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Molecular mechanisms of *Mycoplasma hyopneumoniae* adherence to swine respiratory epithelial cells

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Molecular mechanisms of *Mycoplasma hyopneumoniae*

adherence to swine respiratory epithelial cells

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GENERAL INTRODUCTION

Mycoplasma hyopneumoniae is the etiological agent of mycoplasmal pneumonia of swine (MPS), one of the most important swine diseases all over the world (115). Although the mortality caused by MPS is usually low, the economic impact incurred by MPS is enormous because of retarded growth and poor feed conversion of infected pigs (116). Since the discovery of this mycoplasma in the 1960s, MPS has been the focus for many research groups around the world. Accumulated information generated in the past decades has greatly improved our understanding of the pathogen and the disease. Commercial vaccines against MPS are now available. These vaccines significantly reduce the development of lung lesions in immunized pigs, but do not prevent infection with *M. hyopneumoniae* in the respiratory tract of swine (116). The fact that the virulence mechanisms of the organism have not been well defined is a limiting factor in development of highly efficient control measures for MPS.

Previous in vivo studies with various immunohistochemical techniques demonstrated that *M. hyopneumoniae* cells polarized at the apical surface of the porcine respiratory tract epithelium during infection (4, 99, 140). This organism does not invade the epithelial cells nor penetrate the lamina propria. Colonization of *M. hyopneumoniae* in the respiratory tract leads to an extensive loss of cilia (15, 140, 99, 156), which play an important role in the clearance of harmful substances and infectious agents. Therefore, *M. hyopneumoniae* infection not only induces

typical inflammation in the lungs, it also compromises the defense system in the respiratory tract of pigs, which predisposes to secondary bacterial or viral infections.

Adherence of *M. hyopneumoniae* to ciliated cells is an important initial step in the infection. Previous studies showed that this mycoplasma was able to adhere to many cell types in vitro (154, 157), although it only adheres to cilia of ciliated cells in vivo. Results from these in vitro studies suggest that the adherence process is a multiphasic and complex event, involving both nonspecific hydrophobic interactions and specific receptor-ligand interactions (157, 160). However, the strict tropism of the mycoplasmas for cilia as shown in vivo (99, 140, 156) and in a single ciliated cell adherence assay (160) strongly argue that receptor-ligand interactions are the major factor in the adherence of *M. hyopneumoniae* to ciliated cells. Partially due to the limitations in the established adherence assays and lack of monoclonal antibodies to the surface proteins of *M. hyopneumoniae*, previous work failed to define the molecular mechanisms involved in the adherence process.

This project was designed to evaluate the receptors and adhesins that mediate the interaction of *M. hyopneumoniae* with ciliated cells. There were three specific goals for this study: 1) to establish a specific and convenient adherence model that mimics the in vivo attachment of *M. hyopneumoniae*; 2) to identify and characterize the natural receptors in cilia for *M. hyopneumoniae* adherence; and 3) to define the adhesins of *M. hyopneumoniae*. To achieve the first goal, cilia, which are the in vivo targets of *M. hyopneumoniae* infection, were purified from swine

tracheal ciliated cells and utilized to coat microtiter plates to develop a microtiter plate adherence assay. A number of glycoconjugates and carbohydrates were evaluated in this assay for inhibition of adherence of the mycoplasmas. For the second goal, a thin-layer chromatography (TLC) overlay assay was established to identify the glycolipid receptors in cilia for attachment of *M. hyopneumoniae*. In the third study, various immunochemical techniques along with the microtiter plate adherence assay were utilized to elucidate the adhesins of *M. hyopneumoniae*.

Dissertation organization

This dissertation contains a general introduction, literature review, three manuscripts, general discussion and references. The literature review precedes the first manuscript and a general discussion follows the last manuscript. References cited in a manuscript follows the manuscript, while literature cited in the general introduction, literature review, and general discussion is presented in a separate section which follows the general discussion. The three manuscripts were written in the style of **Infection and Immunity**. The investigations were planned and executed, and the manuscripts written, primarily by the Ph.D. candidate, Qijing Zhang, with the advice of the major professor, Dr. Richard F. Ross, and other co-authors of the manuscripts.

LITERATURE REVIEW

Adherence and its significance in bacterial infections

Adherence, the process by which a microbe binds to tissue surfaces of man and animals, is a prerequisite for initiation of infectious diseases (19). This is especially true for those microbial pathogens that colonize the mucosal surfaces. Bacterial adherence plays several important roles in infection (7, 19, 105): it confers on bacteria the ability to resist and circumvent the flushing and cleaning mechanisms that protect epithelial surfaces in humans and animals; it determines the tissue tropism of a pathogen; it delivers toxic effects on and induces signal transduction across host cell membranes; and it improves acquisition of nutrients from host cells.

There is much evidence showing that adherence is directly involved in infection or pathogenicity. For many bacteria, such as salmonella, escherichia, streptococci, pseudomonas, staphylococci, and neisseria, their ability to adhere to target cells *in vitro* is well correlated with their infectivity *in vivo* (105). Satterwhite *et al.* (122) reported that an isogeneic derivative of an enterotoxigenic strain of *Escherichia coli* (ETEC) which lacked adhering ability and colonizing factor, but retained the ability to produce enterotoxin, was unable to induce diarrhea in humans. In contrast, the parent strain possessing adherence ability produced the disease. This finding indicated that the bacteria must be able to adhere to the

intestinal epithelial cells in order to multiply, colonize the mucosal surface and then produce sufficient amount of enterotoxin to cause diarrhea. In light of the susceptibility to *E. coli* infection, two phenotypes occur among swine populations. One has adhesive intestinal brush borders and is susceptible to K88+ ETEC diarrhea, and the other one has nonadhesive brush borders and is resistant to the disease (126). The difference between the phenotypes is genetically determined (43). The molecular basis for the difference was revealed by Erickson *et al.* (33) who demonstrated that the glycoprotein receptors for K88-mediated adherence are present only in the adhesive brush borders, but not in the nonadhesive brush borders. Older pigs gain resistance to diarrheal disease associated with *E. coli* bearing the K99 adhesin by loss of the receptors for the adhesin (117). Age-related expression of receptors is also responsible for susceptibility to infections caused by ETEC that do not produce K88, K99, F41, or 987P pili (104). The loss of adherence capacity caused by mutation leads to loss of the infectivity of *Mycoplasma pneumoniae*, and reversion of the adherent phenotypes is accompanied by regaining infectivity and virulence (79, 80). These findings strongly indicate that adherence is an important virulence factor of microbial pathogens.

Bacterial adherence: molecular mechanisms

The surfaces of bacteria and eucaryotic cells are overall negatively charged. How can adherence occur between two negatively charged surfaces? A widely

recognized theory for interaction between two negatively charged surfaces was provided by Derjaguin-Landau-Verwey-Overbeek (DLVO, 7, 19). According to the DLVO theory, when two objects with similar charges come into close approximation they are subjected to attractive and repulsive forces which vary with the distance of separation of the two objects. At long distances (>10 nm), the forces of attraction are greater than the repulsive forces, and the two objects are held in a state of mutual attraction. But the forces of attraction are weak at this stage, and adherence may be reversed. At short distances (<10 nm), the absorbed counter ion clouds on each surface cause the objects to repel each other. If the repulsive forces can be overcome and smaller distances (<1 nm) of separation achieved, then the forces of attraction are again greater than the repulsive forces, and the objects are held by firm binding. Bacteria must have specialized mechanisms to counteract the repulsive forces in order to achieve firm adherence. In fact, many pathogenic bacteria express adhesins that mediate adherence by recognizing receptors on the surface of eucaryotic cells.

Adhesins, expressed on the surface of bacteria, are usually protein in nature. Many pathogenic bacteria, such as *E. coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Bordetella pertussis, and Moraxella bovis*, have fimbrial adhesins that are visible under an electron microscope (7, 69). Most fimbriae are proteinaceous polymers composed of major subunits, which have molecular weights ranging from 8,000 to 26,000 daltons, and minor subunits. Recent findings have indicated that these

minor subunits are essential for structure and function of fimbriae (67). In fact, the adhesin subunits (receptor-binding domains) of many *E. coli* fimbriae are minor subunits distinct from the major subunits (52, 102). By using immunoelectron microscopy, the adhesin subunits of S, P, and type 1 fimbriae of *E. coli* were found to be located at the tips of the fimbriae or alongside the fimbriae as distinct globules (67,102,111,48). In contrast, the organization of the K88 fimbriae of swine pathogenic *E. coli* is different from the S, P, or type 1 fimbriae. The major subunit, rather than the minor subunit, of K88 fimbriae is the adhesin, indicating that it may be a polyvalent fimbriae (67). Besides fimbrial adhesins, *E. coli* also have nonfimbrial adhesins that are configured as very thin fibrillae surrounding the bacteria (66).

Genes encoding the major and minor subunits of fimbriae are normally found clustered on the chromosome, together with genes encoding accessary proteins required for export and assembly of the fimbriae. Genetic studies indicate that the gene organization of nonfimbrial adhesins is similar to that of fimbrial adhesins, but lacks the gene encoding the major structural subunits for fimbriae (67). The expression of fimbriae is regulated by environmental conditions, for instance, temperature and growth rate (27). Phase variation between the fimbriated and nonfimbriated state has been observed with type 1 (1), 987P, and P fimbriae (27). Phase variation is also responsible for alternative expression of Q and I fimbriae of *Moraxella bovis* (98).

A fine irregular fuzz, or fibrillae, which covers the streptococcal surface and

is composed of M protein and lipoteichoic acid (LTA), is responsible for adherence of streptococci to human epithelial cells (32). As an amphipathic surface molecule, LTA binds to many eucaryotic cells. A microorganism may have multiple adhesins, providing for increased adherence specificity and strength. *N. gonorrhoeae* has two adherence structures for tissue tropism (138). One is a fimbrial adhesin (pili), and the second is an outer membrane protein known as protein II (P II).

Bordetella pertussis, a human pathogen that causes whooping cough, has 4 potential adhesins: filamentous hemagglutinin (FHA), pertussis toxin (PT), pertactin, and fimbriae (147, 146, 88,151). FHA and PT adhesins mediate the interaction of *B. pertussis* not only with ciliated cells but also with human macrophages (147). FHA is a 220 kDa single polypeptide with a binding specificity to galactose-containing glycoconjugates on cilia. In addition, FHA has a Arg-Gly-Asp (RGD) triplet which binds to the integrin receptor (CR3) on macrophages (146). PT is the only bacterial toxin known to have adhesive capabilities. Both FHA and PT can be cell-associated or secreted into the surrounding environment and are only expressed in virulent strains (146). Pertactin, a 69-kDa protein containing 2 RGD sequences, appears to be involved in the adherence of *B. pertussis* to macrophages. A *B. pertussis* mutant that does not express pertactin but does express other virulence associated proteins (FHA, PT, and fimbriae) adheres less well than the parent wild-type strains to mammalian cells (88). Fimbrial adhesins of *B. pertussis* have been characterized and isolated, but their role in adherence to human cells remains to be elucidated (151).

B. bronchiseptica, a pathogen that infects the upper respiratory tracts of pigs, dogs and rabbits, adheres specifically to nasal epithelial cells (153,13). This adherence appears to be mediated by fimbriae which are 3 to 4 nm in diameter and cross reactive with the fimbriae of *B. pertussis* (153, 87). Sialyl glycoconjugates are reported to be receptors for *B. bronchiseptica* in swine nasal epithelium (60). *B. avium* that colonizes the respiratory ciliated cells of turkey also has fine fimbriae which seem to be responsible for the adherence to tracheal epithelial cells (61). A recent study by Arp *et al.* (6) has shown that the receptors in turkey trachea for *B. avium* may be gangliosides or sialic acid-containing glycoconjugates. Bélanger *et al.* (12) investigated the adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal ring cultures. Their results suggested that lipopolysaccarides might be the adhesin of *A. pleuropneumoniae*. Although fimbriae have been found on *A. pleuropneumoniae* (148), their role in adherence remains to be determined.

