Characterization of the *Mycoplasma pulmonis* soluble hemolysin

by

Becky Ann Esswein

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Iowa State University

This is to certify that the Master's thesis of

Becky Ann Esswein

has met the thesis requirements of Iowa State University

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LIST OF ABBREVIATIONS

A.	*Acholeplasma*
AP	alkaline phosphatase
ATP	adenine triphosphate
BALT	bronchial associated lymphoid tissue
BMM	bone-marrow derived macrophages
BSA	bovine serum albumin
CFU	colony forming units
CMI	cell-mediated immunity
ConA	concanavalin A
DNA	deoxyribonucleic acid
eHL	electroeluted hemolysin
ELISA	enzyme-linked immunosorbent assay
E.	*Escherichia*
FITC	Fluorescein isothiocyanate
g	g force
g	gram
GBSS	Gay's balanced salt solution
GM-CSF	granulocyte-macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HMW	high molecular weight
HL	hemolysin
HPLC	high performance liquid chromatography
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilodalton
LPS	lipopolysaccharide
Mab	monoclonal antibody
M.	*Mycoplasma*
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of flight mass spectrometry

mg	milligram
µg	microgram
MAM	*Mycoplasma arthriditis* mitogen
MHC	major histocompatibility complex
mHL	membrane hemolysin
ml	milliliter
µl	microliter
MIaF	mycoplasma-Ia induction factor
mol. wt.	molecular weight
MRBC	trypsin-treated mouse red blood cells
MRM	murine respiratory mycoplasmosis
NK	natural killer cells
℃	degree Celsius
PBS	phosphate buffered saline
PC	phosphatidylcholine
PG	phosphatidylglycerol
pHL	partially-purified hemolysin
PKC	protein kinase C
PMNL	polymorphonuclear lymphocytes
PMSF	phenyl methyl sulfonyl fluoride
PVDF	polyvinylidene difluoride
PWM	pokeweed mitogen
RBC	red blood cell
RNA	ribonucleic acid
S.	*Spiroplasma*
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sHL	soluble hemolysin
TCR	T-cell receptor
T_H	T-helper cells
TNF-α	Tumor necrosis factor-alpha
TS	Tris saline
URN	upper respiratory nodes

ABSTRACT

Mycoplasma pulmonis contains a membrane hemolysin that is believed to be involved in the acquisition of membrane precursors. This membrane hemolytic activity (mHL) can be released from cell suspensions into the supernatant during long term incubation in a serum-free medium. The kinetics of soluble hemolysin (sHL) release were studied by measuring the amount of sHL activity released as a function of time, by monitoring cell viability, and by measuring cell lysis to determine if cell death and membrane rupture were required for sHL release. After 1.5 hours of incubation, sHL activity could be detected in supernatants, but only about 20% of the original membrane-associated HL was recovered even after 8 hours of incubation at 37°C. Release correlated directly with cell lysis inferring that sHL release was due to degradative processes following cell death and was not a normal physiological process of the organism. The soluble hemolysin (sHL) remained in suspension during ultracentrifugation and failed to pass through a 50,000 mol. wt. cutoff filter, but it passed partially through a 100,000 mol. wt. cutoff filter. Partial purification of the hemolytic activity (pHL) was achieved using gel filtration where the activity was found in the void volume of a G-200 Sephadex column. The hemolytic activity in pHL preparations resolved near the top of 5% native polyacrylamide gels. Several lines of evidence suggested that pHL was a multimeric complex: gel filtration on Sephadex G-200 was not capable of resolving the activity, it was found in the void volume of the column; SDS-PAGE analysis of hemolytically-active pHL bands electroeluted from native gels revealed several protein bands, none of which were large enough to account for the gel filtration results; and separation of its components by isoelectric focusing or treatment with nonionic detergents resulted in permanent inactivation of the hemolytic activity. pHL had potent heat-sensitive chemotactic and chemokinetic activity for J774.A1 mouse macrophages, but it had no effect on the phagocytic ability of either J774.A1 or P338.D1 cells toward FITC-conjugated *E. coli*. It also lacked mitogenic activity towards C3H/HeOuJ mouse splenocytes, but the mitogenic activity of pHL preparations significantly increased after treatment at 70°C for 90 min. Mabs that are directed against *M. pulmonis* mitogen(s) recognized pHL proteins that were lost upon subsequent purification by native polyacrylamide gel electrophoresis, electroelution, and ultrafiltration. There was a change in the immunoblot banding pattern following heat treatment. There was no apparent change in the size of the pHL complex following heat treatment as determined by ultrafiltration. These results suggest that *M. pulmonis* releases a complex from its membrane that has divergent biological activities towards immune effector cells. The hemolytic activity is not related to previously reported mitogenic

activity, but may have macrophage chemotactic activity. In addition, a heat stable mitogenic activity has been identified that is different from previous reports.

GENERAL INTRODUCTION

Rationale and Hypothesis

Mycoplasma pulmonis, the etiologic agent of murine respiratory mycoplasmosis, is one of the more serious infectious agents of laboratory rodents. *M. pulmonis* not only causes its own serious consequences in rodents, but also serves as an important model for human respiratory mycoplasma disease. Mycoplasmas are cell wall-less organisms that require membrane precursors for growth. A membrane-associated hemolytic activity, found in most mycoplasma species, has been proposed to be involved in the acquisition of membrane precursors from eukaryotic cells to which the organism is attached (125, 126). Previous studies have shown that cholesterol may serve as the red cell membrane receptor for the hemolysin (79). Hemolytic activity can be released into serum-free cell suspension supernatants during long term incubation. This mHL activity is trypsin-sensitive, requires bovine serum albumin (BSA) for activity, is inhibited by sterols in a stereo-specific manner, is enhanced with thiol-active substances, and is inhibited with monoclonal antibodies (Mabs) (77, 79). The sHL and the membrane-associated hemolysin (mHL) are essentially identical functionally (78). This project was undertaken to further characterize and define the composition of the sHL. The hypothesis addressed by this research was that the purified sHL of *M. pulmonis* is toxic to eukaryotic cells. In order to address this hypothesis, biochemical and immunological approaches were taken to partially purify the material, to define the mechanism of release, and to observe the effects sHL has on immune effector cells using various immunological assays. Purification of sHL by affinity chromatography using existing Mabs was hampered by the fact that the Mabs also reacted with BSA. The hypothesis was addressed by completing the following specific aims:

- to characterize the kinetics of release of the mHL
- to sufficiently purify the sHL so as not to hamper the characterization assays
- to define the composition of the sHL
- to characterize the sHL activity biologically and immunologically

The purification and characterization of the sHL would add significantly to our understanding of the mycoplasma membrane-associated virulence factors. It could help explain the method of macromolecular acquisition by mycoplasmas, and the various effects mycoplasmas have on the immune system. This hemolytic activity may be one of the key elements in the proposed pathogenesis model of *M. pulmonis*. A timed-hemolysis assay combining the hemolysis assay with the measurement of colony-forming units (CFUs) aided in

determining when the sHL was released during incubation in serum-free buffer. An NADH-oxidase assay was used to determine if cell-death was occurring during this process. The approaches taken to purify the activity involved various protein chemistry methods (HPLC, MALDI/mass spectrometry analysis, isoelectric focusing, protein sequencing, native-PAGE, SDS-PAGE, and electroelution). A combination of G200 Sephadex column chromatography, native-PAGE, electroelution, and SDS-PAGE analysis proved to be the most successful method. Radiolabeling with various radioactive metabolites helped aid in the identification of the composition of the sHL. Blastogenic, chemotactic, and phagocytosis assays were used to characterize the effects of sHL on the immune effector cells J774.A1 and P388.D1.

Thesis Organization

This dissertation is organized in a standard format consisting of four chapters, a General Introduction with Literature Review, Materials and Methods, Results and Discussion and Conclusions. This thesis follows the convention of the American Society for Microbiology.

Literature Review

General introduction

The genus *Mycoplasma* belongs to the order *Mycoplasmatales* of the Class *Mollicutes* which also contains three other orders, *Entomoplasmatales*, *Acholeplasmatales*, and *Anaeroplasmatales* (159). The term mollicute will be used to refer to any member of the class whereas mycoplasma will be used to refer only to members of the family *Mycoplasmataceae*, genera *Mycoplasma* and *Ureaplasma*. The class *Mollicutes* now includes more than 150 species of mycoplasmas (207). Most of these species are parasitic, infecting a vast variety of hosts. They occur in most vertebrates, in numerous insect and other arthropods, and within and on the surfaces of many plants. At the beginning of this century mycoplasmas were considered to be viruses. Later, they were grouped with bacterial L-forms (154). It has been hypothesized that all mycoplasmas arose by degenerative evolution from the *Bacillus-Lactobacillus-Streptococcus* subgroup of Gram-positive sporeforming eubacteria (116).

The advent of new culture techniques has led to the discovery of new species of mycoplasmas in man, insects, and plants. Along with the identification of these new species has been the identification of new characteristics. For example, members of the genus *Mycoplasma* are generally believed to be non-invasive pathogens which adhere to and colonize the epithelial lining of the respiratory and urogenital tracts. Two new species found in man, *M. penetrans* and *M. fermentans* (incognitus strain) (112, 113, 204), are notable exceptions.

These species are capable of penetrating and surviving within animal cells. They also produce fulminating infections (111).

Mycoplasmas are the smallest free-living, self-replicating forms of prokaryotes known. They have several unique features that distinguish them from other prokaryotes. The major defining characteristic of mollicutes is the lack of a cell wall which is one of the principle cellular and morphological structures of prokaryotes. Mycoplasmas contain the minimum number of organelles needed for growth and reproduction like other prokaryotes: a circular double-stranded DNA molecule provides genetic information, ribosomes are the workbench upon which cell proteins are assembled, and a single limiting plasma membrane separates the cytoplasm from the external environment (154). Most mycoplasmas lead a parasitic life style. They have a close association with eukaryotic host cells and can be described as membrane parasites. Because they have a limited genome size, the smallest among known self-replicating organisms (84), mycoplasmas depend on their host for the complete spectrum of amino acids, purines, pyrimidines, fatty acids, cholesterol, and vitamins (154). Consequently, they have a minimal number of structural elements, metabolic pathways, and components of the protein synthesizing machinery. Mycoplasmas express relatively few cell proteins (400-700) with a truncated set of tRNAs. They lack major energy-yielding systems since none of the mycoplasmas characterized so far possess the tricarboxylic acid cycle (154). Most energy requirements are met by the inefficient glycolysis pathway, but some mycoplasmas lack key elements, and thus must depend on even less effective energy-yielding systems. For instance, some mycoplasmas acquire ATP through arginine hydrolysis by the three enzyme arginine dihydrolase pathway (154).

The focus of the research presented here is on *Mycoplasma pulmonis*, the causative agent of respiratory tract disease and arthritis in both rats and mice (34). *M. pulmonis* infection in both rats and mice results in a chronic respiratory disease called murine respiratory mycoplasmosis (MRM). This disease provides an excellent model to study the pathogenesis of both acute and chronic mycoplasmal respiratory diseases. *M. pulmonis* in its own right is an important pathogen to study since it widely contaminates rodent colonies. Even commercially available SPF rats or mice are often mycoplasmal infected.

The mechanisms of mycoplasma pathogenicity are still largely unknown (154). A full understanding of mycoplasma respiratory disease must take into account host defense mechanisms as well as mycoplasma physiology (156). One of the more perplexing problems is how the wall-less, fragile mycoplasmas resist the immune defense mechanisms of their host. Pathogenic mycoplasmas evoke essentially all the immunological responses of the host's defensive repertoire, but they induce nonspecific modulation of host immune responses which

could lead directly to immunosuppression by usurping a more focused, protective host response. A fundamental problem remains unresolved concerning the relationship between host immunity and mycoplasmal pathogenesis: is the lymphoid response in infected lungs directed against the mycoplasma, does an aberrant host immune response aid the mycoplasma in contributing to the lesions characteristic of MRM, or is the mycoplasma itself solely responsible for these lesions through some unknown pathogenic mechanism(s). There is accumulating evidence suggesting that, at least in certain mycoplasma-host interactions, mycoplasmal modulation of the immune response is involved not only in progression of disease and chronicity of infections, but also in the inflammatory, immunopathologic reactions of massive cell proliferation and chemotactic phenomena (84). The lymphoid proliferation seen in most mycoplasma respiratory diseases, the accumulation of macrophages, and the presence of neutrophils for long periods of time suggest that most of the lesions seen in mycoplasma respiratory disease are the result of a frustrated host immune defense. It is well-known that specific anti-mycoplasmal humoral and cellular immune responses are often only partially effective, thus leading to a chronic persistent infection (128). So far it is evident that the immune mechanisms operating during mycoplasma infections are complex and multifactorial. The immune mechanisms between mycoplasmas and the host immune cells depend on both the mycoplasma species and the infected host. The first step taken to elucidate the mycoplasma-induced immune response is to identify and purify the specific mycoplasma epitopes involved. To clarify the mechanism responsible for immune modulation, further studies with purified mycoplasma suppressive and mitogenic components should be pursued (84).

The main emphasis of current mycoplasmal research can be divided into two general areas: the study of the mycoplasmal membrane and the quest for virulence factors. The most studied virulence factors have been the adhesins of mycoplasmas. The goal of the research presented here is to identify and partially characterize a soluble form of a membrane-bound hemolysin (125, 126), another potential virulence factor. The hemolysins found in other bacteria have proven to be important virulence factors. Therefore, this hemolysin is believed to be associated with pathogenesis and could have virulence potential. In order to understand the importance of this *Mycoplasma pulmonis* hemolysin, a more comprehensive review of the recent literature will be addressed. The pathogenesis of MRM is well understood; the mechanisms that cause disease remain unknown. Thus, many studies have been performed on mycoplasma membranes to examine their structure and identify components that could adversely affect host responses to mycoplasma disease. These mycoplasma components could be responsible for the circumvention of innate immune responses which allow the pathogen to establish disease. One

of these *M. pulmonis* components is the hemolysin. This hemolysin has been documented to have some similarities to Gram-positive thiol-activated hemolysins (79).

Disease pathogenesis of *M. pulmonis*

M. pulmonis is the causative agent of a naturally occurring disease in both rats and mice called MRM. It has also been found to be the causative agent of a naturally occurring genital disease in rats. This naturally occurring genital tract disease in mice has not been reported. When injected intravenously into mice and rats, *M. pulmonis* causes arthritis. While the natural occurrence of both respiratory and genital mycoplasmoses seriously restricts the usefulness of rats and mice for other research purposes, they represent useful models for the study of human disease. There are morphological similarities and similar natural histories of chronic bronchitis, bronchiectasis, and emphysema in man (32). Thus rats and mice are useful models for study of the pathogenesis of chronic pulmonary inflammation (32). The pathologic character of arthritis induced by either *M. pulmonis* or *Mycoplasma arthritidis* in rats and mice is also strikingly similar to that of rheumatoid arthritis of man (43).

The exact relationship of genital to respiratory mycoplasmosis is unknown. Each has been detected in the absence of the other. Both genital disease and respiratory diseases caused by *M. pulmonis* progress slowly, are long in duration, and are sometimes clinically silent. In both diseases, successful colonization can result in genital and respiratory tracts that appear normal grossly but show extensive involvement microscopically. Pathologic diagnosis of these diseases is dependent upon microscopic evaluation of the entire respiratory and genital tracts. A noteworthy feature in both diseases is the inconsistency of the lesions produced in rats of the same age, from the same colony, even in animals from the same cage (81).

There are several inherent characteristics of *M. pulmonis* that contribute to the pattern of genital and respiratory diseases. In order for *M. pulmonis* to cause both the genital and respiratory tract disease, it must adhere to the surface epithelial cells and form an intimate contact with the host cell surface. There has been no evidence that *M. pulmonis* penetrates into the submucosa. Adherence in both diseases is thought to be a generalized interaction of the mycoplasma membrane with the host-cell membrane rather than by a specialized attachment tip (33, 34, 108). In order to study the physical process of attachment of *M. pulmonis* to the host, adsorption of the mycoplasma to red blood cells has long been used as a model for mycoplasma attachment to or colonization of epithelial cells. Minion, et. al. (124) has suggested that attachment of *M. pulmonis* is not a simple attachment event, but is a two step process. This colonization results in a wide variety of cellular changes like epithelial destruction (cytoplasmic vacuolization, disruption of mitochondria), epithelial hyperplasia and metaplasia,

giant cell formation, altered tracheobronchial cellular kinetics, and altered mucus production. *M. pulmonis* can also cause ciliostasis of tracheal organ cultures. This renders the host incapable of clearing inflammatory exudates and explains the large accumulation of exudates in major airways *in vivo*. These effects are probably due to mycoplasma-induced alteration of host membranes, mycoplasma utilization of host cell components, and/or the release of toxic metabolic wastes such as hydrogen peroxide (7, 23, 48). Direct damage to epithelial cells, however, does not explain many of the predominant lesions observed during disease (32). These lesions may result from the direct effects of a mycoplasmal component or be an indirect effect due to the development of host inflammatory and immune responses. Researchers need to determine if the cytopathic effect seen *in vitro* with *M. pulmonis* are the same as those seen *in vivo*. Stadtlander, et al., showed that the damage to the epithelium early after infection is due to the direct effects of *M. pulmonis* on the cells. Differences between two virulent strains of *M. pulmonis* seen *in vivo* were not observed *in vitro* suggesting that host responses (indirect effects) may contribute to these cytopathological events. The indirect cellular injuries that are caused by mycoplasmas may better explain these lesions. Since these diseases are chronic in nature and *M. pulmonis* persists for long periods of time, the prolonged accumulation of neutrophils and the release of their lysosomal enzymes could contribute to the severity of the lesions (109). Alternatively, the *M. pulmonis* induction of large amounts of hydrolytic enzymes from macrophages (203) could also contribute. Mycoplasma mitogenicity may be an important factor in disease. The release of lymphokines from nonspecific activation of lymphocytes could explain the lesions observed.

In the 1970's it was shown conclusively that *M. pulmonis* alone could reproduce all of the characteristic clinical and pathological features of the natural respiratory disease MRM. Offspring of infected mothers acquire the organism vertically (from parent to offspring *in utero*) (30) and/or as a result of aerosol transmission in the first weeks of life (horizontally) (34). A slowly progressing respiratory disease is initiated and may resolve or persist for life. These animals then serve as reservoirs of infection, readily transmitting the organism via airborne transmission to cagemates or between adjacent cages (81). *M. pulmonis* quickly overcomes the mucociliary defense mechanisms in the respiratory tract of the host and attaches to the surface of the epithelial cells (108). The preferred sites of natural *M. pulmonis* infections in rats are the nasal passages and middle ears. Thus, the upper respiratory tract then serves as the reservoir of infection from which *M. pulmonis* may be transmitted to other animals or other tissues within the same host (e.g., the lower respiratory tract and/or the genital tract). MRM is a clinically silent infection (108), except for the terminal stages of disease when weight loss, roughened hair coat, nasal and ocular discharges, dyspnea, snuffling, and rales are seen. There

is little correlation between clinical signs and pathologic alterations. The principal gross lesions seen in MRM are acute and chronic rhinitis, otitis media (both of the upper respiratory tract), laryngotrachietis, bronchopneumonia, and bronchiectasis (all of the lower respiratory tract). The upper respiratory tract diseases can exist in the absence of lower respiratory tract disease (36). Ali of these lesions are characterized microscopically by lymphoid hyperplasia and chronic inflammation (33). In general, there are three steps that need to occur as a prerequisite for mycoplasmal disease. These are intimate attachment to the host cell surfaces in order to derive nutrients, the elicitation of an immune response that is both beneficial and detrimental to the host, and induction of chronic infection as a consequence of the mycoplasma's ability to evade or block immunological attack (32). The lymphoid hyperplasia seen in rats is lymphocytic in nature whereas in mice it is plasmacytic in nature. The basic pathology of MRM is well known, but the remaining question is the actual mechanism(s) by which *M. pulmonis* causes chronic disease.

The lesions of MRM have been found to be enhanced by a variety of factors. Complex interactions between host, organism, and environment occur. There are several environmental factors that influence the progression and severity of respiratory disease. These include ammonia (NH_3) (26, 110, 173) presence of sulfur dioxide (102) the administration of hexamethylphosphoramide (141), and the levels of vitamins A and E (208). Another factor that influences MRM is microbial synergy like that seen with *Pasteurella pneumotropica* (24) and Sendai Virus (72). The age of the host (81) also has an effect on the progression of MRM. The last, but most critical determinant of susceptibility to MRM is heredity or genetic factors. There are striking differences seen among species (130, 137) and among strains of the same species.

