Identification of the *Mycoplasma pulmonis* membrane-bound hemolysin

by

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EXPLANATION OF THESIS FORMAT

This thesis has been prepared in the alternative thesis format, and contains two separate manuscripts. Each paper is complete in itself, with an abstract, introduction, materials and methods, results, discussion, acknowledgements and literature cited. The first paper discusses the production of monoclonal antibodies using Mycoplasma pulmonis naturally infected animals. The second paper deals with the identification of the M. pulmonis hemolysin. A current review of relevant literature to this thesis is included in the introduction. A summary and discussion is included after the manuscripts.

GENERAL INTRODUCTION

Murine respiratory mycoplasmosis (MRM), a clinically silent disease indigenous to most conventional and barrier-maintained rodent colonies (25), results from a chronic infection of respiratory tissues by Mycoplasma pulmonis. The pathology of the disease has been well characterized (18,22), but mechanisms of virulence or virulence factors have not been identified. The goal of the research presented was to identify and partially characterize a membrane-bound hemolysin believed to be associated with pathogenesis.

To provide the reader with background material relevant to these studies, three distinct areas will be discussed. The first area will include general characteristics of mycoplasmas, a description of M. pulmonis and the pathogenesis of MRM. Next, membrane antigens and activities of other mycoplasma species will be discussed in hopes of facilitating present and future studies with M. pulmonis. The third discussion area will be a brief general overview of hemolysins from bacteria, including a description and potential mechanisms of action of the M. pulmonis hemolysin.

Mycoplasmas

General Characteristics

Mycoplasmas, which include approximately 50 different species, are the smallest and simplest self-replicating form of Eubacteria (66) and have been shown to colonize and infect animals, insects and plants. In animals, the resulting diseases are usually respiratory or urogenital in nature, but other areas of colonization have been well documented. The following discussion is based upon those species which have animal hosts, although other species have similar characteristics.

Mycoplasmas contain only a minimal set of organizational units essential for cell growth and replication: a plasma membrane to separate the cytoplasm from the external environment; ribosomes to assemble cell proteins; and a chromosome to provide the information for protein synthesis (66). The genome is typically 5×10^8 daltons in size, with a guanine:cytosine ratio in the range of 24-35 mole per cent (56,65). The coding capacity is believed to encode less than 700 different proteins, an insufficient number to supply all metabolic pathways needs (49,56). Thus, some macromolecule precursors must be provided by the environment, host tissues, or the growth medium. For instance, all mycoplasmas lack the orotic acid pathway for pyrimidine synthesis and the enzymatic pathways for de novo synthesis of purine bases (67). Pyrimidine and purine phosphorylases are present however, allowing conversion of nucleotide phosphates to the corresponding free base (66). Nucleotide phosphates or host DNA may be provided by the action of a membrane-associated nuclease (51). Once obtained, the nucleotide bases are readily incorporated into the genome.

In addition, mycoplasmas require exogenous phospholipids and cholesterol for plasma membrane synthesis. These organisms are either partially or totally incapable of synthesizing long chain fatty acids (73). This results in an inability to regulate membrane fluidity by preferential fatty acid synthesis or incorporation, the mechanism used by most bacteria. In mycoplasmas, membrane fluidity is influenced by the composition of the incorporated fatty acids (73) and is maintained and regulated by the incorporation of cholesterol (66). The cholesterol usually remains unaltered once incorporated.

Mycoplasmas contain a truncated respiratory system, located in the cytoplasmic fraction of the cell (66), and are unable to produce ATP via oxidative phosphorylation. They lack quinones and cytochromes, components of the electron transport system (ETS), leaving only two proven means of ATP production. In fermentative mycoplasmas, ATP is formed during glycolysis, whereas in nonfermentative mycoplasmas, the arginine dihydrolase pathway has been proposed as the major source of ATP (72).

In general, mycoplasma replication and protein synthesis resembles typical prokaryotic mechanisms. The mycoplasma DNA polymerase possesses the catalytic properties found in procaryotic DNA polymerase I, but lacks all exo- and endo-DNase activities (56). In addition, the ribosomes from mycoplasmas have sedimentation coefficients, RNA/proteins ratios and ribosomal RNA species similar to other prokaryotes. The major difference is that the rRNA species have a much lower quanine:cytosine content (43-48%) (65). The transfer RNAs, like the ribosomal RNAs, also resemble the corresponding transfer RNAs from other prokaryotes. The mycoplasmas, however, appear to contain fewer isoaccepting transfer RNA species and less modified nucleotides, probably reflecting the limited biosynthetic ability imposed by the small genome size (65).

Mycoplasma pulmonis

M. pulmonis colonizes mice and rats, and has been reported to be found in most, if not all, rodent colonies in the United States (25). It has the general characteristics and nutrient requirements as described above. There have been five membrane-associated activities identified in vitro: lymphocyte mitogenesis, hemadsorption, hemagglutination, hemolysis and a nuclease activity (36,51-54). Of these, hemadsorption

and hydrogen peroxide-mediated hemolysis appear to have no role in the virulence of M. pulmonis (33b). The others have not been characterized as to their role in pathogenesis, although it has been postulated that they may be involved (51,52).

Pathogenesis of Murine Respiratory Mycoplasmosis (MRM)

MRM has been extensively reviewed in recent years (18,20,22, 23,26,27). This disease is often slow in onset and of relatively long duration. Several weeks or months are usually required for visible symptoms to develop. Offspring of infected mothers may acquire M. pulmonis in utero (18) or by aerosol transmission in the first few weeks of life (18). Once infected, the animals serve as reservoirs of infection, transmitting the organism to cagemates and to adjacent cages (9).

After transmission, M. pulmonis quickly overcomes the defense mechanisms of the host and tightly adheres to the surface of epithelial cells, particularly in the nasal passages and middle ear (18). The tight adherence to epithelial cells, often in a continuous blanket 3-5 organisms thick (62), is mediated by a generalized interaction of the mycoplasmal membrane with the host cell membrane. Once attached, M. pulmonis directly injures the epithelial layers and stimulates an immune response. Common types of pathology are ciliostasis, loss of cilia, distention of intracellular spaces, cytoplasmic vacuolization, disruption of mitochondria, epithelial hyperplasia, metaplasia and syncytial giant cell formation (18). These changes are most likely due to the mycoplasmas utilizing host cellular components and the release of toxic metabolic wastes. Indirect injury also occurs to the cells through prolonged chemotaxis of neutrophils, the release of lymphokines, and the

release of large amounts of hydrolytic enzymes from mouse macrophages (18).

The principal lesions resulting from direct and indirect cellular injury are acute and chronic rhinitis, otitis media, laryngotracheitis and bronchopneumonia, all of which are characterized microscopically by lymphoid hyperplasia, neutrophilic exudate and chronic inflammation (22). These observations suggest that the lesions are partially the result of ineffectual immune response in the host. In advanced cases of the disease, entire lung lobes can be afflicted with lesions.

Even with severe lung epithelial injury, the disease often remains clinically silent until the terminal stages of the disease (48a). Most common clinical manifestations are snuffling and rales, which can be heard better than seen. Other symptoms are frequent rubbing of the eyes and, less frequently, head tilt due to labyrinthitis. Terminal stages of the disease often demonstrate weight loss, roughened hair coat, nasal and ocular discharges and dyspnea (48a). Once clinical signs appear, the disease may seem to spread through the colony rapidly (62).

One of the most noteworthy features of the disease is the lack of uniformity between the lesions in the lungs and the severity of the infection, even in animals from the same cages (18). This has been attributed to environmental and genetic influences. In studies with rat colonies, it was demonstrated that the prevalence of pneumonia increased directly with the environmental concentration of ammonia (16), and that the levels of vitamins A and E (18) influenced the disease. In addition, prior infections with Sendai virus enhanced the growth of M. pulmonis and the severity of lung lesions in mice (18). The disease seems to progress more rapidly in older animals (44). Comparison of animals matched for

age, sex and microbial and environmental factors, indicates that heredity is probably one of the most critical determinant for susceptibility to the disease (18).

The prevalence of MRM in rodent colonies is puzzling in view of the fact that rodents respond immunologically, both locally and systemically, to infection with M. pulmonis. In the local response, the submucosa of the respiratory tract and the lung parenchyma are rapidly infiltrated by lymphoid cells which differentiate into plasma cells secreting all of the major classes of antibodies (22). T lymphocytes are essential for this response and may limit dissemination of the mycoplasmas (22). Systemically, spleen cells are activated and antibodies are released into the blood system (18). In many cases, the immune response generated in one animal is able to protect an immunized, uninfected animal from severe disease, but not colonization, during a challenge infection. The mechanism of protection differs though between rats and mice. Mice are sufficiently protected with immune serum (75), whereas rats require immune spleen cells (13).

Survival of M. pulmonis in the presence of a strong immune response has several explanations. The intimate association of M. pulmonis with the host cell surface and the ability of mycoplasmas to bind exogenous proteins readily (34,68) suggests that the organisms might avoid the immune response by acquiring host antigens thereby mimicking "self." Alternative explanations include the release of soluble antigens with the formation of immune complexes which might block specific cellular or humoral effector mechanisms, alteration of lymphocyte responsiveness through interactions with macrophages, and the induction of T or B lymphocyte tolerance (18). In conclusion, the prolonged survival of M.

pulmonis and the continued activation of the immune response may be responsible for all of the observed pathologic phenomena in the respiratory disease.

