to phagocytose a second bacterial target. Examples include Pseudorabies virus (56), Haemophilus somnus (79), and Salmonella choleraesuis (124).

Paradoxically, there are reported findings that mycoplasmas were phagocytosed in the absence of specific antibodies. Webster et al (159) reported that four different mycoplasmas of human origin, M. pneumoniae, M. hominis, M. salivarium and U. urealyticum were engulfed by human neutrophils in the absence of specific antibodies. However, they concluded that mycoplasmas survived within the neutrophils unless they had been opsonized with antibodies prior to engulfment. In addition, Davis et al. (44) reported that rat alveolar macrophages were able to exert an antimycoplasmal effect regardless of the presence or absence of specific antibody. In the same way, reports exist of

failures of phagocytes to phagocytose mycoplasmas even in the presence of specific antibodies (137). This led to the speculation that to explain mycoplasma-phagocytic cell interactions in vivo it is essential to use mycoplasmas, phagocytic cells, and antisera from the natural host (73,75). However, even with such homologous systems, there are conflicting results. It has been reported that M. arthritidis was not phagocytosed by mouse or rat macrophages in the presence of mouse or rat convalescent sera, but phagocytic action was observed in the presence of rabbit antiserum (36). Also, Davis et al. (44) found that mouse alveolar macrophages required hyperimmune rabbit serum but not mouse hyperimmune serum to phagocytose M. pulmonis. In contrast, Taylor and Howard (147) found that convalescent mouse serum promoted the phagocytosis of M. pulmonis by mouse peritoneal macrophages and neutrophils. Also, M. bovis and M. dispar were phagocytosed by bovine alveolar macrophages and mammary neutrophils in the presence of bovine antisera (78). Howard and Taylor (73) suggested that the failure of sera to show opsonic activity may be related to the isotype response to the mycoplasmas or of some other defect of the antibody response since studies have shown that IgG2 antibody was more effective than IgG1 antibody in promoting killing of mycoplasmas by peripheral-blood neutrophils (74).

While extensive study has been reported on the experimental infection of sheep with M. ovipneumoniae (4,5,43,86,87,88,142,145), current knowledge of its interaction with phagocytic cells is extremely limited. Electron microscopic studies by Al-Kassi and Alley (3) showed that, in the absence of specific antibodies, the organisms remained attached to the surface of sheep alveolar macrophages without inducing phagocytosis. The addition of specific antibodies stimulated phagocytosis.

The role of cytokines, in particular tumor necrosis factor (TNF) and interleukin 1 (IL-1) produced primarily by macrophages, in the host defense mechanisms against infectious agents by acting as pyrogens, chemotactic factors and by exerting regulatory activities on other cells such as neutrophils, T cells and B cells is well documented (47,98,107,160). However, chronic release or overproduction of these cytokines may also be detrimental to the host. Macrophages can be activated by several infectious agents with potential release of a number of products including hydrolytic enzymes and cytokines that participate in the mediation of injury through cell recruitment and cytotoxic effects. TNF-alpha has been shown to be an important mediator of endotoxin-induced shock in a variety of mammalian species (84,127). It has also been demonstrated that IL-1 and TNF can have marked pro-inflammatory properties by inducing the synthesis and

release of phospholipase A_2, an enzyme which possesses a wide range of biological activities (116).

It has been reported that M. mycoides subsp. mycoides (LC) induced TNF, and the injection of TNF in goats induced a response similar to that caused by this mycoplasma species (127). Increased TNF-alpha concentrations in the lungs of mice infected with M. pulmonis has also been demonstrated (51). Various other mycoplasmas including M. pneumoniae, and A. laidlawii (13) have been shown to have potent activity for inducing TNF production from macrophages. In a recent study, Almeida et al. (11) reported that encapsulated M. dispar or its capsular material did not induce the production of TNF and IL-1 from bovine alveolar macrophages, but unencapsulated M. dispar did, suggesting that the capsular material could have an inhibitory effect on the production of these cytokines. It was not mentioned whether the mycoplasma species utilized in the previous studies by Rosendal and Faulkner were encapsulated or not. The important consideration is that either exacerbation or inhibition of release of these products from macrophages could have major adverse effects on disease processes.

Mycoplasma-Lymphocyte Interactions Another important characteristic of mycoplasmas is their ability to interact nonspecifically with lymphocytes as first demonstrated by

Ginsburg and Nicolet (62) with M. pulmonis. Consequently, this interaction may contribute to disease pathogenesis by induction of autoimmune antibodies or elaboration of a variety of lymphokines that may induce cell injury (31). Several other species of mycoplasma have been found to share the potential for inducing nonspecific mitogenic activation of lymphocytes. M. pneumoniae was found to exert a mitogenic effect on lymphocytes from human (19) as well as guinea pig (20) and mouse (20,34) lymphocytes. M. arthritidis also was found to be mitogenic for rat (32,33) and human (32) lymphocytes. Many other species including M. fermentans, M. hominis, A. laidlawii, M. arthritidis, M. neurolyticum, M. hyorhinis, M. synoviae, M. felis , M. gallisepticum, M. canis, M. hyosynoviae, M. arginini, and Spiroplasma citri have been reported to have mitogenic activity for mouse lymphocytes and sometimes for human and rat lymphocytes (31). This mitogenic activity has been demonstrated with whole organisms, extracts and membrane preparations (31).

The mitogenic activity produced by mycoplasmas has also been observed in several experiments with either T cells or B cells or both from various animal hosts. This indicates the possible existence of heterogeneity in the mitogenic activity of the various mycoplasma species. M. pneumoniae has been reported to activate either B or T lymphocytes or both from humans (19), mice (20,34) and guinea pigs (20). Activation of

human and murine T-lymphocytes has been also observed (19). Human lymphocytes were activated in vitro by M. pneumoniae, Acholeplasma laidlawii, and M. fermentans as well as by M. pulmonis (109).

Mitogenic activity of mycoplasmas and their products has also been shown with in vivo experimentation. This has led to the speculation that this may contribute to disease pathogenesis. Inoculation of cell membranes or cell membrane components into the respiratory tract of experimental animals resulted in significant lymphocyte infiltration and interstitial pneumonia in some instances (31,106).

Also, the interaction of mycoplasmas with lymphocytes can result in the induction of a variety of soluble products including interferons, interleukins, and chemotactic factors. Rinaldo et al. (120) have reported that several mycoplasma species were capable of inducing interferon production from ovine leukocytes. It has also been reported that human lymphocytes produced interferon in response to M. pneumoniae (35) and M. synoviae (35,140). There is evidence that M. pulmonis membranes were capable of inducing production of significant levels of interleukin-2 from rat lymph node lymphocytes (99). The role of these lymphokines, produced by activated leukocytes or lymphocytes in the disease process depends on their ability to potentiate inflammation by recruiting other cells including lymphocytes, macrophages or

neutrophils and by stimulating differentiation or activation of those cells (31).

Mycoplasma-Associated Immunosuppression While mycoplasma species have been reported to activate lymphocytes, suppressive effects on both humoral and cell-mediated responses have also been reported by several investigators. Kaklamanis and Pavlatos (92) reported that simultaneous injections of M. arthritidis and viral antigens resulted in complete or partial suppression of humoral responses against the antigens. Similarly, M. dispar has also been shown to induce immunosuppression when injected in combination with other antigens (76). Suppression of humoral and cell-mediated responses were reported with M. bovis in calves (17) and M. pneumoniae in human (134).