Unlike with MRM, very little is known about disease pathogenesis and immunogenesis of genital tract disease caused by *M. pulmonis*. Most of the research done on *M. pulmonis* genital tract disease has used a rat model since natural genital tract disease has not yet been reported in mice (36). Female rats acquire genital tract disease early in life and sometimes prior to coitus. One mode of transmission could be due to coprophagia. Females harboring mycoplasma in the upper respiratory passage transfer the infection to the vagina. Another possible mode of transmission could be via the hematogenous route because Juhr (83) has demonstrated a septicemic phase in respiratory disease caused by *M. pulmonis*. Colonization is primarily in the lower genital tract (the vagina and cervix). Here *M. pulmonis* adheres to both squamous and nonsquamous epithelial cells. The predominant gross lesions of the genital tract are peri-oophoritis and salpingitis. These lesions are microscopically characterized by neutrophil exudates in the oviduct lumen, hyperplasia of the epithelium, and lymphoid infiltration into the submucosa. Uteri are usually normal upon gross examination. Occasionally seen are an

underdeveloped or partially resorbed fetus. The lesions of the uteri can range from mild metritis to marked pyometra and endometritis. Epithelial changes seen include hyperplasia, squamous metaplasia, and polyp formation. Genital tract disease in males is much less frequent. In 5% of the males bred with infected females, *M. pulmonis* could be isolated from the urethra, vas deferens, and the epididymis. Genital tract infection with *M. pulmonis* results in both a reduction in fertility and litter size. Studies of this naturally occurring genital disease in rats will hopefully decipher the role of human genital disease caused by *Ureaplasma urealyticum*.

The inability to clear the infection seems to be linked to the inability of the immune response to attack organisms already established on the respiratory epithelium (36). The lymphoid proliferation, accumulation of macrophages, and the presence of neutrophils for long periods of time suggest that most of the lesions seen in *M. pulmonis* diseases are the result of a frustrated host defense (32). There are several ways that mycoplasmas could evade the immune response. Mycoplasmas preferentially reside between the cilia on respiratory epithelial cells and thereby avoid the mucociliary clearance mechanism. There are also at least three general mechanisms by which mycoplasmas can circumvent or block the host immunological defenses: by delaying or preventing antigen recognition (antigenic variation), by altering immune regulatory mechanisms (mitogenesis), or by evasion of effector mechanisms (disguising themselves with host antigens or through the release of soluble antigens and the formation of immune complexes). Mycoplasmas lack cell walls and invoke the host's immunological defense mechanisms, yet they survive an extracellular existence inside the host for extended periods. In the face of an intense immune response *M. pulmonis* can survive for months and even years in the infected host (82). Until this unusual capacity is understood, the most important feature of mycoplasma pathogenicity will remain an enigma.

The immune response generated against mycoplasma is a major determinant of the pathogenic outcome of MRM. Because of this, the interactions or effects of *M. pulmonis* on the immune system are being studied extensively. The immunopathological effects caused by *M. pulmonis* are beginning to take form. The first thing that occurs after *M. pulmonis* infection is the infiltration of mononuclear cells into the submucosa. Killed or live *M. pulmonis* elicit chemotactic responses that specifically recruit circulating lymphocytes. This property is not linked to polyclonal activation, although the two mechanisms may act synergistically. The next step is the development and expansion of the lesions. There are various hypotheses regarding the type of immunological response involved at this stage in disease pathogenesis such as delayed type hypersensitivity or polyclonal activation of lymphocytes during disease. The last process that occurs is the resolution of lesions and includes an inhibition of lesion development from subsequent challenge. It is most likely that specific immune responses to *M. pulmonis* are

responsible for this process. Specific immune responses may act by lowering the numbers of organisms and controlling tissue localization or by inhibiting pathogenic interactions between the organisms and host cells. The severity of the chronic lesions may be influenced by the level of specific immunity present. Several investigators have suggested that pathologic lesions produced in mycoplasma infected hosts are immune injuries (127), but it not yet clear what role is played by these interactions between mycoplasmas and host lymphocytes in either defense or pathogenicity (127).

The hallmark of *M. pulmonis* -induced respiratory disease is the variable susceptibility of infected animals and the inconsistency of disease progression and lesions. The primary reason for this is the organism's variability and the complicated factors encountered in the respiratory and genital tracts of the infected host. The known pathogenic mechanisms are inadequate to explain *M. pulmonis* disease. In order to understand the role of host defenses in mycoplasmal disease, one must identify the membrane antigen(s) or protein(s) responsible for the interactions observed and define the mechanism(s) involved in the mycoplasma-invoked immune responses. Specific mycoplasmal proteins and epitopes involved in inducing and evading the immune response are only beginning to be understood.

Mycoplasma membranes

Mycoplasmas are unable to modify cholesterol and long-chain fatty acids, cannot carry out pinocytosis, and lack a cell wall and intracellular membranous structures. They are unique in that they contain only one membrane, the plasma membrane. This membrane is a single cholesterol-containing phospholipid bilayer which contains a variety of proteins and carbohydrates. Its function is to separate cytoplasmic components from the environment, provide shape, and prevent lysis under changing environmental conditions. Since it is the only membrane in the cell, the mycoplasma membrane is rich in enzymatic activities as well as in specific carriers for the transport of nutrients through the membrane. The plasma membrane contains all of the cellular lipid and, because these cells are small, a substantial fraction of the total cellular protein as well.

Because of their simplistic structure, mycoplasma membranes have been used in many ways to study the general structure and function of membranes. species have been used extensively in the study of membrane structure. The ease of isolation of *Acholeplasma* membranes and the ability to introduce controlled alterations in their membrane composition directly contributed to development of fluid-mosiac model of membrane structure (155). One benefit is that mycoplasma membranes can be purified free of contamination with other membrane types. Since mycoplasmas are partially or totally incapable of synthesizing long-

chain fatty acids and cholesterol, their membrane composition is dependent on the fatty acids derived from the growth medium. This ability to introduce controlled alterations in the fatty acid composition and cholesterol content of mycoplasma membranes has been utilized in studying the molecular organization and physical properties of biological membranes. Razin (153) provides a review on the history behind the progression of mycoplasma membrane research. Reviews on recent mycoplasma membrane research can be also be found in Rottem and Kahane (168) and Maniloff (117).

General composition

The gross chemical composition of mycoplasma membranes is similar to those of other prokaryotic plasma membranes. It consists primarily of proteins (about 60-70% of the membrane mass) and lipid (about 20-30%) with little carbohydrate and small amounts of cytoplasmic contaminants (RNA and DNA) (120). Considerable quantities of Mg^{2+} and small amounts of Ca^{2+} are associated with mycoplasma membranes and probably play a role in the stabilization of the lipid bilayer and the binding of peripheral membrane proteins. Large variations in lipid/protein ratios can be induced by variations in culture conditions, particularly by changes in the type and amount of exogenous fatty acids or sterol added to the growth medium.

The physical properties of mycoplasma membranes have been studied by a variety of physical methods: differential scanning calorimetry, differential thermal analysis, X-ray diffraction, EPR spectroscopy, NMR spectroscopy, light scattering, and fluorescence polarization. The majority of these physical studies firmly established that the bulk of membrane lipids in mycoplasmas constitute a lipid bilayer (fluid-mosiac model). There is a definite transbilayer asymmetry of the mycoplasma membrane with regard to the distribution of membrane phospholipids and glycolipids (121, 151, 167). Cholesterol was found to flip-flop from the outer to inner halves of the lipid bilayer thus leading to an equal distribution in the bilayer (19, 38). These studies are more comprehensively reviewed in Rottem and Kahane (168) and Maniloff (120).

Lipids

Mycoplasma membranes are composed of relatively few lipid species. Several unique lipids occur, most of which are derived from the culture medium and represent absolute nutritional requirements. Some are synthesized *de novo* (188) from fatty acids obtained from the growth medium. The lipid species found are as diverse as the genera and species that make up this class of Mollicutes. The lipid content of the membrane varies between the various species and

depends on the growth phase and growth medium. Lipids are located exclusively in the cytoplasmic membrane and constitute 25-35% of the dry weight of the membrane (168). *Acholeplasma* membranes differ from those of *Mycoplasma* species in their high content of glycosyldiglycerides and phosphoglycolipids.

The major membrane lipids of mycoplasmas are carbohydrate-containing lipids (glycolipids and glycophospholipids), phospholipids, neutral lipids, and sterols. The major endogenous membrane polar lipids in most mycoplasmas are the acidic glycerophospholipids, the neutral glyceroglycolipids, and a wide variety of other minor lipids may be present as well. Glycolipids are notably absent from many mycoplasma species, but when they are present, specifically in *A. laidlawii*, they constitute a significant portion of the membrane lipid. In *A. laidlawii*, the glycolipids are predominantly monoglucosyldiglyceride, and diglucosyl–diglyceride. These carbohydrate-containing membrane lipids are exposed on the external surfaces of several mycoplasma species. In most mycoplasma species, the exogenous phospholipids are incorporated unchanged from the growth medium. In common with most other prokaryotes, the principal *de novo* synthesized phospholipids of mycoplasmas are the acidic glycerophospholipids phosphatidylglycerol and diphosphatidylglycerol. Unlike most eucaryotes and prokaryotes, the ubiquitous aminophospholipids phosphatidylethanolamine or phosphatidylserine are almost completely absent from mycoplasmas. In *Mycoplasma*, not *Acholeplasma*, significant amounts of phosphatidylcholine and sphingomyelin, as well as free and esterified cholesterol from the growth medium, are incorporated into the cell membrane. The neutral lipids are found in small amounts in all mycoplasmas. Neutral glycolipids predominate in *Acholeplasma* species whereas the acidic phosphatides predominate in *Mycoplasma* species. Most mycoplasmas, except for acholeplasmas, are defective in their fatty acid biosynthesis. They cannot synthesize saturated or unsaturated fatty acids, or alter the chain length of either. Thus, most of their fatty acids are supplied to them in their growth medium.

Another type of lipid, a conjugated lipid, has been identified in some, but not all mycoplasma species. This type of lipid, called a lipoglycan, is composed of polysaccharides covalently attached to lipids. These lipoglycans are surface structures that are associated exclusively with the cytoplasmic membrane. Many studies have been done to compare these lipoglycans with the bacterial lipopolysaccharides (186). Lipoglycans are distinguished from Gram negative bacterial lipopolysaccharides in molecular structure (no lipid A backbone) and biological effects on mammalian cells and intact hosts. The lipoglycan identified in *M. neurolyticum* has been found to be mitogenic. Lipoglycans cannot play a role in either the mitogenicity or the Ia-inducing activity of either *M. arthritidis* or *M. pulmonis*, since these organisms have been shown to be devoid of lipoglycans. The lipoglycan of acholeplasmas has

toxicity for mammalian cells. High concentrations of lipoglycan results in hemolysis. The lipoglycans can modulate the immune response of hosts to both soluble and particulate antigens. Despite all of these interactions with eukaryotic cells, the exact role of lipoglycans in mycoplasma cells is not known.

Cholesterol

The members of the class *Mycoplasmataceae* and *Serpulina hyodysenteriae* (189) are the only prokaryotes that require cholesterol for growth. Cholesterol concentrations can reach up to 50 mol% of the total membrane lipids in mycoplasma species, which is comparable to the levels found in plasma membranes of eukaryotic cells. The non-cholesterol requiring acholeplasma has a much lower cholesterol level (up to 10 mol%) (168). None of the mycoplasmas tested so far, including the non-sterol requiring species, are capable of cholesterol synthesis nor or do they metabolize or modify the sterol molecule. Serum lipoproteins (38, 62, 158, 184) are the ordinary donors of the unesterfied cholesterol incorporated from the growth medium. In contrast to mammalian cells, where cholesterol can be synthesized *de novo* or taken up by receptor-mediated endocytosis, mycoplasmas must absorb all of their cholesterol by processes which take place exclusively at the outer surface of the plasma membrane (168). Razin et al. (158) showed that *Mycoplasma* and *Spiroplasma* species incorporate phospholipids along with free and unesterfied cholesterol from the serum lipoproteins. Since mycoplasmas do not use endocytosis or pinocytosis to incorporate cholesterol, they have devised another mechanism to incorporate the cholesterol. The mechanism by which cholesterol is transferred from the serum lipoproteins to mycoplasma membranes involves a simple exchange process which does not involve the adherence or fusion of the particles with the membrane. No significant degradation of the lipoprotein molecule is seen in this process (62, 63, 184). Mycoplasmas, but not acholeplasmas, are thought to have protein receptors for cholesterol (63).

Cholesterol is the major sterol found in all species of mycoplasmas, spiroplasmas, and ureaplasmas. Several different sterol analogues of cholesterol, however, can support mycoplasma growth although they provide a limited degree of functionality as a sterol substitute. In order to permit growth, these sterols must contain a planar nucleus, a free hydroxyl group at the 3β-position, an amphipathic nature, and a branched, aliphatic hydrocarbon side chain at least 8 carbons long. Any steroid containing an aliphatic side chain can be incorporated, but only planar 3-hydroxyl sterols permit growth. These structural requirements for cholesterol lead to an unique orientation of cholesterol in the mycoplasma membrane. Its rigid ring system will be aligned parallel to the hydrocarbon chains of membrane

phospholipids. The phospholipid content of the membrane and their physical state influences the amount of cholesterol taken up (148, 149). The polar hydroxyl group anchors one end of the cholesterol molecule to the polar surface of the bilayer. Extension of the planar hydrocarbon toward the center of the bilayer exerts a condensing effect on lipids at temperatures above the phase transition temperature. At temperatures below the phase transition temperature, cholesterol will prevent the cooperative crystallization of the hydrocarbon chain (166). The hydrophobic interactions between cholesterol and the phospholipids act to order fatty acyl chains above the phase transition and disorder those below the phase transition. This causes the thermal phase transitions to broaden.

By adapting *M. mycoides* subsp. *capri* to grow with minimal amounts of cholesterol (169), one of the primary mechanisms that cholesterol plays in the membrane was discovered. These studies provided the first solid proof that cholesterol functions as a regulator of membrane fluidity (152). Cholesterol has a profound effect on membrane physical properties such as membrane lipid ordering and permeability, condensation of membrane phospholipids, lateral diffusion of phospholipids, lateral phase separations, and phospholipid phase transitions. The incorporation of large quantities of sterol may be necessary to prevent membrane lipids from crystallization at their optimal growth temperatures. Thus, cholesterol creates an intermediate fluid state of the lipid bilayer supporting the function of key membrane enzymes such as the membrane-bound ATPase. Cholesterol helps to overcome the mycoplasmas's inability to control membrane fluidity by fatty acid and complex lipid biosynthesis. Besides altering the thermodynamic properties of lipid bilayers, cholesterol also acts to reduce the membrane surface area by "condensing" the phospholipid molecules and serves to alter bilayer thickness. This architectural/bulk function influences the membrane physical state and is one of the dual roles of sterols in mycoplasma membranes. The other role is regulatory in nature and is fulfilled by minor amounts of sterol which has no measurable effect on bulk physical membrane properties. Apart from the bulk function, cholesterol may act in microdomains to regulate specific metabolic processes (sterol synergism). Also, cholesterol may bind to specific membrane proteins and affect their function (12, 49). Cholesterol may have a role in potassium transport. What distinguishes the regulatory role of sterol from the bulk role is its structural specificity. The bulk role can be fulfilled by a broad spectrum of sterol structures whereas a small amount of cholesterol fulfills a more specialized regulatory role. Despite intensive investigations on sterol-phospholipid interactions in the last decade, the exact role of sterol in mycoplasma membranes remains elusive (168).

Proteins

Protein is a major component of the mycoplasma membrane. The electrophoretic pattern of mycoplasma membrane proteins are reproducible and species-specific. The advantage of these electrophoretic patterns is that they are not significantly affected by variations in *in vitro* growth conditions unlike lipids. The disadvantage of these electrophoretic patterns is that some exogenous proteins (serum proteins from the growth media) are tightly bound to the membrane and may easily be confused with true membrane proteins. Metabolic radiolabeling is an effective way to discriminate cell proteins from exogenous contaminants in mollicutes. The high ratio of membrane lipid to total protein in mycoplasmas is greater than that found in other prokaryotes. This increases the difficulty of purifying mycoplasmal membrane proteins for structural and functional analysis. Many of these proteins are anchored to the mollicute membrane by fatty acid tails. The role of protein membrane components in mycoplasma pathogenicity has become one of the important issues in mycoplasma research.

Essentially two groups of mollicute membrane proteins have been studied. The first group consists of adherence or antigen-variable proteins of the pathogenic species *M. genitalium, M. hyorhinis*, and *M. pneumoniae*. The second group consists of structural proteins and enzymes of *A. laidlawii*, *Spiroplasma* species, and *M. capricolum*. Similar to other biological membrane proteins, the molecular weights of the mycoplasma membrane proteins range from 15 to over 200 kilodalton (kDa). Two-dimensional gel analysis revealed that most mollicute membrane proteins are acidic with pI values ranging from pH 4 to 7 (140). The majority of these proteins are exposed on the inner, cytoplasmic side of the membrane. Highly glycosylated membrane proteins appear to be absent in mycoplasmas. Instead, a significant number of the membrane proteins in this organism (~25) are acylated (138).

A prominent feature of several mollicutes is the large number of membrane proteins that contain covalently attached fatty acyl chains. The reason for this acylation is to anchor proteins which may be trapped in the periplasmic space of conventional bacteria firmly to the membrane. These acylated proteins or lipoproteins are exposed on the cell surface and thus act as important cell antigens. They may be the dominant antigens of mycoplasmas. The genes for several lipoproteins have been cloned and sequenced (20). The function of these lipoproteins has not been determined, but they may serve a functional role to enhance membrane stability, as a system for antigen variation to evade immune surveillance, a host adaptation role or assist in maintaining population diversity.

Lipoproteins, along with some unmodified integral membrane proteins, constitute the major components of the mycoplasmal variable antigenic systems. This is a benefit for mycoplasmas since their small genome size permits them to encode and express relatively few genes

compared to other eubacteria while providing for significant antigenic changes. Antigenic variation provides a possible mechanism by which mycoplasmas survive host defenses causing chronic disease. Phase switching of major membrane antigen genes results in a constantly changing surface antigenicity which is believed to provide an effective escape from rapid elimination by the host immune system (215).

Many mycoplasma species display "families" of membrane proteins encoded by multiple, variant genes that vary both in their expression pattern (phase switching) and in their structural attributes (size variation). Three size-variant membrane surface lipoproteins (Vlps) of *M. hyorhinis* (216) have divergent external domains. These domains undergo size variations by loss or gain of repetitive intragenic coding sequences while retaining a motif with a characteristic negative charge. Experimental evidence exists for the occurrence of antigenic variation *in vivo* (60, 61, 198). Studies of the V-1 antigen of *M. pulmonis* has provided some the first evidence of a role in pathogenesis for variable surface proteins. The *M. pulmonis* V-1 antigen has both structurally and antigenically different forms that exist among strains (210). DNA inversion has been discovered as a possible mechanism by which *M. pulmonis* alters the V-1 structure. There is an interchange of the two variable regions with a single conserved region, and this DNA inversion acts as a switch which allows only one of two different genes to be expressed at any given time (183). The precise function of these antigen variation systems is not known. Simmons et al. suggested that this yet-undefined function of V-1 resides in the variable carboxy region of these proteins. The genes encoding V-1 are similar in structure to genes encoding other mycoplasma surface lipoproteins.

The same lipid-modified proteins responsible for antigenic variation may also act as adhesins in some mycoplasmas (217). Adhesins act in concert with other mycoplasma membrane components in adherence to tissues (9, 85, 86, 157). The proteins involved in adhesion have been the main focus of mycoplasmal research since the late 1970's. Loss of adherence by mutation results in loss of infectivity, and reversion to the cytadhering phenotype results in resumption of infectivity and virulence. The best defined mycoplasma adhesins are the 170 and 30 kDa proteins from *M. pneumoniae* and the 135 kDa protein of *M. genitalium* which is closely related to P1 (9, 157). These and other proteins form an attachment tip organelle in these two species. Auxiliary proteins such as the high molecular weight (HMW) proteins of *M. pneumoniae* play a yet to be determined role, presumably as part of a cytoskeleton. This cytoskeleton appears to be involved in the organization of the adhesins and in the terminal tip structure of the other flask-shaped pathogenic species. Most attention has been given to the adhesins and cytoskeletal elements of *M. pneumoniae* and *M. genitalium*. The main components forming the *M. pneumoniae* cytoskeleton, sometimes referred to as the

Triton shell, have been identified and characterized. They include 30, 40, and 90 kDa proteins along with p65, p200, HMW1, HMW3. They are proline rich and have repeat sequences and other motifs characteristic of eukaryotic cytoskeletal proteins. Efforts have been made to localize these proteins in the membrane and to determine their interactions forming the cytoskeletal network (105, 146). These accessory proteins act in concert to facilitate the lateral movement and concentration of the adhesin molecules at the attachment tip organelle. So far, data indicate that the adhesins and other surface components involved in adherence are not directly responsible for the cytopathic effects, but their proximity to the host cell membrane may form a microenvironment enabling other factors to cause the cytopathic process. Investigators have found adhesins in other mycoplasma species including *M. hyopneumoniae* (217) and *M. hominis* (70). *M. pirum* (205) is thought to have an adhesin that shows 26% amino acid homology with the *M. pneumoniae* P1 adhesin.