In addition to the chronic respiratory disease, M. pulmonis is capable of colonizing the urogenital tract of infected rats (28a). Naturally occurring disease in mice has not been reported but has been produced experimentally (26). The exact relationship of genital infections to respiratory mycoplasmosis is unknown as each has been detected in the absence of the other. Urogenital transmission of the organism may be via a hematogenous route, as M. pulmonis has been shown to invade the blood system (18), or, since rats are copraphagic, by females harboring the organism in the upper respiratory passages transferring the infection to the vagina. Like the respiratory disease, the genital disease progresses slowly, subtle host-parasite interactions predispose to disease, there is an inconsistency in the lesions produced, and genetic factors influence severity (26).

The most common lesions observed in the urogenital tract are perioophoritis and salpingitis (26), which are limited to approximately 30-40% of infected females. Colonization of M. pulmonis occurs on both squamous and nonsquamous epithelial cells. Epithelial changes include hyperplasia, squamous metaplasia and polyp formation. The fallopian tubes and ovaries may also become distended with the occurrence of neutrophilic exudate, hyperplasia and lymphoid infiltration (26). Usually, the uteri appear normal with organisms lining the entire epithelial surface, but occasionally an underdeveloped or partially resorbed fetus can be seen (26). No gross lesions have been observed in the vagina and cervix (18).

In contrast to the percentage of infected females, only 5% of males bred with infected females contracted the infection. In males the organisms colonized the urethra, vas deference and epididymis, and were found in association with chronic inflammation (24).

The urogenital disease results in reduced fertility in 50% of infected animals, which ranges from complete infertility to a 66% fetal loss after implantation (26,35). When pregnancy does occur, the organism is readily transmitted to offspring. Thus, there is a continuous cycle of MRM in all breeding situations.

Efforts to eliminate the disease have involved rigid selection of animals, coupled with administration of antibacterial drugs, or principles of cesarean derivation combined with strict isolation procedures (26,80). As previously mentioned, immunization of mice and rats has been demonstrated, but only in noninfected animals. In those cases, colonization by M. pulmonis was not prevented. These approaches give only temporary eradication of M. pulmonis from rodent colonies.

With the prevalence of MRM and the absence of effective eradication methods, the usefulness of rodents for research purposes may be seriously questioned. This problem is enhanced by the variation in severity of M. pulmonis infection (latency to lethality). Lindsey et al. (48a) and others (22,47) have documented a large number of specific examples where MRM has complicated studies in the areas of respiratory disease, gerontological, nutritional, toxicological and behavior research. In addition, MRM affects many acute studies such as reproductive physiology and immunological studies. These complications have lead to MRM being declared as the number one problem in long term studies (80).

Mycoplasmal Membrane Antigens and Activities

In an effort to define mycoplasma pathogenesis and to produce effective methods of eradication, surface molecules from many mycoplasma species have been identified. These molecules may or may not be involved in virulence but, because they are immunogenic, they may have a potential role in pathogenesis. A few of these molecules have been characterized and at least one is involved in pathogenesis, the P1 attachment protein of M. pneumoniae. Other molecules have been suggested as potential virulence factors based upon in vivo or in vitro biological activities. This review will divide these molecules into three categories: membrane antigens, adherence factors, and membrane activities and potential toxins.

Membrane Antigens

Most mycoplasmal membrane antigens described in the literature were identified using antisera from naturally infected or experimentally immunized animals. Occasionally, the antisera against one mycoplasma species was used to identify cross-reactive surface proteins in another mycoplasma species. A summary of current information is presented in Table 1. The few proteins with characterized activities are noted.

Adherence Factors

In previous years, a large number of studies have been conducted on the identification and characterization of adherence factors and their receptors. Although not directly responsible for disease, adherence is necessary prior to colonization in the animal. Many adherence factors and proteins involved in hemagglutination have been postulated or identified. Razin (64) summarized previous information in 1985. Table 2 is taken from that review, with the addition of current information

Table 1. Mycoplasma membrane antigens

Mycoplasma species	Strain[a]	Molecular[b] weight	Charac-[c] teristics
M. pneumoniae	multiple	170,130,90, 45,35	Stable, 10 years (77)
	M129	165-190	P1 protein involved in cytadherence, C.R. with 140 kDa of M. genitalium (7)
	M129	78	P2 protein, cytadherence (41)
	M129	32	cytadherence(?) (37)
M. genitalium	G-37	110	C.R. with M. pneumoniae (28b)
	G-37,M30	140	MgPa, possibly involved in cytadherence, C.R. with P1 protein of M. pneumoniae (7,40,57a)
M. pulmonis	5782	95,87,79,72	recognized by naturally-infected antisera to M. arthritidis (55a)
	Multiple	150-20, ladder	V-1 antigen comlex, single units of 30 kDa hydrophobic protein forms multiple units, acidic, variable among strains (38,78,79)
	63,47 Negroni 19612	86.5,83.5	heat stable, 0.32 electrophoretic mobility (61)
	63	40,37,30	" "

[a]UK = unknown.

[b]Molecular weight in kilodaltons.

[c]C.R. = cross-reactive.

Table 1 (continued)

Mycoplasma species	Strain	Molecular weight	Characteristics
M. pulmonis	19612	160,59	heat stable, 0.32 electrophoretic mobility (61)
	Negroni	125,28.5,27	" "
M. arthritidis	14124 P10	13-15	Mitogen, secreted or excreted, basic, pI>9, affinity for high high molecular compounds (5)
M. gallicep-ticum	S6 C4	64	hemagglutination (14)
	UK	110	C.R. to M. synoviae (63)
	PG-34	165	C.R. to P1 protein of M. pneumoniae (28b)
M. synoviae	UK	45-50	C. R. to M. gallicepticum (63)
M. bovoculi	UK	94	C.R. to M. dispar and M. arginini (70)
M. dispar	UK	62	C. R. to M. bovoculi (70)
M. arginini	UK	89,85,74	C. R. to M. bovoculi (70)
	G230,23242	74	heat stable (1)
	G230,23243	70	
	G230	44,17	strain specific (1)
	G230	40,29,20	recognized by antisera to a common surface antigen common to most strains (1)
M. hominis	PG-21	102-38 12 antigens	102,87,78,72 recognized by infected human patients, 102 present in 14 strains (21)

Table 1 (continued)

Mycoplasma species	Strain	Molecular weight	Characteristics
M. hominis	UK	77	prominent surface antigen (45)
M. hyorhinis	GDL	23	surface exposed, trypsin sensitive (69)
	GDL	17	trypsin insensitive (69)
	GDL	18	membrane bound portion after trypsin treatment of P23 (69)
	GDL	65,60,44	Lipid modified (15)
	GDL	51,38,46,70	
	GDL	120	surface exposed (13)
M. hyopneumoniae	27719	90,50	surface exposed, trypsin sensitive (45)
	27719	30	trypsin insensitive (45)
	27719	68	surface exposed, trypsin sensitive, related to P26 (45)
	27719	26	trypsin insensitive, related to P68 (45)
	25934	70,50,44	lipid modified (80)
	27719	90,68,50	lipid modified (80)
	25934	65	antisera to protein recognized by multiple species of mycoplasmas (45)
	UK	64	hemagglutination, trypsin insensitive, surface exposed (81)
	25934	41	hydrophilic (45)

Table 2. Adherence and hemagglutination factors

Mycoplasma species	Receptor	Adhesins[a,b] in kDa	HA in[b,c] kDa	Att.[b] org.
M. pneumoniae	glycophorin, sialic acid	P1 (185) P2 (78) ? 32	P1 (185)	+
M. gallicepticum	sailoglycoconjugates, glycophorin	ND	64	+
M. genitalium	sialoglycoconjugates	MgPa (140)		+
M. synoviae	sialoglycoconjugates	ND		-
M. pulmonis	I: trypsin resistant II: trypsin sensitive	ND, trypsin resistant protein(s), trypsin sensitive		-
M. dispar	ND, resistant to neuraminidase, proteases, periodate	protein(s), sensitive to proteases		-
M. hyorhinis	possibly Thy-I, other glycoproteins of lymphocytes	ND		-
M. hominis	proteins, resistent to neuraminidase, inactivated by proteases and formalin	proteins, sensitive to proteases and formalin		-

[a]ND = not determined.

[b]kDa = kilodalton.

[c]HA = hemagglutinin.

[d]Att. org. = Attachment organelle.

Table 2 (continued)

Mycoplasma species	Receptor	Adhesins in kDa	HA in kDa	Att. org.
M. salivarum	similar to *M. hominis*, more readily inactivated by heat and periodate	protein(s), sensitive to proteases, formalin		-
M. hyopneumoniae	ND	ND	64, trypsin sensitive	-

(14,40,54,81). The hemagglutinating proteins and adherence factors have been shown in some cases to be identical, but in others, the relationship between hemagglutination and adherence activities has not been determined.

The P1 protein, the major adherence protein of M. pneumoniae, is the most well-studied mycoplasmal adherence molecule (33a). P1 protein is normally concentrated on the tip of the flask shaped organism, called the attachment tip, but is also found throughout the membrane. This allows M. pneumoniae to have multiple binding sites with the host cell through sialoglycoproteins and glycolipids (41) and is probably responsible for its hemagglutinating activity. Viability is not required for this adherence. Other proteins beside P1 may be involved in adherence, including a 32 kDa protein (37,57b). In addition, monoclonal antibodies (Mab) and monospecific antisera to P1 have been used to identify cross-reactive molecules in M. genitalium and M. gallicepticum (28b), the only other mycoplasma species containing tip structures. In M. genitalium, a 140 kDa protein demonstrated a high degree of cross-reactivity to P1 and this protein also appears to be localized on the tip structure (40,57a). It has not been conclusively shown whether this protein is responsible for adherence, although it is highly suggested.