The effect of certain species of mycoplasma on the responsiveness of lymphocytes to mitogenic stimulation have been studied. Lymphocytes from cattle experimentally infected with M. mycoides subsp. mycoides failed to respond to phytohaemagglutinin (PHA) (121). Similarly, inoculation of active M. arthritidis extracts into mice resulted in decreased responses of lymphocytes to concanavalin A and PHA (2).

Evidence of mycoplasma species induced-immunosuppression has been shown by their ability to potentiate bacterial infections. The potentiating effect of M.

pneumoniae on the development of pneumococcal septicemia in hamsters by Diplococcus pneumoniae was demonstrated by Lui et al. (102). During studies of pyelonephritis in rats, Thomsen and Rosendal (150) showed that inoculation of M. arthritidis followed by an inoculation of E. coli provoked severe suppurative lesions, while inoculation of high doses of E. coli alone did not produce lesions in rats which had not been previously infected with mycoplasmas.

PAPER: INTERACTION OF *Mycoplasma ovipneumoniae* WITH SHEEP ALVEOLAR MACROPHAGES

Interaction of *Mycoplasma ovipneumoniae* with sheep alveolar macrophages

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ABSTRACT

The ability of Mycoplasma ovipneumoniae (field strain) to induce alterations in sheep alveolar macrophage functions was examined in vitro with live and heat killed organisms. Normal alveolar macrophages collected by bronchial lavage of lungs were infected with different preparations of M. ovipneumoniae, and their capability to ingest Staphylococcus aureus and to elicit antibody-dependent cellular cytotoxicity against sensitized chicken red blood cells were tested. Controls consisted of noninfected macrophages in modified brain heart infusion broth or in M199 medium. In addition, the presence of surface receptors on these sheep alveolar macrophages and the effect of live M. ovipneumoniae on their expression were determined by the rosette formation technique. The percentage of S. aureus ingested by nontreated sheep alveolar macrophages (controls) was significantly higher than that of infected macrophages. Live mycoplasmas were more effective in suppressing the ingestion of S. aureus by these macrophages than killed mycoplasmas. Both live and killed mycoplasmas suppressed the cytolytic effect of the sheep alveolar macrophages to a similar degree. About 78% and 45% of the normal sheep alveolar macrophages had IgG and complement receptors, respectively. Infection of these macrophages with M. ovipneumoniae decreased significantly the expression of IgG

receptors but not that of complement receptors. This may explain, in part, the impaired ability of these macrophages to ingest or lyse a second target. Receptors for IgM were not found on any of normal or treated macrophages. Our results suggest that these M. ovipneumoniae-induced alterations in macrophage functions could contribute to the role that this organism may play in sheep respiratory disease.

INTRODUCTION

Ovine pneumonia is considered one of the most common infectious diseases of sheep worldwide regardless of management systems (1,10,13,18,31,41). The disease is caused by a complex interaction of environmental factors producing stress and a variety of microorganisms working synergistically (10,41). Among these microorganisms, Pasteurella sp. are considered the most commonly isolated from both pneumonic and normal sheep. However, challenge experiments have shown that Pasteurella sp. alone have limited ability to produce pneumonia and probably require a predisposing infection by another infectious agent. A number of microorganisms including viruses, mycoplasmas and chlamydia have been suggested to play this role (10,16,26,41).

Among the pulmonary defence mechanisms against infectious agents, the phagocytic potential of alveolar macrophages is of major importance. Mycoplasmas possess various virulence factors that can alter these phagocytic cell functions. Thus, this may facilitate colonization of the respiratory tract by secondary bacterial agents. Previous in vitro studies have shown that in the absence of specific antibodies, several mycoplasma species not only can survive quite well in cultures of phagocytic cells but can also interfere with ingestion and killing of a second bacterial target (5,7,20,21,29,35,39,43).

Despite the fact that M. ovipneumoniae is considered the most common mycoplasma isolated from ovine lungs (4,6,8,34), its role as an important etiologic factor in respiratory disease of sheep is still not clearly defined. While extensive studies on the experimental infection of sheep with this microorganism have been conducted and yielded conflicting results (3,11,27,28,40,42), current knowledge of its interrelationship with the phagocytic cells of ovine lung is extremely limited (2).

The purpose of the present study was to determine if M. ovipneumoniae (isolated from a case of ovine pneumonia) suppressed normal sheep alveolar macrophage functions as a potential mechanism by which M. ovipneumoniae may contribute to respiratory disease in sheep.

MATERIALS AND METHODS

Macrophage Collection

Alveolar macrophages (AM) were collected by bronchial lavage of whole lungs obtained from freshly slaughtered lambs and healthy aged sheep that were sacrificed by intravenous injection of Beuthanasia-D Special containing 350 mg pentobarbitone/ml (Schering-Plough Animal Health Corp., Kenilworth, NJ, 10 ml/animal). The trachea was immediately exposed and tied below the larynx with a piece of cord to avoid any influx of blood. The lungs were then carefully removed and two consecutive bronchoalveolar lavages were performed by infusing 500 ml of sterile warm phosphate buffered saline solution (Dulbecco's PBS, without calcium chloride and magnesium chloride, pH 7.4, Sigma Chemical Company, St. Louis, MO) containing 10 U heparin/ml (Gibco, Grand Island Biological Company, Grand Island, NY), 100 IU penicillin/ml (Gibco Laboratories, Grand Island, NY), 100 μg Streptomycin sulfate/ml (Gibco Laboratories, Grand Island, NY), 50 μg Gentamicin/ml (Sigma Chemical Company St. Louis, MO), and 0.125 μg Fungizone/ml (Gibco Laboratories, Grand Island, NY) into the lungs through the trachea. The lungs were gently massaged and the lavage fluid was poured into a sterile Erlenmeyer flask. The lavage fluid was filtered through a double layer of cheesecloth into a sterile flask. Cells

present in the lavage fluid were washed twice in PBS by centrifugation (300 x g for 10 minutes) and the pellets were gently resuspended in M199 medium (#380-2340, with Earles'salt solution, L-glutamine, sodium bicarbonate and Hepes buffer, Gibco BRL, Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Gibco BRL, Life Technologies, Inc., Grand Island, NY) and antibiotics. The cell suspensions were depleted of red blood cells, if necessary by adding cold, sterile tris-buffered ammonium chloride (0.16M NH_4Cl and 0.17M tris-HCl, pH 7.65) solution for 5 minutes at 37°C, centrifugation, washing and resuspension in M199 medium. A sample of the cell suspensions was streaked on a glass slide and stained with Diff-Quik stain (Baxter Scientific Products, Miami, FL). A differential count was performed. The percentage of viability was determined by trypan blue exclusion. Total cell counts were made in a hemacytometer chamber (Hausser Scientific, Blue Bell, Pa) and the concentration of cells was adjusted as appropriate for each experiment.