Mitogens produced by different mycoplasmas are a heterogeneous group of molecules. They are mainly polyclonal B-cell activators (47, 170). Characterization of a few of these B-cell mitogens showed that lipids do not play a part in their mitogenic activity. However, lipoproteins have also been related to mitogenesis. *M. fermentans* and *M. penetrans* (66) have lipoproteins that appear to act as B-cell mitogens. One exception is the *M. arthritidis* superantigen, MAM, which is a potent T cell activator. It has the ability to induce cytotoxic lymphocytes and lymphocyte proliferation depending on MHC expression. It is the most potent mycoplasma activator of the immune system so far characterized. MAM has been purified (8), cloned and sequenced (42). MAM varies from other bacterial superantigens because it has a preference for the 1-Eα chain of the MHC II. The importance of MAM is that it may provide an explanation as to why mycoplasmal chronic infections often appear as autoimmune reactions. None of these mitogens have been purified, but recently a monoclonal antibody to the *M. pulmonis* mitogen has been reported which might facilitate its purification (104).

In addition to the purified membrane proteins listed, many membrane-associated enzyme activities have been described for mollicutes: transport activities for K^+ and glucose; phosphatase; peptidase; thioesterase; nucleases; enzymes in membrane lipid metabolism (150, 185, 187). The peptidases and nucleases are proposed to be involved in the transport of nutrients of lower molecular weight such as amino acids and nucleosides supplied in the growth medium. Several of these enzymes need a specific lipid environment for proper function. Four membrane enzymes of known activity have been purified. Two of them, NADH oxidase and the $Na^+ + Mg^{2+}$ - ATPase, have been purified from *A. laidlawii*. The ATPase and NADH oxidase activities are localized on the inner cytoplasmic face of the cell membrane. These enzymes play a key role in respiration and energy coupling. Two other membrane

enzymes have been purified from *M. salivarium*; aminopeptidase My is a membrane aminopeptidase, and carboxypeptidase My (218 kDa) is an arginine-specific carboxypeptidase present exclusively in nonfermentative mycoplasmas.

The mollicute membrane proteins exhibit the same structural features as established for membrane proteins in general. Besides certain amino acid sequence similarities for a few membrane proteins, no obvious close relationships to the Gram positive relatives of the mollicutes have been found. Apart from their structural and catalytic roles, they also have a major share in the immunological activities of mycoplasma cells.

Immune responses against *M. pulmonis*

As a group, mycoplasmas have a wide range of immunomodulatory effects. The diverse and multiple effects that mycoplasmas have on the cells of the immune system include polyclonal activation of B cells in vivo (213) and in vitro (132) and T cells (45, 133); induction of expression of major histocompatibility complex class I and class II molecules (164, 195); macrophage activation (193); stimulation of cytotoxic or cytolytic activities of macrophages, T cells (47) and NK cells; and induction of the production of cytokines IL-1, IL-2 (11, 115), IL-4, IL-6, interferons, TNF-α (6), and granulocyte-macrophage colony stimulating factor (GM-CSF) (194).

The chronic nature of MRM suggests that the host's immune response is inefficient in clearing *M. pulmonis*. The basis for this inefficiency is not understood. Differences in susceptibilities between strains of rats (52, 57, 58) and mice (56, 171) has been used to give insights into immune mechanisms operative against *M. pulmonis*. Ultimately, it is hoped that examination of how these rat and mice strains differ in their responses to *M. pulmonis* infection will identify the pathogenic mechanisms influencing mycoplasma respiratory disease expression (65).

Most of the studies of the immune response against *M. pulmonis* have involved LEW and F344 rat strains which differ in their severity and progression of MRM. LEW rats suffer a more severe MRM with more severe upper and lower respiratory tract lesions than do F344 rats (52). In contrast, F344 lung lesions begin to resolve after only 28 days post infection. Also the numbers of lung lymphocytes in F344 rats do not increase overall and only restricted lymphocyte classes increase during the first month of infection (58). LEW lesions continue to increase after 28 days post infection along with increased infiltration of all classes of lymphocytes throughout the course of disease (58). One explanation for this difference is that F344 rats and LEW rats differ in their innate abilities to respond nonspecifically (57, 176) and specifically (175) to immunologic stimuli. Another possible explanation is that one of these two

rat strains have an inadequate serum antibody response. Simecka et al., however, showed that infected LEW rats produce at least as much of each class of specific antibody to *M. pulmonis* as do F344 rats (177). Further studies showed that the level of local antibody production alone, like that of serum antibody, is not responsible for the low susceptibility of F344 rats to MRM (176).

Two mouse strains also differ in their susceptibility to MRM; C3H/HeN mice are susceptible while C57BL/6N mice are resistant to *M. pulmonis* infection. C3H/HeN and C57BL/6N mice differed in the severity of chronic MRM in the lower, but not the upper respiratory tract after infection with *M. pulmonis* (29). Both strains of mice developed the same classes of antibody (29). C3H/HeN have a greater increase in inflammatory cells yet they have poor intrapulmonary killing of *M. pulmonis* (53, 142). Also, C3H/HeN mice and LEW rats produce more total IgG following *M. pulmonis* infection than do the resistant C57BL/6N mice and F344 rats (29). Like the rat strains (177), serum antibody does not account for differences in the severity of chronic respiratory disease in different strains of mice. LEW and F344 rats differ in the IgG subclasses contributing to the total IgG response, unlike C3H/HeN and C57BL/6N mice, and disease severity in rats is probably due to differences in regulator T cell function (177).

It is not known what innate host mechanisms are responsible for limiting the continued growth of organisms and thereby maintaining differences in disease severity between these strains of rats and mice. One possibility is that the differences in susceptibility are due to differences in the innate host defenses. C57BL/6N and F344 have more effective innate clearance mechanisms (53, 56, 142). These studies suggest that adaptive immune responses are ineffective in clearing the organism (29). The other possibility is that basic genetic differences between the two strains may predetermine recovery from MRM. In fact, receptors for mycoplasma attachment are under genetic control, suggesting that certain hosts are more susceptible to both specific and nonspecific immune stimulation (67). Lai et. al. used the difference in mouse strains to determine that an autosomal single dominant gene (MP^r) confers resistance to *M. pulmonis* growth in the lungs (100). This gene confers resistance to early stages of infection before the development of significant humoral or T cell-mediated responses to *M. pulmonis* (100).

Innate immunity

Innate immunity is the first defense line mycoplasmas contact as they initiate infection. In the lungs, mucociliary clearance and intrapulmonary killing are the major processes responsible for nonspecific resistance. Alveolar macrophages are the principal mediators of intrapulmonary

killing of bacteria (178). Other components that play a part in intrapulmonary killing are neutrophils or polymorphonuclear leucocytes, nonspecific opsonins such as fibronectin, serum components, and complement, and unidentified fluid factors (178). The chronicity of mycoplasma infections appears to be related to the ability of the organisms to evade these nonspecific defense mechanisms (50, 73). In general the innate responses that occur with mycoplasmal respiratory diseases are the mass infiltration of alveolar macrophages (the predominant phagocyte in the normal, noninflamed respiratory tract) and polymorphonuclear leukocytes (neutrophils), and the persistence of mycoplasma antigen in the lung before the onset of antibody production (119). In vivo studies have shown that several factors affect the innate responses: 1) The strain of mycoplasma (50, 73), mouse (56), or rat (177) used is critical. 2) Different types of phagocytic cells have varying degrees of mycoplasmacidal activity (74). Alveolar and peritoneal macrophages differ in their metabolism, enzyme content, and bactericidal activity. Alveolar macrophages from rats exert their anti-mycoplasma effect regardless of the presence or absence of specific antibody. 3). The presence of NK cells in the lungs is also critical. It has been shown that NK cells are the principal cells responsible for mycoplasmacidal activity in the respiratory tract of mice (50, 95-97, 99). 4) Environmental factors like NO_2 (53, 142) may also play a role.

Complement

Complement has a confirmed role as a noncellular immune defense factor. Complement aids in antibody- and macrophage-mediated killing of mycoplasmas (199, 203). Several mycoplasma species both bind antibody rapidly and directly activate complement through either the classical or alternative pathway as well as interacting with C1 component directly (211). Mycoplasmas are also susceptible to lysis by activated complement (22).

Phagocytes (macrophages and neutrophils)

Neutrophils and macrophages are the main mediators of immunity to mycoplasmas at the onset of infection (119). Some comprehensive reviews detailing the earlier studies on mycoplasma macrophage reaction are (31, 47, 74, 75). Neutrophil-rich exudates are incapable of clearing *M. pulmonis* from the peritoneal cavity of mice whereas macrophage exudates can do so (74). Overall, the fact that mycoplasmas are phagocytosed but still cause disease in antibody deficient subjects suggests that phagocytosis is not an adequate defense mechanism (211).

Mycoplasmas persist in the lung parenchyma despite the presence of phagocytes (73, 201) which has caused an interest in studying the interaction of mycoplasmas with macrophages. Most of the in vitro studies showed that mycoplasmas attach and multiply on the surface of macrophages, and without the addition of specific antibody (67, 201), the macrophages were incapable of ingesting mycoplasmas (199). *M. pulmonis* can also induce the release of hydrolytic enzymes from macrophages (203). Several experimental techniques like immunoflourescence (109), and recently colloidal carbon clearance from the lungs (87), have been developed to observe the in vivo effects of mycoplasmas on the phagocytic activity of macrophages in rats and mice. Recent in vivo studies contradict these in vitro studies, and suggest that specific antibody is not necessary for killing of mycoplasmas in the lung (142).

A recent review discusses the stages of phagocytosis that could potentially be avoided by mycoplasmas and thus explain why phagocytosis is not a protective defense mechanism in mycoplasmal infections (119). In vitro experiments have shown that in the absence of opsonins, *M. pulmonis* attaches to the surface of macrophages but is not ingested. During this first stage of attachment and engulfment, specific attributes of mycoplasmas offer multiple explanations for escape. Mycoplasmas can avoid this first stage by their filamentous morphology (118) or by their possession of antiphagocytic proteins on their membrane. For *M. pulmonis* this resistance to ingestion is trypsin-sensitive suggesting the presence of such a surface protein. Also, their capsular material could mask their high hydrophobicity and thus decrease phagocytosis. Additionally, several mycoplasmas have the ability of binding the Fc portion of immunoglobulins (3, 182). The most obvious reason for inefficient phagocytosis is localization of mycoplasma between cilia and cytoplasmic processes. This makes them inaccessible to most phagocytes. The second stage that mycoplasma can affect phagocytes is in the phagosome. They can affect the integrity of the phagosome membrane by causing phagocyte metabolism and activation or respiratory burst (119). Mycoplasmas do this by producing proteases, lipases and phospholipases (13), and oxygen radicals (7). The third stage at which mycoplasmas can have an effect is the phagosome-lysosome fusion. This process could be impaired by the production of ammonia by mycoplasmas. The last, most unlikely scenario is that mycoplasmas may just survive phagocytosis (211) and escape into the cytoplasm through an unknown mechanism. Evidence so far does not prove whether phagocyte activation is induced by mycoplasmas directly or indirectly via cytokine production. Alveolar infiltration of macrophages and polymorphonuclear lymphocytes have been observed in mice injected with live, killed, or membrane preparations of MP suggesting that mycoplasma antigen alone may be sufficient to attract phagocytes to foci of infection thereby stimulating inflammatory responses (35, 176). Stuart et al. showed that *M. arginini* and *M. arthritidis* act

on bone-marrow derived (BMM) macrophages by indirectly stimulating the production of soluble factors (194).

In addition to all of these direct effects of mycoplasmas have on macrophages, there are several nonspecific effects mycoplasma exert. Interestingly, all of these mycoplasmal nonspecific effects can be seen also on B cells. These include mitogenic and chemotactic effects, and induction of class II MHC. Although most mycoplasmas stimulate primarily B cells, mycoplasma species differ in their ability to induce proliferation of BMM macrophages (194). *M. pulmonis* did not induce proliferation of BMM macrophages. Ross et al (165) demonstrated that *M. pulmonis* membrane preparations possess a potent chemoattractant activity for peritoneal and alveolar macrophages from fresh rat serum. These studies have been corroborated by Meier et al. (122) and Komatsu (91). Some species, including *M. pulmonis*, have been shown to induce Class I and II MHC surface expression on a variety of cell types including primary bone marrow macrophage cultures (193), and cultures from several different murine and human macrophage cell lines (193). This factor has been designated MIaF (mycoplasma-Ia induction factor) (193). MIaf could be a membrane component or a secreted factor consisting of a protein, glycoprotein, lipoglycan, or lipoprotein. It is resistant to heat treatment like some mitogens of mycoplasma species (47). The mechanism(s) responsible for the increase in MHC expression is not known. Evidence shows that it probably does not involve either the production cytokines IFN-γ, IL-4 or GM-CSF (192). The induction of MHC expression in macrophage cell lines and cultures is due directly to an increase in the transcriptional activity of MHC genes (164, 193, 195). The significance of this event is that it enhances the antigen presenting ability of the cells involved. This could cause Ia expressing cells to be capable of presenting a variety of antigens to host T cells which is not necessarily beneficial because it enhances not only a specific immune response to mycoplasmal antigens but also to host cells expressing elevated levels of Ia. This might result in the inappropriate presentation of self-antigen to T cells and the development of autoimmune disease.

One beneficial consequence of this mycoplasma-macrophage interaction in the host is that certain mycoplasma species can cause macrophages to be tumoricidal (28, 114, 197). It has been shown that activated macrophages maintain the ability to specifically recognize and lyse tumor cells in vivo. *M. gallisepticum* in concert with IFN-γ causes macrophages to be tumoricidal (197). The macrophage activating factor, Mf-B, is stable biochemically and has been shown not to be a protein (197). A purified highly hydrophobic active component from *M. capricolum's* membrane also activates macrophages to tumor cytotoxicity (28). It has also been shown that *M. orale* and *Spiroplasma* activate the tumor killing activity of macrophages.

The mechanism by which mycoplasmas activate macrophages has been shown to be different from that of LPS (174, 196), but little else is known.

Natural Killer (NK) cells

One of the less well-documented, but recent findings on mycoplasmal immunomodulatory effects, is that IFN-γ and natural killer (NK) cells are involved in resistance to *M. pulmonis* infection (95, 99). NK cells are a subpopulation of lymphoid cells which spontaneously lyse a limited number of tumor cells and virus-infected cells in vitro without prior sensitization of the host. Humoral and cellular immunity are inhibited by *M. pulmonis*, but NK function is enhanced within 24-hours after infection (95, 101). Activated NK cells function in resistance to early stages of infection with *M. pulmonis* (95). NK cells of infected mice can directly inhibit colony formation by *M. pulmonis* in vitro and can eliminate viable *M. pulmonis* from the lungs in vivo (99). The most convincing evidence that stimulated NK cells can indeed have anti-*M. pulmonis* activity is the reduced resistance to infection of C.B-17 SCID mice when NK cells and their products were depleted (99).

The mechanism by which NK cells lyse *M. pulmonis* lysis is unknown (99). *M. pulmonis* may augment NK cells in mice by inducing the secretion of interferons and/or interleukin-2 (27, 106, 160). One possible mechanism is that the NK cells secrete IFN-γ on exposure to *M. pulmonis*. IFN-γ secreted from NK cells has the ability to activate mouse macrophages in vitro and in vivo which would lead to macrophage activation and mycoplasmal killing. Another possible mechanism is the association of a protease or lysolecithin, or both, with lower NK activity are directly responsible for the killing of *M. pulmonis*.

Adaptive immunity

Adaptive immune mechanisms are better understood than the innate immune mechanisms, yet the interaction of circulating antibodies, local secretory antibodies, NK cells, and cell mediated immunity (CMI) during mycoplasmal disease is not known. Even the type of immune response, humoral or cellular, that is most effective in the protection against or resolution of the infection is not known (176). Mice and rats resolve the disease process differently (37, 108, 109), and in mice, a humoral response appears to be necessary for the alleviation of the disease process and prevention of future infection. This is supported by the fact that the passive transfer of serum antibodies to mice intranasally or intravenously protected these mice against intranasal challenge with *M. pulmonis* (75, 199, 202). This passive transfer of serum antibodies did not affect rats. Instead, cellular immunity in rats appears to play the major role in

the prevention of disease and the resultant of lesions. Adoptive transfer of immunity by spleen cells, not sera, was protective in rats (34, 75, 98). Although a humoral response can be detected in rats during infection, it appears to have no effect in the progression of disease (58).

Humoral immunity

Antibody could protect against mycoplasma infection by several mechanisms: inhibition of attachment, inhibition of growth or metabolism, opsonization, or promotion of complement killing. One of the major functions of antibody is the conferral of resistance against infectious agents. Recovery from naturally occurring or experimentally-induced MRM results in immunity; reinfection can occur but there is protection against development of subsequent MRM. Also, specific antibody of any immunoglobulin class present in the respiratory tract seems to be capable of conferring resistance to mycoplasma respiratory infections (199). Humoral antibody also serves as another advantage in both humans and animals by preventing dissemination of mycoplasma infections to systemic tissues (177). In the absence of antibody but the presence of complement, it has been reported that neutrophils or PMNL's phagocytose mycoplasmas and disseminate them to joints where the mycoplasma survives in the absence of antibody. The presence of antibody seems to be prevent this systemic spread of the infection (211).

Systemic antibody responses to mycoplasma have been studied extensively. Serum IgM responses appear first and remain throughout infection (35, 177, 182). Serum IgG responses are usually detected next, but this varies with the mycoplasma, the host, and the route of infection. There is a preference in some animals for the production of a particular immunoglobulin subclass. F344 rats preferentially produce serum IgG2b, but LEW rats do not show a similar preference (175). This preference for a particular IgG subclass has been described for *M. bovis* infections in cattle as well. Regulation and development of IgG subclass responses are probably important in defense mechanisms against many mycoplasma diseases (117), but the development of serum antibody does not result in recovery from chronic lung disease in mice or rats (29).

Since serum antibody is ineffective in clearing mycoplasma infections, the local immune mechanisms may play a greater role in the resistance to mycoplasma infections of the respiratory tract. Secretory immunity serves to protect the mucosal surfaces from disease by inhibiting adherence of bacteria to epithelial surfaces and by neutralizing bacterial products such as toxins and enzymes (190). Antibody-producing (plasma) cells are present in the lungs of

animals infected with *M. pulmonis* (35). In rats, the upper respiratory nodes (URN) are the initial and major sites of antibody production throughout the course of disease (180, 181).

Many studies have included experimental designs to examine both systemic and secretory responses. These studies showed that IgG antibody is frequently the predominant class in lung washings, particularly following systemic and combined systemic and local routes of antigen presentation. Even following local infection only, IgG is a major component of the local response (35, 200). Rose and Cebra (162) showed that B cells display an isotype commitment of IgG1 after different modes of mucosal stimulation with *M. pulmonis*. This is an isotype that is not ordinarily predominant after mucosal stimulation. Chronic stimulation with *M. pulmonis* lead to an increase of antigen-specific B cells in all lymphoid tissues (163). The majority of T-dependent clones generated in vitro by primed B cells in vivo secrete exclusively IgG1 (163). Thus, it appears that IgG1 is the predominant isotype expressed by antigen-specific B cells after chronic mucosal stimulation or stimulation via other routes in normal and athymic mice. This predominance of IgG1 secretion by clones from *M. pulmonis* primed B cells must be due, at least in part, to the peculiar stimulatory effect of the *M. pulmonis* antigens on specific B cells. The majority of *M. pulmonis* specific B cells are $sIgG1^+$ and $sIgD^-$ (163). To complicate the situation, recent articles have showed that some mycoplasmas have Fc receptors (3) and contain IgA proteases. In summary, antibody responses play a limited role in the host's recovery from infection (182) since even in mice the production of local and systemic antibodies does not prevent the chronic nature of MRM.

Cell-mediated immunity (CMI)

Since humoral immunity cannot prevent the chronic nature of MRM, studies on cell-mediated immunity (CMI) have been performed to determine what effects it has on MRM pathogenesis. It has been shown that *M. pulmonis* functions as both a T-dependent and T-independent antigen (162). CMI has three main functions: 1) T cell proliferation and elaboration of mediators in response to specific antigens. Both IL-2 (106) and IFN (192) production are induced by *M. pulmonis* - demonstrating that T cells are involved; T cells proliferation and differentiation into specific cytotoxic killer cells; and 3) T cells serve as helper or suppressor cells regulating both T-cell functions and B-cell antibody production (75). The role of T-cell subsets in pathogenesis needs to be explored, particularly in relationship to resistance to mycoplasmal disease. A predominant feature of the histopathology in many mycoplasma infections is an accumulation of lymphoid cells, the majority of which contain immunoglobulin (35). In F344 rats infected with *M. pulmonis*, the proliferating lymphocytes

appear to be mainly T cells and null cells (55, 58). Differences in the proportion of T_H cells in rats appears to be related to the severity of *M. pulmonis* lung disease (57); there is a greater proportion of T_H cells found in susceptible strains of rats. To further determine the effects of T lymphocytes during *M. pulmonis* infection, athymic nude mice were used to assess the chronicity of induced arthritis (89). It was concluded that the T-cell element of the immune response to *M. pulmonis* was important in both the prevention of and recovery from disease since impaired T-cell function predisposed the mice to a severe degree of chronic arthritis as a result of their failure to eliminate the causative organisms (89). Rats and mice that are deficient in T cells also develop less severe respiratory disease than do immunocompetent animals after infection with *M. pulmonis* (59). To develop a full-fledged interstitial pneumonia, one requires the participation of T cells and their products (106, 131).