Attachment mechanisms of mycoplasma species which do not contain attachment tips (all species other than M. pneumoniae, M. gallicepticum, and M. genitalium), have been studied less extensively. M. pulmonis seems to adhere to host cells via a multifactorial process. The proposed model of adherence involves an initial recognition event, the exposure of additional binding sites by the induction of membrane protein diffusion

on the eukaryotic cell surface, and the recognition and binding to these additional receptors by the mycoplasmas (54).

Membrane Activities and Potential Toxins

Other than adhesions, mycoplasmas contain many membrane-bound activities involved in cell maintenance. These activities may have toxic properties under certain conditions. This area has been extensively reviewed by Gabridge (36). Tables III and IV are taken from this review with the addition of current data (2,17,51,54,60,70,75). Most of these molecules have not been described in detail. The exception is the M. arthritidis mitogen (MAM).

The 15 kDa heat-stable mitogen of M. arthritidis is the only identified mitogen in mycoplasmas, although several other species have membrane-associated mitogenic activities (Table III). M. arthritidis is also unique in that the mitogen is either excreted or released from the membrane. M. arthritidis mitogen (MAM) strongly activates H-2^k bearing T lymphocytes and is dependent upon Ia-bearing, radioresistant adherent accessory cells bearing the alpha chain of the I-E encoded molecule of the murine major histocompatibility complex (MHC) (5,29-31). The alpha chain of the I-E molecule appears to be the receptor for MAM (8). Additionally, a non MHC-encoded gene is required for T lymphocytes to be responsive to MAM (29). Processing of the mitogen by the accessory cells is not essential for activation. The MAM also stimulates both lymphocyte proliferation and the production of interferon. In summary, activation by MAM requires three different genes and the T cell receptor does not appear to be involved (8).

A second activity that may be involved in pathogenesis is the membrane-bound hemolysin from M. pulmonis. In all cases except M.

Table 3. Biological activities of nonviable mycoplasma lysates (LY) and membrane (MB) preparations.

Mycoplasma species	Prep.[a]	Effect
M. mycoides	MB	Casein cleavage
	LY	Synovitis in calves
M. fermentans	MB	Lethality in mice Cytotoxicity in thymocytes
	LY	Mitogenic for mouse lymphocytes and human B and T lymphocytes
M. pneumoniae	MB	Ciliostasis, necrosis, decreased ATP content and decreased metabolism in tracheal explants, nonspecific attachment to tracheal cells, catalase inhibition
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		- [illegible] guinea pig lymphocytes, mitogenic for mouse lymphocytes
M. arthritidis	MB	Immunosuppression in rabbits, suppression of interferon production in mice
	LY	Inflammation in rabbit synovium, cytotoxicity, immunosuppression, inhibit mitosis, inhibit antibody production, suppression of interferon in mice, joint inflammation in sensitized rabbits
M. synoviae	LY	Mitogenic for mouse lymphocytes
M. gallicepticum	MB	Binding to specific receptors on erythrocytes
	LY	Mitogenic for mouse lymphocytes, binding to specific receptors on erythrocytes

[a]Preparation.

Table 3 (continued)

Mycoplasma species	Prep.	Effect
M. neurolyticum	MB	Mitogenic for rat B lymphocytes
M. hyopneumoniae	MB	Cytotoxicity in fibroblasts
M. hyorhinis	MB	Mitogenic
M. pulmonis	MB	Mitogenic for rat B and T lymphocytes, suppression of interferon production in mice, pneumoniae and tracheitis in rats, hemagglutination and hemolysis of erythrocytes, nuclease activity
	LY	Inflammation in rabbit synovium, suppression of interferon production in mice

Table 4. Potential toxins of Mycoplasmas

Mycoplasma species	Product[a]	Biological Effect
M. gallicepticum	Putative	CNS inflammation, polyarteritis, toxicity in turkeys
M. orale	Arginine deiminase	Lysis of L5178Y mouse lymphoma cells
M. fermentans	Heat stable, trypsin sensitive lipid/protein complex	Lethal for mice
M. mycoides	Galactan	Inflammation and edema in cattle
	Diffusable toxin	Stimulates connective tissue proliferation
	Phenol extract, lipoglycans	pyrogenic, leukopenia
M. pneumoniae	Aggregates of membranes, sensitive to sonication	Cytotoxicity and ciliostasis in hamster tracheal explants
	NaCl extract, >20 proteins plus carbohydrates	Ciliostatic; hemagglutinating, proteolytic; attachment inhibition
M. pulmonis	Protein from membrane, protease sensitive, heat-labile	Mitogenic for rat B and T lymphocytes
	Protein from membrane, protease sensitive	Hemagglutinating
	" "	Hemolytic activity

[a]kDa = kilodalton.

Table 4 (continued)

Mycoplasma species	Product	Biological Effect
M. bovis	Ethanol extract polysaccharide, heat stable, not a protein or lipid, 73 kDA	Eosinophilic mastitis in cattle
M. neurolyticum	Thermolabile protein, >200 kDa	Neurologic syndrome and death in mice
	Lipoglycans	Mitogenic for T and B lymphocytes, hemagglutinating
	Proteins, heat-labile, sensitive to oxidation, periodate	Mitogenic
M. arthritidis	13-15 kDa protein nondialyzable	Mitogenic for rat lymphocytes

pulmonis, mycoplasma-associated hemolytic activities have been attributed to hydrogen peroxide production and are unrelated to pathogenesis or virulence, although cellular injury does occur (33b,36). In M. pulmonis, two forms of hemolytic activity have been documented; hemolysis due to hydrogen peroxide and hemolysis caused by a membrane protein. Membrane-associated hemolysis is dependent upon a helper factor (i.e., bovine serum albumin), temperature, and is more effective against trypsin-treated red blood cells (52,53). There is evidence that this activity may play a role in the acquisition of cholesterol and phospholipids from the membranes of red blood cells (50). The studies reported here show that the hemolysin is an 88.5 kDa protein that remains bound to the membrane both in viable cells and membrane fragments.

Microbial Hemolysin

Bacterial hemolysins vary in size and mechanisms of action, with most being secreted. The extracellular hemolysins with known mechanisms of action can be divided into three categories: 1. thiol-activated and pore-forming hemolysins; 2. phospholipases; and 3. surface active toxins. Several other extracellular hemolysins have been identified, but their mechanisms of action are unknown. These are not included in this discussion. Finally, two membrane-bound hemolysins have been identified and are discussed in the last section.

Thiol-Activated and Pore-forming Hemolysins

The thiol-activated or oxygen-labile hemolysins are produced by gram-positive bacteria. Best studied of these molecules are the streptolysin O (Streptococcus pyrogenes), theta toxin (Clostridium perfringes), cerolysin (Bacillus cereus), pneumolysin (Streptococcus pneumoniae) and tetanolysin (Clostridium tetani) (10). These hemolysins

share several properties. They are lethal and cardiotoxic, are inactivated under reducing conditions or by small amounts of cholesterol or other related sterols, and have similar pH and temperature optima for hemolytic activity (10,11). Most of them have a molecular weight between 45-75 kDa (10). The initial step of lysis with these hemolysins is the binding to cholesterol, the membrane receptor. Lysis occurs either by the formation of hemolysin-cholesterol rings accompanied by a lipid phase transition, or by the formation of lysin pores (4).

Besides the thiol-activated hemolysins, other extracellular products have been shown to cause pores in cell membranes. The best studied of these is the Staphylococcal alpha toxin (4). This toxin causes lysis by inserting itself into the membrane and forming hexamers, resulting in a pore. The mechanism of lysis has been termed "colloid-osmotic." Aerolysin from Aeromonas hydrophila has also been shown to function by this mechanism (10).

Phospholipases

Several different types of phospholipases have been identified in bacteria (10,55b). Phospholipase A is usually cell bound, and is nonhemolytic (55b), as is phospholipase D (55b). Phospholipase C is the most common bacterial phospholipase and all are extracellular in nature. This enzyme is produced by both gram-positive and gram-negative species of bacteria, including Acinebacter, Pseudomonas, Staphylococcus and Clostridium (55b). Hemolysis is induced by cleavage of membrane phospholipids resulting in disruption of the normal membrane bilayer structure. The specificities of the bacterial phospholipases have been described in detail (4,6).

Surface Active Toxins

Surface active toxins are a group of small peptides capable of producing cell lysis either by forming aqueous channels which span the membrane or by increasing membrane permeability through the formation of other structures (4). These structural formations depend directly on the composition of the toxic molecules and its conformation in the membrane. Staphylococcal delta toxin is the best known bacterial toxin in this category (4,10). Most of the other toxins are found in snake venom and from insects.

Membrane-bound Hemolysins

Most bacterial hemolysins are excreted or secreted, as was noted above. In contrast to these are the membrane-bound hemolysins of Serratia species (46) and M. pulmonis (52). A third hemolysin which remains associated with the membrane is streptolysin S (32). In the latter case, the 32 amino acid peptide can be released from the membrane by exposure to a carrier molecule, such as RNA.

The hemolysins from both Serratia and M. pulmonis remain bound and active in the cell membrane (46,52), but the mechanisms of action have not been determined. Hemolysis caused by Serratia requires viable organisms and the activity stimulates the release of histamine and the generation of leukotrienes. In contrast to Serratia, M. pulmonis hemolysin does not require viable organisms since membrane fragments retain hemolytic activity (this study). The requirements for M. pulmonis membrane-bound hemolysis also differ: the presence of serum or helper factors, trypsin-treated red blood cells, and 37°C (52). The hemolysin is thought to be involved in cholesterol and phospholipid uptake from red

blood cells (50). Both Serratia hemolysins and the hemolysin from M. pulmonis are thought to be involved in pathogenesis (46,52).