Mycoplasma Preparation

The M. ovipneumoniae isolate used for this study was obtained from a pneumonic sheep lung by culture of lung tissues. The organism was identified as M. ovipneumoniae by a fluorescent antibody (FA) test. Culture impressions were made

on glass slides, dried in air and fixed with acetone for 7 minutes. One to 2 drops of diluted (1:100) primary anti M. ovipneumoniae antibody (Ab) (equine origin, prepared by immunizing a horse with formalin-killed M. ovipneumoniae) was added. After 30 minutes incubation at 37°C, the slides were washed twice with PBS, rinsed with distilled water and dried in air. After that, two drops of diluted (1:20) secondary fluorescein-labeled, rabbit-anti equine globulin (Accurate Chemical and Scientific Corp., Westbury, NY) were added. The slides were again incubated, washed as previously described, and mounted in glycerinated PBS (one part of PBS in 9 parts of glycerol) for observation using a fluorescent microscope. Colonies of M. ovipneumoniae and M. bovis (courtesy Dr. R. Rosenbusch, Veterinary Research Institute, College of Vet. Med., Iowa State University, Ames, IA) were included as (positive and negative) controls, respectively.

A stock culture of M. ovipneumoniae was prepared by growing the organism in autoclaved modified brain heart infusion broth medium (MBHI, pH 7.6: brain heart infusion, 25 g; neopeptone, 2.5 g; bacto-casitone, 2.5 g (Difco Laboratories, Detroit, MI); dextrose (Fisher Scientific, Fair Lawn, NJ), 2 g; yeast extract (Difco Laboratories, Detroit, MI), 20 g; distilled water, 800 ml) supplemented with 200 ml of horse serum (HyClone Laboratories, Inc. Logan, Utah). Stock solutions of bacitracin and thallium acetate (Sigma Chemical

Company, St. Louis, MO) were made by adding 2 g of bacitracin or 1 g of thallium acetate to 100 ml of distilled water. After filtration through a 0.45 μm membrane filter (MSI, Westboro, MO), 10 ml of each solution was added to the one liter of medium. The number of organisms/ml was determined by the plate count method and aliquots were stored at -70°C until used. The viability of the stock preparation of M. ovipneumoniae was determined at the time of use in each experiment.

Staphylococcus aureus Ingestion Assay

The aim of this experiment was to determine if M. ovipneumoniae could influence the ingestion of S. aureus by normal sheep alveolar macrophages. Macrophage suspensions adjusted to 5 x 10^6 cells/ml were infected with different numbers of M. ovipneumoniae to give a ratio of 1:1, 1:10, 1:100, 1:1000, or 1:4000 macrophage to mycoplasmas. In addition, macrophage suspensions were treated with heat-killed M. ovipneumoniae (56°C in water bath for 30 minutes) at a ratio of 1:1000 macrophage to mycoplasmas. Controls consisted of macrophages in MBHI medium or macrophages in M199 medium. Since no major differences between the 2 controls were observed during the experiments, only MBHI controls were included in the results. After an infection period of 30, 60, 90, or 120 minutes at 37°C, the macrophages were washed twice with PBS by centrifugation (100 x g for 5 minutes) at 37°C,

their viability checked by trypan blue exclusion (greater than 80%), resuspended in M199 medium and assayed for ^{125}I-labeled S. aureus ingestion as described previously (38) with some modifications. Briefly, 100 µl of ^{125}I-labeled S. aureus, 50 µl of diluted (1:4) bovine anti-S. aureus serum (courtesy Dr. J. Roth, Dept. MIPM, College of Vet. Med., Iowa State University, Ames, IA.), and 300 µl of Earle's balanced salt solution (EBSS) were added to the tubes and incubated for 10 minutes at 37°C. Then, 50 µl of a suspension of macrophages treated as indicated were added and the tubes were centrifuged (100 x g for 5 minutes) and incubated at 37°C for 15 minutes. After vortexing, 500 µl of PBS solution containing 0.5 unit of lysostaphin was added and incubation allowed to proceed for a further 30 minutes. Approximately 2 ml of cold PBS was added to stop the reaction, and the tubes were centrifuged at 1250 x g for 10 minutes in a refrigerated centrifuge. The supernatant was discarded, the cells resuspended in cold PBS and again sedimented by centrifugation. The supernatant was discarded and the tubes containing the final pellets were placed in a gamma counter to determine the counts per minute (CPM) of radioactivity present. Each assay included a pair of standard tubes which contained S. aureus but no macrophages or lysostaphin. Also, a pair of background tubes which contained all reactants except macrophages were included. All samples

were set up in duplicate. The percentage of S. aureus ingested was calculated by using the following formula:

$$\% \text{ ingestion} = \frac{(\text{CPM in test tube}) - (\text{CPM in background tube})}{(\text{CPM in standard tube}) - (\text{CPM in background tube})} \times 100$$

Cytotoxicity Assay

The antibody-dependent cell-mediated cytotoxicity (ADCC) assay was conducted with ^{51}Cr-labeled chicken red blood cells (CRBC) as target cells as described previously (30) with minor modifications.

Preparation of ^{51}Cr-Labelled Chicken Red Blood Cells

CRBC were collected in an equal volume of Alsever's solution, washed twice in PBS by centrifugation (1500 x g for 10 minutes), and resuspended in PBS at concentration of 12.5 x 10^6 cells/ml. Then, one ml of washed and resuspended CRBC was added to 10 ml of PBS and centrifuged as before. The supernatant was discarded, the cells resuspended in 0.5 ml of M199 medium, and 50 μl (50 μCi) ^{51}Cr (Amersham Corporation, Arlington Heights, IL) were added and the cells were incubated overnight at 37°C in a 5% CO_2 humidified atmosphere. After this incubation period, the cells were washed twice in PBS (1500 x g for 10 minutes), and resuspended in 10 ml M199 medium to a final concentration of 1.25 x 10^6 cells/ml.

Cytotoxicity Procedure

For the cytotoxicity assay, 100 μl of macrophages suspended in M199 medium at a concentration of 3.5 x 10^6 cells/ml were placed in each well of a plate (#3596, 96-well, Costar Corporation, Cambridge, MA). Then, 50 μl of diluted (1:4) bovine anti-CRBC antiserum (courtesy, Dr. J. Roth, Dept. MIPM, College of Vet. Med., Iowa State University, Ames, IA) and 100 μl of ^{51}Cr-labeled CRBC were added to each well. After a 7 hour incubation at 37°C in a 5% CO_2 humidified atmosphere, ^{51}Cr release was measured by harvesting the supernatant onto cotton plugs (Skatron cell harvesting equipment, Skatron, Norway). The cotton plugs were transferred into tubes, placed in a gamma counter and counted for 2 minutes to determine the CPM of radioactivity present.