Although numerous investigations have addressed the question of host defense in mycoplasmal infections of the respiratory tract (131, 177), none as yet has defined the essential mechanisms involved in protective immunity against these organisms. Ongoing studies are evaluating whether lymphocyte responses play a protective role in preventing dissemination of *M. pulmonis* to extrapulmonary tissues and death, or whether lymphocyte responses contribute to disease severity and clinical symptoms. The humoral response could contribute to disease pathogenesis through the development of hypersensitivity responses or the deposition of immune complexes (182). The high serum IgG responses within C3H/HeN mice and LEW rats correlated with more severe lung disease. This specific immune response contributed to disease by an unknown mechanism (29). Cartner et. al. (29) suggested that effective innate host defenses are more important than the adaptive immune responses.

Nonspecific interactions with host defenses

Nonspecific effects of mycoplasmas on B and T cells

Mycoplasmas are able to modulate immune responses nonspecifically. One of the ways they accomplish this is through polyclonal activation of lymphocytes. Lymphocytes react with mycoplasmas in a variety of ways. Both stimulation and suppression can occur as a result of antigenic-specific or mitogenic (nonspecific) responses by the immune cells involved (1, 2, 67, 101, 170). It is well-documented that mycoplasmas and or substances isolated from mycoplasmas have the potential to activate lymphocytes from their original host as well as lymphocytes not from their original host (14, 16, 17, 40, 132). A wide variety of pathogenic and nonpathogenic mycoplasmas have these mitogenic properties (137). The subpopulations of lymphocytes (B cells, T cells, or both) that are polyclonally activated also vary with species

(14-16, 127, 129, 133-135, 213). In addition, different mitogenic capacities are exhibited by various strains of the same mycoplasma species tested in parallel (137). These mycoplasmal mitogens not only differ in the lymphocyte subpopulation(s) they activate, but also in the resistance of their mitogenic activity to heat treatment (16, 40, 69, 135, 137). The majority of these mitogens identified cause polyclonal activation of B cells (132, 137). The B cell blasts generated mature into terminally differentiated plasma cells secreting immunoglobulins of specificities not related to the stimulating agent. Nonspecific T cell activation by mycoplasma is less common (67). *M. arthritidis* mitogen (MAM) is the most well-characterized mycoplasma T-cell mitogen (42). Even though mitogenesis is nonspecific, the regulation of these organism-host interactions is not random (42, 67). The control mechanisms for lymphocyte activation by MAM are well described (41, 44-46) and those for the *M. pulmonis* mitogen(s) are beginning to be understood.

The original report by Ginsburg and Nicolet (69) proved that in vitro-cultured rat lymphocytes were stimulated by *M. pulmonis*. Later it was shown that *M. pulmonis* has mitogenic activity towards both rat B- and T-lymphocytes. *M. pulmonis* also induces the development of antibody producing cells (APC) (133). *M. pulmonis* has a stronger effect upon B cells and less so on T cells (133). Only a portion of the lymph node T-cell population responding to either ConA or PWM is activated by *M. pulmonis*. The biochemical characterization of *M. pulmonis* showed its mitogenic activity was abolished upon heating (135). Whereas the mitogens of *M. arthritidis, A. laidlawii*, and *M. pneumoniae* were heat stable (127). Outer surface proteins appear to be major constituents of the *M. pulmonis* mitogenic factor(s) (127). Membrane carbohydrates, but not lipids, may also be involved in the mitogenicity of *M. pulmonis* (127, 135). It is still not certain whether the same membrane mitogenic factor(s) are responsible for the activation of both B and T cells. Different mitogens within the membrane may stimulate different subpopulations of lymphocytes (127). Mitogenicity (as defined by in vitro assays) (181, 213) of *M. pulmonis* resides within its membranes, but in vivo tests of mitogenicity (57, 129, 131) are the ones that will indicate a correlation of mitogenicity and pathogenicity. Naot et al. used nonviable *M. pulmonis* cells to show that nonspecific activation of T cells was a major cause of the pneumonia seen in MRM, but they failed to show a clear role of B cell activation in *M. pulmonis* pathogenesis (129). Nonspecific activation and recruitment of B cells occur during the development of MRM (181). Its possible that the mitogenic and differentiative activities of *M. pulmonis* surface proteins may promote nonspecific proliferation and differentiation to antibody-producing plasma cells in vivo (165).

Mitogenic stimulation of host lymphocytes by *M. pulmonis* membrane mitogens is believed to be a major factor in the development and severity of lung diseases produced by this organism in rats (129). The crucial question is whether or not nonspecific, polyclonal lymphocyte activation plays any role in mycoplasma disease (131, 178). Evidence supporting the role of mitogenesis in *M. pulmonis* pathogenesis is the following: 1) Rat strains which give the strongest mitogenic response to *M pulmonis* develop the most severe respiratory lesions. LEW rats (the more susceptible rat strain) develop greater in vitro lymphocytes responses to *M. pulmonis* mitogens than do F344 rats (57, 176). 2) Administration of mitogenic *M. pulmonis* membranes induces pulmonary lesions characterized by lymphoid infiltrates predominantly in the alveolar region rather than in the bronchial regions, as occurs with natural disease (129).

There is also evidence that it is unlikely that all of the phenomena seen in mycoplasma infections are directly related to this nonspecific lymphocyte activation (214). The role of nonspecific activation by *M. arthritidis* in disease pathogenesis remains unclear. The ability of *M. pulmonis* to directly activate polyclonally and cause proliferation and differentiation of B lymphocytes may play a role in the large number of B cells and plasma cells seen in lung lesions of MRM. There are alternative hypotheses, however, describing the purpose or consequences of this mitogenic effect of mycoplasmas. For instance, polyclonal activation could stimulate the development of autoimmune disease. Another potential consequence of polyclonal activation is altered immune responses to non-cross reacting antigens resulting in the generation of antibody responses to unrelated antigens (181, 213). Both enhancement and suppression have been documented following infection with *M. pulmonis* and *M. arthritidis*. Suppression of responses to unrelated antigens may be related to disruption of immunoregulation (31). The mechanisms and consequences of this polyclonal activation have yet to be defined. It is still controversial whether this activation of host immune cells leads to the development of specific autoimmunity or whether it represents simply an aggravated inflammatory response (193).

In addition to activating lymphocytes, *M. pulmonis* is capable of producing chemotactic factor(s) for B cells and macrophages (165). This is the first description of a bacteria-derived chemoattractant for B lymphocytes. The chemotactic activity of *M. pulmonis* may explain one of the most prominent pathological signs seen in a the majority of chronic mycoplasma infections, the infiltration of large numbers of mononuclear cells. This chemotactic ability could provide an explanation as to why B cells form an early persistent component of the lesions of MRM (165). The chemotactic factor is sensitive to trypsin and protease treatment (165). Currently it is thought that this activity is mediated by surface membrane proteins of *M. pulmonis* because the major variable surface antigen of *M pulmonis*, V-1 (210), possesses this

in vitro B lymphocyte recruitment activity to a marked degree. This chemotactic factor is resistant to heat treatment and solubilization, unlike the *M. pulmonis* mitogen. Thus, chemotaxis and mitogenesis are not necessarily linked to the same mycoplasmal component. One model would specify that the role of the chemotactic factor is to promote the initial recruitment of B lymphocytes to the lung during the early stages of MRM where the mitogen would then promote lymphocyte blastogenesis perpetuating chronic pulmonary inflammation (182).

M. pulmonis has been shown to up-regulate MHC class II expression on resting murine B lymphocytes by an as yet undetermined mechanism (164). This investigators suggest that the induction of Ia hyper expression may be a necessary event for B-cell proliferation. Hyper expression of Ia on macrophages and B cells may prepare them for interaction with T_H cells (178). The Ia induction by mycoplasma is polyclonal and non-antigen specific, and thus, may lead to an increased potential for response to self-antigens and autoimmunity (178). Alternatively, this increase in MHC class II expression might promote a more efficient immune response to the organism by increasing macrophage and B cell cooperation with T_H lymphocytes (178).

The cumulative effects of all of these nonspecific interactions may be substantial (182). All of the above interactions of *M. pulmonis* with the host defense system may be involved in lesion development and the survival of the organism since most mycoplasma infections are chronic (182). The mechanisms and consequences of the polyclonal activation, chemotaxis, and induction of MHC class II expression on lymphocytes have yet to be defined. The mitogenic activity for B cells, ability to enhance Ia expression, and modulation of other host responses may promote in situ proliferation of lymphocytes and macrophages to perpetuate chronic pulmonary inflammation (165). The potential consequence of this level of nonspecific polyclonal activation of lymphocytes may determine the severity of lesions through either the release of chemotactic factors and lymphokines thus leading to increased infiltration and activation of lymphoid cells or the activation of autoimmune clones. However, polyclonal activation of lymphocytes need not actually play a direct role in disease pathogenesis since the immunoregulatory mechanisms which affect polyclonal activation may also function to control the development of other immune responses such as hypersensitivity reactions (176). Additional work in this area is needed to demonstrate the significance of these phenomena in vivo.

Cytokines

Cytokines are soluble molecules produced by immune cells that function as transmitters for intercellular communication (192) and thereby modulate the host immune response. They have both stimulatory and inhibitory effects on cells involved in the immune response (192). The purpose of studying mycoplasma effects on cytokine production is to determine whether a specific effect is due directly to the action of a mycoplasmal product on the cell or indirectly through the stimulation of cytokine production (170, 192). The ability of mycoplasmas to induce cytokine production in immunocompetent cells has been known for some time, but little information has been reported about the production of cytokines or their involvement in the pathogenesis of respiratory mycoplasmal infection (65). It has been shown that cytokines are important mediators in both lung defense and inflammation (88). Cytokine production is also involved in the pathogenesis of lung inflammatory disease (65). The capacity of mycoplasmas to induce proinflammatory cytokines seems to be a general phenomenon shared by very distant species (147).

Various mycoplasma species have various effects on the induction of cytokines: IL-1, IL-2 (11, 115), IL-4 and IL-6, TNF-α, IFN-α, -β, and -γ (7, 10, 18), GM-CSF (68, 194). Several mycoplasma species or mycoplasmal components have been found to induce proinflammatory cytokines like TNF-α (4, 107, 174, 196), IFN-γ (90, 99), IL-1 (4, 90), and IL-6 in vitro by monocytes and resident macrophages (25, 92, 174).

Recent studies have demonstrated that the genes of several cytokines are expressed in mice following experimental infection with either *M. pulmonis* (139) or *M. pneumoniae* (143). Faulkner, et. al. (65) showed the sequential appearance of cytokines in the lungs of infected mice; TNF-α was first followed by IL-1, IL-6, and finally IFN-γ. Since different mouse strains (C3H/HeN & C57BL/6N) display different susceptibilities to *M. pulmonis*, it was of interest to determine if this could be related to cytokine production. C3H/HeN mice had higher and more persistent concentrations of TNF-α and IL-6 in bronchial alveolar lavage fluid than did C57BL/6N mice. This suggests that TNF-α and IL-6 may be important factors in pathogenesis of acute *M. pulmonis* disease in mice. Pathologic findings may in part be attributed to TNF-α released from macrophages (6, 174, 196). *M. pulmonis* was also found to trigger rat lymphocytes to secrete IL-2 (106). The significance of IL-2 production after exposure of cells to *M. pulmonis* mitogens is not the production of cytotoxic lymphocytes to mycoplasma-infected cells, but as an amplifier of the inflammatory response through the augmentation of B cells and the T_H response.

The mechanism by which mycoplasmas induce the production of cytokines has yet to be determined. It has been reported that protein-kinase C (PKC) and intracellular Ca^{2+}-related

biochemical events play key roles in the activation of cytokine production by immune cells (76, 93). Yet, no one has addressed the specific mechanisms by which mycoplasmas induce the production of proinflammatory cytokines. Previous studies have shown that the cytokine induction by *M. fermentans, M pneumoniae, M penetrans*, and *M. arginini* was restricted to the lipid membrane constituents or lipoprotein fractions (147). Researchers have also compared the activities of different mycoplasma preparations with that of LPS (147, 196). The signal transduction pathways involved in cytokine induction by mycoplasmas has been studied and showed that mycoplasma activates a biochemical pathway different from that triggered by LPS (196). LPS uses a PKC pathway to stimulate TNF production in mouse peritoneal macrophages (185). Mycoplasma-induced TNF production involves a Ca^{2+}-dependent, but not a PKC dependent, biochemical pathway. This induction of TNF production by mycoplasmas is not mediated by prostaglandin E_2 (PGE_2) secretion from cells nor by a cyclic nucleotide acting as an intracellular second messenger (196). It was also shown that mycoplasma induces TNF production at the level of transcription of the gene. Further studies showed that tyrosine phosphorylation is a key event in the mycoplasma-mediated induction of proinflammatory cytokines IL-1β, TNF-α, IL-6, like LPS (147). Unlike LPS, however, mycoplasmas were unable to induce the phosphorylation of some high molecular weight proteins. Thus, mycoplasmas can induce cytokine production by specific pathways which may overlap with, yet remain distinct from, those triggered by LPS (147).

This research proves that many of the activities associated with the modulation of immunocompetent cell activity and function by mycoplasmas have been shown to be due, at least in part, to the induction of immune reactive cytokines (192). Most likely an interplay between multiple immunologic processes, and not a single immunologic response, ultimately determines the severity and progression of MRM (176). Further studies are required to fully characterize the types of immune responses involved, and to determine how they interact.

Bacterial protein hemolysins (cytolysins)

Extracellular proteins with hemolytic or cytolytic activities are produced by a large variety of gram-positive and gram-negative bacteria (5, 80, 191) . Much work has been done on red blood cells even though hemolysis is rarely ever a feature of the diseases caused by the pathogens which elaborate these toxins. Red blood cells are convenient to obtain and handle in large quantities, and they possess a simple and sensitive indicator system to demonstrate membrane damage when it occurs. Since many bacterial hemolysins act on cells other than red blood cells, the term cytolysin describes more accurately the range of their biological activities. The principal sources of cytolytic peptides are bacteria, higher fungi, cnidarians (coelenterates)

and the venoms of snakes, insects, and arthropods. The mechanism of action and the toxin's relation to pathogenesis is an active area of research today (39, 161). One of the larger, well-characterized group of hemolysins are the pore-formers (21). This review will focus on the predominant Gram positive hemolysins (cytolysins) known.

Pore-forming hemolysins

Gram positive pore-forming toxins are generally produced as soluble proteins that aggregate into supramolecular complexes of various sizes on the membrane and ultimately end up as membrane-associated complexes forming pores of a defined size (209). As more of these toxins are discovered and studied, classes based on similarities in mechanism and/or primary sequence are becoming evident. Some cytolytic proteins have clearly evolved from a single progenitor gene (convergent evolution) and others may share mechanistic features, but lack sequence similarities (divergent evolution). The staphylococcal δ and α toxins are both pore-formers with quite different physical characteristics that seem to be derived by the divergent evolution. *Clostridium perfringens*, *Staphylococcal aureus*, and *Streptococcal pyogenes* produce several different cytolysins.

Thiol-activated cytolysins

This group of cytolysins make up the largest group of related cytolytic proteins produced by the Gram positive bacteria. These cytolytic proteins are found in four genera of Gram-positive bacteria - *Clostridium*, *Streptococcus*, *Listeria*, and *Bacillus*. This group of pore-formers share many characteristics: they are inactivated by mild oxidation and reactivated by reduction with thiol reagents or sulfhydryl compounds; they are irreversibly inactivated by small amounts of cholesterol and stereo-specifically related sterols (suggesting they use cholesterol as their receptor); they are lethal and cardiotoxic; apart from their much studied hemolytic property, thiol-activated cytolysins are known to interact with a variety of leukocytes, in particular those involved in defense mechanisms (191); and they display immunological cross-reactivity. The proposed mechanism of action of these pore-formers is that they initially bind to cholesterol, a temperature independent step, and then upon warming to 37°C, they oligomerize and form supramolecular complexes varying in structure from arcs and C-shapes to closed rings composed of a variable number of toxin monomers. A complete listing of the sequenced thiol-activated hemolysins has been compiled (209). The most studied of these are LLO (listeriolysin O; from *Listeria monocytogenes*), PLY (pneumolysin; *Streptococcus pneumoniae*), SLO (streptolysin O; *Streptococcus pyogenes*), and PFO

(perfringolysin O; *Clostridium perfringens*) (21). In contrast to the other thiol-activated cytolysins, PLY is not secreted (209). Sequence analysis of many of the genes for these toxins have shown that all but IVO (ivanolysin; *Listeria monocytogenes*) have a single cysteine in the mature protein. This cysteine is invariably present in a conserved 11-residue peptide with the sequence ECTGLAWEWWR. Multiple studies have been performed mutagenizing this 11-residue peptide to find the purpose of the conserved cysteine (209). These studies clearly showed that the sulfhydryl of the single cysteine was not required for activity, but it contributes to the overall structure of this domain. Cysteine substitution mutants were no longer subject to oxidative inactivation and thiol-activation, suggesting that the cysteine residue is responsible for these properties (21). Although the sulfhydryl function of the essential cysteine is not necessary for the activity of these toxins, the cysteine is clearly in a sensitive site that cannot withstand chemical modification. There is no obvious role for the highly conserved 11-residue region. This region may interact with the sterol after membrane binding or may trigger a post-binding conformational change that readies the protein for oligomerization (209). Thus, the term "thiol-activated" is no longer a true description of these toxins (209).

Lytic peptides which alter permeability to allow passage of ions (colloid osmotic lysis)

Hemolysis reactions by thiol-activated cytolysins, unlike several other cytolysins, do not involve a colloid osmotic mechanism. Both *S. aureus* α toxin and the Gram-negative toxin aerolysin form pores and cause lysis of the colloid-osmotic type. Being the first cytolytic protein to be identified as a pore-forming toxin, *S. aureus* α toxin is the best characterized toxin in this group. This toxin forms a hexameric oligomer on the membranes (209). Like the thiol-activated cytolysins, α toxin binds to the membrane, diffuses laterally, and oligomerizes with other toxin monomers to form a pore in the membrane. Unlike the thiol-activated toxins using cholesterol as their receptor, no specific receptor is required for α toxin. Band 3 protein of erythrocytes may be the a toxin receptor (209). Aerolysin has been found to use a specific glycoprotein receptor (12).

Channel-forming agents

This group of toxins are small, secreted peptides which display a detergent-like action. Most toxins in this group are found in arthropods (bees, wasps, hornets, bumblebees, and ants), fungi, and sea anemones (12). The δ toxin from *Staphylococcal aureus* belongs to this group. This toxin is a 26 residue secreted peptide, contains no cysteine in its sequence, and is

composed of a high proportion of hydrophobic amino acids unlike most pore-formers which are mainly hydrophilic in nature. Other unique characteristics are its thermostability, inhibition by serum lipoprotein and phospholipids, and low specificity for red blood cells of different species. The δ toxin is a relatively weak toxin that forms a pore of monomers into a barrel-stave structure (209) thus forming channels in lipid bilayers.

Phospholipases

Phospholipases are classified according to their site of action on the phospholipid molecule. They are a group of bacterial membrane-active toxins with enzymatic activities. They also disrupt membranes and lead to lysis of cells by enzymatic degradation of membrane bilayer phospholipids. Hydrolysis of the phospholipids in a cell does not necessarily lead to cell disintegration, however. Phospholipase A is membrane-associated and is present in the venoms of most snakes and in the venoms of a considerable number of arthropods (12). It produces limited hydrolysis of erythrocyte phospholipids but is not hemolytic itself. Some Mycoplasmas have been found to contain phospholipase A_2. Phospholipase D is also not cytolytic. It is produced by *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* and is a constituent of the venom of the brown recluse spider (12). Phospholipase D is specific for sphingomyelin and lysophosphatidylcholine. Cytolytic as well as non-cytolytic Phospholipases C have been described for many other bacterial species (12). One of the most studied, that of the gas gangrene causing *Clostridium perfringens* is one of the most thoroughly studied of the Phospholipase Cs.

Cytopathic effects caused by mycoplasmal toxins

In contrast to the gram-positive and gram-negative bacteria, most species of mycoplasmas do not produce toxins (154, 179). *M. neurolyticum, M. gallisepticum, M. arthritidis, M. pulmonis* , *M. hyopneumoniae, M. bovis*, and *M. mycoides* are the most familiar species of mycoplasmas that are known to be toxic for the animals in which they are pathogens. The toxin that *M. neurolyticum* makes produces the well-researched "rolling-disease" in mice. Both the toxins of *M. neurolyticum* and a variant of *M. gallisepticum* have been identified as neurotoxins. The neurotoxin of both of these mycoplasma strains appear to have similar in vivo biological effects, except that the *M. gallisepticum* neurotoxin has not been shown to be an exotoxin like the neurotoxin of *M. neurolyticum. M. arthritidis* and *M. pulmonis* both have been found to be toxic for mice and rats. Their toxins share similar attributes to *M. gallisepticum* except that there are no specific neurological manifestations.