The animal cell surface is covered with a layer of carbohydrates associated with plasma membrane proteins or lipids. This is especially true for epithelial cells of mucosal surfaces (106). These surface components play an essential role in controlling many cellular activities, for instance, development, differentiation and inter- or intracellular communication (106). Bacteria, bacterial toxins and viruses exploit these surface structures as attachment sites during infection (70). In most cases, the receptors on animal cells for microbial pathogens are glycoconjugate in nature. They can be either glycoproteins or glycolipids, in which the carbohydrate

moieties serve as receptor epitopes. The nature and the precise orientation of the carbohydrate moieties, as well as the neighboring molecular structures, determine the specificity of adhesin-receptor interactions and tissue tropism (70). In some cases, proteins, such as fibronectin, may serve as receptors for bacterial attachment, especially for Gram-positive bacteria and intracellular microbial pathogens (139).

It is well known that different types of fimbriae of *E. coli* recognize distinct carbohydrate sequences. Type 1 fimbriae of *E. coli* bind specifically to mannose-containing glycoconjugates. Mannosides inhibited type 1 fimbriae-mediated adherence and blocked infection of *E. coli* in the urinary tract (5). The P fimbrial adhesins of *E. coli* bind to the Galα1-4Galβ residue of glycolipids, while the S fimbriae recognize the α-sialyl-(2-3)-β-Gal sequence (45). Sialic acid residues of glycoproteins or glycolipids (gangliosides) are common moieties for attachment of bacteria, viruses and mycoplasmas. Using monoclonal antibodies and various receptor analogues, Tuomanen *et al.* identified galactose or galactose-glucose-containing glycoconjugates in human ciliated cells as receptors for adherence of *B. pertussis* (145). One of their interesting findings was that the receptors were only present on ciliary tufts, which are the *in vivo* sites for *B. pertussis* attachment, but not on cell bodies, indicating that the distribution of receptors for bacteria could be polarized in specific regions on host cell membranes.

Development of a thin-layer chromatography (TLC) overlay assay has greatly facilitated the identification and characterization of glycolipid receptors for

microbes (49). In this assay, glycolipids separated on a TLC chromatogram are overlaid with bacteria, viruses, or toxins. Specific binding to a glycolipid receptor is detected by either immunological labels or radioactive labels. Combination of TLC overlay assay with modern analytical methodologies, such as mass spectrometry and nuclear magnetic resonance spectroscopy, results in resolution of the molecular structures of glycolipid receptors (71). The carbohydrate moieties of glycolipids are simpler in structure and closer to the membrane surface than those of glycoproteins. Therefore, bacteria bound to glycolipid receptors are intimately associated with the host cell surface.

Most glycolipid receptors belong to three categories: gangliosides, sulfated glycolipids, and lacto-series glycolipids (85). Many pulmonary pathogens, such as *Pseudomonas aeruginosa, Haemophilus influenzae, and Streptococcus pneumoniae,* recognize the terminal or internal GalNAcβ1-4Galβ1 sequence found in gangliosides (84). This binding is independent of the sialic acid residues despite their presence in gangliosides. A recent study by Alphan *et al.* demonstrated that sialic acid-containing lactosylceramides on oropharyngeal epithelial cells and erythrocytes are responsible for the fimbriae-mediated binding of *H. influenzae* (3). It should be indicated that in many cases a carbohydrate sequence recognized by microbes is an internal sequence rather than a terminal sequence of oligosaccharides (84), implying that the accessibility of the sequence may affect the interaction with adhesins. Usually, sialic acid residues are located at the termini of oligosaccharide chains of glycoconjugates. Production of neuraminidase by some

bacteria during infection may play a role in unmasking the internal sequence for binding. There may be a redundancy of receptors on eucaryotic cells corresponding to the existence of multiple adhesins of a microorganism. For example, *Helicobacter pylori*, a causative agent of chronic gastritis and gastric ulcers, binds specifically to three different receptors: sialylated glycoconjugates (34), sulfatides (121), and Lewis[b] antigens (17) on the surface of gastric epithelial cells.

Integrins are a class of cell surface molecules with important physiological functions in cell-cell adhesion, cell-substratum adhesion, cell migration, cell differentiation, and signal transduction (31). Structurally, the integrins are transmembrane proteins consisting of α and β chains. Some bacteria utilize integrins as adherence receptors. One of the examples is *B. pertussis* that utilizes the integrin as a port of entry to the intracellular compartment of macrophages (123). The invasion of macrophages is mediated by the interaction of FHA of *B. pertussis* and the integrin (CR3) on macrophages. Mutation of the Arg-Gly-Asp (RGD) sequence in FHA resulted in loss of the ability to enter macrophages, indicating that FHA has two different adhesin epitopes, one for ciliated cells and another for macrophages. The uptake of *B. pertussis* by macrophages did not result in bacterial killing, and therefore, provided a sequestered reservoir of bacteria within the macrophages. These findings suggest that the RGD-mediated adherence to the integrin on macrophages contributes to persistence of *B. pertussis* in human lungs. Another important finding in *B. pertussis* adherence is

that PT shares significant homologies in structure and function with the eucaryotic

selectins, a family of glycoproteins that are expressed on the surface of endothelial

cells and direct the movement of leukocytes (51,144). Usually, ligation of selectins

with their corresponding ligands on leukocytes leads to increased expression of

integrins on leukocytes. Based on the information, Tuomanen proposed that the

adherence of *B. pertussis* could be a cooperative process in which secreted PT first

interacts with macrophages and induces the expression of the integrin CR3, and

then, FHA binds to CR3 resulting in entry to macrophages (144). Type 1-fimbriated

E. coli has also been shown to utilize the CD11/CD18 integrin as a receptor for

entry of phagocytes (38). Fibronectin and collagens are usually the receptors for

Gram-positive bacteria such as Staphylococci and Streptococci (139). However,

recent studies have indicated that some Gram-negative bacteria are also capable

of binding to extracellular matrix proteins. Thus, the fimbriae of enterobacteria

mediate adherence not only to epithelial cells by protein-carbohydrate interaction,

but also to extracellular matrix by protein-protein interaction (66). It is believed that

the two different interactions are mediated by different components of the fimbriae.

For example, the adhesin subunit (35 kDa) in P fimbriae of *E. coli* is responsible for

adherence to epithelial cells, whereas a distinct minor subunit (16.5 kDa) mediates

the attachment of *E. coli* to fibronectin and collagen; a 17 kDa subunit in the S

fimbriae plays the same role as the 16.5 kDa subunit in the P fimbriae (66). This

type of adherence may play a role in bacterial colonization of wounded tissues or

in penetration of cells and tissues.

Adherence-mediated host response

The adhesins of microbial pathogens not only play a role in tropism for tissues, but also induce host responses directly. For example, type 1 fimbriae of *E. coli* have been shown to stimulate T cell-independent proliferation of human B lymphocytes and secretion of immunoglobulins from resting B cells (107, 108). This stimulation is mediated by the mannose-specific adhesin subunit of type 1 fimbriae because α-methyl mannoside inhibits the response, and FimH- (without the adhesin subunit) type 1 fimbriae was nonstimulatory. Both *in vitro* and *in vivo* studies have shown that the P fimbriae of *E. coli* induce production of Interleukin 6 by urinary epithelial cells, while the P fimbriae lacking the receptor-binding domains do not have the activity (91, 50). These findings indicate that the binding of carbohydrate receptors by the adhesins triggers cellular responses. It is reasonable to speculate that the adhesin-receptor interaction induces signal transduction across the cell membrane of epithelial cells, leading to expression of IL-6 molecules. Since IL-6 plays a role in inflammation by increasing the production of acute phase proteins and acting as endogenous pyrogen, it is possible that the fimbriae of *E. coli* are directly responsible for development of mucosal inflammation. These findings illustrate the newly discovered roles of fimbrial adhesins in the pathogenic mechanisms of bacteria. Considering the diversity of adhesin-receptor interactions, it is expected that more and more host tissue responses to microbial adhesins will be discovered in the future.

Enteropathogenic *E. coli* (EPEC) cause diarrhea in man and animals. Intestinal epithelial cells infected by EPEC exhibit an extensive loss of microvilli and a proliferation of filamentous actins beneath the regions of intimate bacterial attachment; this process is known as "attaching" and "effacing" (76). The mechanisms underlying the attaching and effacing process have been the focus for many research groups. Recent studies have suggested that adherence-induced signal transduction may play a role in microvillous effacing and rearrangement of cytoskeletal proteins (30). It was found that adherence of EPEC to eucaryotic cells leads to elevation of intracellular calcium; restoration of intracellular calcium levels prevented formation of attaching and effacing lesions, suggesting that second messengers are involved in lesion development (30, 9). Donnenberg and Kaper have speculated that elevated calcium concentrations lead to cleavage of actin within the microvillous core, resulting in effacement of microvilli (30). Adherence of EPEC to HEp-2 cells also results in phosphorylation of several cellular proteins, including myosin light chain, vaculin, α-actinin, and unidentified proteins (97, 112), which link the membrane receptors to cytoskeletal components. Inhibitors of tyrosine kinase blocked EPEC invasion (114). These observations strongly imply that protein phosphorylation and changes in intracellular calcium levels may be responsible for the ultrastructural lesions caused by EPEC infection.

Adherence is essential not only for extracellular pathogens, but also for intracellular pathogens. In fact, adherence-mediated signal transduction is essential for many intracellular bacteria, such as shigella, yersinia, and salmonella,

to gain entry into mammalian cells (59, 16). For example, yersinia and salmonella encode an invasin which binds to the integrins or other membrane receptors on eucaryotic cells (16). This binding initiates a cascade of intracellular signals, leading to changed cell functions, especially those associated with the cytoskeletal networks, and uptake of bacteria. Many regulatory proteins, such as protein tyrosine kinases and MAP kinases, and intracellular messengers have been found to be involved in bacterial entry into host cells.

Prevention of diseases by interfering with adherence

Because of the importance of adherence in bacterial infections, blockage of adherence should be a feasible approach to prevention of infectious diseases. One of the means is to immunize animals with vaccines prepared with purified adhesins. Vaccines prepared with purified fimbriae from various strains of *E. coli* have been successfully utilized to induce protective immunity against diarrhea in domestic animals. Rutter and Jones immunized pregnant sows with purified K88 fimbriae and challenged the newborn piglets with the homologous strain of ETEC (119). Most of the piglets from immunized sows survived the challenge, whereas 70% of the piglets from nonimmunized sows died of diarrhea, indicating that a protective passive immunity was induced by the fimbrial vaccine. A similar approach was applied to immunization of dams with K99, 987P, F41 or type 1 fimbriae, and the antibodies to the fimbriae in the dams' colostrum protected

calves, lambs and piglets from subsequent challenges (2, 103, 128, 118, 68).

These vaccines depend on passive protection of suckling neonates via colostral

antibody to fimbrial adhesins induced by vaccination of the dam during pregnancy.

Although the protection is short-lived, it is practically useful because of the age

specificity of ETEC infections. It is known that these fimbrial vaccines only induce a

protective immunity to a homologous challenge; there is no cross protection

between *E. coli* with different fimbriae. Multi-valent vaccines containing K88, K99,

and 987P fimbriae provided additive protection against diarrhea of calves (22).

These types of vaccines may be more useful than single-valence vaccines

because of the existence of multiple fimbriated *E. coli* in natural infections. K88 and

K99 vaccines have been produced by recombinant DNA technology (125).

Vaccines consisting of CFA1, CS1, CS2, or CS3 fimbriae were also proven to

protect humans from ETEC diarrhea (75). Experiments conducted in monkeys

showed that immunization with P fimbriae offered protection against experimentally

induced urinary tract infection caused by a P-fimbriated strain of *E. coli*, which

causes pyelonephritis in humans (75).