To determine if M. ovipneumoniae could interfere with the capacity of normal sheep AM to mediate ADCC, macrophage suspensions at a concentration of 3.5 x 10^6 cells/ml were incubated with live and heat-killed M. ovipneumoniae diluted in M199 mdium (without antibiotics) to give macrophage: mycoplasma ratios of 1:10 and 1:100. After 120 minutes, these macrophages were washed twice in PBS by centrifugation (100 x g for 5 minutes), their viability checked by trypan blue exclusion and assayed for cytotoxic activity. Each assay included a pair of standard wells which contained 150 μl of 1% Triton X-100 and 100 μl of ^{51}Cr-labeled CRBC but no macrophages

or anti-CRBC antibodies. Also, a pair of (control) background wells which contained all reactants except macrophages were included. All samples were set up in duplicate. The percentage of ^{51}Cr-labeled CRBC lysed was calculated by using the following formula:

$$\% \text{ lysis of target cells} = \frac{(\text{CPM sample}) - (\text{CPM background})}{(\text{CPM standard}) - (\text{CPM background})} \times 100$$

Assay for Surface Receptors

Preparation of Antibody-Coated and Complement-Coated Sheep Red Blood Cells

Sheep red blood cells (SRBC) were collected in an equal volume of Alsever's solution (110mM glucose, 70mM NaCL, 20mM trisodium citrate, 3mM citric acid) and washed 3 times in normal saline solution by centrifugation at 500 x g for 20 minutes. The supernatant was discarded and the packed cells were resuspended to a final 1% v/v concentration in PBS^+ (Dulbecco's PBS with calcium chloride and magnesium chloride, pH 7.3, Sigma Chemical Company, St. Louis, MO). Erythrocyte-antibody complexes were prepared by mixing equal volumes of 1% SRBC suspensions with rabbit IgG or IgM anti-SRBC serum (Cappel, Organon Teknika Corp., West Chester, PA) that were diluted in PBS^+ at a subagglutinating titer (1:320). The mixtures were incubated for 30 minutes at 37°C and for an additional 30 minutes at 4°C. These complexes were designated

as erythrocyte-IgG (EG) and erythrocyte-IgM (EM), respectively. For the preparation of erythrocyte-complement (EC) complexes, EM at a 1% concentration were washed once in PBS^+. Freshly collected sheep serum (known to not agglutinate SRBC alone) was added at a rate of 100 μl/ml as a source of complement. The mixture was incubated for 30 minutes at 37°C. Unsensitized SRBC (E) were prepared by incubating washed SRBC in PBS^+ in the absence of Ab or C for 30 minutes at 37°C and for an additional 30 minutes at 4°C. The various SRBC preparations were centrifuged at 300 x g for 5 minutes, and cells resuspended to a final concentration of 0.5% in M199 medium. These cells were used immediately in the rosetting assays.

Rosette Formation

Three ml of a suspension of macrophages (1 x 10^6/ml) were dispensed into a polystyrene 25 cm^2 tissue culture flask (Corning Glass Works, Corning, NY) and incubated at 37°C in a 5% CO_2 humidified atmosphere to allow macrophages to adhere. After a 4 hour incubation period, the cell monolayers were washed gently with warm sterile PBS to remove non-adherent cells. The number of adherent macrophages was estimated by using 0.2% trypsin (Difco Labs., Detroit, MI) to release the cells in one flask. The number of cells was then counted in a hemacytometer. The flasks were reincubated overnight (about 10

hours) in M199 medium containing 10% FCS and antibiotics. Macrophages in some flasks were infected with live M. ovipneumoniae diluted in M199 medium (without antibiotics) to give a macrophage:mycoplasma ratio of approximatively 1:10 and incubated overnight as well. Observations indicated that the viability of macrophages was not affected by the 10-hour infection with mycoplasmas. For the rosetting assay, adherent macrophages were washed once with PBS, and 2 ml of a SRBC preparation (EG, EM, EC, and E) were added to an individual flask (in duplicate). Incubation was allowed to proceed 45 minutes at 37°C in a 5% CO_2 humidified atmosphere. At the end of this time, the flasks were gently rinsed twice with PBS to remove unattached SRBC, and the macrophages were stained with Diff-Quik stain. The bottom of each flask was removed, and the cells observed through an oil immersion microscope at a 100 X magnification. A macrophage with a least 3 SRBC associated with its membrane was considered a rosette. A total number of 200 macrophages were counted randomly for each flask, and the percentage of rosette forming cells (RFC) was calculated as:

$$\% \text{ RFC} = \frac{\text{Number of RFC}}{\text{Total number of counted cells (200)}} \times 100$$

Statistical Analysis

Results were analyzed by analysis of variance (ANOVA). Values are reported as mean percentages ± standard error of means (SEM). The significance of differences between means was accepted when the p value was at least less than 0.05.

RESULTS

Although conditions for identification of the cell suspensions were not quite optimal (based only on the morphological criteria) the Diff-Quik staining showed generally more than 87% of the cells had a morphology compatible to that of macrophages, the major contaminating cells being lymphocytes. The percentage of viability was greater than 95%.

The effects of live and heat-killed M. ovipneumoniae on the ingestion of S. aureus by sheep AM are indicated in figures 1 and 2. The percentage of S. aureus ingested by non-treated control AM was significantly higher ($p<0.05$) than that of all preparations of AM pretreated with M. ovipneumoniae except for the 1:1 ratio at the 120 minute post-infection period. Also, live mycoplasmas were significantly ($p<0.05$) more effective in suppressing the ingestion of S. aureus by sheep AM than killed mycoplasmas (figure 2). Increasing the ratio of M. ovipneumoniae to sheep AM from 1:1 to 4000:1 did decrease significantly ($p<0.05$) the percentage of S. aureus ingested by sheep AM. The suppression was directly related to the number of mycoplasma utilized to infect sheep AM. However, varying the time intervals of sheep AM infection with M. ovipneumoniae from 30 minutes to 120 minutes did not influence

significantly the ingestion of S. aureus by sheep AM (Figure 3).

The effect of M. ovipneumoniae on ADCC activity of sheep AM was determined by the ability of normal sheep AM (controls) and sheep AM treated with live or heat-killed M. ovipneumoniae to lyse ^{51}Cr-labeled CRBC (Figure 4). The percentage of ^{51}Cr released from CRBC by normal sheep AM (42%) was significantly higher ($p<0.05$) than that for sheep AM treated with live or heat-killed M. ovipneumoniae (about 12% and 16%, respectively). There was no significant difference ($p>0.05$) between live and heat-killed mycoplasmas in depressing sheep AM ADCC function. Similarly, increasing the ratio of M. ovipneumoniae (live and heat-killed) to sheep AM from 10:1 to 100:1 did not significantly affect the ability of these AM to perform ADCC.

Both noninfected sheep AM and those infected with live M. ovipneumoniae failed to form rosettes with IgM-sensitized or with nonopsonized SRBC, while rosettes were observed with IgG-sensitized and C-sensitized SRBC (Figures 5, 6). About 78% and 45% of the normal sheep AM formed rosettes with IgG-sensitized or C-sensitized SRBC, respectively. The mean percentage of infected sheep AM forming rosettes with IgG-sensitized SRBC was 58%. This was significantly lower ($p<0.05$) than that of noninfected sheep AM. C-sensitized SRBC formed rosettes with

49% of AM infected with M. ovipneumoniae which was similar to the value of noninfected AM.