A membrane-associated hemolysin (mHL) in *M. pulmonis* has been studied as a possible toxin or virulence factor. Initial studies showed that the mHL required a serum cofactor such as BSA for activity, was trypsin-sensitive and was only active at temperatures above 22°C (125). This mHL activity is a common activity in the Mollicutes (126). Further biochemical analyses showed that this mHL was inhibited by a sulfhydryl inhibitor (NEM) and oxidizing agents (iodoacetate, and hydrogen peroxide); it was activated by sulfhydryl activating compounds (cysteine, dithiothrietol, β-mercaptoethanol, sodium thiosulfate); and cholesterol as well as other sterols inhibited mHL activity (79). These characteristics share strong similarities to the gram-positive thiol-activated cytolysins. The only difference is they both display different stereo-specificity requirements for cholesterol inhibition (79).

Four hypotheses have been proposed as a mechanism of action of this activity (123). Soluble metabolic products such as H_2O_2 that can damage RBC membranes in relatively high concentrations, but mHL activity was not diffusible and could not be inhibited by catalase or pyruvic acid (125). Alterations of RBC lipids by a mycoplasma-associated phospholipase could result in membrane damage, but previous studies have failed to correlate phospholipase C activity in mycoplasmas to hemolytic activity (126). The mHL could form a pore-like structure upon transfer to the RBC membrane or cause a destabilization of the RBC membrane through some unknown mechanism. This theory is partially supported by the similarity that exists between the mHL and the thiol-active cytolysins (79), but further studies are needed to prove or disprove this possibility. Finally, mycoplasmas could use this activity to acquire membrane components from their host thus causing membrane destabilization and lysis. Previous studies with a fluorescent cholesterol probe lend credence to this hypothesis by demonstrating significantly enhanced transfer of the probe under conditions conducive to lysis by the mHL (123). The research presented here focuses on further characterization of a soluble form of the mHL which, like the *M. neurolyticum* toxin, emerges as a broad, single peak in the void volume of a G-200 Sephadex column.

MATERIALS AND METHODS

Mycoplasmas

M. pulmonis UAB6510 was used throughout this study. Cultures were routinely grown from a stock culture maintained at -70°C by inoculation into a modified dialyzed medium (145) at a 1:10 dilution and incubated statically at 37°C. These cultures were maintained by serial passage. Organisms were harvested by centrifugation (8000 x *g*, 15 minutes) at the mid to late log phase of growth, washed once with 0.01 M sodium phosphate - 0.14 M sodium chloride, pH 7.4 (PBS). Mycoplasmas were used immediately in the assays. Protein concentrations were determined with the Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, Calif.) using bovine serum albumin (BSA) as a control.

Mice

Pathogen-free BALB/cByJ, C3H/HeJ and C3H/HeOuJ mice were obtained from Laboratory Animal Resources (LAR) which is a division of the College of Veterinary Medicine, Iowa State University, Ames, IA. These mice are monitored and tested quarterly for mycoplasma contamination by serological screening. These colonies are negative for all pathogens, and all animals were negative for IgG and IgM anti-*M. pulmonis* antibodies at the time of the experiment.

Mycoplasma Media

M. pulmonis was grown in a modified dialyzed medium to minimize medium contamination (145). The dialyzed medium base was prepared by dialyzing 220 g of brain heart infusion (Difco Laboratories) and 110 g of yeast extract (Difco) (dissolved in 1,100 ml of water) against 8 L of 0.5% NaCl for 6 h at 80°C and continuing at 4°C overnight. Phenol red (0.001%) was added, and the pH was adjusted to 8.0 before autoclaving. The base was then completed by supplementing with 10% agamma horse serum (GibcoBRL), 0.5% glucose (Fisher, Fairlawn, NJ), and 25 µg per ml Cefobid (Roerig-Pfizer, New York, NY). Cultures were harvested at mid to late-log phase as indicated by a color change in the media. For determining colony forming units (CFUs), mycoplasmas were grown on Broth A agar plates prepared as described (51).

Radiolabeling of *M. pulmonis*

Radiolabeled *M. pulmonis* was prepared by pelleting 100 ml of mid log-phase cultures grown in dialyzed media and resuspending the pellet in 25 ml of Hanks Balanced Salt Solution

(HBSS) labeling medium (HBSS–1% glucose–10% dialyzed horse serum–25 mM HEPES, pH 8.0). Horse serum was prepared by dialysis against 40 volumes of PBS or Tris saline (TS, 0.01 M Tris–0.14 M NaCl, pH 7.4) overnight at 4°C, followed by filter sterilization. Fifteen micocuries per ml of Trans[^{35}S]label (ICN Radiochemicals, Irvine, Calif.) or 50 μCi per ml of [^{14}C]amino acid mixture (ICN Radiochemicals), or 1 μCi per ml of [^{32}P]Na_2HPO_4 (ICN Radiochemicals) was added to the labeling medium, and the cells were incubated for 4 h at 37°C. The label was chased for 2 h with an equal volume of dialyzed media. When radiolabeling with [^{32}P]Na_2HPO_4, extra care was taken to avoid any solutions containing phosphates. The culture was then pelleted, washed with PBS-1% glucose (PBSG), and resuspended in 1 ml of RPMI 1640 and incubated at 37°C for 8 h. The suspension was then centrifuged at 15,000 x *g* for 20 min. The pellet was resuspended in 400 μl of water and the supernatant was diluted with 1 ml of RPMI 1640. Efficiency of radiolabeling was determined by precipitating the proteins by adding trichloroacetic acid (100 μl of a 72% solution) and deoxycholate (100 μl of a 0.15% solution) and counting the radioactivity of the pellet and the supernatant. From that information, percentage of incorporation was determined.

Soluble Hemolysin Preparation

The fresh mycoplasma whole cell pellet obtained from 1 liter of culture was washed with PBS and resuspended in RPMI 1640–25mM HEPES–2 g Na HCO_3 at one fiftieth of the culture volume and incubated for 8 h at 37°C. This suspension will be referred to as a RPMI mycoplasma suspension. The supernatant was prepared by centrifuging the suspension at high speed (12,000 x *g*, 20 min) and then by ultracentrifugation (40,000 x *g*, 2 h). The resulting supernatant will be referred to as the soluble hemolysin (sHL).

Hemolytic Assays

Blood from mice was collected by cardiac puncture, and the mouse red blood cells were prepared for use in the hemolytic assay in the following way. Blood was collected in Alsever's anticoagulant, and the mouse red blood cells were washed in PBS–25 mM HEPES four times, treated with diphenyl carbonyl chloride-treated trypsin (T-1005, Sigma) for 1.5 h (1:1000 dilution, 4% suspension) at 37°C with gentle agitation. The trypsin-treated mouse RBCs (MRBCs) were then washed in PBS–25 mM HEPES four more times and resuspended in the same buffer to a final suspension of 10%. This suspension was kept at 4°C for no more than three days before use.

The HL assay has been described previously (125) except that MRBCs obtained from BALB/cByJ, C3H/HeOuJ or C3H/HeJ mice were used. The HL assay mixture consisted of

MRBCs in RPMI 1640–25 mM HEPES–1% BSA (pH 7.4) at a concentration of 0.5% MRBCs (optical density at 600 nm of 0.8-0.9) and varying amounts of mycoplasma soluble protein in a total volume of 3 ml. The tubes were placed in a shaking water bath and incubated at 37°C. The optical density of the RBC suspension was measured at the beginning and end of the experiment, usually 90 min, and the hemolytic activity was calculated as follows:

$$[(OD_S - OD_E) / (0.5 \times OD_S)] / (T \times mg)$$

where OD_S = optical density at the start of the incubation, OD_E = optical density at the end of the incubation, T = time in hours, mg = mg of mycoplasma protein added to the reaction mixture. Experiments were repeated a minimum of three times. Controls consisted of MRBCs alone (negative control), freshly harvested mycoplasmas and MRBCs in assay medium (positive control).

For blood agar plates, 2.4 g of SeaPlaque GTG low melting temperature agarose (FMC BioProducts, Rockland, Maine) was dissolved in 120 mls of RPMI 1640–25 mM HEPES. This was allowed to cool, and then 6.06 mls of 30% BSA (Sigma), 15 mls of MRBCs (10% suspension), and 600 µl of 0.25% Cefobid was added. This mixture was then poured into 8 petri-plates (Fisher) and set in the refrigerator for 1 h to allow solidification. Plates were then brought to room temperature and wells were punched in the agar with a pipetman. Ten to fifteen µl of sample were placed into the wells and the plates were placed in a 37°C incubator. After 4 h, the plates were examined for zones of lysis. These plates were also used to locate the region of hemolytic activity following native- polyacrylamide gel electrophoresis (PAGE) by placing the upper half of the 3 mm gels on the blood agar and observing lysis after 4 h of incubation at 37°C.

Soluble Hemolysin Release Assay

In order to determine the relationship between viability of the mycoplasmas in RPMI buffer, cell lysis and the release of sHL activity, a modified assay was used. One ml aliquots from a 20 ml RPMI mycoplasma suspension was removed every 30 min for a total of 8 h. Each aliquot was then centrifuged at 15,000 x *g* for 15 min, and the pellet was carefully resuspended in 1 ml of PBS. Five hundred µl of the supernatant and 500 µl of the cell pellet suspension were tested in a hemolysis assay. Colony forming units (CFUs) were determined by spotting 20 µl of 10-fold dilutions (10^7 - 10^{14}) of cell suspensions on Broth A agar plates and incubating the plates at 37°C for 4 - 5 days.

The appearance of NADH oxidase activity in the supernatant fraction was used as an indicator of cell lysis. The NADH oxidase assay was performed as follows. Two hundred µl aliquots (0.4 - 0.6 mg per ml protein) was removed from the incubating mixture at 30 min

intervals for a total of 7 to 8 h, and the whole cells were removed by centrifugation as described above. The assay mixture contained 200 µl of supernatant, 100 µl of solution A (0.5 M HEPES buffer–75 mM KCl, pH 7.5), and 600 µl of H_2O. The reaction was initiated by the addition of substrate (100 µl of 0.64 mM β-NADH) and the tubes were incubated at room temperature. The optical density at 340 nm was recorded with a UV 1604 Shimadzu spectrophotometer by following the decrease in absorbance using the Time Scan Mode for 300 seconds as described (144). A decrease in optical density indicated the presence of NADH oxidase. The slope of the curve is indicative of the enzyme concentration.

Purification Schemes

Preparative isoelectric focusing

High resolution preparative isoelectric focusing using the Rotofor (BioRad Laboratories) was used in attempts to purify the sHL. The apparatus containing the 15 ml cell was assembled per manufacturer's instructions and the sample was loaded in a total volume of 40 ml. The sample was prepared by mixing 40 ml of diluted sHL with a carrier ampholyte (Ampholine 3.5-9.5, Sigma catalog number A-4549) at a final concentration of 2% (v/v). Total protein was relatively low ($\leq$ 0.5 mg). Running conditions were as follows: the cell was focused for 4 h at 11W constant power at 4°C. The initial voltage was 154V, and at equilibrium, the voltage rose to 400V where it remained constant. The anodic buffer was 0.1M H_3PO_4 and the cathodic buffer was 0.1M NaOH. The gradient was harvested as twenty fractions, and their pH values measured. A 2.13 to 11.23 pH gradient was formed.

The Rotofor fractions were dialyzed using Spectra/Por 2 dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, Calif.) against 100 volumes of 1M NaCl for 1 to 1.5 h and then against PBS (pH 7.4) overnight at 4°C. After removal of ampholytes, aliquots were analyzed for the presence of HL activity using a blood agar plate assay. The presence of antigen in the harvested, dialyzed Rotofor fractions was determined by enzyme linked immunosorbent assay (ELISA) using monoclonal antibody 1D4G12 shown previously to block *M. pulmonis* hemolytic activity (77).

High performance liquid chromatography

High pressure liquid chromatography (HPLC) was also used in attempts to purify the hemolysin. A reverse-phase analytical C-18 column (21 mm x 22 cm) (Vydac, Hesperia, Calif.) was used for most of the HPLC separations using a Waters 600E HPLC system. Before the sample was loaded, the column was equilibrated with 2 column volumes of degassed HPLC-grade water. After this step, the column was then equilibrated with 2 column

volumes of Buffer A (1% TFA, trifluoroacetic acid). An elution gradient was then run repeatedly from 0-100% Buffer B (0.8% TFA in acetonitrile) until no more peaks eluted from the column. Five hundred µl of sHL was injected into the column, and the parameters for separation were as follows: Flow rate = 0.2 ml per min at a pressure of 700 psi; elution program: 0-5 min 100% Buffer A; 5-20 min 0-20% linear gradient Buffer B; 20-25 min 20-45% linear gradient Buffer B; 25-45 min 100% Buffer A. Proteins were detected at a wavelength of 214 nm with a sensitivity of 1.7 absorbance units full scale.

A Bio-Gel SEC-30 XL (7.8 cm x 30 cm) column (Bio-Rad) was also used in the HPLC separations. This column was washed with 20% dimethylsulfoxide for 45 min at a flow rate of 1 ml per min before use. The same procedure parameters and elution programs as above were used. Both analytical and preparative runs were performed. The preparative run flow rate was 0.6 ml per min. PBS–1mM β-mercaptoethanol–0.1 mM phenyl methyl sulfonyl fluoride (buffer A) and PBS–12% acetonitrile (buffer B) were used as the mobile phases.

Proteins eluted from the C-18 reverse-phase and the Bio-Gel SEC-30 XL columns were collected by the peak detection method, dried with a Speed Vac concentrator (Savant Instruments, Inc., Farmingdale, N.Y.) and dissolved in 60 µl of PBS. Blood agar plates were used to detect hemolytic activity. The presence of the HL antigen was determined by both ELISA and immunoblots using the hemolysin-blocking monoclonal antibody 1D4G12. The HPLC analysis was performed at the Iowa State University Protein Facility.

Low pressure column chromatography

In order to determine if hemolytic activity bound nonspecifically to various chromatography media, Sepharose (Sigma), Sephacryl S-200 (Pharmacia), and Sephadex G-200 (Sigma) were incubated with sHL and the remaining hemolytic activity in the supernatant fraction assayed. To accomplish this, the various media were rehydrated, washed several times with PBS and resuspended to 50% in PBS. One ml of sHL was placed into microfuge tubes and various amounts of the media (50, 100, 200, and 400 µl) were added to the sHL. These tubes were then rocked for 45 min at 4°C and then spun down slowly. Four hundred µl of the various supernatants were analyzed for remaining hemolytic activity using a standard assay. Supernatant not reacted with chromatography media was used as a control. In some studies, 5, 10, or 20% glycerol (final concentration) was added to the sHL prior to the incubation with the chromatography media in attempts to prevent nonspecific adherence of the sHL activity.

Gel filtration chromatography was performed using Sephadex G-200. To prepare samples for gel filtration, 100 mls of supernatant from soluble hemolysin preparations were concentrated to 5 mls by using Diaflo membranes (XM50; Amicon, Beverly, Maine). Three

mls of the concentrated sample were applied to a 2.5 x 18 cm Sephadex G-200 column (Amicon, Beverly, Maine) at an operating temperature of 4°C. Samples had $\leq$ 5 mg protein per ml. The eluting buffer was PBS. One and a half ml fractions were collected, and 400 µl of each fraction was assayed for activity at a final dilution of 1:6.25. Fractions containing hemolytic activity were referred to as partially-purified hemolysin (pHL).

Electrophoresis

Washed *M. pulmonis* was suspended in water to achieve a concentration of 2-5 mg protein per ml. sHL, pHL, and heat-inactivated pHL were concentrated to approximately 250 µg protein per ml. Samples were prepared by heating at 100°C for 1-2 min in sodium dodecyl sulfate (SDS)-PAGE sample buffer (4% SDS–10% β-mercaptoethanol–125 mM Tris–20% glycerol, pH 6.8), cooled, and then layered on the stacking gel. SDS-PAGE slab gel electrophoresis was performed by the method of Laemmli (94) using a 3% stacking layer (pH 6.8) and a 10% resolving gel (pH 8.8). All gels were allowed to polymerize overnight at 4°C. A Bio-Rad model Protean II slab gel apparatus was used with a Bio-Rad model 3000xi power supply (Bio-Rad). Gels were electrophoresed for 3 to 4 h at 20 mAmps per slab gel. After electrophoresis, the gels were stained with an aqueous solution containing 0.025% (wt/vol) Coomassie blue (Bio-Rad)–50% (vol/vol) methanol–12% (vol/vol) acetic acid. Gels were destained in the same solution without Coomassie blue.

For radioactive samples, gels were impregnated with 1 M sodium salicylate (Fisher, Itasca, Ill.) for 30 min to enhance the radiographic signals, and then dried under a vacuum using a Hoefer gel drying apparatus. Radiolabeled proteins were identified by exposing the radioactive gels to preflashed X-Omate-AR film (Eastman Kodak Co., Rochester, N.Y.) for 3 to 6 weeks at -70°C. Molecular weights were calculated from known high range molecular weight standard markers (GibcoBRL).

Similar procedures were followed for preparative native-PAGE gels except that all SDS in the buffers and solutions were excluded and the samples were not heated. Following electrophoresis on a 5% 3 mm slab gel, the upper half of the gel was placed on a blood agar plate for a maximum of 4 h until a zone of lysis was observed indicating the location of the hemolytic activity. This particular band, about 1.5 cm from top of gel, was excised and the hemolytic activity was electroeluted using a Centrilutor Microelectroelutor (Amicon) for 6 h at 200 V at 4°C. Nondenaturing 1X Tray buffer (3 g Tris–14.5 g glycine per L) was used as the electroelution buffer. Centricon-50 concentrators (Amicon) were used to collect and concentrate the electroeluted protein(s). The electroeluted hemolytic activity from the native gel will be referred to as eHL.

Immunoblots

Following electrophoresis for 3-4 h at 20 mAmp constant current, proteins were transferred to nitrocellulose (Midwest Scientific, St. Louis, Mo.) following the procedure of Towbin et. al. (206). A Hoofer model TE 42 Transphor apparatus with a model TE-50 power supply was used for this transfer process. The electrode buffer contained 25 mM Tris–192 mM glycine–20% methanol (vol/vol), pH 8.3. A current of 1 Amp was applied for 2 h to insure efficient transfer of all proteins to the nitrocellulose. Cooling was maintained by running the apparatus at 4°C.

Nitrocellulose blots were removed from the transfer apparatus, and unreacted protein binding sites were blocked by incubation of the nitrocellulose in 1% gelatin (BioRad) for 2 h at room temperature. The blots were rinsed once with TS buffer and incubated at room temperature for 18 h with monoclonal antibody 3.3.10.2 (10 μg per ml) that blocks mitogenic activity of *M. pulmonis* (provided by Dr. Yehudith Naot, Technion-Israel Institute of Technology, Bat Galim, Haifa, Israel). Blots were washed once in TS buffer for 10 min, twice with TSN buffer (TS–0.05% Tween-20) for 20 min, and once again with TS buffer for 10 min to remove unbound antigen. The antigen-antibody complexes were detected by reacting the blot for 1.5 h at room temperature with goat antibody to rat IgG (whole molecule) conjugated to alkaline phosphatase (Cappel, Organon Teknika Corp., Westchester, Penn.) at a 1:1000 dilution in TS buffer. The blot was washed twice with TS buffer for 15 min each, twice with TSN buffer for 20 min each, and once with TS buffer for 15 min. The blot was developed by using 0.03 g Napthol AS-MX phosphate (Sigma) plus 0.06 g Fast Red Tr (Sigma) dissolved in 40 ml of 20 mM Tris, pH 7.5.

For analysis of HPLC or Rotofor fractions, 15 μl of each HPLC or Rotofor fraction was dried by vacuum (Savant) for 20 min to evaporate off the acetonitrile in the case of HPLC fractions or to concentrate the fractions. The pellets were the resuspended in 6 μl of PBS, and then 5 μl of each sample was dotted onto a nitrocellulose membrane and left to air dry. These blots were blocked with milk blocking buffer (PBS–5% Carnation instant milk–0.05% Tween-20–0.2g Thimersol) for 1-2 h. Undiluted 1D4G12 Mab was incubated with each blot overnight at 4°C. The same washing steps were then followed as described above. Horseradish peroxidase labeled goat anti-rat IgG (H+L) conjugate (Bio-Rad) was diluted 1:1000 with TSN and placed on the blots for 75 min. The same washing and development steps were followed as described above.