The characteristics and requirements of the M. pulmonis hemolysin indicate that the molecule is unique among bacterial hemolysins. It is unlikely to be a pore-forming hemolysin or a surface active toxin because it is not released from the membrane. An understanding of the mechanism involved may depend upon the identification and purification of the protein. The studies reported here identify the membrane protein involved in hemolysis and develop the reagents needed for future studies.

In summary, M. pulmonis is a surface parasite of rodents and is dependent upon nutrients derived from the host for survival. One of the major requirements is cholesterol, which maintains membrane fluidity in the absence of a cell wall. The role of the hemolysin in cholesterol uptake has been suggested (50), but additional studies need to be performed before the mechanism of action and the role of the hemolysin in pathogenesis can be determined.

SECTION I. PRODUCTION OF RAT ANTI-MYCOPLASMA MONOCLONAL ANTIBODIES FOLLOWING IN VITRO STIMULATION OF SPLEEN CELLS FROM NATURALLY INFECTED ANIMALS

Production of Rat Anti-Mycoplasma Monoclonal Antibodies Following In Vitro Stimulation of Spleen Cells from Naturally-Infected Animals

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ABSTRACT

Spleen cells derived from rats naturally infected with Mycoplasma pulmonis were stimulated in vitro, and then fused with a mouse myeloma cell line. The resulting hybridoma supernatants were screened for mycoplasma-specific Mabs by ELISA and for HL-blocking activities. Fusions performed with in vitro stimulated SPCs yielded larger numbers of growth-positive wells and antibody-secreting cells than unstimulated SPCs from the same animals. Mabs capable of inhibiting M. pulmonis HL activity were obtained, indicating that B cell priming during mucosal infections can provide SPCs suitable for hybridoma production.

Key Words

Rat monoclonal antibodies, naturally infected rats, in vitro immunization, mycoplasma

Abbreviations

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HL, hemolysis; HS, horse serum; LPS, lipopolysaccharide; Mabs, monoclonal antibodies; MRBC, mouse red blood cells; PBS, phosphate buffered saline (pH 7.5); PBSG, PBS-0.5% glucose; PBST, PBS-0.05% Tween 20; SPCs, spleen cells.

INTRODUCTION

Despite the continued efforts of many researchers, several factors hinder the identification of antigens contributing to the pathogenicity of microorganisms. For example, pathogens often express virulence determinants only in vivo or at greatly reduced levels in vitro. Also, epitopes may be lost during Western blotting and thus are not recognized by convalescent antisera. Finally, bacterial products may be immunosuppressive, preventing humoral immune responses against key virulence determinants.

The identification of virulence-related proteins using Mabs derived from immunized animals has become routine. Recently, Takahashi et al. (11) described an in vitro immunization technique using purified antigens to obtain specific Mabs. An adaption, discussed in this paper, takes advantage of the SPC priming which occurs during a natural infection. SPCs primed systemically by a natural infection were further stimulated in vitro by a modification of the method of Borrebaeck and Moller (2). In vitro immunization has several advantages; the immunization period is shortened (3 days), smaller quantities of antigen are required (0.1-10 ug), Mabs can be produced against antigens or epitopes that are weakly immunogenic (11), and the primed B cells may be removed from a potentially inhibiting environment. Additionally, our modification does not require the immunization of infected animals, which may result in death or potentiation of adverse reactions such as immunosuppression (1) or tolerance.

Mycoplasma pulmonis colonizes the respiratory and genital tracts of rodents, inducing local and systemic immune responses (3). Minion and Goguen (7) identified a membrane-bound hemolytic activity which may have

a role in cholesterol acquisition (6) and may be virulence-related. In the studies reported here, hemolysin blocking antibodies were found to be present in sera from infected animals, but absent in hyperimmune antisera. In order to identify this protein, a series of hybridoma fusions were performed using SPCs from naturally infected rats. The SPCs were either used directly or stimulated in vitro with mycoplasma antigen and adjuvant. Fusions incorporating in vitro treated SPCs demonstrated higher percentages of growth-positive wells and antibody-secreting hybridomas than fusions using untreated SPCs. The resulting Mabs were screened for their ability to block hemolytic activity, and are currently being used to identify the hemolysin.

MATERIALS AND METHODS

Mycoplasma Cultures

M. pulmonis strains UAB6510 (9) and ISM3001 were cultured in PPLO broth or on PPLO agar media supplemented with 10% gamma globulin-free horse serum (Gibco Laboratories, Grand Island, NY). Overnight mycoplasma cultures were harvested in mid-log phase (pH 7.2-7.4). Organisms were pelleted, washed, resuspended in PBSG, and protein concentrations of cell suspensions determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Cal.) with BSA as the standard according to manufacturer's directions.

Animals

Sprague-Dawley rats (SASCO, Inc., Omaha, Neb.) and BALB/cByJ mice (Jackson Laboratories, Bar Harbor, Ma.) were housed at the Laboratory Animal Resource Division of the College of Veterinary Medicine, Iowa State University.

Antigen Preparation

Antigens used for mouse immunizations and for the rat in vitro stimulation assays consisted of UAB6510 whole cells suspended in H_2O and frozen at -70°C. BALB/cByJ mice were intraperitoneally immunized with 50 ug antigen in incomplete Freund's adjuvant intraperitoneally and boosted with an identical dose 14 days later. Rabbits were immunized with whole cell lysates or purified membranes as previously described (9).

Experimental Mouse Infections

BALB/cByJ mice were infected with 50 ul of mid-log culture of UAB6510 intranasally. One week post infection, blood was obtained by retino-orbital puncture, and sera were tested by ELISA. Two weeks later,

animals were anesthetized with methoxyflurane (Pitman-Moore Inc., Washington Crossing, NJ) and blood obtained by cardiac puncture.

Monoclonal Antibody Production

Rat SPCs and mouse myeloma P3X63-Ag8.653 cells were mixed together at a 5:1 ratio, fused with polyethylene glycol 1500 (Boehringer Mannheim, West Germany) and plated at a cell density of 5×10^5 cells/well in 24 well plates (Corning 25820). Selective medium consisted of RPMI 1640 medium (Gibco Laboratories, Grand Island, N.Y.) containing 15% heat-inactivated FCS, 10% conditioned medium, 1.2% MEM Non Essential Amino Acids (Gibco), 0.3 g/L L-glutamine, and hypoxanthine-aminopterin -thymidine media supplement (Sigma Chemical Co., St. Louis, Mo.). Conditioned medium was prepared by suspending BALB/cByJ mouse SPCs (1×10^6 cells/ml) in RPMI 1640 medium containing 10% FCS and L-glutamine for three days. The resulting supernate was collected, filter-sterilized, and stored at -20°C until use. Hybridomas were screened for mycoplasma-specific antibody production by ELISA (see below). Subcloning was performed using the Autocloner accessory on an EPICS VII fluorescence activated cell sorter (EPICS model 752). All hybridomas and subclones were frozen in medium containing 15% dimethylsulfoxide in liquid nitrogen and supernates from confluent cell cultures were stored at -70°C.

In Vitro Stimulation

Rat SPCs were obtained from naturally-infected animals and suspended at a concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 15% heat-inactivated FCS, 10% conditioned medium, 1.2% MEM Non Essential Amino Acids (Gibco), 0.3 g/L L-glutamine and 5×10^{-5} M 2-mercaptoethanol (tissue culture grade). Cells were plated into 24 well plates (Corning 25820, 0.5 ml/well), and the following were added to each well: 10 ug

indomethicine, 10 ug of sterile UAB6510 whole cell antigen and 5 ug E. coli K235 LPS. The plates were rocked for three days at 37°C in an atmosphere of 83% N_2 - 7% O_2 - 10% CO_2, and fed daily with the above medium. After incubation, the cells were collected and fused with the mouse myeloma line as described above.

ELISA

ELISA plates were prepared by coating 96 well serocluster "U" vinyl plates (Costar 2797) with 100 ul of mycoplasma antigen (5 ug/ml) suspended in coating buffer (0.1 M sodium carbonate - 0.02% sodium azide, pH 9.6) for 5 h at 37°C or overnight at room temperature. Plates were washed three times with wash solution (0.9% NaCl - 0.05% Tween 20), incubating 5 minutes at room temperature between washes, and stored at -70°C until use.

Thawed ELISA plates were washed twice with wash solution, and blocked with PBS - 0.5% BSA for 30 minutes at room temperature. The plates were then washed three times with wash solution, and undiluted hybridoma supernate (100 ul/well) or polyclonal sera (100 ul/ml, 1:100 dilution in PBST) was added to the wells, and the plate incubated overnight at room temperature. The plates were again washed three times prior to the addition of rabbit anti-rat IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md) or goat anti-mouse IgG (BioRad Laboratories, Richmond, Ca) horseradish peroxidase conjugate (100 ul/well, 1:2,000 dilution in PBST). The plates were incubated for 1 h at 37°C, washed four times, and 3,3',5,5'-tetramethylbenzidine (Kirkegaard and Perry Laboratories, Inc.) was used as substrate according to manufacturer's directions. ELISA plates were read on a Bio-Tek Automated Microplate Reader (Model EL310).