Since frequent phase variation or antigenic variation occurs for adhesins of

some bacteria, an adhesin vaccine may not be protective in such circumstances.

This limitation led to development of synthetic vaccines which consist of synthetic

peptides corresponding to conserved regions of an adhesin (124). This approach

was successful with the P fimbriae of *E. coli*, but failed with the fimbriae of

gonococci (124). The M protein of streptococci is highly variable at the N-terminal,

while the surface exposed C-terminal is conserved. Synthetic peptides corresponding to residues within the C-terminal were linked to the mucosal adjuvant cholera toxin B subunit (14). This conjugate vaccine significantly reduced the colonization of streptococci in pharyngeal mucus of mice.

Mice immunized intraperitoneally or intramuscularly with purified FHA of *B. pertussis* had significantly reduced bacterial colonization in the lungs (73). This protection was mediated primarily by serum antibodies to FHA, which transudate to respiratory secretions, because intravenous inoculation of anti-FHA antibodies alone produced a similar protection. The protective efficiency of FHA vaccines was further confirmed by mucosal immunization, in which mice immunized intranasally or intraduodenally with FHA prior to *B. pertussis* aerosol challenge resulted in a 2 to 3 log reduction in the number of bacteria recovered from the lungs and the tracheas (127). This result indicated that gut mucosal immunization with a major adhesin of a respiratory pathogen generated a protective immune response in the respiratory tract.

There are a few examples showing that receptor analogues have the potential to block in vivo adherence and infection. In a mouse infection model, mannosides reduced the colonization of *E. coli* in the urinary tract (5, 157). Globotetraose, a receptor analogue for P fimbriae of *E. coli,* also decreased bacterial persistence in the same model (136). Lactoseries oligosaccharides in human milk were shown to inhibit adherence of pneumococci, such as *H. influenzae* and *S. pneumoniae*, to respiratory tract epithelial cells (137). In vivo

studies demonstrated that babies receiving milk with high activity of the receptor

analogue had reduced nasopharyngeal colonization and frequency of otitis

compared to children receiving milk with low inhibitory activity (137). Unlike

vaccination, the application of receptor analogues in prevention of bacterial

infection is still in the experimental stage.

Mycoplasma adherence

Mycoplasmas are wall-less organisms, and many of them are mucosal

pathogens. Membrane-membrane interactions with host cells, especially epithelial

cells, plays an important role in the initial stage of mycoplasmal diseases (110).

Most of the studies on mycoplasmal adherent mechanisms have been focused on

the human mycoplasmas, *M. pneumoniae* and *M. genitalium* (110, 143). *M.*

pneumoniae attaches to host cells through a specialized tip-structure which was

first described in the early 1970s (20, 21). Treatment of *M. pneumoniae* with trypsin

resulted in loss of a 170 kDa protein designated as P1 and concomitant loss of

adherence of the mycoplasma to eucaryotic cells, while regeneration of P1

restored adherence of the mycoplasma, indicating that P1 is associated with

adherence (44, 53). Further studies demonstrated that P1 is localized at the tip-

structure of *M. pneumoniae* (55, 36, 11); monoclonal antibodies against P1

inhibited adherence of the mycoplasma (55, 36); solubilized P1 was able to bind to

host cells (78); and *M. pneumoniae* organisms that lack P1 or were unable to

cluster it at the tip organelle failed to attach to respiratory epithelium (79, 11). These findings strongly indicate that P1 is a major adhesin of *M. pneumoniae*.

The gene encoding P1 adhesin was identified and characterized. Cloning and sequencing of the P1 gene (135, 58) indicated that P1 has 20 UGA codons for tryptophan, which hampers the production of P1 proteins by recombinant technology because UGA is recognized as a stop codon in *E. coli*. P1 gene also has an unusually long signal sequence consisting of 59 amino acids for translocation, no cysteine, and a high content of proline, particularly at the C-terminus region. About two thirds of the structural gene for P1 was found in multiple copies in the genome of *M. pneumoniae*, suggesting that genetic rearrangement mechanisms may function for inducing variation in adhesins (135, 132). However, the cell-binding domain of P1 is a single-copy region containing 13 amino acids (24). These findings imply that the cell-binding domain is conserved, while the multiple-copy regions display considerable sequence divergence. Examination of P1 genes from different isolates of *M. pneumoniae* revealed significant differences in the nucleotide sequences (133, 134).

The P1 gene is part of a three-gene operon, P1 operon, which has three open-reading frames, ORF-4, P1, and ORF-6 (57, 58). ORF-4 encodes for a 28 kDa protein, P1 for a 170 kDa protein and ORF-6 for a 130 kDa protein. The function of the 28 kDa protein is unknown at present. The 130 kDa protein is cleaved into two proteins during maturation, 90 kDa and 40 kDa (129). Both of the proteins are absent in the nonadherent avirulent *M. pneumoniae* strain (B176), indicating the

product of ORF-6 is also involved in adherence of *M. pneumoniae*. A recent study has shown that the 90 kDa and 40 kDa proteins are surface exposed and are localized at the terminal tip organelle, further confirming their role in adherence (37). Coordinated expression of these genes in the operon may be required for successful adherence.

Analysis of adherent and nonadherent mutants of *M. pneumoniae* suggested that multiple proteins might be involved in adherence, although P1 was the major adhesin (10, 77). In addition to P1, a 30 kDa protein (P30) has also been identified as an adhesin of the mycoplasma, because 1) P30 is clustered at the tip-structure of *M. pneumoniae* (10); 2) antibodies to P30 inhibited adherence of the mycoplasma to erythrocytes (10); 3) a class of nonadherent mutants lack P30, while it is present in wild-type *M. pneumoniae* (110); and 4) P30 shares considerable DNA and protein sequence homology with P1 (23). Besides P1 and P30, other membrane proteins are also required for *M. pneumoniae* adherence. Proteins designated HMW1, HMW2, HMW3, HMW4, and HMW5 (with molecular weights ranging from 140 to 340 kDa) and proteins A, B, C (72, 85, and 37 kDa, respectively) were missing in nonadherent mutants, while re-acquisition of these proteins lead to reversion of the adherence-positive phenotype (80, 130). The functions of these proteins are not defined. The HMW proteins appear to be part of the cytoskeleton-like Triton shell of *M. pneumoniae* and are localized mainly in the filamentous extensions of the mycoplasmal cells, in proximity to the tip organelle (130). It has been proposed that the HMW and A, B, and C proteins are not real

adhesins but are accessory proteins which play a role in the lateral movement and concentration of the adhesin molecules at the tip-structure, and consequently are required for the proper functioning of adhesins (110).

M. genitalium has a major adhesin similar to P1 of *M. pneumoniae* (54). This adhesin, designated as MgPa and with a molecular weight of 140 kDa, is localized at the tip structure of *M. genitalium*. MgPa cross-reacts with P1 immunologically, and shares extensive homology with P1 at both DNA and protein levels (26, 54, 90), leading to a speculation that the P1 and MgPa were the product of a single ancestral gene which was conserved during evolution. Monoclonal antibodies against MgPa inhibited adherence of *M. pneumoniae* (54). The MgPa gene is also a part of a three-gene operon consisting of three open reading frames (56), ORF1 (29 kDa), ORF2 (160 kDa, MgPa), and ORF3 (114 kDa). Thus, there is a strong resemblance to the P1 operon of *M. pneumoniae*. The protein sequences encoded by the MgPa operon have considerable homologies with those of the P1 operon (56).

M. gallisepticum, an avian respiratory pathogen, is another mycoplasma with a specialized adherence organelle (89, 141). Antibodies to P1 cross-reacted with a 155 kDa protein of *M. gallisepticum*. However, adherence-inhibiting monoclonal antibodies to P1 and MgPa did not react with the 155 kDa protein. Homology between P1 gene and the genomic DNA of *M. gallisepticum* was demonstrated with southern hybridization (25). Polymerase chain reaction, conducted by using the oligonucleotide primers derived from the conserved region of P1 and MgPa

genes, amplified a 583 base-pair DNA fragment from *M. gallisepticum*. Subsequent cloning and sequencing indicated that this fragment encoded a 193-amino acid peptide which shares considerable homology to the expected regions of P1 and MgPa proteins. This fragment may be a portion of an adhesin gene of *M. gallisepticum*. A common receptor (100 kDa) in human fibroblasts for *M. gallisepticum*, *M. pneumoniae*, and *M. genitalium* bound to a 139 kDa protein of *M. gallisepticum* (41). The relationship between the 155 kDa protein and the 139 kDa protein and their roles in *M. gallisepticum* adherence are unknown. Recent studies indicated that a 64 kDa protein of *M. gallisepticum* might be an adhesin of the organism since monospecific antiserum inhibited adherence of the mycoplasma to chick tracheal ring organ cultures by as much as 80% (8). The same antiserum also caused growth inhibition of *M. gallisepticum*. Interestingly, the 64 kDA protein cross reacted serologically with a 66 KDA protein of *M. pulmonis*, which is a putative attachment protein of *M. pulmonis* (86).

Many other mycoplasmas, such as *M. bovis*, *M. dispar* and *M. pulmonis*, do not have specialized adherence structures, although they are able to bind to a wide variety of eucaryotic cells (110). Inhibition studies with monoclonal antibodies suggested that a 26 kDa surface protein of *M. bovis* might mediate the adherence of the mycoplasma to embryonic bovine lung cells (120). Washburn *et al.* (150) showed that *M. arthritidis* was able to attach to rat synovial fibroblasts, lung cells, and skin cells. A trypsin-sensitive protein (90 kDa) of *M. arthritidis* seemed to be involved in the adherence because it is absent in nonadherent mutants and

antibodies against it inhibited the adherence (150).

The chemical nature of host receptors for some mycoplasmas has been characterized. Treatment of host cells with neuraminidase indicated that sialic acid-containing glycoconjugates were receptors for *M. pneumoniae, M. genitalium, M. gallisepticum, M. synoviae, and M. bovis* (120, 109). Further studies with purified sialylated glycoproteins or glycolipids confirmed the role of sialic acids in adherence of *M. pneumoniae* (94, 113). More importantly, Loveless and Feizi (96, 95), utilizing immunofluorescence and immunoelectron microscopy, identified the sialo-receptors in human bronchial epithelium, which is a primary site for *M. pneumoniae* infection. They demonstrated that the receptors were polarized at the cilia and apical microvillar domains of ciliated cells, whereas the secretory cells and mucus lacked the receptors. However, sialylated glycoconjugates are not the sole receptors for mycoplasmas. A non-sialylated glycoprotein from human fibroblasts was also described as a receptor for *M. pneumoniae* (40). By utilizing thin-layer chromatography overlay assay, Krivan *et al.* (83) reported that *M. pneumoniae* specifically bound to sulfatide and other sulfated glycolipids including seminolipids and lactosylsulfatide. This binding was independent of sialic acid moieties and was inhibited by dextran sulfate. A consensus binding sequence in the glycolipid receptors was identified as a terminal $Gal(3SO_4)\beta1$ residue. This type of receptor was found in human trachea, lung and Widr cells. Sulfated glycolipids are common constituents of cell membranes, present in many types of cells (46), and play a role in cell adhesion in eucaryotes (35). Mycoplasmas that utilize

sulfated glycolipids as receptors include *M. pneumoniae, M. hominis, M. genitalium, M. salivarium, M. orale, M. hyorhinis, M. arginini, M. pulmonis, and Ureaplasma urealyticum* (85, 93).