Protein Sequencing

The 10 µl of retentate from the electroelution process was separated on a 10% SDS-PAGE gel at 15 mA. This gel was soaked in sequencing transfer buffer [10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)–10% methanol, pH 11.0] for 15-20 min to allow diffusion of the Tris-glycine buffer. The proteins were transferred to Immobilon-P^{SQ} PVDF (Millipore Corp., Bedford, Maine) for 2 h at 1 Amp. The PVDF membrane was stained with Coomassie blue for 2 min and then destained. The bands were excised, washed with HPLC grade water and loaded onto a pre-conditioned filter for sequence analysis. The N-terminal sequence analysis was performed with an Applied Biosystems, Inc., Protein Sequencer Model 477A equipped with an on-line PTA amino-acid analyzer model 120A. Repetitive Edman degradations were performed on the eHL sample using this automated system. The PTH amino acids were then passed through a reverse-phase Vydac C-18 (21 mm x 22 cm) HPLC column and detected at 270 nm. Their retention time was then compared with the PTH amino acid standards both visually and by computer data analysis programs. This N-terminal sequencing was performed by the Iowa State University Protein Facility (Ames, Ia).

Enzyme Linked Immunosorbent Assay (ELISA)

ELISAs were performed as previously described (71). Rotofor and HPLC fractions were dried under vacuum (Savant) and resuspended in 50 µl of PBS. ELISA plates for the HPLC and Rotofor fractions were prepared by coating a 96-well serocluster 'U' vinyl plate (Costar, Cambridge, Maine) with 75 µl of coating buffer (0.1 M sodium carbonate, pH 9.6) and 25 µl of either HPLC or Rotofor sample to each well. These plates were allowed to incubate for 4 h at room temperature or at 37°C. The plates were washed 3 times with ELISA wash buffer (0.9% NaCl–0.05% Tween 20), waiting 10 min between each wash. The wells were then blocked for 1 h at room temperature with PBS–0.5% BSA. The wells were then washed 4 times with ELISA wash buffer, and 100 µl of undiluted 1D4G12 Mab was incubated in the wells overnight at 4°C. Plates were washed three times with ELISA wash buffer prior to the addition of 100 µl of horseradish peroxidase labeled goat anti-rat IgG (H+L) conjugate (Bio-Rad) per well at a 1:3000 dilution in PBS–0.1% BSA. The plates were incubated for 1 h at 37°C, washed three times with ELISA wash buffer, and developed using TMB peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) according to the manufacturer's directions. ELISA plates were read at 450 nm using an automated microplate reader (BioTek, Model EL310, Winooski, Vt.).

Cell Culture

The murine monocytic/macrophage cell lines J774.A1 and P388.D1 were obtained from American Type Culture Collection (Rockville, Md.). J774.A1 cells were grown in 37°C in Dulbecco's minimum essential medium (Gibco Laboratories) supplemented with 4.5 mg D-glucose–5% fetal bovine serum–5 ml of gentamicin (0.82% solution, Sigma)–5 ml of PKS (0.627% penicillin (Sigma)–1% kanamycin (Sigma)–1% streptomycin)–0.3 g L-glutamine, per liter. The J774.A1 cells were maintained as monolayers in petri dishes (Fisher brand #08-757-13). Liquid policeman (PBS–0.2 g per liter EDTA) was used to harvest the adherent J774.A1 cells from the petri dish.

P388.D1 cells were grown at 37°C in RPMI 1640 (Gibco Laboratories)–15% fetal bovine serum–5 ml of gentamicin–5 ml of PKS–0.3 g L-glutamine, per liter. This cell line was also maintained as monolayers in bacteriologic treated petri dishes and harvested using a disposable cell scraper (Fisher). All cell cultures were initially tested for mycoplasmal contamination using the Hoescht stain kit (Flow Laboratories, Inc., McLean, Va.). To keep a stock of all cell lines, they were frozen in their respective medium containing 15% dimethylsulfoxide (Fisher) in liquid nitrogen.

Chemotaxis Assay

Chemotaxis of J774.A1 cells was assayed in a 48-well micro-chemotaxis chamber (Neuroprobe, Bethesda, Md.) as previously described (64). J774.A1 cells were washed once in DMEM media and resuspended to 2.6×10^6 cells per ml in chemotaxis medium (Gay's Balanced Salt Solution (GBSS, Gibco Laboratories)–0.5% BSA) prior to use. The upper wells of the chamber were filled with J774.A1 cell suspensions (0.035 ml), and the bottom wells contained 0.025 ml of various concentrations of *M. pulmonis* pHL (0.1 - 50 µg protein per ml) obtained by purification through a G-200 Sephadex column as described above or human recombinant C5a (Sigma) at a concentration of 1 nM. The final dilutions of all attractants were prepared daily in chemotaxis medium. Chemotaxis medium and PBS served as negative control stimuli. Six replicates of each stimulus were placed in the bottom wells. The chamber was incubated for 3 h at 37°C in humidified 5% CO_2. The polycarbonate filter membrane (Nucleopore - 5.0 µm) was then removed, fixed, and stained. The migrated macrophages were counted with a 100X objective. Two fields were counted per well, and the mean and standard deviation of triplicate wells was determined.

Blastogenesis Assay

Mitogens

Various concentrations of pHL (0.1 - 50 μg protein per ml) prepared as described above were tested for mitogenic activity. The final dilutions of all mitogens were prepared daily in RPMI 1640 (GIBCO)–25 mM HEPES–0.2% $NaHCO_3$ –2 mM L-glutamine. For some studies, the pHL was heated at 70°C for 1.5 hrs to abolish hemolytic activity. Heat inactivated pHL was tested at 5 and 0.1 μg protein per ml in the blastogenesis assay. As a positive control, *M. pulmonis* cell cultures were harvested in the late-log phase of growth by centrifugation for 15 min at 8,000 x *g* at 4°C. The pellets were washed with PBS by centrifugation and then UV irradiated for 30 min. These nonviable suspensions were then sonicated on ice for 5 min to disrupt the membranes. The sonicate was then aliquoted and stored at -70°C until use. Protein was determined by using the BioRad Assay as described above. Concentrations used for blastogenesis ranged from 0.125 - 20 μg per ml protein. LPS-endotoxin (K235 *E. coli* endotoxin; Sigma) was diluted to concentrations ranging from 0.1 to 10 μg per ml. Concanavalin A (ConA, Sigma) was diluted to 0.25 to 1 μg per ml.

Lymphocytes

Three- to 4-week-old C3H/HeJ and C3H/HeOuJ mice were killed by asphyxiation. Their spleens were removed aseptically, minced, and pressed through a sterile metal screen (mesh size, 250 μm) into cold RPMI media (1% fetal bovine serum). Red blood cells were lysed using a mixture of 90 ml of 0.2 M NH_4Cl and 10 mls of 0.24 M Tris, pH 7.2. Most of the macrophages were removed by adherence by placing the spleen cell suspensions in tissue-cultured treated plates (Falcon #3003, Becton Dickinson) and incubating at 37°C for 2 hrs. The resulting lymphocytes were washed twice and resuspended to a final concentration of $5x10^6$ cells per ml using RPMI media.

Assay

Immediately before use, the mitogens were prepared by diluting them to the appropriate protein concentrations listed above using RPMI 1640–25 mM HEPES–10% fetal calf serum, and 20 μg per ml gentamicin. $5x10^5$ spleen cells per well (0.1 ml volume) were cultured in a humidified atmosphere containing 95% air and 5% CO_2 for a total of 48 hrs in a 96-well flat-bottomed microtiter plate (Costar, Cambridge, Maine) along with 0.1 ml of mitogen giving a final volume of 0.2 ml per well. Controls included medium plus cells alone (negative control) and cells with *M. pulmonis* mitogen, LPS-endotoxin or ConA (positive controls). Twenty-four hours before harvest, 0.5 μCi of [^{3}H]thymidine was added to each culture well. The cells were

collected onto glass fiber strips, and washed with distilled water using a PHD cell harvester (Cambridge Technology, Inc.). The strips were then air dried and counted using ScintiVerse (Fisher) scintillation cocktail in a Packard 1500 Tri-Carb liquid scintillation counter (Meriden, CT). All assays were run simultaneously in triplicate so that the results could be compared.

Phagocytosis Assay

Fluorescence labeling of *E. coli*

Fluorescein-labeled *E. coli* were used as the physiological targets for phagocytosis. *E. coli* K-12 strain L929 was grown overnight in Luria-Bertoni media (172), washed by centrifugation, and the cells were resuspended in PBS at a concentration of 1.0×10^9 cells per ml. The cell suspension was heat treated by incubation at 60°C for 70 min, and the cells were washed with 1 ml of HBSS and then resuspended in 1 M $NaHCO_3$ to achieve a concentration of $5x10^6$ cells per ml. Fluorescein isothiocynate (FITC) isomer-1 (Sigma) was added to a final concentration of 0.3 µg per ml, the tube was covered in foil, and then incubated at 25°C on a rotator for 60 min. Bacteria were washed twice with 1 ml of HBSS and frozen at -20°C in 80% glycerol. The cells were thawed and washed one time the day of the experiment. Uniformity of staining was confirmed by flow cytometry.

Assay

The phagocytic assay was performed as follows. J774.A1 and P388.D1 mouse monocytic/macrophage cells were harvested, washed and resuspended to a final concentration of 2×10^5 cells per ml. Fifty µl of each cell suspension were injected into flow cytometer tubes (Falcon # 2002. Becton Dickinson, Lincoln Park, N.J.) along with 67 µl of various concentrations of pHL (0.1 - 50 µg protein per ml). The tubes were kept in an ice-water bath for 15 min to allow cells and pHL to acclimate. Twenty µl of FITC-conjugated *E. coli* was then injected into each tube and vortexed. Except for the negative control maintained at 0°C for the duration of the experiment, the tubes were shifted to a 37°C water bath and incubated for 10-12 min. The tubes were removed immediately and placed in the ice-water bath to stop phagocytosis. One hundred µl of ice-cold quenching solution (Trypan blue, 4 mg per ml) was added to each tube, the tubes were vortexed and the cells were fixed by the addition of 2 ml of PBS–0.5% paraformaldehyde within 10 min. These tubes were vortexed and centrifuged at 150 x *g* for 5 min. Supernatants were removed leaving about 1 ml of fluid. Two ml of PBS–0.1% sodium azide was added to each tube, vortexed, and the tubes were centrifuged at 150 x *g* for 5 min. Supernatants were again removed except for 1 ml. Two hundred µl of PBS–0.1% paraformaldehyde was then added to each tube. Propidium iodide (Sigma) at a final

concentration of 50 μg per ml was added to all tubes just before cytometric analysis. All samples were analyzed by flow cytometry (Cell & Hybridoma Facility, Iowa State University, Ames, Ia.). Bacteria that were not engulfed by the macrophage cell lines were gated out from bacteria that were engulfed by differential intensities of red propidium iodide. Macrophages that did not engulf bacteria were distinguished from macrophages that did by assessing the different intensities of green florescence of FITC.

RESULTS AND DISCUSSION

Kinetic Studies

The initial studies undertaken were to determine the kinetics of release of sHL in order to maximize its production. By correlating loss of viability, cell lysis (appearance of NADH oxidase) and appearance of sHL in the culture supernatant it might also be possible to shed light on whether release of sHL was a normal physiological process of the organism or it resulted from degradation following cell death. This correlation was accomplished using a timed release assay as described above. By calculating total HL activity in the cell suspension prior to incubation with the amount released during incubation, it was possible to estimate the percentage of released activity by *M. pulmonis* whole cells in RPMI. Approximately 20% of the activity in whole cells was released into the supernatant during the incubation period.

Figure 1 shows the release of sHL activity during incubation in RPMI media over time. Also shown is the viability of the cells (CFUs) and the remaining mHL in the cell pellet. A gradual release of sHL activity was observed for the first 5 h with a sharp increase at this time. Viability in the cell suspension was steady for about 3 h and then decreased during the next 4 h until little viability remained. The mycoplasma-associated mHL activity remained steady for about 3 h and then sharply decreased until a basal level was reached and maintained throughout the experiment.

Aliquots were obtained every 30 min from the experiments above and the supernatants measured for NADH oxidase activity. Figure 1 also shows the results of those experiments. There was no NADH oxidase activity released until 2 h when a sharp increase was observed (shown by the increase in the negativity of the slope). The correlation of NADH oxidase release with a decrease in cell viability indicated that cell lysis occurred immediately upon cell death during sHL preparation. This also suggested that hemolytic activity was not secreted continuously by the organism. Release of sHL may have been due to the loss of membrane potential upon cell death or it could have occurred following actual membrane rupture. Since it was not possible to separate cell death from cell lysis, further examination of this phenomenon was not possible at this time.

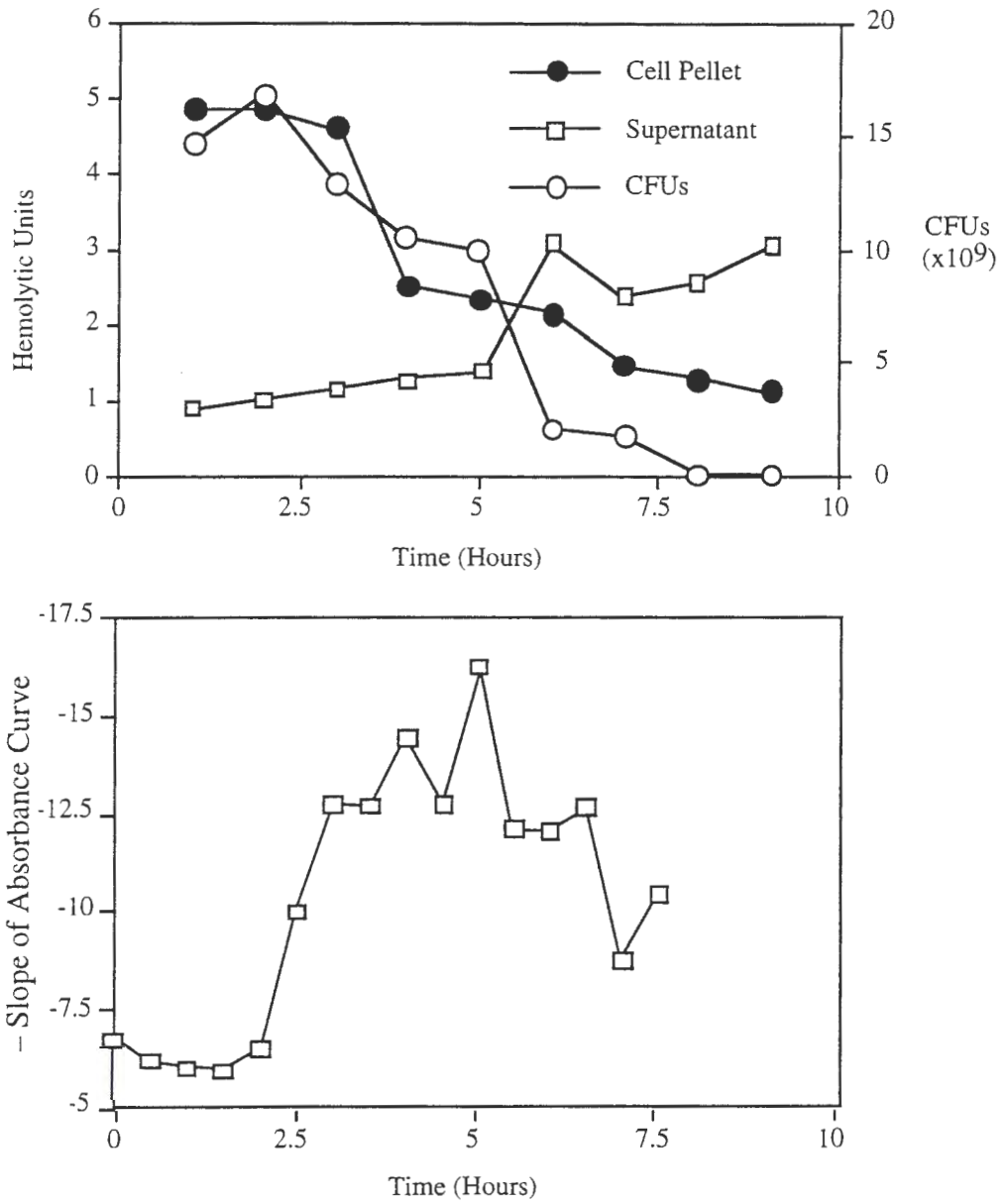


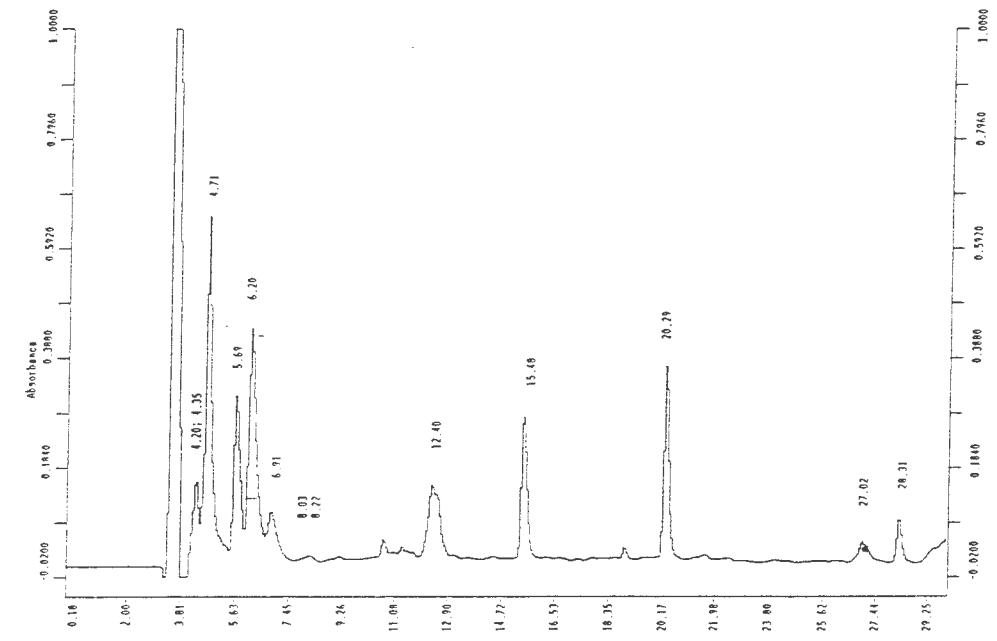
Figure 1. Correlation of hemolysin release with cell viability and lysis. Upper panel. Inverse correlation of sHL release with cell viability. Mycoplasma cell suspensions in RPMI buffer supplemented with 50 mM HEPES buffer (pH 7.5) were incubated at 37°C over the indicated time interval and samples taken at 30 min time points to measure cell viability, sHL activity released into the supernatant and mHL activity remaining in the cell membranes. The data are from a single experiment, but are indicative of the trend observed in at least three different experiments. Lower panel. Release of NADH oxidase during incubation of *M. pulmonis* in RPMI at 37°C. The assay is described in the Materials and Methods. Data represents the mean of three experiments.

Soluble Hemolysin Purification

In order to gain a better understanding of the nature of the sHL, attempts were made to purify the activity and biochemically characterize the mycoplasmal components involved. Multiple purification approaches were attempted because of the unusual nature of the hemolytic activity and its requirement for BSA (125). It was absolutely essential to maintain activity during purification since it was not clear if Mab 1D4G12 bound to a mycoplasmal component or inhibited hemolysis through a steric hindrance mechanism. In previous studies, it had not been possible to identify a specific mycoplasmal component using 1D4G12 either by immunoblot or in immunoprecipitation assays (78). The Mab had some reactivity to BSA as determined by ELISA, but it was clear from these earlier studies that reaction with the sHL or cell associated activity was stronger than to BSA alone (78). Thus, there was no clear marker for mycoplasmal hemolytic components during purification other than its activity. It was not possible to use detergents to assist in the purification scheme since incubation of sHL with 1% CHAPS detergent followed by dialysis to remove the detergent resulted in a loss of activity (78). Therefore, purification schemes had to use nondenatured proteins in the absence of even mild detergents. In the situations where denaturation was likely to occur, HPLC and preparative isoelectric focusing, fractions were assayed for hemolytic activity and were also monitored for positive responses to Mab 1D4G12.

Purification by HPLC

Because of the high resolution available with HPLC, sHL purification was first attempted in this way. Initially, a reverse-phase C-18 column was used to separate sHL fractions as described in Materials and Methods. The proteins were allowed to separate for a total of 30 min. Figure 2 shows the typical peaks that were detected with each run. The majority of the peaks appeared to be hydrophilic with a few appearing to be more hydrophobic. A size-exclusion column HPLC column (Bio-Gel SEC-30 XL) was also used with a lower percentage of acetonitrile (12%) then was used with the C-18 reverse-phase column (Figure 2). This was done to determine if the acetonitrile used was interfering with or destroying the hemolytic activity of the sHL. The sample protein concentration for each HPLC run ranged from 200-400 µg per ml. Proteins eluted from both columns were collected by the peak detection method. Concentrated protein fractions were dotted onto nitrocellulose membranes and analyzed for the presence of Mab-reactive antigens as well as analyzed for HL activity. These same protein fractions were also used to coat a 96-well ELISA plate. None of the fractions eluted from the HPLC columns had HL activity or showed immunoreactivity with Mab 1D4G12.