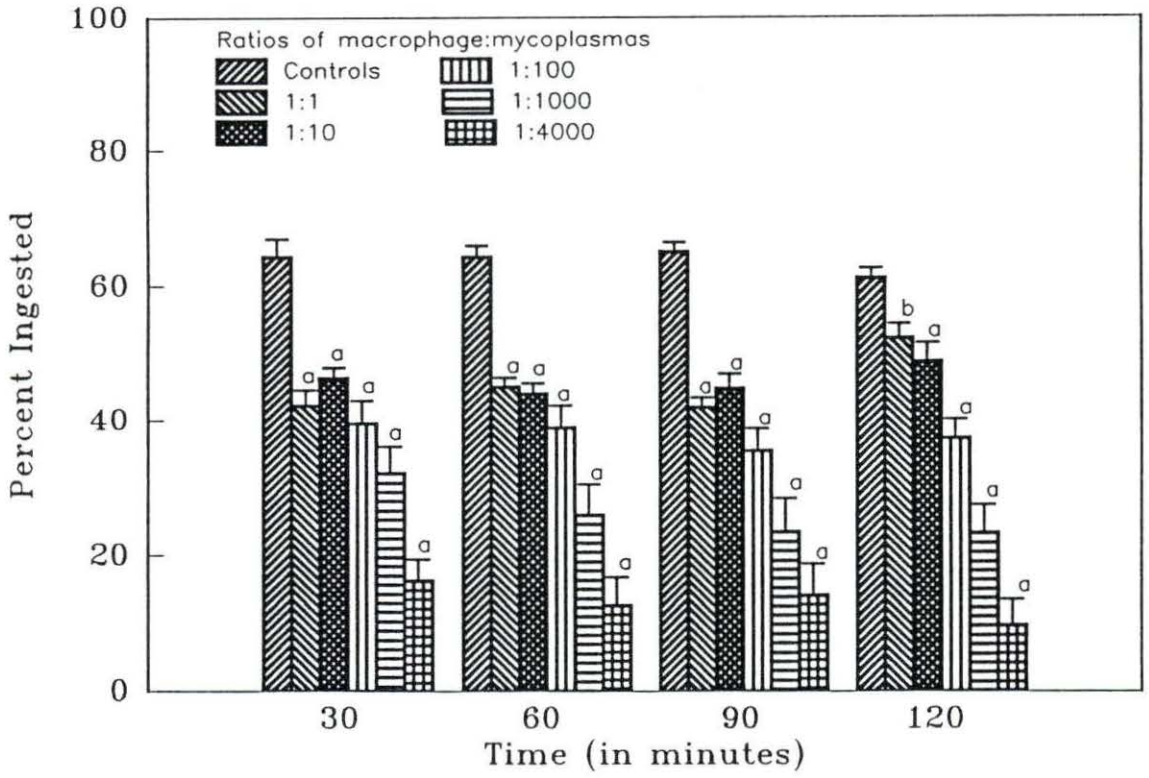

Figure 1. Effects of M. ovipneumoniae treatment of sheep AM on S. aureus ingestion. Sheep AM were exposed to live M. ovipneumoniae at ratios of 1:1, 1:10, 1:100, 1:1000, or 1:4000 macrophage to mycoplasmas. Controls consisted of untreated sheep AM in MBHI btoth. Values represent mean percentages (±SEM) of 5 separate experiments. Statistical differences from control values are indicated as; a=$p<0.05$, and b=$p>0.05$

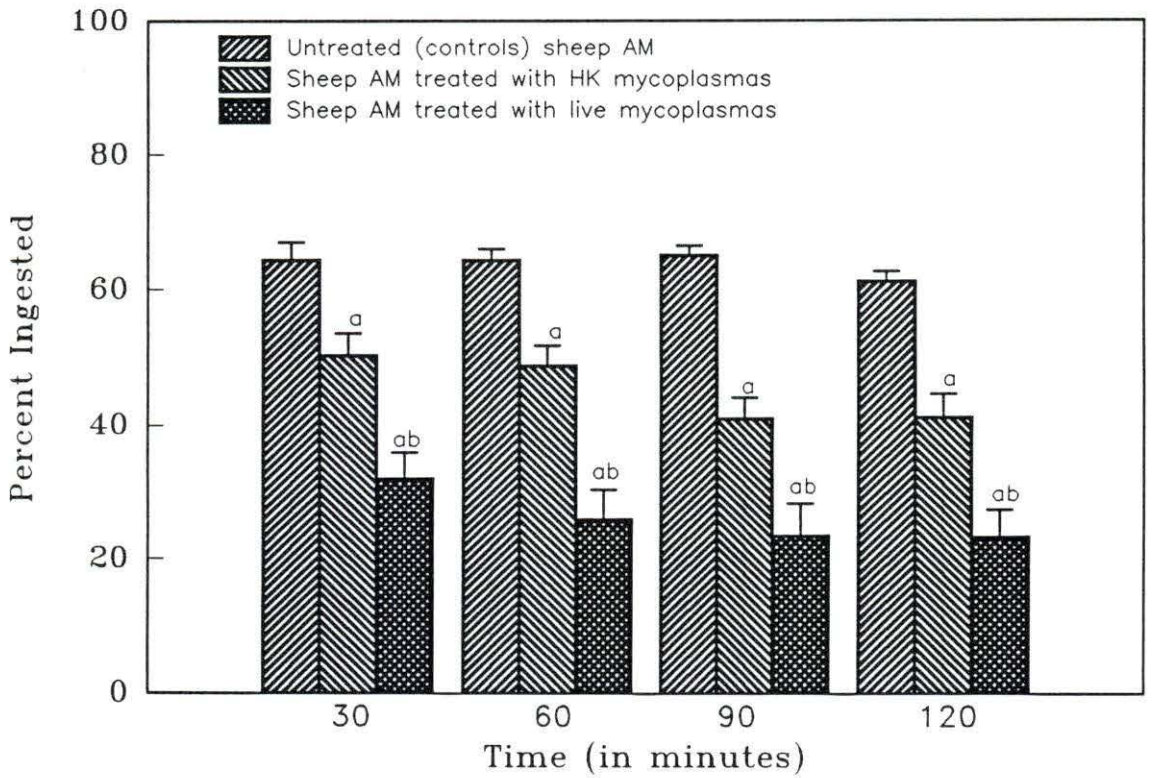

Figure 2. Effects of heat-killed (HK), or live M. ovipneumoniae on S. aureus ingestion by sheep AM at macrophage:mycoplasma ratio of 1:1000. Controls consisted of untreated sheep AM in MBHI broth. Values represent mean percentages (±SEM) of 5 separate experiments. Statistical differences are indicated as a=$p<0.05$ from control values, and b=$p<0.05$ for live vs HK mycoplasmas.