Because of the importance of adherence in the initiation of mycoplasmal diseases, immunization with adhesins should be a plausible approach to prevention of mycoplasmal diseases. As an integral and hydrophobic membrane protein, the P1 adhesin of *M. pneumoniae* was very difficult to purify (109). Jacobs *et al.* (64, 63) utilized preparative SDS-PAGE to obtain enough purified P1 protein to immunize guinea pigs. Guinea pigs immunized intranasally or intraperitoneally elicited strong antibody responses to P1; however, these animals were not protected from subsequent challenge with virulent *M. pneumoniae*. In fact, immunized guinea pigs had more severe histological lung lesions, manifested by infiltration of lymphoid cells, than control animals, indicating that P1 induces not only a strong humoral response but also an undesired strong cellular response which is responsible for development of lung lesions (110). Evaluation of sera from patients infected with *M. pneumoniae* revealed that most of the anti-P1 antibodies were not against the adherence-mediating epitope but to other sites of P1 molecules, suggesting the adherence-mediating epitope is not immunogenic (62). This finding may provide an explanation for the failure of immunization of guinea pigs with P1 adhesins. As Razin and Jacobs proposed (110), the most efficient P1 vaccine should be one that, on the one hand, induces a strong antibody response to the adherence-mediating epitope, and, on the other hand, elicits a low but

specific cell-mediated immunity to avoid adverse effects.

In summary, the adherence of mycoplasmas to host cells is a complex process, involving the specific interactions of mycoplasmal adhesins and their receptors on eucaryotic cells. Mycoplasmal adhesins are surface proteins, and some of them are clustered at a specialized attachment organelle. A mycoplasma may have multiple adhesins. Adhesins from different species of mycoplasmas may share homology in structure. Two common types of receptors identified for mycoplasmas are sialylated glycoconjugates and sulfated glycolipids. Existence of multiple adhesins and receptors may provide mycoplasmas with enormous flexibility in counteracting the host defense mechanisms.

Pathogenic mechanisms of *M. hyopneumoniae*

M. hyopneumoniae causes mycoplasmal pneumonia of swine (MPS), which is one of the most important swine diseases worldwide (116). Even though the mortality caused by MPS is low, enormous economic losses may occur due to high morbidity, retarded growth and poor feed conversion of infected herds (115). Commercial vaccines are now available, but these vaccines do not prevent infection, although they significantly reduce development of lung lesions (116). Lack of knowledge of the pathogenic mechanisms and virulence factors of the mycoplasma has limited the development of more efficient measures for control of MPS.

The incubation period of MPS is about 10 days to 3 weeks (116). Most obvious lung lesions are usually observed at 4 to 6 weeks after infection. Observations with electron microscopy and immunofluorescence microscopy have demonstrated that *M. hyopneumoniae* colonizes the surfaces of ciliated cells in trachea, bronchi, and bronchioles of pigs, whereas few mycoplasmas can be observed in alveoli (4, 99, 156, 15). Invasion of epithelial cells by the mycoplasma is not found. In experimental infections, mycoplasmas can be observed on the surface of cilia as early as 7 days after inoculation (99). Highest numbers of mycoplasmas are obtained at 4 to 6 weeks after infection (4, 156). Thereafter, mycoplasmas gradually decrease in number and are hardly detectable in the more advanced stages of the disease. Attachment of *M. hyopneumoniae* leads to progressive and extensive damage to cilia during in vivo infection (99, 156, 15, 140). Under the circumstances without secondary bacterial infection, cilia can be regenerated in the late stage of the disease (156, 15). *M. hyopneumoniae* tends to form microcolonies and accumulates over remaining ciliated cells (99, 156, 65). As a matter of fact, this mycoplasma only adheres to the cilia of ciliated cells but not to other epithelial cells, indicating that adherence to cilia may play an important role in colonization and tropism.

Studies with various in vitro models have generated valuable information on the pathogenic mechanisms of *M. hyopneumoniae*. Adherence and lesions similar to in vivo infection were demonstrated in porcine tracheal ring organ cultures (65,28). DeBey evaluated ciliostasis and loss of cilia induced by *M.*

hyopneumoniae in tracheal organ cultures and found that the damage to cilia

decreased with in vitro passage of the mycoplasma, which usually reduces the

pathogenicity of *M. hyopneumoniae* (28). The damage to cilia was diminished by

porcine antibodies to *M. hyopneumoniae*, and a 0.1 μm membrane that separated

mycoplasmas from the epithelial cells blocked the cytotoxicity, suggesting that *M.*

hyopneumoniae does not secrete toxins and that direct adherence of the

mycoplasma to ciliated cells is essential for production of ciliary damage.

Young *et al.*, who established a hemagglutination assay to characterize the

adherence mechanisms of *M. hyopneumoniae*, reported that this organism

agglutinated turkey red blood cells rather than red blood cells from pigs, guinea

pigs, sheep, or human beings (154). The hemagglutination was increased by

treatment of turkey RBC or mycoplasmas with trypsin, suggesting the

hemagglutinin of the mycoplasma was trypsin-insensitive and the receptor (s) on

RBC was cryptically presented. Thirteen carbohydrates evaluated in the study did

not inhibit the hemagglutination. Two monoclonal antibodies to 64 kDa and 41 kDa

proteins of *M. hyopneumoniae* blocked the hemagglutination, indicating that the

two proteins might be hemagglutinins.

M. hyopneumoniae also adhered to cell monolayers including human and

porcine lung fibroblasts and porcine kidney cells (157). The adherence appeared

to be mediated by receptor-adhesin interaction because the adherence was

saturable and inhibited by nonradiolabeled mycoplasmas. Unlike

hemagglutination, treatment of mycoplasmas with trypsin diminished adherence to

cell monolayers, suggesting that the adhesin (s) mediating adherence to cell

monolayers was different from the hemagglutinins. More recently, Zielinski and

Ross established a model using single ciliated cells to study adherence of *M.*

hyopneumoniae (160). Their results showed that adherence of the mycoplasma

was polarized at the ciliary tufts of porcine tracheal ciliated cells, and few

mycoplasmas attached to cell bodies of porcine ciliated cells and to tracheal

ciliated cells from sheep and rabbit, further confirming the adherence specificity of

M. hyopneumoniae. Treatment of mycoplasmas with trypsin decreased adherence

in this model, indicating the adhesin (s) was trypsin-sensitive. However,

convalescent serum, hyperimmune serum, lung lavage fluid, and many

carbohydrates did not inhibit the adherence. Tetramethyl urea which disrupts

hydrophobic interactions and sulfur containing compounds, including dextran

sulfate, ammonium sulfate, magnesium sulfate, and methionine, inhibited the

adherence, which led to the conclusion that adherence to porcine ciliated cells was

mediated by receptor-adhesin interaction as well as hydrophobic interactions. A

recent study indicated that the cell surfaces of *M. hyopneumoniae* were weakly

hydrophobic (159). Since the natural target cells (porcine tracheal ciliated cells) for

M. hyopneumoniae were utilized in the single ciliated cell assay, information

generated from this model should be more meaningful than that from the

hemagglutination assay and the cell monolayer assay. However, the complicated

procedures and subjectivity involved in evaluation of results are drawbacks of the

assay and limit its application in characterization of adherence mechanisms. Thus,

improvement of this assay is required for future studies.

A typical structure of *M. hyopneumoniae* in ultrathin sections consists of ribosomes and nuclear materials in cytoplasm, a trilayered cell membrane, and undefined layer of materials outside the cell membrane (140, 155). No specialized organelles, such as the tip structure of *M. pneumoniae*, were observed with *M. hyopneumoniae*. Tajima and Yagihashi compared the ultrastructure of an in vivo-passaged virulent strain and an in vitro-passaged nonvirulent strain (140). They demonstrated that the two strains were very similar in general morphology. However, long fibrils and a thicker capsular material were observed outside the membrane of the virulent strain. No fibrils and a thinner layer of capsules were associated with the nonvirulent strain, which grew poorly in porcine lungs and was unable to produce MPS. More importantly, the fibrils and/or the capsular material appeared to bridge the interaction of mycoplasmas with cilia during infection (15, 140). These findings suggested that the fibrils and/or capsule might play a role in adherence or virulence of *M. hyopneumoniae*. The nature of the fibrils and capsule remains to be defined.

Although *M. hyopneumoniae* does not secrete toxins, membrane-associated toxins may exist. Geary and Walczak reported that whole cells or purified membranes of *M. hyopneumoniae* were cytopathic to porcine lung fibroblasts and human lung fibroblasts (42). The cytotoxicity was located in the mycoplasmal membranes not in the cytoplasm, and was sensitive to pronase. Further studies indicated that the cytopathic factor was a 54 kDa membrane protein with an

isoelectric point of 6.2 (39). The effect of the cytopathic factor on porcine ciliated cells has not been defined. Minion *et al.* have recently reported nuclease activities associated with *M. hyopneumoniae* membranes and proposed that, by degrading host DNA, the nucleases may play a role in obtaining nucleotide precursors for mycoplasmal growth (101). The significance of nucleases in pathogenicity remains to be determined.

Several surface proteins of *M. hyopneumoniae*, including p70, p65, p50, and p44, were identified as lipoproteins (152). The last three lipoproteins were shown to be major immunogens of the mycoplasma as detected with convalescent swine serum. Recombinant p65 (partial fragments) were expressed in *E. coli*, and mapping with various polyclonal and monoclonal antibodies indicated that the immunogenic region of p65 was located in the C-terminal (72). A monoclonal antibody to p44 inhibited *M. hyopneumoniae* growth in vitro (81). The Mab to p70 recognized a 70 kDa protein in strain J and a 73 kDa protein in strain VPP11, indicating that size variation of surface proteins occurs in *M. hyopneumoniae* (152). The biological functions of these lipoproteins are unknown at present.

Another surface protein with an estimated molecular weight of 74.5 kDa was characterized because it was also a major immunogen of *M. hyopneumoniae* (142). The genomic DNA for the antigen was cloned in E. coli, and sequence analysis indicated that it shares homology with the 70 kDa family of heat shock proteins. Thus, the authors proposed that the 74.5 kDa protein was a heat shock protein of *M. hyopneumoniae* and designated it as HSP74. Interestingly, a recent

study showed that recombinant HSP74 bound to sulfated glycolipids which were putative receptors for many mycoplasmas, suggesting that HSP74 may play a role in the adherence of *M. hyopneumoniae* (92). However, solid evidence indicating that HSP74 is an adhesin has not been presented.

The gene encoding a 36 kDa protein of *M. hyopneumoniae*, which elicits an early and strong immunologic response in pigs, was cloned by Strasser *et al.* and the recombinant protein was expressed in *E. coli* in its full length (131). Immunocytochemical study showed that the 36 kDa protein was localized in the cytoplasm of *M. hyopneumoniae* cells. DNA sequencing indicated that the p36 is a lactate dehydrogenase of the mycoplasma (47). Its role in pathogenicity is unknown.

A typical microscopic lesion of MPS is the peribronchial and perivascular infiltration of lymphocytic cells. Since overreaction of immune cells is directly responsible for development of inflammation, there is a general notion that the infiltrated lymphocytes play a role in causing pneumonia. This hypothesis was supported by some evidence derived from in vitro studies in which mitogenic activity to porcine lymphocytes was demonstrated with intact *M. hyopneumoniae* cells or purified membranes (82,100). The identity and the functions of the infiltrated lymphocytic cells are not clear at present. Elucidation of these questions may provide valuable information on the pathogenesis of MPS. Other studies indicated that *M. hyopneumoniae* was suppressive to mitogen-induced transformation of swine lymphocytes (74). In vivo infection of *M. hyopneumoniae*

did not affect the blastogenic response of porcine peripheral lymphocytes to various mitogens, but decreased the humoral response to sheep erythrocytes (149). Suppressed functions of alveolar macrophages were found in pigs infected with *M. hyopneumoniae* and then with *A. pleuropneumoniae* (18).