Absorbance
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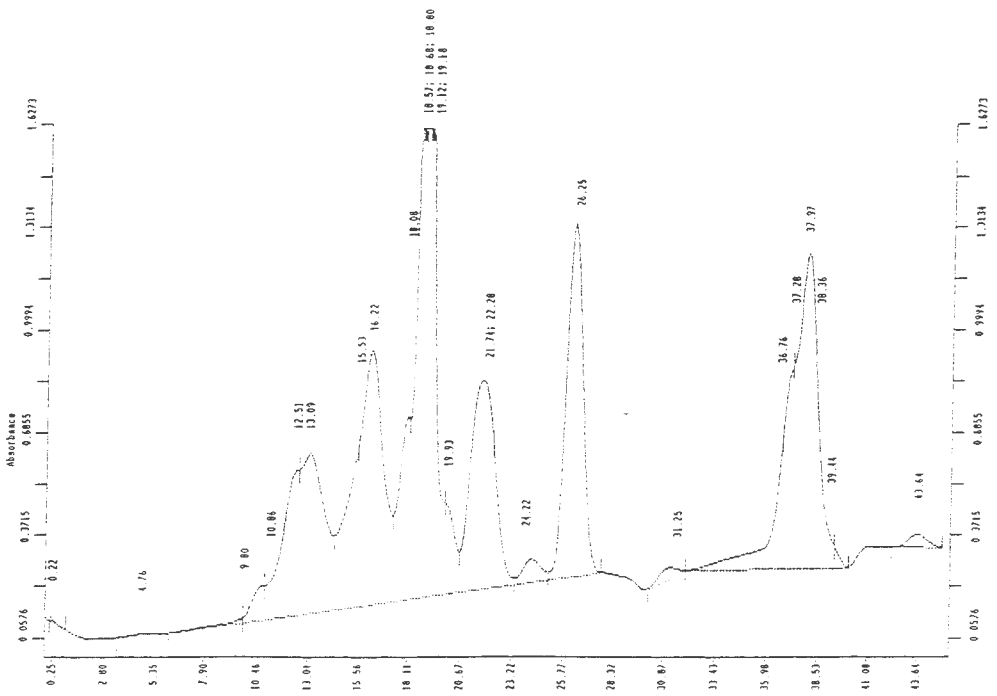
4.20; 4.35
5.69
6.20
6.91
8.03
8.22
12.40
15.49
20.29
27.02
28.31


Absorbance
9.80
10.86
12.51
13.09
15.53
16.22
18.08
18.57; 18.68; 18.80
19.12; 19.18
19.93
21.74; 22.28
24.22
26.25
31.25
34.76
37.28
37.97
38.36
39.44
43.64

Preparative isoelectric focusing

Isoelectric focusing (IEF) is a high resolution technique capable of resolving proteins that differ in pI by fractions of a pH unit. Since HPLC failed to resolve hemolytic activity due to inactivation during separation, nondenaturing techniques were next attempted. Isoelectric focusing in the absence of detergents is a powerful technique to isolate proteins in their native conformations. The Rotofor preparative apparatus has a focusing chamber divided into 20 compartments located between a cation exchanger (anode H_3PO_4) and an anion exchanger (cathode NaOH). During a typical Rotofor run, the protein(s) is eletrophoresed through an established pH gradient which is rapidly formed during the early stages of electrophoresis by small, charged molecules or ampholytes. Proteins migrate in the electrical gradient based on their net charge until they reach the pH where their net charge is 0 where they focus. The voltage drops when the ampholytes are moving and then will gradually increase while the proteins are focusing. When all the proteins are focused the voltage becomes steady.

As a control, ampholytes (2%) were mixed with sHL, dialyzed and tested for HL activity. Addition of ampholytes did not inactivate the hemolytic activity. Ten to twelve mls of sHL were then loaded on the 15 ml Rotofor cell. Twenty samples were collected with each run, and their protein concentrations were measured. The final pH range established for each run was 1.72–11.97. All of the samples were dialyzed and tested for hemolytic activity on blood agar plates and the presence of Mab-reactive antigen by dotting the individual samples onto nitrocellulose or by coating an ELISA plate. Fractions 1 - 4 in the acidic portion of the gradient showed positive HL activity on blood agar, but were not recognized by the Mab. Only fraction 10 in the neutral portion of the gradient gave a positive response with the Mab.

These results demonstrate the limitations of this approach and the unavailability of adequate immunoreagents for the detection of purified sHL. The presence of even small amounts of ampholytes resulted in lysis of red cells on blood agar so it is not clear if fractions 1-4 actually contained active sHL or if residual amounts of ampholytes remained in the samples after dialysis. The protein concentration of the samples was too low to analyze by SDS-PAGE. Also, previous studies showed that a majority of the proteins from *M. pulmonis* focused in this region of the cell which would make it difficult to identify a specific mycoplasmal component associated with HL activity. Because of the lack of confidence in the technique, it was decided to attempt purification by other techniques.

Gel filtration

Preliminary studies were performed to determine which gel filtration chromatographic media would be best suited for purification of sHL, Sephacryl (spherical allyl dextran and

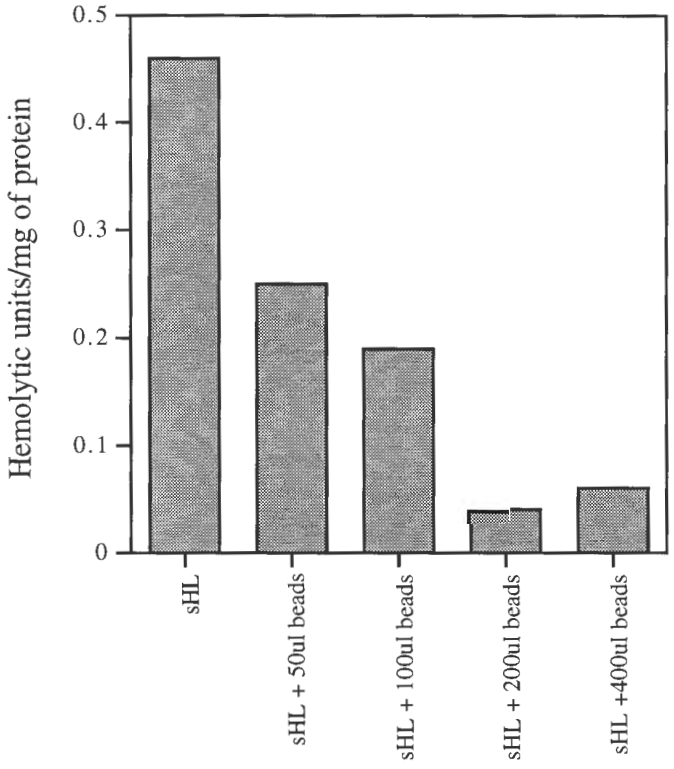


Figure 3. Effect of Sephacryl S-200 on sHL activity. sHL containing fractions were mixed with Sephacryl S-200 chromatography media and then examined for HL activity as described in the Materials and Methods. With increasing amounts of Sephacryl S-200, activity decreased indicating nonspecific adsorption to the chromatography media. Data are from one experiment.

N,N′-methylene bisacrylamide), Sepharose (cross-linked agarose), or Sephadex (cross-linked dextran). Initial tests proved that sHL activity nonspecifically adsorbed to both Sephacryl S-200 (Figure 3) and Sepharose (Figure 4). Even when the combination of sHL and glycerol were reacted with Sepharose/Sephacryl beads the hemolytic activity did not reach the levels of hemolytic units as when compared to the sHL alone (Figure 5). Sephadex G-200 beads seemed to be the only column-chromatography matrix that did not nonspecifically adsorb any of the sHL hemolytic activity.

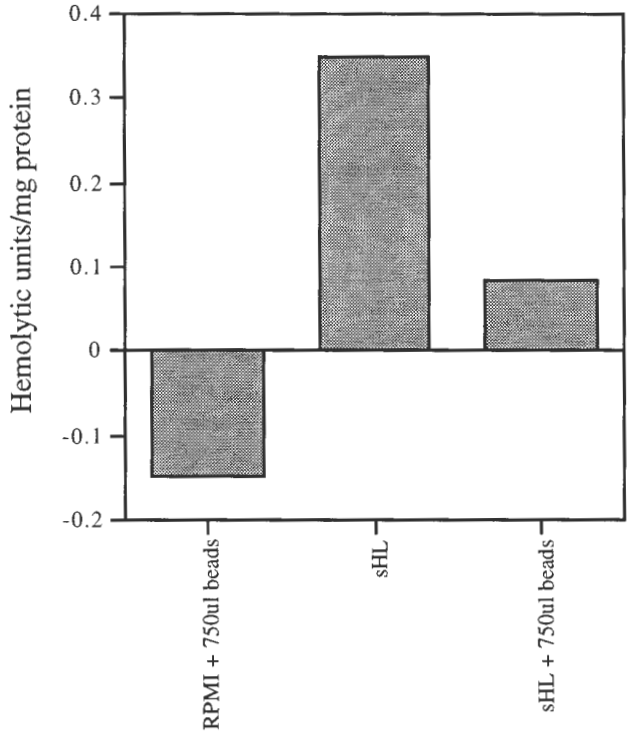


Figure 4. Effect of Sepharose beads on sHL activity. sHL containing fractions were mixed with Sepharose chromatography media and then examined for HL activity as described in the Materials and Methods. Data are from one experiment.

Concentrated sHL was loaded onto a Sephadex G-200 column as described in Materials and Methods. All hemolytic activity was detected in the fractions containing the void volume with an estimated size of 200 kDa or larger (Figure 6), suggesting that the sHL is either a large, multimeric complex or that the protein or complex is nonglobular in shape. When analyzed by SDS-PAGE, the pHL fraction gave a single prominent band with several smaller bands (Figure 7). The combination of ultrafiltration followed by gel filtration lead to a partially-purified hemolytic preparation designated as pHL.

Native-PAGE gel purification

To further purify the pHL obtained by gel filtration chromatography, 2 mls of pHL was loaded onto a 5%, 3 mm, preparative native-PAGE gel. A prominent band of MRBC lysis (Figure 8) was identified by laying the gel on a blood agar plate to determine the location of the

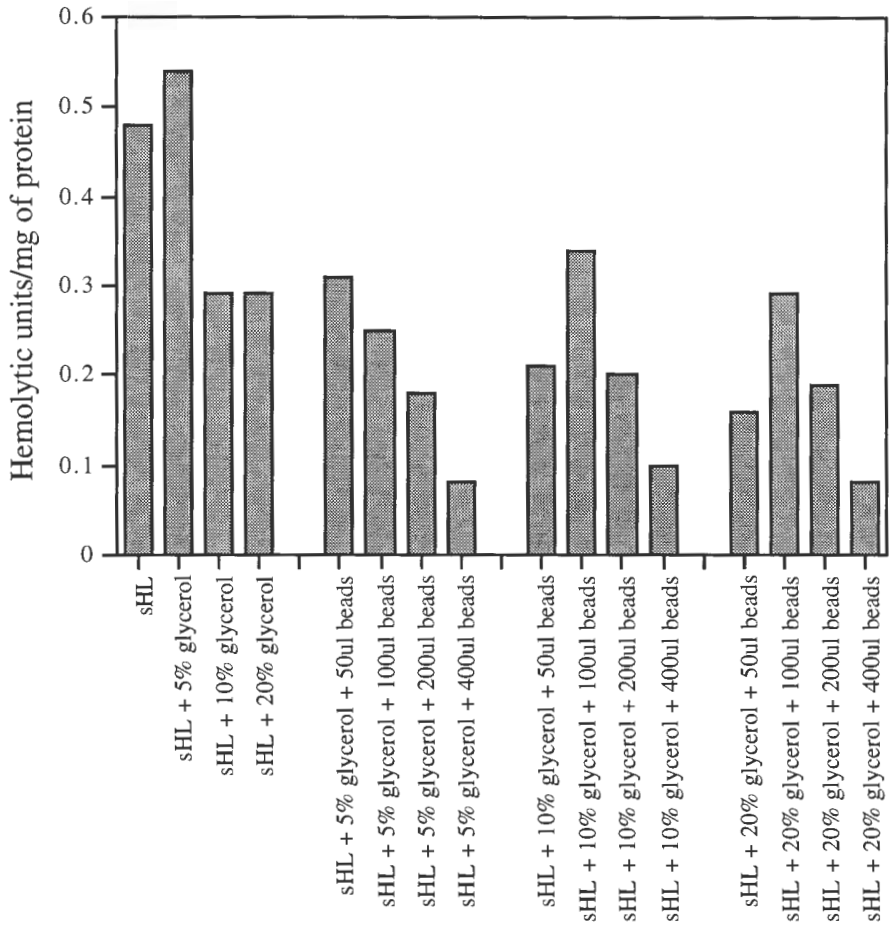


Figure 5. Effect of glycerol on the nonspecific adsorption of sHL to Sephacryl S-200 chromatography media. Increasing concentrations of glycerol (5, 10 or 20%) was added along with increasing amounts of Sephacryl S-200 beads to sHL containing fractions, incubated and the remaining HL activity assessed as described in the Materials and Methods. Data are from one experiment.

hemolytic activity approximately 1.5 cm from the top of the gel. Initial lysis of the blood agar happened within 4 hours of incubation at 37°C but diffused quickly throughout the agar over time (data not shown). This zone of lysis corresponded directly with a single band seen on native gels of pHL when stained with Coomassie blue (Figure 9). When electroeluted, this band is referred to as eHL. When comparing the Coomassie stained protein patterns of sHL loaded onto a native gel and pHL loaded onto a native gel (Figure 9), multiple bands can be seen on the sHL gel and only one band on the pHL gel. These results also support the hypothesis that the sHL is a large, multimeric complex.

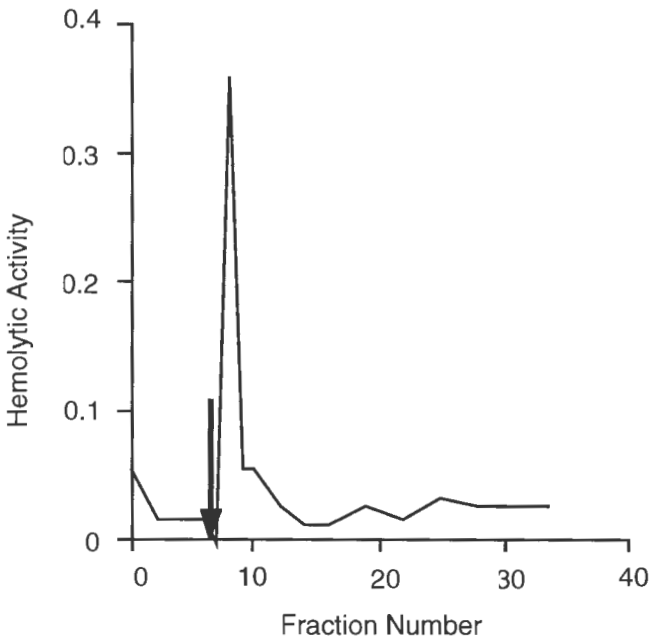


Figure 6. Profile of hemolytic activity from Sephadex G-200 column. Concentrated sHL supernatant was separated on a Sephadex G-200 column, and the fractions analyzed for hemolytic activity using the standard hemolytic assay. The arrow indicates the beginning of the void volume. Results are from a typical gel filtration experiment.

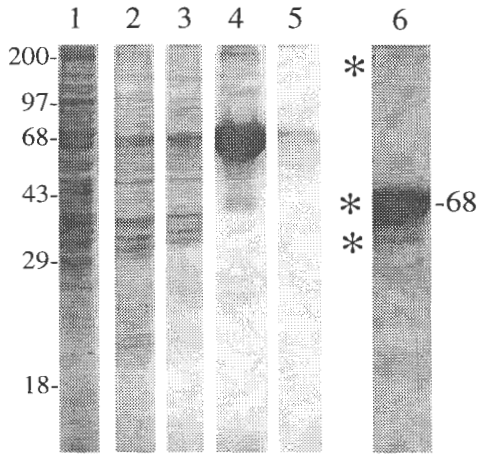


Figure 7. SDS-PAGE analysis of partially purified sHL fractions. A 10% SDS-PAGE resolving gel was used to separate hemolytic fractions at the various stages of purification. The gel was stained with Coomassie blue. Lane 1, *M. pulmonis* whole cell proteins; lane 2, sHL; lane 3, pHL; lane 4, eHL; lane 5, heat-treated pHL; lane 6, eHL fraction used for protein sequencing (from mini-gel). Asterisks indicate bands sequenced.

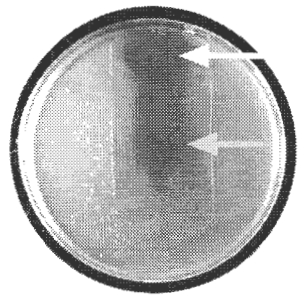


Figure 8. Analysis of pHL on MRBC agar when separated by native-PAGE. The upper arrow (white) indicates the hemolytic activity associated with the top region of the native gel. The lower gray arrow shows activity that is sometimes observed after prolonged incubation of the gel on the MRBC agar.

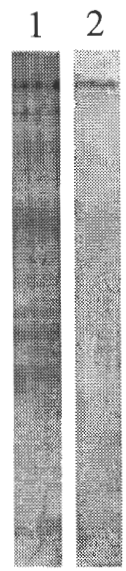


Figure 9. Coomassie blue stained native PAGE gel of hemolytically active sHL and pHL fractions. Lane 1, sHL fraction; lane 2, pHL fraction.

Protein sequencing

SDS-PAGE analysis of the active band(s) electroeluted from native gels revealed that the pHL was a multi-protein complex which may still contain contaminating proteins (Figure 7). The proteins were transferred to PVDF membrane and three of these bands (indicated by arrows) were excised and sequenced by the Iowa State University Protein Facility. The prominent middle band gave a sequence of DTAK(S+S′)EIAHRFKDCG. The upper band had several sequences present, suggesting either that proteolytic degradation had occurrred or that there are several N-terminal sequences possible for this peptide/protein. The most likely

sequence for the upper band was K?PKSEIAHR. The sequence for the lower band was not interpretable due to low protein concentration. Cycles with a "?" indicate that there seemed to be no major amino acid present for the cycle. This may indicate either a cysteine which cannot be detected unless it is pyridylethylated, a glycosylated amino acid, or a modified amino acid. BLASTP searches of the protein databases showed that the upper band had the highest homology (35%) with alpha 1-proteinase inhibitor. The middle band showed high homology (81%) to bovine serum albumin suggesting that this component is horse albumin derived from the serum component of the medium.

From the results above, the best purification procedure for the *M. pulmonis* sHL involved a combination of methods. Ultracentifugation was used to remove the *M. pulmonis* membrane fraction followed by ultrafiltration to concentrate the sHL. Gel filtration of the concentrated material on Sephadex G-200 was then performed collecting the sHL in the void volume. The final purification step which still retained hemolytic activity was native-PAGE. Active fractions could be electroeluted from native gels and analyzed further, but the use of any procedure that disrupted the complex resulted in the loss of hemolytic activity. Figure 7 shows how the combination of these methods yielded a partially-purified sHL by comparing *M. pulmonis* whole cell proteins, sHL, pHL, and eHL (electroeluted HL from a native-PAGE gel).

Radiolabeling Studies

The results mentioned previously clearly show that sHL is a large complex and that serum albumin is a major constituent of that complex. This suggests that the hemolytic activity is composed at least partially of a serum contaminant, but other studies clearly show that a mycoplasmal component is also required for activity. In the initial studies identifying the *M. pulmonis* hemolysin, Minion and Goguen showed that trypsin treatment removed the hemolytic activity from the mycoplasmal cell surface (125). Since the assay is performed in 1% BSA, trypsin treatment did not simply degrade the serum albumin of the membrane-associated complex. This could have been quickly replaced by the albumin in the assay buffer. Minion and Goguen also showed that following trypsin treatment, hemolytic activity could be regenerated, but only under conditions where protein synthesis was occurring (the protein synthesis inhibitor chloramphenicol prevented restoration of hemolytic activity) (125). Thus, a mycoplasmal protein component must be involved in the membrane-associated hemolysin activity. There is also the unanswered question of how the hemolytic complex is maintained in solution and whether a mycoplasmal protein component is involved in this structure or just in maintaining its membrane association during cell growth. These studies were undertaken in

order to determine if a mycoplasma protein or other macromolecule could be identified in the sHL complex through intrinsic radiolabeling procedures.

^{35}S methionine and cysteine metabolic radiolabeling

The first radiolabeling experiments involved incorporation of Trans [^{35}S] radiolabel (Amersham) metabolically into proteins via methionine or cysteine residues. This approach seemed reasonable since the mHL was proven to be sensitive to thiol-active compounds and oxidizing reagents suggesting a role for disulfide residues in the active site or in maintaining the complex's structure (79). Following radiolabeling, pHL was purified by gel filtration using disposable 0.8 x 4 cm columns (BioRad) and the void volume was analyzed by SDS-PAGE and autoradiography as described in the Materials and Methods. The results of this study are shown in Figure 10.