Hemolysis Assay

The standard HL assay mixture consisted of assay medium (RPMI 1640, 20 mM HEPES, 1% BSA, pH. 7.5), 0.5% trypsin-treated MRBCs (final concentration) prepared according to Minion and Goguen (7), and freshly prepared UAB6510 in a total volume of 1 ml. This mixture was rocked slowly for 1.5 h at 37°C and lysis determined by measuring optical density at 630 nm. The minimal amount of mycoplasma protein needed to lyse the MRBCs was determined for each mycoplasma culture to adjust for day to day variation. The HL inhibition assay was performed by statically treating this concentration of mycoplasmas with antibody for 1 h 37°C prior to the addition of MRBCs. Sera from immunized or infected animals were diluted 1:25 in assay media. Hybridoma supernatants were used undiluted (300 ul). After incubation with antibodies, MRBCs were added and the total volume adjusted to 1 ml with assay medium. Optical density readings were taken at times 0 and 1.5 h. Controls consisted of MRBCs alone (negative control), mycoplasmas and MRBCs in assay media (positive control), and mycoplasmas and MRBCs with a known HL blocking antisera (HL blocking control). Percent inhibition was defined as $1 - [T_0 - T_{1.5h}\ \text{experimental}]/[T_0 - T_{1.5h}\ \text{positive control}] \times 100$.

RESULTS

A Sprague-Dawley rat colony presenting typical mycoplasma disease symptoms was screened for mycoplasma infection by standard culture techniques (4). Mycoplasma isolates from these animals were filter-cloned (12) and positively identified as M. pulmonis by using a fluorescent antibody test (5). One of these isolates was designated ISM3001. Animals from this colony displaying typical mycoplasmal disease symptoms were used in the hybridoma fusion experiments. All mice experimentally infected with UAB6510 demonstrated clinical signs of the disease and had high serum antibody titers as determined by ELISA.

Hybridoma Fusions

The results of fusions 1 and 2, which were performed using SPCs taken directly from mycoplasma-infected animals, are shown in Table 1. Fusion efficiencies were low (8-25%) and most wells did not contain mycoplasma-specific antibodies as determined by ELISA. To test whether in vivo primed SPCs could be expanded in vitro and used efficiently in hybridoma fusions, cells from the same source as cells used in fusions 1 and 2 were cultured in vitro in the presence of antigen and LPS for three days prior to fusion (Table 1, fusions 3 and 4). In vitro stimulation increased both the percentage of growth-positive and antigen-specific (ELISA positive) wells.

Hemolysis Inhibition Assay

Figure 1 shows the results of the HL blocking assay. Hyperimmune rabbit and mouse sera failed to block M. pulmonis-mediated HL activity, but both experimentally infected mouse and naturally infected rat sera were capable of blocking HL. Hybridoma supernatants with more than 50% inhibitory activity were considered positive.

Table 1. Effect of in vitro stimulation on production of *M. pulmonis*-specific rat hybridomas

Fusion Number[a]	Percent Growth Positive[b]	Percent ELISA Positive
1	8	2
2	8	0
3	88	69
4	74	16

[a]Fusions 1 and 2 used unstimulated naturally infected rat SPCs; fusions 3 and 4 used in vitro stimulated, naturally infected rat SPCs from the same animals as fusions 1 and 2, respectively.

[b]Data are presented as the percentage of growth positive or ELISA positive wells from the total number of wells plated for each fusion.

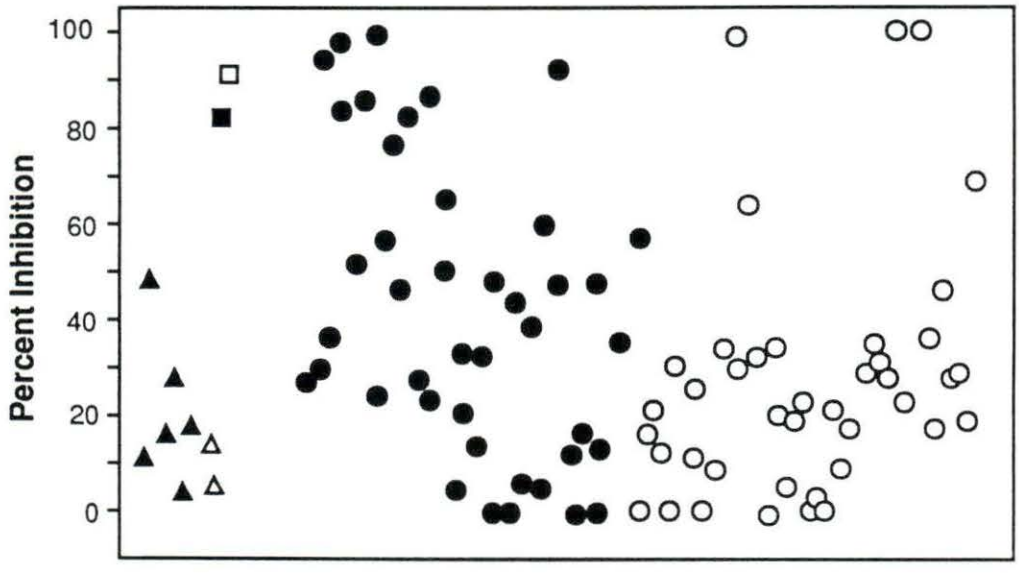

Figure 1. Hemolysin blocking activities of rabbit, rat, and mouse antisera, and rat Mabs. (▲) hyperimmune rabbit antisera; (△) hyperimmune mouse antisera; (■) experimentally infected mouse antisera; (□) naturally infected rat sera; (○) rat Mabs; (●) mouse Mabs. Data are presented as percent inhibition as defined in the text.

DISCUSSION

Previous studies on the membrane activities of M. pulmonis demonstrated the presence of a trypsin-sensitive hemolysin (7) which may be involved in cholesterol uptake from adjacent eukaryotic membrane surfaces (6). Identification of the HL protein was hindered by the lack of hyperimmune antisera capable of blocking its activity (Figure 1). The results with sera from naturally or experimentally infected animals, however, showed that antibodies could block hemolytic function.

Rats used in these experiments were from a Sprague-Dawley colony with a history of chronic respiratory disease. The M. pulmonis strain detected in this colony by culture and immunofluorescence, ISM3001, was antigenically similar to our laboratory strain UAB6510 by Western blot analysis (8). Importantly, the sera from these animals blocked the HL activity of strain UAB6510 (Figure 1).

Initial fusion experiments with untreated SPCs from naturally infected rats resulted in few ELISA positive, mycoplasma specific hybridoma supernatants (Table 1). This indicated that some antigen specific B cells were present in the rat SPC preparations although their numbers were low. Since only a few primed B cells give rise to successful hybridomas, it was necessary to increase the numbers of primed antigen specific B cells, and consequently increase the likelihood of obtaining hemolysin specific Mabs. This was accomplished by exposing the cells to mycoplasma antigen and an adjuvant, E. coli LPS, in vitro for three days (Fusions 3 and 4), resulting in an increase in both the number of growth- positive wells and antigen-specific hybridomas (Table 1). Mabs which block hemolytic activity have been identified (Figure 1) supporting the hypothesis that in vitro stimulation can be used

effectively to produce Mabs to antigens recognized primarily during infectious processes. This is further substantiated by the observation that approximately 50% of the Mabs obtained were of the IgG_{2b} isotype (data not given), and thus primed B cells were present prior to the three day in vitro stimulation.

The ability to produce Mabs from SPCs obtained from naturally infected animals offers a practical solution to many problems in hybridoma research. A natural host may not be required as long as colonization of the host and stimulation of its humoral defense occurs. With the widespread use of rodents for the study of pathogenic mechanisms and the use of alternate fusion partners (i.e., rabbit - mouse or swine - mouse, etc., 10), the development of Mabs to virulence determinants not generally recognized by rodents may become possible. Further enhancement of this technique through the use of in vivo grown organisms to aid in the screening of Mab libraries may lead to the discovery of previously unrecognized virulence-related antigens.

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SECTION II. IDENTIFICATION OF THE *MYCOPLASMA PULMONIS* MEMBRANE-ASSOCIATED HEMOLYSIN

Identification of the *Mycoplasma pulmonis* Membrane-Associated Hemolysin

by

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ABSTRACT

Mycoplasma pulmonis lysis of trypsin-treated red blood cells in suspension requires contact between the mycoplasma and the red blood cell, and is mediated by a trypsin-sensitive membrane protein. In order to identify this protein, acute and hyperimmune sera were screened in a hemolytic blocking assay. Sera produced by infected rodents, but not by animals immunized with broth grown organisms were able to block hemolytic activity. Monoclonal antibodies (Mabs) were also produced to M. pulmonis membrane proteins using spleen cells from naturally infected rats and mice. Several Mabs which demonstrated anti-hemolysin activity were used in radioimmune precipitation assays to identify the hemolysin, a 88.5 kilodalton protein. These Mabs were shown to be specific for the hemolysin by the following criteria: they reacted strongly in an enzyme-linked immunosorbent assay using intact whole cells, purified membranes, or tryptic peptides released from the mycoplasma surface as antigens; these tryptic peptides could interfere with the anti-hemolytic activity of the Mabs; Fab fragments of these Mabs were 50-70% effective in blocking hemolysis; and intact Mabs were unable to block attachment of the organism to red blood cells.

INTRODUCTION

Hemolysins (HLs) which are diverse in size, structure and mechanism of action, have been shown to be important virulence determinants in a variety of bacterial species (2,16,17,26). Several demonstrate phospholipase activity (1,3,4), and at least one is membrane bound (17). Many mycoplasmas are important animal and plant pathogens, and have demonstrated a nonvirulence-related agar plate hemolytic activity (5,12, 14) resulting from production of hydrogen peroxide (11). A second hemolytic mechanism involving a trypsin sensitive membrane protein has been described for Mycoplasma pulmonis (21,22). This mechanism differs from agar plate hemolysis in that only trypsin-treated red blood cells (RBCs) are lysed, a helper or carrier molecule is required, and lysis occurs rapidly in suspension presumably when the M. pulmonis membrane contacts or attaches to the RBC surface (22). The role of this activity in virulence has not been established, but it is thought to be involved in the acquisition of membrane precursors from eukaryotic membranes (21).