In summary, multiple virulence factors may be involved in the pathogenesis of *M. hyopneumoniae* infection. One of the most important requirements for development of MPS is the adherence of *M. hyopneumoniae* to porcine ciliated cells. The molecular mechanisms of *M. hyopneumoniae* adherence to porcine ciliated cells have not been defined. The adhesin (s) of the mycoplasma and the corresponding receptors on cilia remain to be identified and characterized. More efficient measures to control MPS may be developed based on the information obtained from adherence studies.

MICROTITER PLATE ADHERENCE ASSAY AND RECEPTOR ANALOGS

FOR *MYCOPLASMA HYOPNEUMONIAE*

Qijing Zhang, Theresa F. Young and Richard F. Ross[2]

Abstract

A microtiter plate adherence assay for *Mycoplasma hyopneumoniae* was established by using purified swine tracheal cilia which contained receptors for the mycoplasma. *M. hyopneumoniae* bound specifically to plates coated with solubilized cilia. The binding was dependent on both the concentration of cilia and the number of mycoplasmas. Dextran sulfate, heparin, chondroitin sulfate, laminin, mucin, and fucoidan significantly inhibited binding of the mycoplasma. The six inhibitors also disrupted the adherence of the mycoplasma to intact ciliated cells. Preincubation with either mycoplasmas or cilia indicated that heparin, mucin, fucoidan and chondroitin sulfate interacted with the adhesive molecules on the surface of mycoplasmas, whereas laminin blocked the receptors in cilia. The basis for inhibition induced by dextran sulfate was unknown. Treatment of cilia with

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neuraminidase appeared to promote adherence of the mycoplasmas, whereas treatment of cilia with sodium metaperiodate decreased the binding. These results indicate that receptors for *M. hyopneumoniae* in the ciliated epithelium of the respiratory tract of pigs are glycoconjugate in nature.

Introduction

Mycoplasma hyopneumoniae is the etiological agent of mycoplasmal pneumonia of swine (MPS), an economically important and widely spread swine disease (22). This organism is an extracellular pathogen and colonizes the respiratory epithelium of pigs. Immunofluorescence tests (1) revealed that large numbers of mycoplasmas are primarily located on the luminal surface of trachea, bronchi, and bronchioles. Few of them can be found in alveoli. Electron microscopy (3, 17, 30) demonstrated that *M. hyopneumoniae* attaches only to ciliated cells, not to other types of epithelial cells, and that the organism causes progressive damage to cilia during infection. These data strongly indicate that cilia have specific receptors for mycoplasmal adherence. Damage to the muco-ciliary system results in inflammation and compromises the defense mechanisms of the porcine respiratory tract, predisposing to infections by other bacteria and viruses (22). Virulence factors of *M. hyopneumoniae* have not been characterized. However, adherence to ciliated cells is considered an important event in determining tropism and colonization.

M. hyopneumoniae adheres only to ciliated cells in vivo, but it can attach to different types of cells in vitro, including turkey red blood cells (29), cell monolayers (33), and single ciliated cells of the swine respiratory tract (31). Several adherence models have been established based on these findings. However, turkey red blood cells and cell monolayers may not represent the natural target cells to which *M. hyopneumoniae* adheres. The single ciliated cell adherence assay (SCCAA), utilizing the natural target cells and mimicking the in vivo adherence of *M. hyopneumoniae*, provides a very useful model for characterization of adherence mechanisms of *M. hyopneumoniae* (31). However, the complexity in the procedure and the relatively subjective evaluation of results limit its routine use in the laboratory. Due to lack of a reliable adherence model, receptors and adhesins involved in adherence of *M. hyopneumoniae* have not been characterized. In this study, a microtiter plate adherence assay (MPAA) was established by utilizing purified cilia which contain receptors for *M. hyopneumoniae.* Receptor analogues that block adherence were identified with the MPAA. By defining the nature of molecules involved in adherence, we expect to provide more information on the pathogenesis and further insight into the development of control measures for MPS.

Materials and methods

Chemicals and reagents. Antibodies to *M. hyopneumoniae* were produced by immunizing rabbits with immunogens prepared in rabbit muscle infusion medium supplemented with 20% rabbit serum (19). Peroxidase-conjugated goat anti-rabbit IgG was purchased from Cappel (Durham, NC). Peroxidase substrate (ABTS) was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Fluorescein isothiocyanate (FITC)-conjugated antibodies against *M. hyopneumoniae* were supplied by B. Erickson, Iowa State University, Ames, Iowa. Various sugars and glycoconjugates were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Mycoplasma. *M. hyopneumoniae* strain 232 LI27 was originally derived from strain 11. Passages 1 to 5 were cultured in Friis mycoplasmal medium (9) for 24 h at 37°C. Mycoplasmas were harvested by centrifugation at 25,000 x *g* for 15 min. Color changing units (CCU) representing the number of mycoplasmas were determined by serial dilution in tubes containing Friis medium. The pellet of mycoplasmas was resuspended to 1/10 of the original volume in RPMI 1640 medium containing 1% gelatin (adherence buffer, AB). This preparation was further diluted in AB before addition to microtiter plates. For investigation of the effect of in vitro passage on adherence, the infected lung homogenate (LI27) was passaged in Friis medium. Cultures at passages 3, 10, 20, 30, 40, 50, 60, and 70 were collected, washed, and adjusted to contain 12.5 and 25 µg/ml protein.

Preparation of cilia. Specific-Pathogen-Free (SPF) pigs free of *M. hyopneumoniae*, 7 to 12 weeks old, were obtained from a herd maintained at the Iowa State University Animal Resources Station. The herd was originally established from cesarean-born, isolation-reared swine and was a mixture of Yorkshire and Hampshire bloodlines. Swine tracheas were collected as described (31). Briefly, the pigs were euthanatized, and the tracheas and lungs were exposed. The tracheas were clamped at the larynx and separated from the lungs by cutting at the bifurcation. Other connective tissues were trimmed from the outer surface of tracheas, and then the tracheas were washed three times by immersing in RPMI 1640 medium before they were cut into small fragments. Ciliated cells were collected by scraping the inner surface of tracheas with a sterilized stainless steel laboratory spoon. Cilia were extracted from ciliated cells according to methods by Tuomanen et al. (28). Briefly, ciliated cells were suspended in 40 ml buffer containing 20 mM Tris, 10 mM EDTA, and 125 mM sucrose (TES, pH 7.2). Two washings with the TES buffer were conducted by centrifugation at 300 x g for 5 min. The cell pellet was resuspended in 6 ml AES (80 mM acetate, 10 mM EDTA, 125 mM sucrose, pH 6.8) buffer and incubated for 5 min at 25°C. Then, 0.2 M CaCl$_2$ was added to a final concentration of 10 mM. The mixture was vortexed for 10 min, diluted with 20 ml TES buffer, and then centrifuged at 500 x g for 10 min. The sediment which contained mainly cell bodies was saved, and the supernate containing cilia was harvested by centrifugation at 18,000 x g for 15 min at 4°C. The ciliary pellet was washed twice with phosphate-buffered saline (PBS) and stored at

-70°C until use. Purity of cilia was ascertained by light microscopy. Protein

concentration was determined using the BCA* protein assay reagent (Pierce,

Rockford, IL.) according to the instructions provided with the product. Usually, 6 to 8

mg of ciliary proteins were obtained from a trachea collected from a pig weighing

100 to 150 pounds.

Coating plates. Purified cilia (2 mg/ml in PBS) were solubilized with

sodium dodecyl sulfate (SDS, 1 mg/mg protein) at 37°C for 45 min. This

preparation was further diluted with sodium carbonate buffer (0.1 M, pH 9.5) to a

final concentration of 10 µg/ml protein. To each well of a flat bottom microtiter plate

(Immulon®2, Dynatech Laboratories, Inc., Chantilly, VA) was added 100 µl of the

solution. The plates were incubated overnight at room temperature and stored at -

70°C without removal of the coating solution. Coated plates were stored at -70°C for

at least one year without loss of adherence ability. Porcine albumin (fatty acid free,

10 µg/ml) and gelatin (10 µg/ml) were coated onto plates under conditions used for

cilia as negative controls. After deciliation, cell bodies of ciliated cells were also

solubilized and immobilized onto microtiter plates (10 µg/ml protein).

Microtiter plate adherence assay. After 4 washings with PBS (pH 7.4),

the cilia-coated plates were blocked with 200 µl/well of AB for 2 h at 37°C. Then,

100 µl of *M. hyopneumoniae* cells resuspended in AB were added to each well,

and the plates were incubated at 37°C for 90 min. Non-adherent mycoplasmas

were removed by four washings with PBS. Subsequently, 100 µl of rabbit

antibodies to *M. hyopneumoniae* was added and incubated at 37 °C for 60 min.

The wells were washed 4 time with PBS and then incubated with 100 μl of goat anti-rabbit peroxidase conjugate for 60 min at 37°C. After 4 washings with PBS, 100 μl ABTS was added to each well and incubated at 25 °C for 10 min. Optical density (OD) values at 405 nm were measured with an automated microplate reader (Model EL310, Bio-Tek Instruments, Inc., Winooski, VT). Binding of mycoplasmas to cell body-coated plates was detected as described above.

Adherence inhibition. For inhibition assays, various carbohydrates and glycoconjugates (Table 1) diluted to appropriate concentrations in AB were mixed with mycoplasmas and added to cilia-coated plates. After incubation and washings, binding of mycoplasmas to the plates was detected with rabbit anti-*M. hyopneumoniae* antibodies, and goat anti-rabbit conjugates as described previously. In order to optimize the sensitivity to interference, 2.5 x 10^{8} CCU of mycoplasmas were utilized in each well so that the OD values from the non-inhibitory controls were about 0.5, which was in the linear range of the adherence curve (Fig. 1). Two approaches were utilized to study competitive inhibition; 1) competitors were preincubated with mycoplasmas at 37°C for 1 h, unbound competitors were removed by differential centrifugation (25,000 x *g* for 15 min), and incubated mycoplasmas were applied to cilia-coated plates; 2) cilia-coated plates were preincubated with competitors at 37°C for 1 h, unbound competitors were removed by four washings, then mycoplasmas were added to the plates. Thereafter, procedures for both approaches were identical, including sequential addition of antibodies to mycoplasmas, secondary antibody conjugates, and

peroxidase substrates. Percentage of inhibition was calculated as follows: % inhibition = (OD value from AB - OD value from an inhibitor)/OD value from AB. I^{50} representing the concentration of a competitor that resulted in 50% inhibition was determined from the inhibition kinetics obtained with multiple concentrations of a competitor.

Single ciliated cell adherence assay. Substances determined to have inhibitory activity with the MPAA were further evaluated using the SCCAA (31). Briefly, single ciliated cells were brushed off the epithelial surface of a trachea from a SPF pig and resuspended in RPMI 1640 medium. Cells were washed, counted, and resuspended in the medium to 10^5 cells/ml. One ml of ciliated cells was mixed with 25 μl of mycoplasmas (10^{10} CCU/ml) and 25 μl of inhibitor diluted to the appropriate concentration (Table 4). After incubation in a water bath for 90 min, free mycoplasmas were removed by centrifugation (500 x g for 10 min). Cell pellets with associated mycoplasmas were washed twice with PBS and pipetted to glass slides. Air-dried, methanol-fixed slides were stained with FITC-conjugated antibodies to *M. hyopneumoniae* and observed under UV light with a Nikon epifluorescence microscope. The fluorescent score (an estimation of the number of mycoplasmas bound to a ciliated cell) for each sample was calculated as reported previously (31).

Treatment of cilia with neuraminidase and sodium metaperiodate. Purified cilia were incubated with 10 mM or 100 mM sodium metaperiodate in 10 mM sodium acetate (pH 5.0) at 37°C for 30 min, then washed

three times with PBS, solubilized with SDS, and coated onto microtiter plates

according to methods described previously. Cilia treated with 10 mM sodium

acetate only were utilized as the controls. Adherence of *M. hyopneumoniae* to

treated cilia was evaluated by the standard procedures. For treatment with

neuraminidase, purified cilia were incubated with neuraminidase (1 unit/ml in 10

mM sodium acetate, pH 5.0) at 37°C for 30 min. After three washings with PBS, the

treated cilia were solubilized with SDS and coated onto microtiter plates.