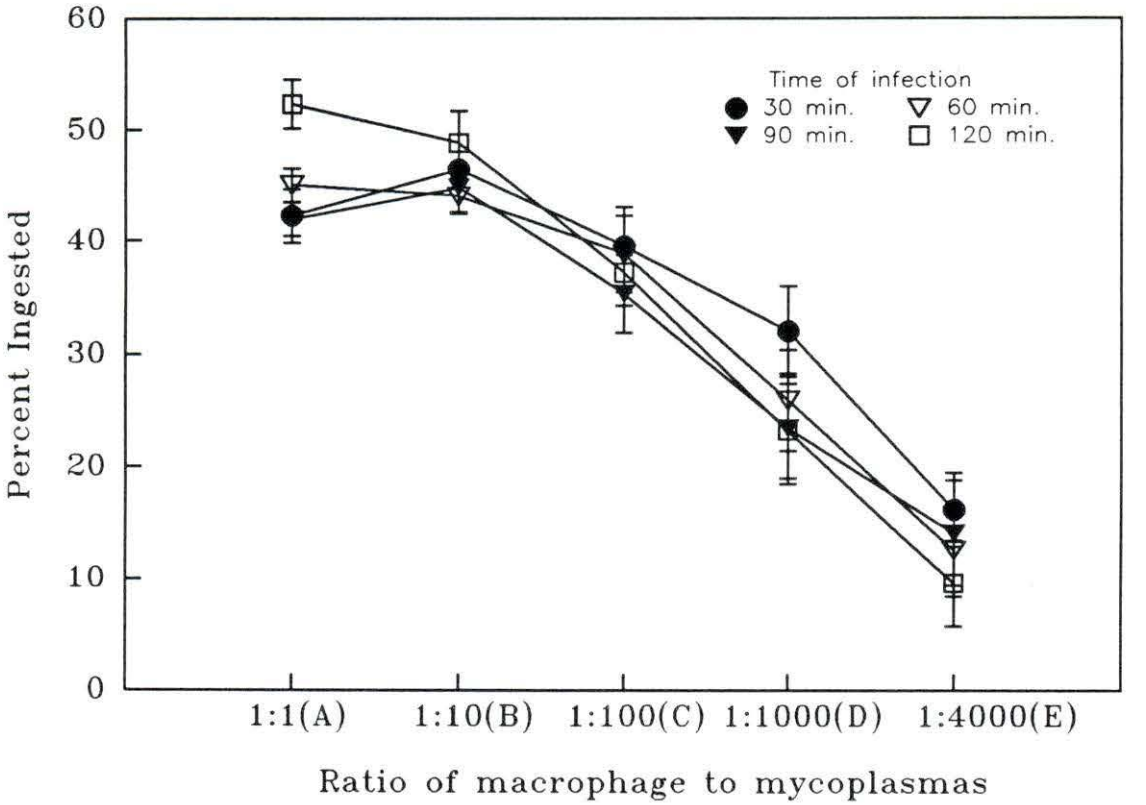

Figure 3. Effects of varying doses of M. ovipneumoniae (A,B,C, D,E) and times of infection (30, 60, 90, or 120 minutes) of sheep AM on ingestion of S. aureus. $p>0.05$ for A vs B,C, and $p<0.05$ for A vs D,E.

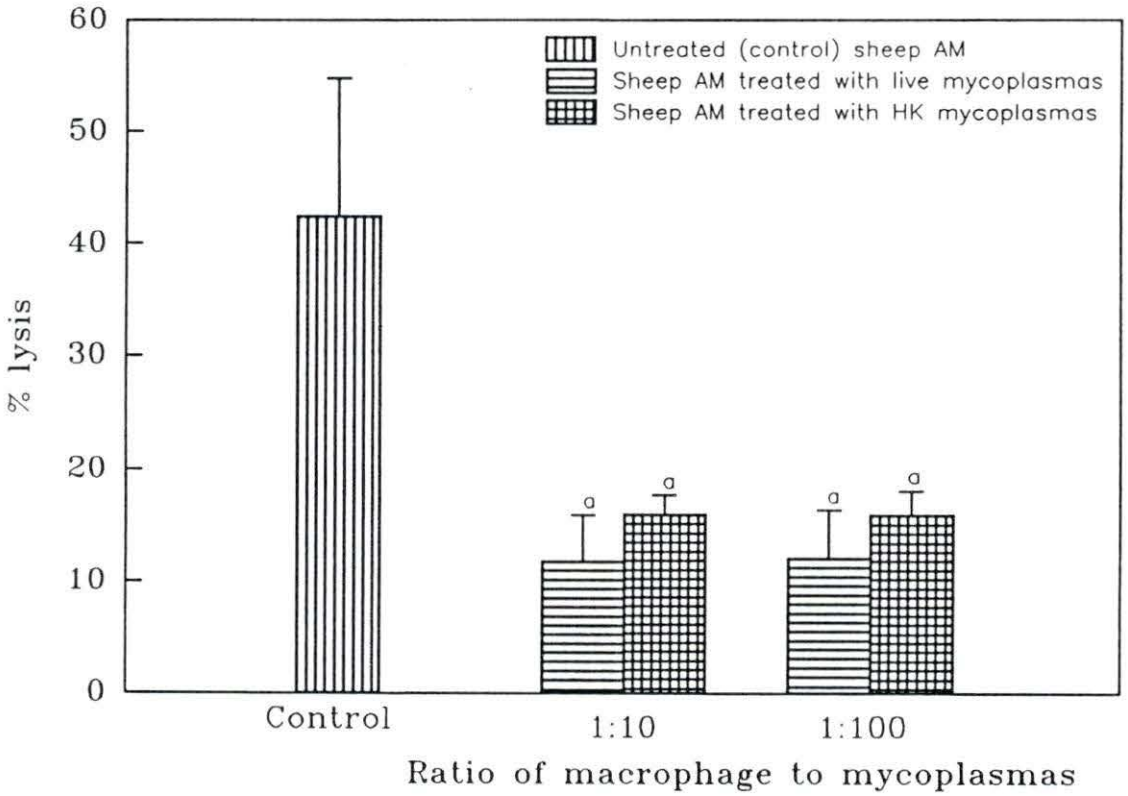

Figure 4. Effects of live and heat killed (HK) M. ovipneumoniae on sheep AM cytotoxic activity at ratios of 1:10, or 1:100 macrophage to mycoplasmas. Values represent mean percentages (±SEM) of 3 separate experiments. Statistical differences from control values are indicated as; a=$p<0.05$.