Understanding the mechanism of membrane-associated hemolysis is complicated by the fact that M. pulmonis has other multifactorial membrane properties. For instance, adherence has been shown to involve at least two different mechanisms, a trypsin resistant form (Type I) and a protein mediated form (Type II) (23). Differentiation between the hemolysin and the Type II adhesion is difficult because both activities are observed using trypsin-treated RBCs and both are protease sensitive. Therefore, both activities may be part of the same multifunctional protein molecule (22). Another membrane activity, hemagglutination, may also be associated with the Type II adhesion (22).

The goal of this study was to identify the protein involved in hemolysis and determine its relationship to Type II adherence. To accomplish this goal, a monoclonal antibody (Mab) library to M. pulmonis proteins was produced and screened for anti-hemolytic activity. Several Mabs capable of blocking HL activity were obtained and three were chosen for further study. These Mabs recognized an 88.5 kilodalton (kDa) membrane protein by radioimmune precipitation (RIP) assays, and failed to block attachment to trypsin-treated RBCs in adherence assays, thereby differentiating the membrane hemolysin and Type II adherence properties.

MATERIALS AND METHODS

Culture Conditions and Antigen Preparations

M. pulmonis strains UAB6510 (24) and ISM3001 were cultured in PPLO broth without crystal violet (Difco Laboratories, Detroit, Mich.) supplemented with 10% serum, 0.5% glucose, 2.5% fresh yeast extract (Flow Laboratories, Inc., McLean, Va.), and 25 ug of Cefobid (Pfizer, Inc., New York, N.Y.) per ml. Agar plates contained 1% Noble agar (Difco). Serum supplements consisted of gamma globulin-free horse serum (GIBCO Laboratories, Grand Island, N.Y.) or rabbit serum (GIBCO). Cultures were incubated at 37°C, and harvested in mid log phase (pH 7.3). Mycoplasmas were cultured from rat lungs and uterine horns using standard techniques (8), filter-cloned (27), and identified as M. pulmonis by plate epiimmunofluorescence (15a).

Mycoplasmal Antigens

Mycoplasma whole cell immunizing antigen consisted of organisms grown in PPLO medium supplemented with 10% serum. These organisms were pelleted by centrifugation (10,000 x g, 20 min), washed three times with 10 mM Na_2HPO_4 - 140 mM NaCl, pH 7.5 (PBS), and lyophilized or resuspended in sterile PBS (approximately 1 mg protein per ml) and stored at -70°C until use. Membranes were prepared from washed organisms by osmotic lysis as previously described (23).

Trypsin-released peptide (TRP) antigen was prepared by resuspending washed, whole organisms from 2 L of broth in 10 ml of PBS - 0.5% glucose (PBSG), and treating with 1 ml of immobilized trypsin (Cooper Biomedical, Inc., Malvern, Pa.) overnight at 37°C with gentle agitation. The immobilized trypsin and the organisms were removed by centrifugation (10,000 x g, 10 min), and the resulting supernate concentrated in a

SpeedVac (Savant Instruments, Inc., Farmingdale, N. Y.) and filter-sterilized. Protein concentrations were determined using the dye binding protein assay (Bio-Rad Laboratories, Richmond, Calif.) and bovine serum albumin as the standard.

Mycoplasma enzyme-linked immunosorbent assay (ELISA) lysed antigen was prepared as above except that cells were washed and resuspended in water at 2 - 3 mg protein per ml prior to dilution in carbonate lysis buffer. Trypsin-treated ELISA antigen was prepared by treating whole cells resuspended in PBSG with trypsin (100 ug trypsin per mg M. pulmonis protein) for 1 h at 37°C, and washing the cells three times with PBSG. These antigens were stored at -70°C prior to use.

Immunoreagents

New Zealand White rabbits (Small Stock Industries, Pearidge, Ark.), Sprague-Dawley rats (SASCO Inc., Omaha, Neb.) and BALB/cByJ mice (Jackson Laboratories, Bar Harbor, Maine) were housed at the Laboratory Animal Resources Division of the College of Veterinary Medicine, Iowa State University. Rabbits were immunized with rabbit or horse serum grown antigen suspended in PBS at 0.1 mg protein per ml as previously described (6). Experimentally infected mouse sera was obtained by inoculating six BALB/cByJ mice intranasally with 1 X 10^8 colony forming units of UAB6510 in 0.05 ml of mycoplasma broth supplemented with horse serum. The mice were sacrificed after two weeks and sera pooled. Sera were also obtained from M. pulmonis naturally infected Sprague-Dawley rats.

Hybridoma Production

BALB/cByJ mice were immunized with 100 ug of mycoplasma whole cell antigen suspended in PBSG or 10 ug of TRP antigen. Primary injections were given intraperitoneally and secondary boosters given 14 days later

intravenously. Blood was obtained on days 1, 14, and 17 by retino-orbital puncture. Spleen cells were also obtained from untreated rats naturally infected with M. pulmonis.

Myeloma fusions were performed with rat or mouse spleen cells (SPCs) and mouse myeloma P3X63-Ag8.653 cells (ATCC CRL 1580) using standard techniques. Hybridomas were screened for mycoplasma-specific antibody production by ELISA. Subcloning was performed using the Autocloner accessory on an EPICS VII fluorescence activated cell sorter (model 752). Antibody isotypes were determined using the Mouse Subtyping Kit (BioRad), or the Rat Isotyping Kit (Serotek, England) according to manufacturers directions.

In Vitro Stimulation of Rat Spleen Cells

Rat SPCs obtained from naturally infected animals were suspended at 2×10^6 cells per ml in RPMI 1640 supplemented with 15% heat-inactivated FCS, 10% conditioned medium, 1.2% MEM Non-essential amino acids (GIBCO), 0.3 g L-glutamine per liter and 5×10^{-5} M 2-mercaptoethanol (tissue culture grade). Cells were cultured into 24 well tissue culture plates (Corning 25820, 0.5 ml per well), with the following additions per well: 10 ug indomethicine, 10 ug of UAB6510 whole cell antigen and 5 ug E. coli K235 LPS. The plates were rocked for three days at 37°C in an atmosphere of 83% N_2, - 7% O_2 - 10% CO_2, and the wells fed daily with the above medium. After incubation, the cells were collected and fused with the mouse myeloma line as described above.

Monoclonal Antibody Concentration and Fab Fragments

Hybridomas were adapted to grow in HL-1 medium (Ventex, Fisher Scientific Co., St. Louis, Mo.) with a 0.5% fetal calf serum concentration, and culture supernates concentrated using an Amicon

concentrator with 15,000 molecular weight cutoff filters (Amicon Division, W. R. Grace & Co., Danvers, Maine). The antibodies were precipitated with 36% sodium sulfate, washed twice with 18% sodium sulfate by centrifugation, and dialyzed against PBS overnight at 4°C. Antibodies were again concentrated using the Amicon concentrator, protein concentration determined, and their activity assessed by ELISA prior to use. Fab fragments were produced using immobilized papain (Pierce Chemical Co., Rockford, Ill.) according to manufacturer's suggestions. The extent of digestion was determined by analyzing samples of the digests by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (23).

ELISA

The ELISA was performed with carbonate buffer-lysed antigen as described (7). Plates for the whole cell ELISA were prepared by treating 96 well serocluster "U" vinyl plates (Costar, Cambridge, Mass.) with PBS - 0.1% glutaraldehyde for 4 h at room temperature or overnight at 4°C. Plates were then washed three times with PBS, coated with intact organisms suspended in PBS - 0.1% glutaraldehyde (5 ug per ml, 100 ul per well) for 4 h, and the wells blocked for 1.5 h at room temperature with PBS - 1% BSA. Undiluted hybridoma supernates or antisera diluted in PBS - 0.1% BSA (1/100) was incubated in the wells overnight at 4°C. Plates were again washed three times with PBS - 0.1% BSA prior to the addition of horseradish peroxidase labeled goat anti-mouse IgG (H+L) (Bio-Rad), 100 ul per well, 1:3000 dilution in PBS - 0.1% BSA. The plates were incubated for 1 h at 37°C, washed three times with PBS, and 3,3',5,5'-tetramethylbenzidine (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) was used as substrate according to manufacturers

directions. ELISA plates were read on a automated microplate reader (BioTek, Model EL310, Winooski, Vt.).

RBC Preparation and Hemolytic Assays

Mouse RBCs (MRBCs) for the hemolytic assay were prepared essentially as described (23). Blood was collected in Alsevers anticoagulent, washed in PBSG three times, treated with trypsin for 30 min (1 mg per ml, 8% suspension) with gentle agitation, washed in PBSG three more times, and resuspended in the same buffer. The standard HL assay mixture consisted of assay medium (RPMI 1640 - 20 mM HEPES - 1% BSA, pH. 7.5), 0.5% trypsin-treated MRBCs (final concentration), and freshly prepared UAB6510 (approximately 10 - 15 ug protein) in a total volume of 1 ml. The mixture was rocked slowly for 1.5 h at 37°C and lysis determined by measuring optical density (630 nm) at 0, 45 and 90 min. The minimal amount of mycoplasma protein needed to completely lyse the MRBCs was determined for each mycoplasma culture to adjust for variation in hemolysin concentration. The HL inhibition assay was performed by treating this concentration of mycoplasmas with antibody (sera diluted 1:25 in assay medium or 300 ul of undiluted hybridoma supernate) for 1 h statically at 37°C prior to the addition of MRBCs to bring to final volume. Controls consisted of MRBCs alone (negative control), mycoplasmas and MRBCs in assay medium (positive control), and mycoplasmas and MRBCs with a known HL blocking antisera (HL blocking control). In some experiments 20 - 30 ug TRPs were added along with the Mabs. In these experiments, mycoplasmas with TRPs, and TRPs with MRBCs only served as additional controls. After incubation with antibodies, MRBCs were added and the total volume adjusted to 1 ml with assay medium. Percent inhibition was defined as: (DC - DE)/DC x 100 where DC = optical density

(O.D.) at 0 min - O.D. at 90 min for the positive control, and DE = O.D. at 0 min - O.D. at 90 min for the experimental.