Adherence was evaluated according to the standard procedures.

Statistics. Analysis of variance with the Tukey contrast at a significance

level of 0.05 was utilized for comparison among multiple treatments in an assay.

The Student t test was used if only two treatments were evaluated.

Results

Development of MPAA. *M. hyopneumoniae* bound specifically to cilia-

coated wells but not to control wells coated with gelatin or porcine albumin (Fig. 1).

OD values obtained with the control wells were always less than 0.08. The degree

of binding was influenced by both the number of mycoplasmas and the

concentration of cilia utilized for coating plates. The optimum concentration of cilia

for coating plates was 1 µg protein per well. About 2×10^8 mycoplasmas were

required for half-maximal binding at 37°C. Saturated binding was observed with

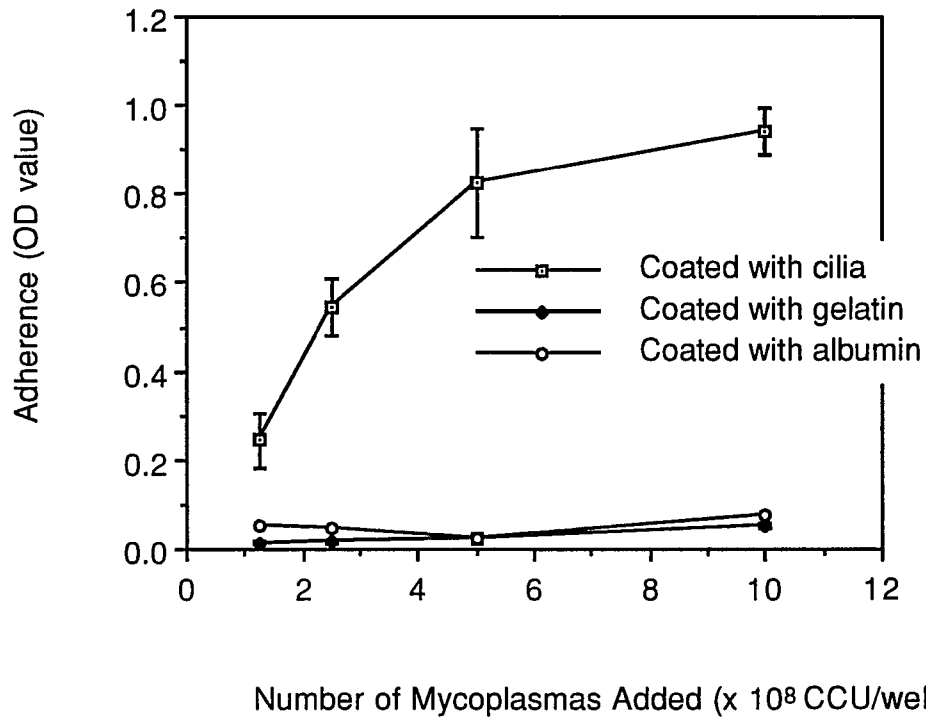more than 1×10^9 CCU mycoplasmas per well. Compared to 37°C, the binding

FIG. 1.   Dose-dependent binding of mycoplasmas to immobilized cilia in MPAA. Data represent mean OD ± SD in three independent experiments. Mycoplasmas bound to cilia-coated wells but not to control wells.

activity of *M. hyopneumoniae* was about 3 times lower at 25°C and minimal at 4°C. Similar adherence patterns were observed with solubilized mycoplasmas at different temperatures (37 °C, 25 °C and 4 °C). The adherence was also time-dependent, with an optimum incubation time of 90 min (data not shown). Prolonged incubation resulted in increased background values. Well-to-well or plate-to-plate difference was negligible (<5%). Mycoplasmas resuspended in PBS or RPMI 1640 medium adhered equally well to cilia-coated plates (data not shown), although the latter was utilized throughout this study. Mycoplasmas solubilized by SDS or sonication also had the ability to adhere. In fact, solubilized mycoplasmas had higher adherence activity than intact mycoplasmas (data not shown). Heating of mycoplasmas (both intact cells and solubilized mycoplasmal proteins) at 56°C for 30 min resulted in an 80% reduction in adherence. With increased passage level, *M. hyopneumoniae* gradually decreased in adherence activity (Fig. 2), with a significant decrease starting at passage 50 (P<0.01). Solubilized mycoplasmas displayed a similar trend of decreased adherence with increase in passage level (data not shown). Mycoplasmas bound equally well to cell body-coated wells and to cilia-coated wells (data not shown).

Adherence inhibitors. Since host cell surface receptors for mucosal pathogens are usually carbohydrate in nature, we evaluated the binding of the mycoplasma in the presence of various sugars and glycoproteins. In order to facilitate screening, competitors and mycoplasmas were added at the same time to cilia-coated plates. Among the carbohydrates and glycoconjugates evaluated in
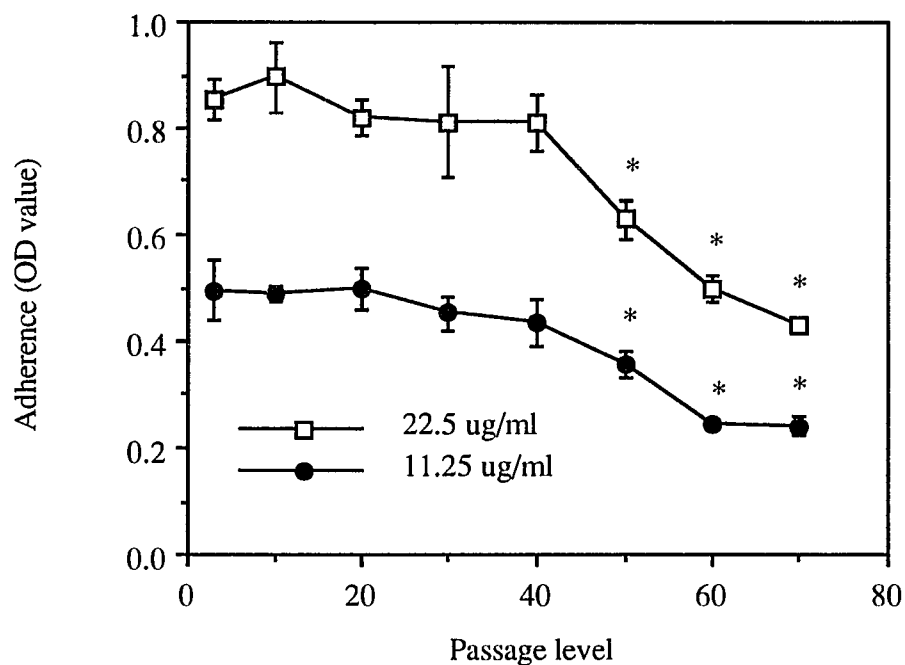
FIG. 2.   Effect of passage level on adherence. Mycoplasmal proteins of
          different passages were adjusted to 11.25 and 22.5 µg/ml. Adherence
          was conducted in cilia-coated plates. Data represent mean OD ± SD
          of three experiments. Asterisk (*) indicates the passages at which
          significant reduction in adherence was observed compared to
          passage 3 (P<0.01).

this experiment, dextran sulfate, heparin, fucoidin, chondroitin sulfate, mucin and laminin inhibited adherence of *M. hyopneumoniae* (Table 1). Three different molecular weights of dextran sulfate produced similar inhibition kinetics and inhibited adherence as much as 90%, but dextran was not an efficient competitor. Other sulfated compounds including D-galactose-6-sulfate, D-glucose-6-sulfate, N-acetyl-glucosamine-3-sulfate and N-acetyl-glucosamine-6-sulfate had no effect on binding of *M. hyopneumoniae*. Tetramethyl urea, which disrupts hydrophobic interactions, magnesium sulfate and methionine did not inhibit adherence. Mucin from porcine intestine produced 75% inhibition. When compared at concentrations of 20, 200, and 1,000 µg/ml, mucin from bovine submaxillary glands produced substantially lower inhibition than mucin from porcine intestine (data not shown). Actually, inhibition by bovine mucin never exceeded 30% at concentrations of up to 2 mg/ml. Remarkable differences in inhibition were obtained among chondroitin sulfate A, B, or C. Chondroitin sulfate B and chondroitin sulfate A had an I^{50} of 20 µg/ml and 80 µg/ml, respectively, while no inhibition occurred with chondroitin sulfate C at concentrations of up to 2 mg/ml. Sialic acid had no effect on adherence. Partial inhibition (<50%) was obtained with fetuin, a glycoprotein rich in sialic acids. Asialofetuin and asialomucin, which were derived from fetuin and mucin (bovine submaxillary glands), respectively, and chemically modified to remove surface sialic acids, had significantly higher inhibition activity than fetuin and mucin (Table 2).

Table 1. Glycoconjugates evaluated for inhibition of adherence

Name	$I_{50}{}^a$
Asialofetuin	*b
Asialomucin (bovine submaxillary glands)	*
Chondroitin sulfate A	80
Chondroitin sulfate B	20
Chondroitin sulfate C	_c
D-fucose	-
D-galactose	-
D-galactose-6-sulfate	-
D-glucose	-
D-glucose-6-sulfate	-
D-mannose	-
Dextran	-
DEAE dextran	-
Dextran sulfate MW 5,000	<1
Dextran sulfate MW 8,000	5
Dextran sulfate MW 500,000	<1
Diacetylchitobiose	-
Fetuin	*
Fucoidan	<1
Galactopyranosyl-galacto-pyranose	-
Heparin	<1 unit/ml
Hyaluronic acid	-
L-fucose	-
Lactose	-
Laminin	50
Lactoferrin	-
Melibiose	-
Mucin (bovine submaxillary glands)	*
Mucin (porcine intestine)	40
N-acetyl-galactosamine	-
N-acetyl-glucosamine	-
N-acetyl-neuramin-lactose	-
N-acetyl-mannosamine	-
N-acetyl-lactosamine	-
N-acetyl-glucosamine-3-sulfate	-
N-acetyl-glucosamine-6-sulfate	-
Sialic acid	-
MgSO4	-
Methionine	-
Tetramethyl urea	-

[a] Concentration (μg/ml, unless indicated) that resulted in 50% inhibition.
[b] Partial inhibition was observed, but not more than 50% at the maximum concentration (2 mg/ml) tested.
[c] No inhibition was detected at concentrations of up to 2 mg/ml.

Table 2. Effect of sialylation on adherence

Concentration (μg/ml)	% inhibition[a] by:			
	Mucin	Asialomucin	Fetuin	Asialofetuin
10	2.9 ± 2.9	6.6 ± 4.8	ND	ND
50	ND	ND	3.9 ± 7.1	19.3 ± 4.6[b]
100	25.9 ± 3.3	30.9 ± 1.6[c]	ND	ND
500	ND	ND	27.2 ± 4.3	48.9 ± 3.4[b]

[a] Mean % inhibition ± SD in three experiments.

[b] $P < 0.01$ versus fetuin as determined by student t test.

[c] $P < 0.05$ versus mucin as determined by student t test.

ND: not determined.