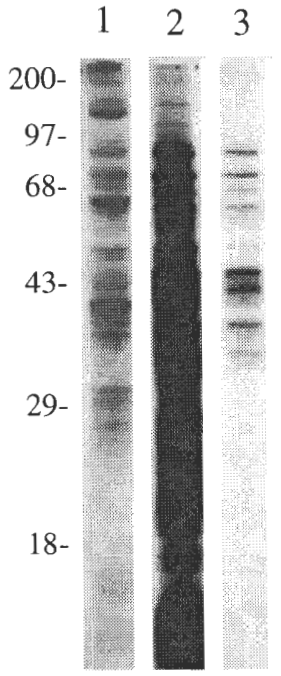


Figure 10. SDS-PAGE analysis of ^{35}S-labeled whole cell protein, sHL and pHL fractions. Lane 1, whole cell protein (2 hour exposure); lane 2, sHL fraction (3 day exposure); lane 3, pHL fraction (3 day exposure).

^{14}C amino acid radiolabeling

In an attempt to improve the radiolabeling of the mycoplasma proteins by either increasing the specific activity of the labeling (all amino acids are radiolabeled as opposed to only methionine and cysteine) and potentially the number of proteins labeled (some mycoplasma proteins may not contain either methionine or cysteine), *M. pulmonis* cultures were radiolabeled with a [^{14}C] amino acids mixture. pHL fractions were then prepared from these radiolabeled cells using disposable columns and analyzed by SDS-PAGE as described in the Materials and Methods. The *M. pulmonis* ^{14}C-labeled whole cell protein when compared with the *M. pulmonis* ^{35}S-labeled whole cell protein, lane 1 in the [^{35}S] autoradiograph (Figure 10) showed more protein bands indicating that the labeling was more universal. The specificity of labeling was much lower, however, and it was not possible to identify specific proteins in either the sHL or pHL fractions radiolabeled with ^{14}C (data not shown).

^{32}P Na_2HPO_4 radiolabeling

Protein modification by phosphorylation has only been identified very recently in mycoplasmas (212). Therefore, it was of interest to determine if the hemolytic complex also contained phosphorylated mycoplasmal proteins. [^{32}P] Na_2HPO_4 was used to radiolabel *M. pulmonis* cultures which should label both phosphorylated proteins and possibly some lipids as well. To increase labeling efficiency, phosphates were first removed from the media by dialysis against Tris buffer prior to radiolabeling. The organisms were radiolabeled as described in the Materials and Methods and sHL and pHL preparations obtained as described for ^{14}C- and ^{35}S-labeled preparations above. To confirm the proteinaceous nature of the radiolabeled bands, portions of the preparations were subjected to trypsin digestion. These preparations, including the trypsin-digested material, were then analyzed by SDS-PAGE. Only the whole-cell pellet lane contained radiolabeled bands, mostly at the stacking/resolving layer interface; no radiolabel was detected in either the sHL or pHL lanes (data not shown).

Biological Activities of pHL on Immune Effector Cells

Studies were undertaken to further delineate the functional nature of this pHL activity. Following successful colonization of host tissues by invading microorganisms, interactions between mycoplasmas and the host immune system are initiated. Various cellular assays were performed to examine the biological effects of the hemolysin on chemotaxis, blastogenesis, and phagocytosis.

Chemotaxis

A wide range of concentrations of pHL (0.1 to 50 µg/ml) were tested for their chemotactic potential for J774.A1 mouse macrophages. The results of this study are shown in Figure 11. A strong chemotactic activity was observed with concentrations from 5 to 50 µg per ml of pHL. The 30 µg per ml concentration gave the strongest chemotactic dose response when compared to the positive control GBSS + 1 µg per ml C5a. A checkerboard analysis was then done to determine whether chemokinesis (increased random motility) contributed to the observed J774.A1 chemotactic (directed migration) response. This was done by placing 10 µg per ml of pHL on both sides of the filter thus abolishing the concentration gradient of pHL. Since enhanced migration was not dependent upon a gradient of pHL (compare 10 pHL with 10 pHL (T&B), Figure 11), it appeared that pHL is both chemokinetic and chemotactic. This is not surprising since C5a, a well-known, potent chemoattractant, also shows chemokinetic properties. Heat inactivation decreased the chemotactic activity by approximately 50% which is in agreement with previous studies by Ross et al. (165).

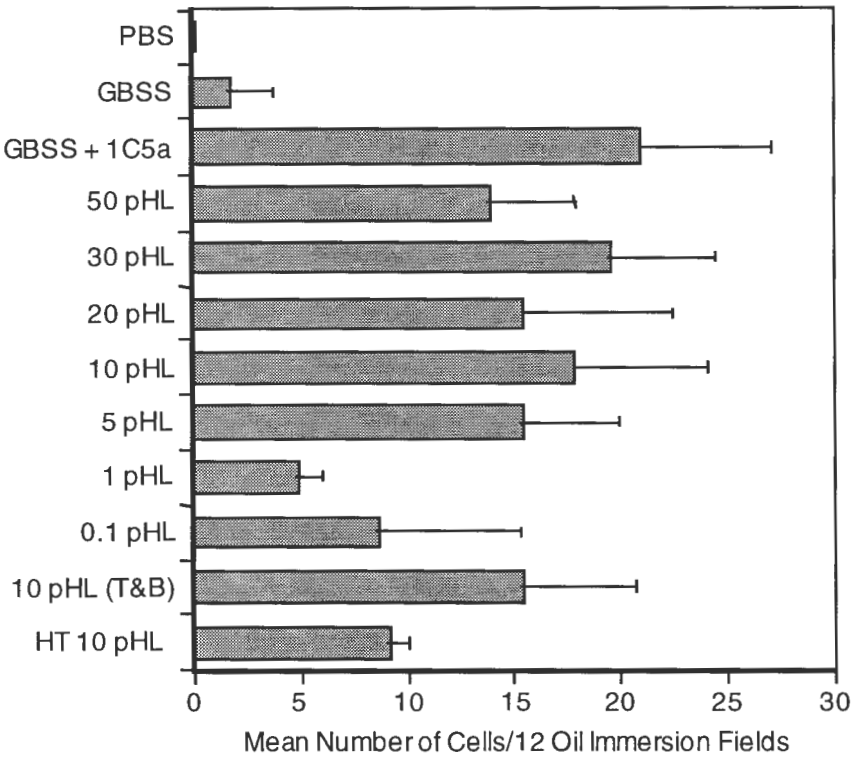


Figure 11. Effect of pHL fractions on J774.A1 migration. Data are expressed as the number of cells per 12 oil immersion fields (magnification, 100X). Data represent mean ± standard error of three experiments, 6 replicates per experiment. T&B, pHL in both top and bottom wells; HT, heat treated (70°C, 90 min).

Blastogenesis

In vitro stimulation of C3H/HeOuJ mouse splenic lymphocytes with *M. pulmonis* lysate, pHL, and heat-inactivated pHL was determined by the uptake of [^{3}H]thymidine. The blastogenic activity of these preparations, except for pHL, was found to be comparable in strength to those of ConA and LPS-endotoxin (Table 1). These results show that mouse splenic lymphocytes respond strongly to ConA, LPS-endotoxin (positive controls), and *M. pulmonis* lysate. In contrast, they were not significantly simulated by nondenatured pHL which was surprising due to the presence of potent mitogens in the *M. pulmonis* membrane. Once pHL was heat treated (70°C, 90 min), however, the in vitro proliferative response of mouse spleen cells to pHL was enhanced compared with the untreated pHL preparations.

The effect of heat-treated pHL on mouse splenic lymphocyte responses to ConA and LPS-endotoxin was also studied. Heat-treated pHL had no effect on LPS-endotoxin stimulation at the 0.1 and 10 μg per ml concentrations and only a slight effect at the 1 μg per ml concentration (Table 2). In contrast, heat-treated pHL (1 μg per ml) + ConA (0.5-1 μg per ml) showed an increase in proliferation in comparison to the ConA alone treatment. Heat-treated pHL at a concentration of 5 μg per ml had no effect on ConA stimulation suggesting that increased levels of heat-treated pHL adversely affected thymidine uptake in the presence of ConA.

Various mycoplasmas differ not only in the lymphocyte subpopulation(s) they activate but also in their resistance to heat treatment. In most studies, the mitogenic activity of *M. pulmonis* was abolished upon heating (136, 137), although a recent report by Lapidot and Naot cites a heat stable mitogenic activity in *M. pulmonis* (103). Their studies discuss the use of Mab 3.3.10.2 to study the *M. pulmonis* mitogen (103, 104). Therefore, it was of interest to determine whether this Mab reacted with sHL and pHL fractions and if heat treatment had an effect on this reactivity. These studies were performed by immunoblot using *M. pulmonis* lysate, sHL, pHL and heat treated pHL fractions resolved by 10% SDS-PAGE gels. The results of this study are shown in Figure 12. The Mab recognized two bands in the lysate sample, a band of approximately 97 kDa and a lower molecular weight band of approximately 33 kDa. The sHL had multiple bands in the 75-95 kDa range; the upper band had the highest intensity probably indicating a higher concentration of this material. The pHL sample also had the multiple banding pattern of sHL, but with slightly different staining intensities; the lower 75 kDa band had the same intensity as the upper 95 kDa band. Upon heat treatment, the multiple banding pattern was resolved to a single band of 75 kDa. These results suggest that sHL and pHL fractions contain a subset of the immunoreactive components of *M. pulmonis* recognized

Table 1. Mouse lymphocyte blastogenesis induced by various *M. pulmonis* preparations[a]

Mitogen derived from: (μg per ml)	Thymidine Uptake (cpm ± SD)[b]
lysate	
2.0	4143 ± 260
1.0	7148 ± 1398
0.5	10874 ± 745
0.25	15296 ± 1046
0.125	11190 ± 936
pHL	
5.0	1337 ± 296
3.0	2105 ± 734
2.0	1781 ± 550
1.0	1446 ± 387
0.5	1666 ± 909
0.1	1054 ± 339
0.01	1940 ± 255
Heat-treated pHL	
5.0	12139 ± 902
1.0	6584 ± 1661
ConA	
1.0	10348 ± 1150
0.5	12598 ± 2138
0.25	25111 ± 2846
LPS	
10.0	17290 ± 2444
1.0	10839 ± 946
0.1	3137 ± 544
RPMI	1658 ± 382

[a] C3H/OuJ mouse spleen cells ($5x10^5$ cells per well) were cultured in the presence of various concentrations of *M. pulmonis* lysate, pHL, heat inactivated (HIA) pHL, ConA, and LPS-endotoxin.

[b] Results represent the mean ± standard deviation (SD) of three independent experiments each performed in triplicate.

by Mab 3.3.10.2. Lapidot et al. also suggest that the prolonged heat treatment (70°C, 90 min) of pHL had a substantial effect on the size of the complex components as determined by SDS-PAGE (104). This could be due to changes in the conformation of the immunoreactive components or to their modification during heat treatment. It is important to note that Mab 3.3.10.2-reactive components in pHL was lost upon further purification by native PAGE (data

not shown). This shows that the hemolytic activity of pHL has no relationship to the Mab 3.3.10.2-reactive components of the partially purified material.

Since heat treatment of pHL significantly altered its immunoreactivity to Mab 3.3.10.2, it was of interest to determine if a change in the overall size of the complex could be detected following heat treatment. pHL fractions, before and after heat treatment, were passed through 10,000, 30,000 and 100,000 molecular weight cutoff ultrafilters and the retentate and filtrate were tested for blastogenic activity as described above. Blastogenic activity of the heat-treated pHL failed to pass through the 10,000 and 30,000 molecular weight cutoff filters, but passed partially though the 100,000 molecular weight cutoff filter (Table 3). This was in agreement with previous studies showing that sHL hemolytic activity could pass partially through a 100,000 molecular weight cutoff filter (78). Thus, it appeared that heat treatment of pHL (70°C, 90 min) did not significantly alter the size of the complex.

Table 2. Effects of heat-treated pHL on ConA and LPS-endotoxin[a]

Mitogens (μg/ml)	Thymidine Uptake (cpm ± SD)
LPS-endotoxin	
10	15936 ± 968
1	4750 ± 1365
0.1	3173 ± 388
LPS + HT pHL (5)	
10	11092 ± 80
1	7832 ± 1105
0.1	5820 ± 539
LPS + HT pHL (1)	
10	15556 ± 269
1	9949 ± 291
0.1	3574 ± 779
ConA	
1	9009 ± 1495
0.5	12598 ± 2138
0.25	24099 ±3082
ConA + HT pHL (5)	
1	10440 ± 289
0.5	12595 ± 511
0.25	18666 ± 1296
ConA + HT pHL (1)	
1	18720 ± 238
0.5	23640 ± 4060
0.25	23031 ± 1809
RPMI	1993 ± 248

[a]Results are means of three independent experiments each performed in triplicate. HT, heat treated.

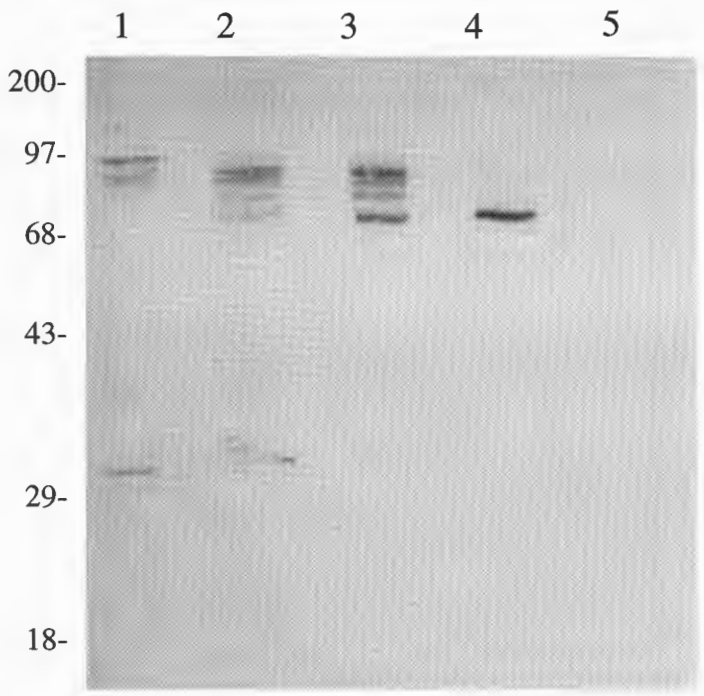


Figure 12. Immunoblot analysis of *M. pulmonis* lysate, sHL, pHL and heat-treated pHL using Mab 3.3.10.2. Each sample was treated at 100°C for 2 min in SDS-PAGE sample buffer. Heat-treated pHL was treated at 70°C for 90 prior to addition of sample buffer. Lane 1, *M. pulmonis* lysate; lane 2, sHL; lane 3, pHL; lane 4, heat-treated pHL; lane 5, eHL. Molecular weight standards are given in kilodaltons on the left.

Table 3. Effect of ultrafiltration on heat-treated pHL blastogenic activity.

Mol. Wt. Cutoff Size	100,000	30,000	10,000
Retentate	6257 ± 1191	11550 ± 5871	13066 ± 2037
Filtrate	7678 ± 1181	2105 ± 378	1990 ± 39
Heat-treated pHL			
5.0		12139 ± 902	
1.0		6584 ± 1661	
RPMI		1658 ± 382	

[a]Results are means ± standard deviation of one experiment in triplicate.

Phagocytosis

Previous studies of *M. pulmonis* interactions with alveolar macrophages suggested that these organisms contained a trypsin-sensitive anti-phagocytic factor on the cell surface (54). Therefore, it was of interest to determine if the trypsin-sensitive hemolytic activity (pHL) could serve this purpose. pHL was examined for its ability to stimulate phagocytic activity of J774.A1 and P388.D1 cell lines. The results of these studies is shown Figure 13. The percent phagocytosis of either cell line was not affected by pHL at any concentration used. Thus, it appears that pHL is not related to the anti-phagocytic activity observed previously by Davis et al. (54).

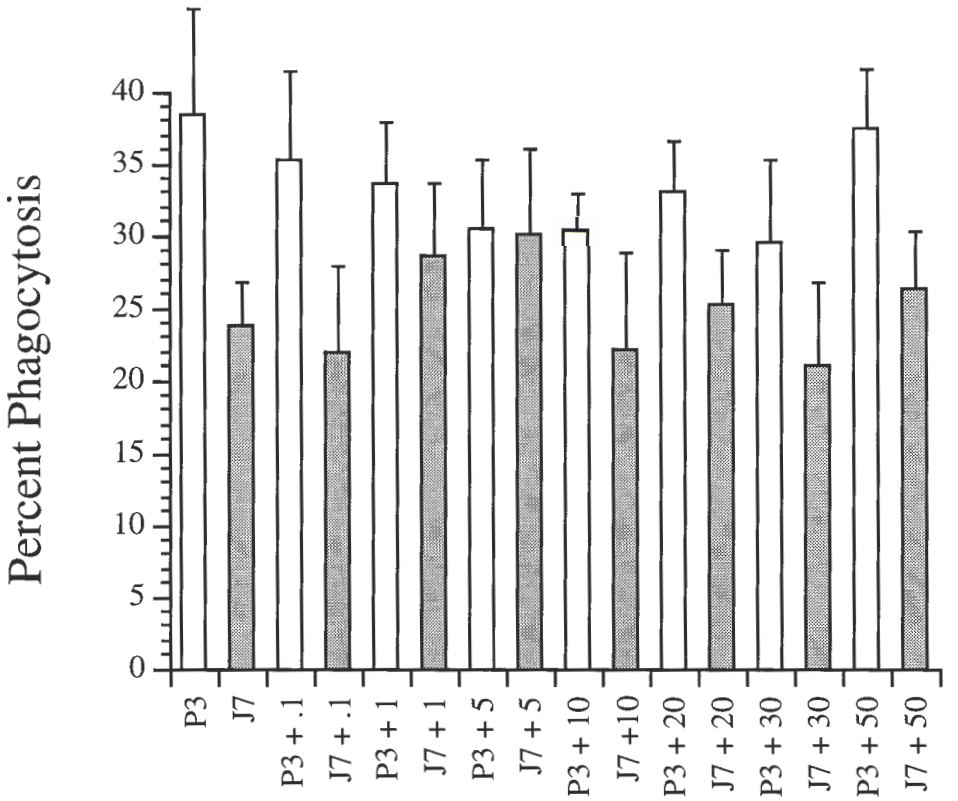


Figure 13. Effect of pHL on phagocytic activity of J774.A1 and P388.D1 cell lines. Numbers represent µg of pHL. White bars are results with P388.D1 cells, gray bars are results with J774.A1 cells. Data represent means ± standard error of three experiments, duplicates within each experiment.

CONCLUSIONS

Based upon the results of these studies, the following conclusions have been made:

1) Incubation of mycoplasma suspensions under conditions that inhibit cell division results in the release of soluble hemolytic activity (sHL).
2) Appearance of hemolytic activity (sHL) in the cell suspension supernatant occurs after 5 hours of incubation.
3) The release of sHL correlates with cell death, probably occurs as a consequence of membrane rupture and is not a normal consequence of in vitro cell growth.
4) The increase in NADH oxidase activity observed in the cell suspension supernatants correlated directly with the loss of viability indicating that cell lysis occurs even in an isotonic buffer.
5) sHL appeared to be a large complex because it was found in the void volume of a Sephadex G-200 column. By that criteria, the complex had a molecular size of 200 kDa or greater. Ultrafiltration data, however, indicated that the complex had a size of approximately 100 kDa. This suggests that the complex is not globular in shape, but may have a rod-like shape which could explain both observations.
6) The main component of pHL appeared to be serum albumin, a requirement for hemolytic activity.
7) Intrinsic radiolabeling demonstrated that mycoplasmal components were also incorporated into the hemolytic complex, but it was not possible to identify a specific component associated with the hemolytic activity.
8) The anti-mitogenic Mab 3.3.10.2 recognized several bands in the sHL, pHL and heat-treated pHL fractions. This reactivity was lost upon further purification by native PAGE demonstrating that Mab 3.3.10.2-reactive components had no relevance to the hemolytic activity.
9) The complex had the property of nonspecific adherence to various surfaces including two chromatography media, Sepharose and Sephacryl.
10) The pHL showed strong chemotactic and chemokinetic activity for J774.A1 macrophages which approached the activity of C5a. The concentration of 30 μg per ml was the most active of the concentrations tested.
11) Although it enhanced the migratory activity of macrophages, pHL had no effect on their phagocytic activity.

12) Untreated pHL had no effect on mouse splenic lymphocytes, but heat-treated pHL (70°C, 90 min) was a potent blastogenic factor. Since heat-treated pHL (1 μg per ml) enhanced the ConA response (0.5-1 μg per ml), the blastogenic effect of pHL appears to reside with mouse T cells. This mitogenic activity is different from previously reported activities.

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