Radioimmune Precipitation Assay

Intrinsically radiolabeled organisms were prepared as described (22) using ^{35}S-labeled amino acids (Trans ^{35}S, ICN Biomedicals, Inc., Costa Mesa, Calif.). The RIP assay was performed as described (23) except that 1 - 3 ug protein of purified Mab were used in some assay mixtures. Rat and mouse antibodies were precipitated using biotinylated rabbit anti-mouse IgG + IgM (H+L) (Sigma) or rabbit anti-rat Ig (IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, IgM, IgA, Fc and Fab) (Nordic Immunological Laboratories, Capistrano Beach, Calif.) and Streptavidin agarose (Sigma). Biotinylated antibodies were prepared fresh using photoactivatible biotin (CLONTEK Laboratories, Inc., Palo Alto, Calif.) according to the manufacturers directions. Rabbit antibodies were precipitated using Sepharose-Protein A (Sigma) as described (23).

Adherence Assay

The mycoplasma - RBC adherence assay was performed essentially as described (24) using both untreated and trypsin-treated human RBCs. The effect of antisera, hyperimmune rabbit, naturally infected rat, and anti-hemolysin Mabs was examined by incubating the organisms in a 1:10 dilution of antisera or undiluted hybridoma supernates for 1 - 2 h at 37°C. MRBCs were then added directly to the mycoplasma - antibody mixture. Controls consisted of untreated organisms, and organisms treated with antibodies without RBCs. Effect on adherence was determined by taking the difference in the 0 and 60 min time points for each treatment and subtracting the treated organisms less RBC control for each

treatment. This accounted for any aggregation of the organisms as a result of antibody treatment.

Electrophoresis

Polyacrylamide gel electrophoresis was performed by a modification of the method of Laemmli (19) using an upper tray buffer of 3 g Tris per L, 14.5 g glycine per L, and 1 g SDS per L (pH 8.5), and a lower tray buffer diluted 1:1 with water. Proteins were separated using a 3% acrylamide stacking and a 10% acrylamide resolving layer. Molecular weight standards consisted of high molecular weight standards (Bethesda Research Laboratories, Gaithersburg, Md.) and rainbow standards (Amersham Corp., Arlington Heights, Ill.). Proteins were visualized by Coomassie blue or silver staining (20) for determination of apparent molecular weights and by autoradiography for identification of radiolabeled proteins. Gels with ^{35}S-labeled proteins were impregnated with 1 M sodium salicylate for 30 min prior to drying to enhance the radiographic signals.

RESULTS

Rats and Mice

Sera from naturally infected rats were obtained from a colony with severe mycoplasma disease. Sera antibody titers to M. pulmonis UAB6510 were high (>2.0 optical density units above background controls), lungs had extensive lymphocytic involvement (9), and mycoplasmas were cultured from lung tissues and identified as M. pulmonis by colony epifluorescence (15). Mice had low positive mycoplasma antibody titers by ELISA which is typical of low level, clinically silent mycoplasma disease (10). After immunization, their antibody titers to UAB6510 were equal to or greater than the titers from the infected rats.

Hybridoma Fusions

Spleen cells from untreated naturally infected rats were stimulated in vitro with mycoplasma antigen and E. coli lipopolysaccharide resulting in an 8 - 10 fold increase in fusion efficiency as compared to nonstimulated SPCs (data not given). Both the percentage of growth-positive wells and antigen-specific (ELISA positive) wells demonstrated similar increases (data not given). Additional fusions were performed using mouse SPCs from immunized animals as noted above. Mouse SPCs were not stimulated in vitro prior to fusion.

Monoclonal Antibodies

Mouse and rat hybridomas were screened using different ELISA antigens. Mouse Mabs which demonstrated high ELISA values, reactivity in the whole cell ELISA, or decreased binding to trypsin-treated antigen were studied further. All of the rat hybridoma supernates were included in these studies. Antibody isotypes included mouse IgA, IgM, and all IgG subtypes, and rat IgM and IgG_{2b} (Table 1).

Hemolytic Activity of M. pulmonis

A typical hemolysis curve is shown in Fig. 1. Optical density readings were taken at 0, 45 and 90 min as was suggested by previous studies which indicated complete lysis of MRBCs by M. pulmonis in 90 min (22). Naturally infected rat sera and the two Mabs shown prevented lysis of MRBCs (Fig. 1). Mouse and rabbit hyperimmune antisera, however, failed to block hemolysis as indicated by the similarity of the lysis curve to the positive control. Rabbit antisera produced against whole mycoplasma cells, purified membranes, and cytoplasmic proteins were tested and all failed to block hemolysis (data not given). The hyperimmune sera and the naturally infected rat sera reacted strongly in the ELISA and on Western blots (data not given) indicating their recognition of multiple M. pulmonis antigens, some of which were external membrane proteins (data not given).

Effects of Monoclonal Antibodies on HL Activity

Test samples were compared with three controls: mycoplasmas plus MRBCs, MRBCs only, and mycoplasmas treated with naturally infected rat sera plus MRBCs (HL-blocking control). Only those which demonstrated 75% or greater inhibition were considered for further study. Three hybridomas (1C5B9, 1B3G1, and 1D4G12) demonstrating high inhibitory activity were partially purified. These Mabs recognized a tryptic peptide released from the M. pulmonis membrane as evidenced by the ability of TRP preparations to block their anti-HL activity and by a positive ELISA reaction to these peptides (Table 1). Fab fragments from these Mabs retained greater than 50% of the hemolysis blocking activity. Several other Mabs which were positive in the whole cell ELISA failed to block anti-HL activity (2C3F2, 7D2B10, 12C3B1, 12D2A11, and 11C2A5)

demonstrating that anti-HL activity by Mabs was due to antibody binding to a specific antigen and not to steric hindrance, or other nonspecific effects.

Effect of Antisera and Mabs on Adherence to Human Red Blood Cells

To accommodate the inclusion of an antibody incubation step in the adherence assay, control tubes containing only organisms and antibodies were monitored in each treatment to eliminate any effect antibody-mediated agglutination may have had on the assay. Hyperimmune rabbit and naturally infected rat sera, and the monoclonal antibodies did not increase the background counts demonstrating that the counts at the 60 min interval represented actual binding of organisms and not aggregates of mycoplasmas. When compared to mycoplasmas alone, anti-hemolysin Mabs consistently failed to block adherence of M. pulmonis to either untreated or trypsin-treated human RBCs (data not shown). Naturally infected rat sera blocked binding to trypsin-treated RBCs, but not to untreated RBCs (data not shown).

Identification of the M. pulmonis Hemolysin

The HL protein was identified as an 88.5 kDa protein by three anti-HL Mabs using the RIP reaction (Fig. 2, lanes 1 - 3). The trypsin sensitivity of the hemolysin was confirmed by treating intrinsically radiolabeled whole cells with trypsin prior to solubilization of proteins in RIP buffer. This resulted in the disappearance of the 88.5 kDa protein from the gel (lane 4). For comparison, the radiolabeled antigen preparation (lane 6) and trypsin-treated preparation (lane 5) are also shown.

Table 1. Results of Mab ELISA tests and hemolysis inhibition assays.

Number[c]	Sp.[d]	Iso[e]	Antigen[a] LAg	Try	WC	Memb	TRP	Percent Inh.[b] Mab	Fab	TRP
1C5B9*	M	IgM	+++	+++	+	+	+	82	50	17
1C5E6	M	IgM	+++	+++	-	nt	nt	42	nt	nt
2C3F2	M	IgG_1	++	nt	+	nt	nt	14	nt	nt
7D2B10	M	IgG_1	+++	nt	+	nt	nt	17	nt	nt
12C3B1	M	IgG_{2b}	++	nt	+	nt	nt	5	nt	nt
12D2A11	M	IgA	++	nt	+	nt	nt	19	nt	nt
11C2A5	M	IgA	++	-	+	nt	nt	0	nt	nt
1B3G1*	R	IgG_{2b}	++	++	nt	+	+	100	70	19
1D4G12*	R	IgM	+	+	nt	+	+	100	50	11
3D3C4	R	IgG_{2b}	+	nt	nt	-	-	17	nt	nt

[a]LAg = carbonate buffer lysed antigen; Try = trypsin-treated antigen; WC = whole cell antigen; Memb = purified membranes; TRP = trypsin-released peptides. nt = not tested. Data represents relative ELISA values: (+++) = ELISA O.D. greater than 2.0; (++) = $0.5 < O.D. < 2.0$; (+) = $0.1 < O.D. < 0.5$; (-) $O.D. < 0.1$.

[b]Data represent percent HL inhibition as defined in text. Purified intact Mabs (Mab), Fab fragments (Fab), or Mabs + trypsin-released peptides (TRPs) were added to hemolysis assays and percent inhibition was determined (see Materials and Methods).

[c]Number = Mab number. (*) indicates Mabs used to identify HL by RIP reactions.

[d]M = mouse; R = rat.

[e]Isotype.