Inhibitory mechanisms. It was speculated that positive results obtained in the adherence assay with various carbohydrates and glycoconjugates could have resulted either by interference with adhesins of mycoplasmas or by interference with receptors on cilia. The effects of the six competitors were further evaluated by preincubating them with mycoplasmas or with cilia. When preincubated with mycoplasmas, fucoidin, heparin, chondroitin sulfate B and mucin significantly blocked binding of the mycoplasma; laminin (6%) and dextran sulfate (16%) resulted in limited inhibition (Table 3). When the six competitors were pre-incubated with cilia, laminin produced 75% inhibition, whereas fucoidan produced 27% which was much less than that from preincubation with mycoplasmas; the other 4 competitors had no inhibitory effect. The six competitors also significantly reduced attachment of *M. hyopneumoniae* to intact ciliated cells in the SCCAA (Table 4). The effect of the six competitors on the detection of *M. hyopneumoniae* with antibodies was evaluated by ELISA. Microtiter plates coated with *M. hyopneumoniae* were incubated with the detecting antibodies in the presence of heparin, mucin, dextran sulfate, fucoidan, chondroitin sulfate, and laminin. There was no significant difference in OD values between the wells with the competitors and the control wells without the competitors, indicating that these substances impacted the mycoplasma-cilia interaction rather than the detection process.

Effect of neuraminidase and sodium metaperiodate on adherence. Pretreatment of cilia with neuraminidase resulted in a trend toward increased binding of *M. hyopneumoniae* (Fig. 3), although the increase was not

Table 3. Inhibitory mechanisms of different competitors

Treatment (μg/ml)	% inhibition[a] produced by preincubation with	
	cilia	mycoplasmas
Laminin (200)	74.8 ± 0.6[b]	6.2 ± 5.8
Mucin (200)	-6.6 ± 6.6	78.3 ± 3.1
Dextran sulfate MW 500,000 (100)	0.5 ± 6.7	16.4 ± 20.2
Chondroitin sulfate B(200)	-1.3 ± 0.4	45.6 ± 15.5
Fucoidan (100)	27.0 ± 3.5	77.6 ± 6.8
Heparin (10 units/ml)	-2.2 ± 6.2	93.4 ± 11.5

[a] % inhibition was calculated versus adherence buffer.

[b] Mean % inhibition ± SD in triplicated assays.

Table 4. Inhibition of mycoplasmal adherence to intact ciliated cells[a]

Inhibitor (μg/ml)	Adherence (fluorescence score)[b]		
RPMI 1640	21.6	±	6.0
Dextran sulfate MW 500,000(10)	1.8	±	1.4[c]
Heparin (10 units/ml)	8.2	±	4.7[c]
Laminin (200)	12.3	±	5.3[c]
Fucoidan (100)	1.1	±	0.8[c]
Mucin (200)	2.1	±	1.7[c]
Chondroitin sulfate B (100)	9.4	±	4.9[c]

[a] Adherence was conducted with the SCCAA model in the presence of the inhibitors.

[b] Means ± SD in duplicate experiments.

[c] Significantly different from RPMI 1640 as determined by analysis of variance with the Tukey contrast ($P<0.05$).
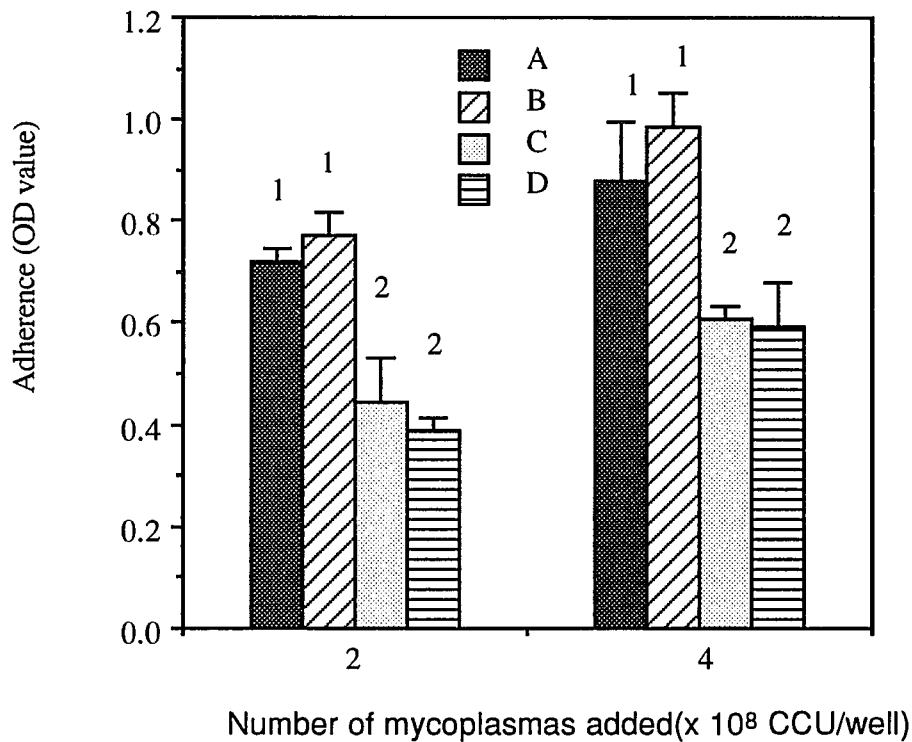
Number of mycoplasmas added(x 10^8 CCU/well)

FIG. 3. Effect of treatment of cilia with neuraminidase and sodium metaperiodate on adherence. Cilia were treated with sodium acetate buffer (A), neuraminidase (B), 0.01 M sodium metaperiodate (C), or 0.1 M sodium metaperiodate (D). Bars represent mean OD ± SD in triplicate assays. Bars with different numbers were significantly different as determined by analysis of variance with the Tukey contrast at a significance level of 0.05.

significant. In contrast to neuraminidase, pretreatment of cilia with sodium metaperiodate, which perturbs carbohydrate structure, significantly reduced attachment (Fig. 3).

Discussion

Results from this study indicated that the MPAA is specific and reproducible. Compared with other assays, this model is more objective and sensitive. MPAA provides a convenient method for evaluation of adherence mechanisms of *M. hyopneumoniae*. It is not feasible to screen large numbers of adherence competitors by the SCCAA because of its limitations. However, many samples can be easily analyzed using the MPAA, resulting in identification of receptor analogues. Furthermore, the nature of receptors may also be determined using this model.

Data from the study demonstrated that adherence of *M. hyopneumoniae* is temperature and dose dependent. Lysed mycoplasmas had higher adherence activity than intact mycoplasmas, indicating that viability of *M. hyopneumoniae* is not required for successful binding and that solubilization of mycoplasmal membranes may expose more binding sites. Similar findings were reported with *Mycoplasma gallisepticum* (12). Unlike *M. pneumoniae,* which did not adhere well in nutrient-deficient medium (14), *M. hyopneumoniae* did not require glucose for binding because mycoplasmas resuspended in RPMI and PBS bound equally well.

This observation was consistent with a previous finding in which metabolically active *M. hyopneumoniae* were not required for binding in the SCCAA (31). Heating (56°C for 30 min) mycoplasmas before adherence remarkably decreased binding, indicating that adhesin (s) of *M. hyopneumoniae* is sensitive to heat. This sensitivity to heat could have resulted from conformational changes in binding sites of mycoplasmal adhesins rather than an impact on metabolic activity because *M. hyopneumoniae* need not be metabolically active for successful binding. Reduced adherence at low temperatures (4 °C and 25 °C) could be caused by decreased thermal motion of particles or molecules, the number of molecular collisions, and consequently, the speed of specific ligand-receptor interactions. A high passage culture of *M. hyopneumoniae* was reported to be unable to cause pneumonia in pigs (32). Attenuation of virulence through successive in vitro passages was also obtained with other mycoplasmas (4, 27). In this study, the adherence capability of *M. hyopneumoniae* to SDS-solubilized cilia was gradually decreased by passage in Friis medium (Fig. 2). This change in adherence appears to parallel the decrease in pathogenicity of the mycoplasma (32). We speculated that a selective variation of mycoplasmal adhesins might occur with the increase in passage level, therefore leading to reduced capability of attachment.

Six adherence inhibitors were identified in this study (Table 1). Four of them, fucoidan, heparin, chondroitin sulfate and mucin, bound to the ligands on the surface of mycoplasmas and therefore were receptor analogues. They are all sulfated glycoconjugates, which indicates that sulfonation of carbohydrate chains is

important for receptor activity. However, sulfate alone was not sufficient for mycoplasmal binding since other sulfated carbohydrates including D-glucose-6-sulfate, D-galactose-6-sulfate, N-acetyl-glucosamine-3-sulfate, N-acetyl-glucosamine-6-sulfate and SO_4^{-2} had no effect on attachment. The inhibitors are all polyanionic molecules. The only structural difference between chondroitin sulfate A and C is the position of sulfate (24). Chondroitin sulfate A inhibited adherence of the mycoplasma, whereas chondroitin sulfate C did not. This finding indicates that the inhibition is probably not due to ionic interaction since chondroitin sulfate A and chondroitin sulfate C have similar charge properties. Chondroitin sulfate B, which contains iduronic acid instead of glucuronic acid found in chondroitin sulfate A (24), caused significantly higher inhibition than chondroitin sulfate A. These results suggest that the binding affinity of *M. hyopneumoniae* to cilia is greatly affected by the nature of carbohydrates and positions of sulfate on carbohydrate chains.

The animal cell surface is covered by various forms of carbohydrates (13). Many viruses (7), bacteria (23), and bacterial toxins (8) utilize eucaryotic cell surface carbohydrates as attachment sites, which is an essential process for establishment of colonization and production of toxic effects. In this study, the inhibition of adherence by several carbohydrates and the decreased binding caused by treatment of cilia with sodium metaperiodate strongly suggest that the receptors for *M. hyopneumoniae* are sulfated glycoconjugates. Both sulfated complex carbohydrates and sialylated glycoconjugates were demonstrated in rat (26), dog and human (25) respiratory epithelium and in bronchial goblet cells of

pigs (6). These data indicate that sulfated carbohydrates are available in vivo for attachment of pathogens that colonize respiratory epithelium.

Many types of eucaryotic cells have membrane-associated heparin or heparin-like polysaccharides, which facilitate cell-cell interactions and cell-extracellular matrix interactions (5, 21). It is not known if ciliated cells have surface exposed heparin or heparin-like polysaccharides. *M. hyopneumoniae* may utilize this natural substratum for attachment. However, further experiments are required to verify this hypothesis. Dextran sulfate produced more than 90% inhibition when mixed together with mycoplasmas and cilia, but it caused limited inhibition (16%, Table 3) when preincubated with mycoplasmas. We speculated that this discrepancy might be caused by the low affinity of dextran sulfate for mycoplasmas. After preincubation, dextran sulfate could be removed from mycoplasmas by the washing step in the adherence assay. Laminin, a glycoprotein with a large molecular weight, did not interact with molecules on mycoplasmas. It was reported that laminin binds specifically to sulfated glycolipids (20). Also, sulfated glycolipids are receptors for several species of mycoplasmas (14, 15). Perhaps, laminin binds to glycolipid receptors in swine cilia and therefore inhibits adherence of *M. hyopneumoniae*. Sialic acid has been reported to be a receptor site for bacteria (11), viruses (16) and mycoplasmas (10, 18). Results of this study demonstrated that sialic acid is not involved in adherence of *M. hyopneumoniae*. In fact, removal of sialic acids from cilia appeared to promote adherence of *M. hyopneumoniae* (Fig. 3). This trend is correlated with increased inhibition from asialomucin and

asialofetuin (Table 2). Sialic acid is usually on the terminal position in oligosaccharide chains. Removal of it may either change the charge properties on cilia or uncover more receptor epitopes for the mycoplasma. It is not known whether *M. hyopneumoniae* produces neuraminidase during infection.

Secretion of large amounts of mucus during MPS seems to be a host defense mechanism to trap microbes such as *M. hyopneumoniae*. However, this mycoplasma causes extensive damage to cilia (3, 17, 30) by unknown means, which compromises the major clearance system in the swine respiratory tract. Thus, adherence to mucin may promote the infection of *M. hyopneumoniae* since secretions can not be efficiently excluded from the respiratory tract due to malfunction of the muco-transportation system. DeBey et al. (6) quantitated bronchial goblet cell secretions of pigs by image analysis of sections stained with high iron diamine/Alcian blue. The results revealed that bronchial goblet cells of pigs infected with *M. hyopneumoniae* contained significantly less sialomucin and more sulfomucosubstances than goblet cells of control pigs. This finding suggests that goblet cells of pigs altered production of type of mucosubstances in response to infection with the mycoplasma.