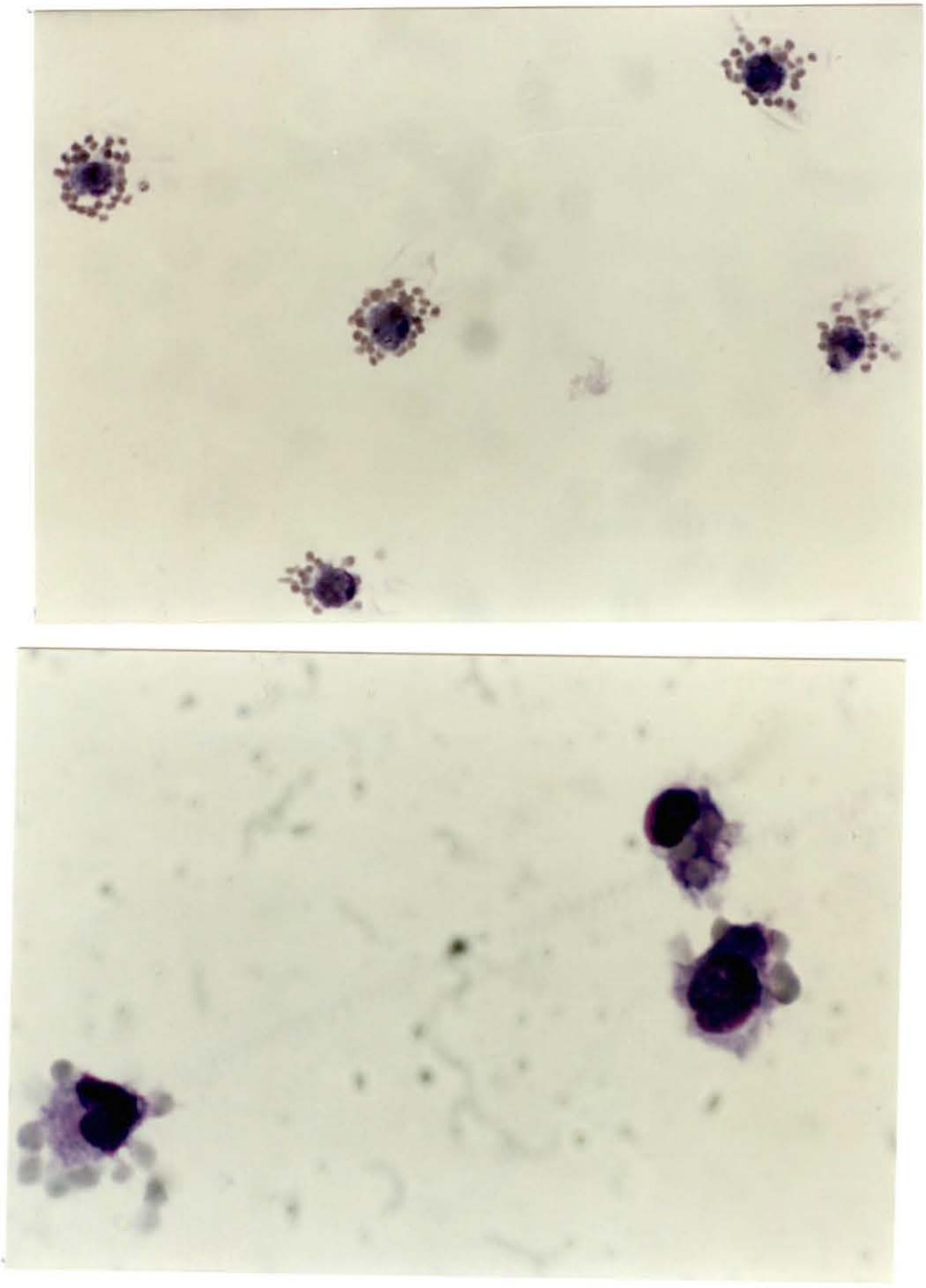

Figure 5. Sheep AM forming rosettes with IgG-sensitized sheep RBC.

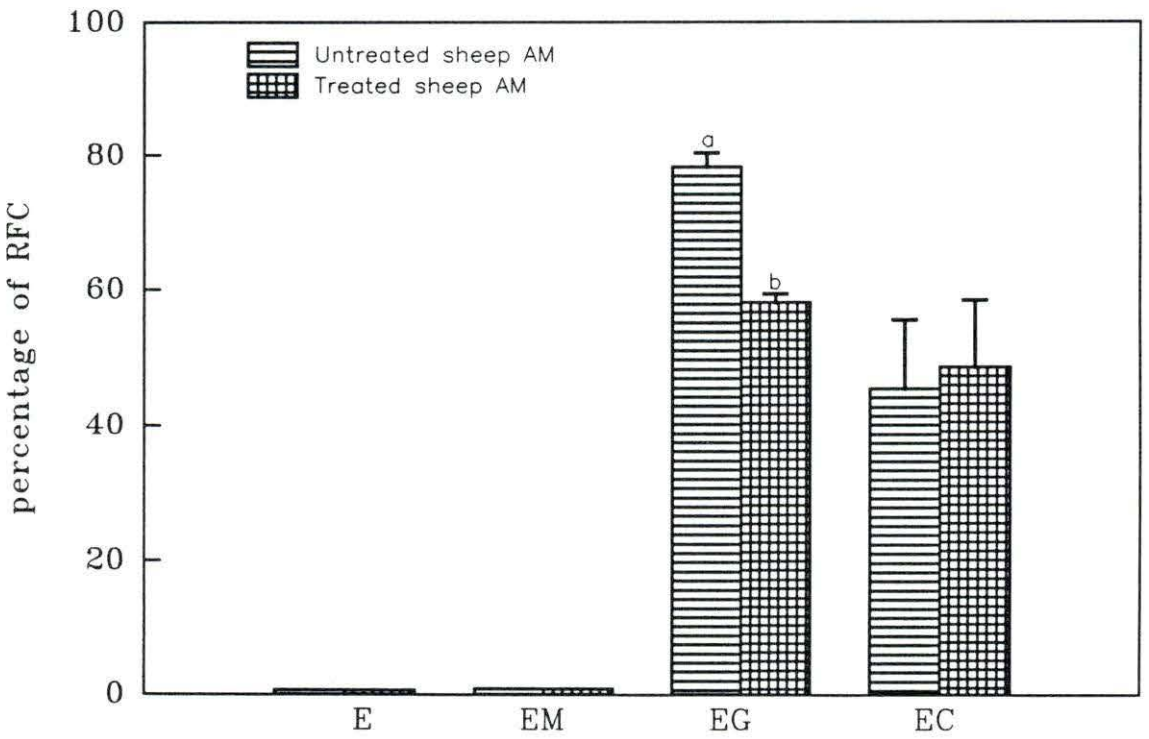

Figure 6. Percentages of sheep AM forming rosettes with various SRBC preparations. Sheep AM were either untreated or exposed to live M. ovipneumoniae. SRBC preparations were: E= nonsensitized, EM= sensitized with IgM, EG= sensitized with IgG, and EC= sensitized with complement. Values represent mean percentages (±SEM) of 10 separate experiments. The difference between a and b was significantly different ($p<0.05$).

DISCUSSION

The phagocytic effectiveness of phagocytic cells depends heavily on their membrane receptors which mediate both attachment and ingestion of foreign particles. Evidence is presented here to suggest that normal sheep AM express receptors for IgG and C but not for IgM. IgG receptors were present on about 78% of the normal sheep AM, while only 45% of these cells exhibited C receptors. This is quite similar to the IgG and C receptor percentages reported in equine (12), swine (17), and guinea pig (25) normal AM. However, in man and rabbits (36) the percentages of C receptors reported were 93% and 80%, respectively which contrast with our findings. The absence of AM forming rosettes with IgM-sensitized SRBC found in the present study has been previously reported in swine (17) and equine (12). Few reports have been concerned with surface receptors on AM from ruminants. McGuire and Babiuk (32) indicated that almost 100% of bovine AM that had been cultured in vitro for at least 48 hours expressed receptors for both IgG and C, but when examined earlier only 29.9% and 53% of these cells had IgG and C receptors, respectively. These authors suggested that the use of unfractionated rabbit antisera accounted for the low percentages seen in these early cultured cells, but as the cells matured in vitro, the expression and the number of cells displaying both Fc and C

receptors increased. Fleit et al. (14) used a fluorescence assay technique and reported that 62.7% of AM obtained from 5 day-old lambs had receptors for IgG, but they did not investigate the expression of C receptors. Our results indicate that 78% of normal AM obtained from adult sheep expressed IgG receptors. The technique employed and age-related differences may account for the difference between these two percentages.