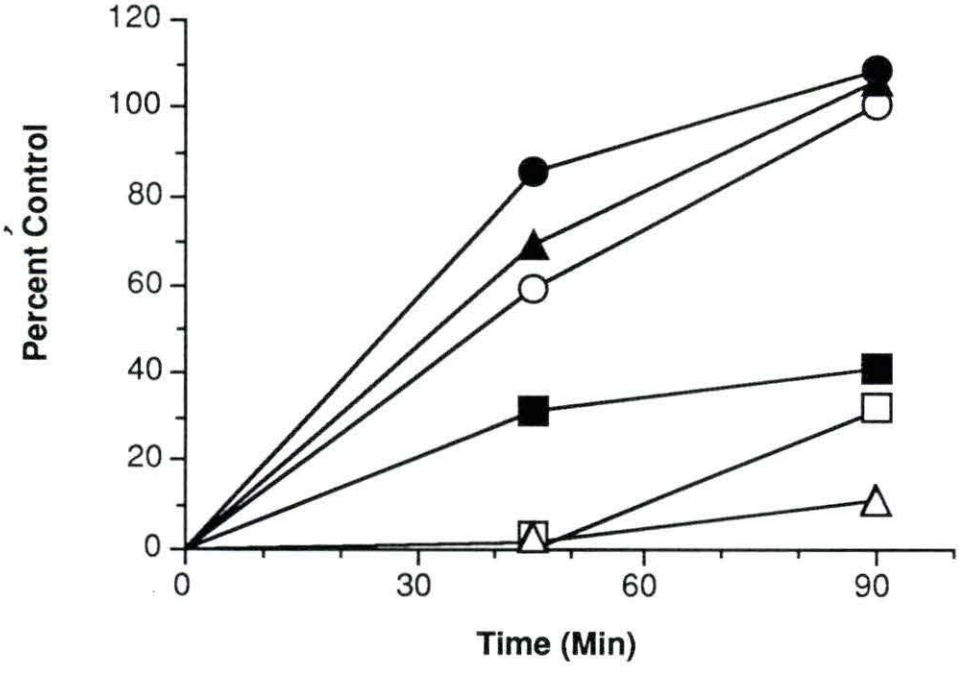

FIGURE 1. Hemolysin blocking activities of rabbit and mouse hyperimmune antisera, naturally infected rat antisera, and rat and mouse Mabs. Symbols: ●, positive control (no antibodies); ▲, hyperimmune rabbit antisera; ○, hyperimmune mouse antisera; ■, naturally infected rat sera; □, mouse Mab 1C5B9; △, rat Mab 1B3G1. Data are presented as percent lysis: [(O.D. T_x/ O.D. TC_{90} x 100] where T_x = O.D. at 0, 45 or 90 min for each reaction, and TC_{90} = O.D. control at 90 min.

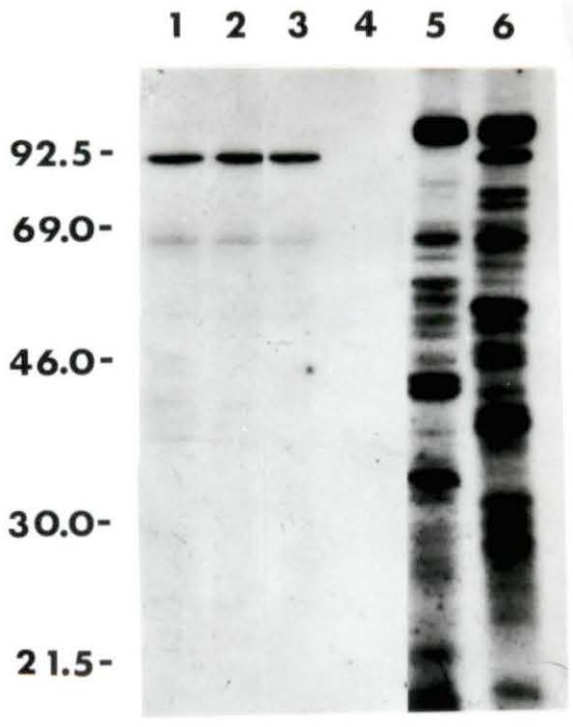

FIGURE 2. Immunoprecipitation of the M. pulmonis hemolysin. Mabs 1C5B9 (mouse, lane 1), 1B3G1 (rat, lane 2) and 1D4G12 (rat, lane 3) were used to precipitate proteins from a radiolabeled M. pulmonis antigen preparation (lane 6). The single band in lanes 1 - 3 represents the M. pulmonis hemolysin (88.5 kDa). Lanes 4 and 5 contain radiolabeled proteins from trypsin-treated whole cells. Trypsin treatment of radiolabeled whole cells prior to immunoprecipitation with Mab 1D4G12 resulted in the loss of the 88.5 kDa hemolysin from the gel (lane 4) and this band was also missing in the trypsin treated antigen preparation (lane 5). Molecular weight markers are shown on the left in kilodaltons.

DISCUSSION

The M. pulmonis hemolysin (HL) is a unique molecule which can be differentiated from other mycoplasmal hemolytic activities by its association with an external membrane protein (22). To study this protein further, HL-specific Mabs were produced and used to determine its size (88.5 kDa, Fig. 2), and confirm its membrane location and trypsin sensitivity. The antigenic epitope recognized by these three Mabs resides on a soluble tryptic peptide, and the antigenicity of this fragment is retained in solution as evidenced by the ability of TRPs to block anti-HL activity and their recognition in the ELISA by hemolysin specific Mabs.

Mycoplasmas seem to adhere and interact with cell surfaces through complex mechanisms. M. pneumoniae is the most extensively studied mycoplasma, and contains a large membrane protein as the primary mediator of attachment to eukaryotic cell surfaces, but a 32 kDa protein has also been implicated in attachment (15b). Studies of this protein, P1, have shown that it contains lectin-like activity for sialic acid residues. Cytoadherence of M. pneumoniae to hamster tracheal rings or chicken RBCs can be inhibited with P1 specific rabbit antisera or Mabs (18,25), but Mabs to lipids enhanced binding to RBCs (25). Attachment of M. pneumoniae to glass can also be inhibited with antisera (13).

The adherence mechanism of M. pulmonis seems to be more complex, however, involving both specific and nonspecific activities (24). In order to differentiate membrane hemolysis from trypsin sensitive Type II adherence, attempts were made to block adherence using Mabs and various sera. Anti-HL Mabs failed to block Type II adherence to trypsin-treated human RBCs in contrast to hyperimmune rabbit sera, naturally infected rat

sera, and a membrane positive, HL negative Mab. This indicated that the activity of the anti-HL Mabs was due to specific antibody interactions and not to nonspecific effects arising from the blockage of binding of the organism to the MRBCs. It also suggested that hemolysis and Type II adherence were separable properties of the M. pulmonis membrane. These studies lend credence to the idea that binding of M. pulmonis to RBCs is a complex multiphasic interaction (24) because antibodies failed to block Type I adherence to untreated RBCs (data not shown). In fact, increased binding was often observed supporting previous reports of increased binding of mycoplasmas to RBCs in the presence of specific antibodies (25). Further studies are required to give a better understanding of the differences between Types I and II adherence.

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GENERAL SUMMARY AND DISCUSSION

The M. pulmonis hemolysin was previously shown to be a membrane-bound, trypsin-sensitive protein whose activity was temperature sensitive, required a carrier molecule, and was capable of lysing trypsin-treated (RBC) more effectively than nontreated RBCs (52). This study increases our knowledge about this molecule by showing that the hemolysin is 88.5 kDa in apparent molecular weight, remains membrane-bound and active in membrane fragments, has unusual antigenic properties, and can be differentiated from Type II adherence properties. The molecule is capable of eliciting an immune response in infected but not in systemically immunized animals. In addition, antigenic epitopes are located in an outer tryptic fragment.

Several differences exist between the M. pulmonis hemolysin and other characterized bacterial hemolysins. Most notable is the membrane location; all but one of the bacterial hemolysins are extracellular. The membrane-bound hemolysin from Serratia, however, is only active in viable cells. In addition, none of the other bacterial hemolysins, including the hemolysins from Serratia, are temperature-sensitive or require a helper molecule. Finally, the observation that the M. pulmonis is more effective in lysing trypsin-treated RBCs has not been demonstrated with other hemolysins. Thus, the hemolysin from M. pulmonis is a unique molecule, with a different mechanism of action.

These studies shed little light on the mechanism of action because of the peculiarity of its association with a membrane. There has been a suggestion of a role in cholesterol uptake (50) and this may be the mechanism whereby RBCs are lysed. A more defined study, which may

require the construction of specific mutants, is required before the exact mechanism is elucidated.

Studies using this approach might proceed by purifying the protein using the immunoreagents generated during these studies. Purified protein could then be used to generate monospecific antisera for use in identifying the specific sequences in genomic libraries. Using recombinant DNA technology and recently developed methods for introducing specific mutations in M. pulmonis (48b,48c), a series of mutations could then be constructed in the hemolysin gene. The resulting mycoplasma mutants could be studied for phenotypic characteristics, pathogenic potential, etc. These mutants may also serve as suitable vaccine candidates. Other studies might include screening of antisera from acute, chronic and convalescent animals to test for the presence, quantity and isotype of antibodies to the hemolysin which arise during the course of infection.

In conclusion, the hemolysin from M. pulmonis is a unique molecule unrelated in characteristics and possibly mechanism of action to other known bacterial hemolysins. It has a possible role in the acquisition of cholesterol and phospholipids, and may be involved in the pathogenesis of MRM. The further characterization of this molecule may result in determining the mechanism of action and producing an effective vaccine against M. pulmonis infection of rodent colonies.

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