The effect of M. ovipneumoniae on the expression of Fc and C receptors on normal sheep AM was investigated in the present study. Treatment of sheep AM with live mycoplasmas resulted in about a 20% decrease in sheep AM expressing IgG receptors, while the percentage of AM exhibiting C receptors remained almost constant, as compared with the normal sheep AM. The mechanisms involved and why C receptors were not affected remain undetermined. However, it is likely that this suppression of the IgG receptors by M. ovipneumoniae can explain, in part, the impaired ability of these sheep AM to ingest S. aureus or to lyse ^{51}Cr-labelled CRBC when pretreated with M. ovipneumoniae as demonstrated by the phagocytosis and cytotoxicity assays during the present study.

Exposure of normal sheep AM to M. ovipneumoniae in a low number of one AM to 10 mycoplasmas decreased up to one-third the bacterial uptake by these phagocytic cells. Decreased activity was also observed when sheep AM were exposed to heat-

killed M. ovipneumoniae but to a more limited degree as compared to live mycoplasmas. Also, a steady decline in S. aureus uptake by sheep AM was observed when these macrophages were pretreated with increasing concentrations of M. ovipneumoniae. This suggests a dose-related response. However, varying the time interval of sheep AM infection with M. ovipneumoniae did not affect the ingestion of S. aureus by sheep AM. An inhibitory effect in vitro of several mycoplasma species on the ability of phagocytic cells to cause phagocytosis of a second bacterial target has previously been reported (5,20,39,43). Similarly, certain microorganisms other than mycoplasmas have also been reported to interact with normal phagocytic cells in vitro, resulting in an impaired ability to phagocytose a second bacterial target (15,22,37).

The role of phagocytic cells in the host-defence mechanisms depends also on their ability to lyse cells infected with various microbes through antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity. Results of this experimentation indicated that normal sheep AM were quite effective in lysing ^{51}Cr-labelled CRBC (in the presence of specific antibody) as target cells. When treated with both live and heat-killed M. ovipneumoniae, a significant loss of ability of these sheep AM to lyse the target cells was observed. To our knowledge, no such experiment with mycoplasma species has been reported. However,

since mycoplasmas in general are known to induce alterations in phagocytic cell functions, it is not surprising that M. ovipneumoniae could inhibit sheep AM cytotoxic effect.

Little is known about the mechanisms that allow mycoplasmas to suppress phagocytic cell functions. However, evidence exists that the capsular material may exert an anti-phagocytic effect and also inhibit the ability of phagocytic cells to ingest and kill a second bacterial agent. In a recent study, Almeida et al. (5) demonstrated that encapsulated M. dispar or its purified capsule inhibited the phagocytosis of S. aureus or Serratia marcescens by bovine AM. Also, it is speculated that the ability of mycoplasmas to inhibit phagocytosis is due to their ability to bind firmly to the cell membranes of these phagocytes. Thus, this may mask or prevent stimulation of appropriate receptors (19). While it is not known whether or not M. ovipneumoniae possesses a capsule, electron microscopic studies by Al-Kassi and Alley (2) showed that, in the absence of specific antibodies, the organism remained attached to the surface of sheep AM without being phagocytosed.

The finding in the present study that M. ovipneumoniae decreases the number of IgG receptors on sheep AM may be correlated, at least in part, to the impaired ability of these macrophages to ingest S. aureus or to lyse CRBC as target cells. Several conditions have been shown to modulate Fc and C

receptor expression. Harmsen and Jeska (17) reported a 45% increase in C receptor expression in pigs experimentally infected with Toxoplasma gondii, while the percentage of AM exhibiting IgG receptors remained constant compared to the noninfected control pigs. However, they concluded that this augmentation of C receptors did not independently enhance the ability of these pig AM to endocytose SRBC. These results differ from those that we have found. Species variations or conditions of experimentation may explain these differences. Caruso and Ross (9) reported that the percentage of AM phagocytosing opsonized sheep RBC was not affecty by in vivo infection of pigs with M. hyopneumoniae alone but, was suppressed by a combined infection with M. hyopneumoniae and A. pleuropneumoniae.

Other studies have shown that prolonged exposure of guinea pigs to corticosteroids in vivo can cause alterations in membrane Fc receptor function of AM (24) and the loss of these Fc receptors caused a marked decrease in cytotoxic effector function of these macrophages (23). Similarly, administration of glucocorticoids decreased the number of IgG receptors on phagocytic cells from man (33). While our data seem consistent with these findings, the manner in which this inhibitory effect is exerted remains to be determined. Perhaps there is release of certain substances that might alter the phagocytic cells' surface membrane or metabolism. However,

even with such an hypothesis, why the C receptors were not also affected is puzzling.

In the lung, alveolar macrophages play an important role in bacterial clearance mainly by their phagocytic activities. Any alteration of this function may compromise the host pulmonary defence mechanisms against secondary bacterial agents. Results of the present study suggest that M. ovipneumoniae can induce alterations in sheep AM functions. These in vitro inhibitory effects exerted by M. ovipneumoniae on the ability of sheep AM to ingest a second bacterial agent, to lyse a target cell, and the diminished expression of IgG receptors on these macrophages define a potential role that this organism may play in sheep respiratory disease. However, the mechanisms involved, and possible components of the organism responsible for these inhibitory effects need to be investigated further.

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SUMMARY AND CONCLUSIONS

The main objective of the present study was to elucidate the negative effect that M. ovipneumoniae (isolated from a case of ovine pneumonia) exerts on some important functions of sheep AM as a potential mechanism by which this organism may initiate respiratory disease in sheep. The experimentation included the ability of M. ovipneumoniae to suppress the capability of sheep AM to ingest S. aureus, to mediate ADCC, and to express membrane surface receptors.

It was found that exposure of normal sheep AM to M. ovipneumoniae in a low number of as many as 10 mycoplasmas per macrophage decreased significantly the bacterial uptake and the cytotoxic activity of these macrophages. Also, it was found that normal sheep AM expressed receptors for IgG and C but not for IgM. Treatment of these macrophages with live M. ovipneumoniae resulted in about a 20% decrease in the number of sheep AM expressing IgG receptors. This suggests that the impaired ability of these sheep AM to ingest or to lyse a second target is due, in part, to the loss of Fc receptors on their surface membrane. However, the role of other mycoplasmal components, such as capsular material in preventing the uptake of a second bacterial target by phagocytic cells, should be investigated, as it has been demonstrated with M. dispar (11). Another possible mechanism is the binding process with the

phagocytic cell membrane that may mask the surface receptors, as has been speculated (72). Several species of mycoplasma, including M. ovipneumoniae have been shown to attach to the surface of phagocytic cells without being phagocytosed, in the absence of specific antibody (3,89,115). The possible mechanism that allows M. ovipneunmoniae to alter the expression of Fc receptors for IgG was not determined in the present study. Perhaps the organism is capable of releasing certain substances that might alter the surface membrane or metabolism of the phagocytic cell. The present in vitro findings provide a basis for further investigations towards understanding the role of M. ovipneumoniae in sheep respiratory disease.

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