Adherence of *M. hyopneumoniae* to swine ciliated cells is a complicated process. In order to reach ciliated cells, the mycoplasma must overcome the sweeping force and penetrate the mucus gel which covers the surface of epithelium. Receptor-ligand interactions as well as hydrophobic interactions may contribute to this process (31). It was speculated (31) that the hydrophobic

interactions initiated the first phase of adherence (nonspecific and low affinity), and the adhesin-receptor interaction mediated the second phase (specific and high affinity). Tetramethyl urea, which disrupts hydrophobic interactions, partially inhibited adherence in the SCCAA model (31), but it did not inhibit adherence in the MPAA (Table 1). This discrepancy might suggest that in the MPAA, where the ciliary membranes were solubilized and the receptor (s) was directly exposed to mycoplasmas, the hydrophobic interactions were no longer necessary for the adherence. Under the conditions utilized with the MPAA, adherence is mainly adhesin-ligand mediated since 1) *M. hyopneumoniae* did not bind significantly to microtiter plate wells without coated cilia (Fig. 1); 2) receptor analogues inhibited the adherence by as much as 90%; and 3) tetramethyl urea did not interfere with adherence (Table 1).

M. hyopneumoniae also attached to the cell body of ciliated cells in the MPAA model, indicating presence of receptors in the plasma membrane of ciliated cells. Although the distribution of some membrane surface components may be polarized at different locations in the plasma membrane of ciliated cells, it seemed that the receptor (s) for *M. hyopneumoniae* was present in both the ciliary membrane and the luminal membrane of ciliated cells. This finding was not consistent with results obtained by the SCCAA model in which this mycoplasma predominantly bound to the ciliary tuft and only a few to cell bodies (31). This contradiction may be explained by varied accessibility of receptors on different locations of ciliated cells. These findings suggest that host receptors that interact

with adhesins of a pathogen are not the sole factor for tissue tropism.

The six inhibitors obtained with MPAA also blocked the attachment of *M. hyopneumoniae* to intact ciliated cells (Table 4), indicating adherence mechanisms involved in the MPAA were comparable with those operating in the SCCAA. In the SCCAA, intact ciliated cells instead of solubilized cilia were utilized to react with mycoplasmas. This result confirmed the specificity of the receptor analogues which interrupt the intimate associations between molecules on the surfaces of mycoplasmas and ciliated cells. No antibodies against *M. hyopneumoniae* have been identified that block the adherence, which makes isolation of adhesins difficult. Thus, these receptor analogues provide a valuable alternative approach for purification and characterization of mycoplasmal adhesins in the future. In addition, novel approaches to the prophylaxis of MPS may be developed by utilizing the receptor analogues. It was reported by Aronson et al. (2) that mannose, a receptor analogue for type I fimbriated *E. coli*, successfully decreased episodes of cystitis when instilled with the organism into the mouse bladder.

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GLYCOLIPID RECEPTORS FOR ATTACHMENT OF *MYCOPLASMA HYOPNEUMONIAE* TO PORCINE RESPIRATORY CILIATED CELLS

A paper submitted to Infection and Immunity

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Abstract

Glycolipid receptors for *Mycoplasma hyopneumoniae* attachment were analyzed by using a thin-layer chromatography (TLC) overlay assay. *M. hyopneumoniae* bound specifically to sulfatide, globoside, and monosialoganglioside GM3. No binding was detected to sphingomyelin, cerebroside, lactosyl ceramide, ceramide trihexoside, monosialogangliosides GM1 and GM2, disialogangliosides (GD1a, GD1b, and GD3), trisialoganglioside (GT1b), cholesterol, cholesterol sulfate, palmitic acid, tripalmitin, or cholesteryl palmitate. Total lipids extracted from cilia of the swine respiratory epithelium also were separated on TLC plates and overlaid with mycoplasmas. *M. hyopneumoniae* bound specifically to three ciliary glycolipids identified as La, Lb, and Lc. Binding to Lc was stronger than to La and Lb. All three lipids were sulfated glycolipids as determined by laminin binding. Lc had a mobility similar to that of sulfatide on TLC plates, but it was barely detectable when reacted with orcinol ferric chloride

reagent. Homogeneous ciliary glycolipid receptors were successfully purified from a ciliary lipid mixture and immobilized onto microtiter plates. Sensitive and dose-dependent binding of *M. hyopneumoniae* to La, Lb, and Lc was detected. Lipid-extracted cilia had substantially lower adherence activity than did non-extracted cilia. These results indicate that the sulfated glycolipids, La, Lb, and Lc, were the major native receptors for *M. hyopneumoniae* attachment in vivo.

Introduction

Mycoplasmal pneumonia of swine (MPS), caused by *Mycoplasma hyopneumoniae,* is a worldwide, economically important swine disease (24). Lack of knowledge about the pathogenic mechanisms and virulence factors involved in *M. hyopneumoniae* infection is the limiting factor in the development of highly effective vaccines for control of MPS. *M. hyopneumoniae* colonizes the surface of ciliated cells in the tracheas, bronchi, and bronchioles of pigs and does not invade epithelial cells (1, 24). Intimate attachment of this organism to cilia during infection has been well documented (2, 20, 29). This association leads to extensively progressive loss of cilia, desquamation of epithelium, and development of pneumonia (24). Both in vitro and in vivo studies have shown that *M. hyopneumoniae* only adheres to cilia of swine respiratory ciliated cells but not to non-ciliated cells of the porcine respiratory tract (2, 20, 29, 30). These data indicate that cilia of the porcine respiratory epithelium have receptors for *M.*

hyopneumoniae.

Mucosal surfaces are the ports of entry and major sites of many infectious agents. Many pathogens, including viruses (6, 19), bacteria (25), and bacterial toxins (8), bind to specific carbohydrate moieties on the mucosal surfaces, enabling colonization and infection, and potentially leading to toxic damage to the host cells. These carbohydrate moieties may be present in either glycoproteins or glycolipids. Although several in vitro adherence models have been established for *M. hyopneumoniae* (27, 28, 30, 31), the adhesin(s) of the mycoplasma and its host receptors has not been identified. In a previous study (28), several carbohydrates and glycoconjugates including dextran sulfate, heparin, fucoidin, chondroitin sulfate, mucin, and laminin inhibited adherence of this mycoplasma to ciliated cells; treatment of cilia with sodium metaperiodate before mycoplasmal binding reduced the attachment of mycoplasmas. These data strongly indicate that glycoconjugates on the surface of the ciliary membrane are involved in the attachment of *M. hyopneumoniae.* In this study, the native glycolipid receptors for *M. hyopneumoniae* were identified by by use of a TLC overlay assay.

Materials and Methods

Chemicals and reagents. Neutral glycolipids (cerebroside, sulfatide, sphingomyelin, lactosyl ceramide, ceramide trihexoside, and globoside), gangliosides (GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, and asialo-GM1) and

non-polar lipids (cholesteryl palmitate, tripalmitin, palmitic acid, cholesterol, and

cholesterol sulfate) were obtained from Matreya, Inc. (Pleasant Gap, PA). Laminin,

rabbit anti-laminin antibodies and orcinol ferric chloride spray reagent (Bial's

reagent) were from Sigma Chemical Company (St. Louis, MO). Polyclonal

antibodies to *M. hyopneumoniae* were produced by immunizing rabbits with

mycoplasmal immunogens prepared in rabbit muscle infusion medium

supplemented with 20% rabbit serum (21). Peroxidase-conjugated goat anti-rabbit

IgG was purchased from Cappel (Durham, NC). Peroxidase substrates, ABTS and

4-chloro-1-naphthol, were from Kirkegaard & Perry Laboratories, Inc.

(Gaithersburg, MD).

Mycoplasma. *M. hyopneumoniae* strain 232 LI27 was cultured in Friis

mycoplasmal medium (10) for 24 h at 37°C. Color changing units (CCU),

representing the number of mycoplasmas in the culture, were determined by serial

dilution in tubes containing Friis medium. Mycoplasmas were harvested by

centrifugation at 25,000 x *g* for 15 min. The pellet of mycoplasmas was

resuspended to 1/10 of the original volume in RPMI 1640 medium containing 1%

gelatin. This preparation was further diluted in the buffer for use in the TLC overlay

assay and the microtiter plate adherence assay.

Extraction of total lipids from swine cilia. Ciliated cells were

harvested from specific-pathogen-free swine trachea as described (30), and the

cilia were extracted according to the methods of Tuomanen et al. (26). Briefly,

ciliated cells were harvested from the trachea and suspended in 40 ml TES buffer

(20 mM Tris, 10 mM EDTA, and 125 mM sucrose, pH 7.2). The cells were washed twice with TES by centrifugation (300 x g for 5 min). The cell pellet was resuspended in 6 ml AES buffer (80 mM acetate, 10 mM EDTA and 125 mM sucrose, pH 6.8) and mixed with 6 ml, 20 mM $CaCl_2$. This mixture was vortexed for 10 min at 25 °C, diluted with TES to 40 ml, and then centrifuged at 500 x g for 5 min. The supernate containing extracted cilia was sedimented by centrifugation at 18,000 x g for 20 min. Cilia were washed twice with phosphate-buffered saline (PBS, pH 7.2) before extraction of lipids. Extraction of total lipids from cilia was performed as reported by Magnani et al. (18). Briefly, 0.5 gram wet weight of cilia was homogenized in a mixture of 1.5 ml water, 5.4 ml methanol, and 2.7 ml chloroform. The homogenate was centrifuged at 5,000 x g for 20 min. The supernate was collected, and the pellet was extracted twice more with 0.5 ml water, 2.7 ml methanol and 1.35 ml chloroform. The supernates from three extractions were pooled and evaporated. The residue was dissolved in chloroform-methanol (2:1) and extracted 2 or 3 times until nonsoluble substances (proteins) were totally removed. The total lipid extract was resuspended in 0.5 ml chloroform-methanol and stored at -20°C. The nonsoluble substances in chloroform-methanol (2:1), mainly containing ciliary proteins, were dissolved in PBS and stored at -70°C. Some of the lipid extract was subjected to Folch partition in six volume chloroform-methanol (2:1) and one volume water (9). The upper phase was collected, and the lower phase was partitioned twice more with six parts chloroform-methanol (2:1) and one part water. The upper phases from each partition were pooled,

evaporated, dissolved in chloroform-methanol (2:1), and stored at -20°C.

TLC overlay assay. The TLC overlay assay was performed as described (16) with some modifications. Various lipid standards and total lipid extract of cilia were applied onto polyester-backed silica gel TLC plates (Sigma Chemical Company, St. Louis, MO). The lipids were separated with chloroform/methanol/0.25% $CaCl_2$ in water (60:35:8). Two chromatograms were developed in parallel on the same plate; one was sprayed with Bial's reagent to display any glycolipids, and one used in the overlay assay. After drying, the plates for the overlay assay were dipped in 0.5% polyisobutylmethacrylate (Polysciences, Inc., Warrington, PA) in hexane for 2 min and air-dried. The coated plates were immersed in RPMI 1640 medium containing 1% gelatin at 37°C for 2 h. Then, the plates were overlaid with a suspension of *M. hyopneumoniae* (5×10^8 CCU/ml) and incubated at 37°C for 5 h in a humid atmosphere. Unbound mycoplasmas were removed by three washings in PBS, 3 min each time with gentle shaking. Rabbit anti-mycoplasma antibodies were added and incubated for 1 h at 37°C. After three washings with PBS, the plates were overlaid with goat anti-rabbit IgG conjugated with peroxidase at 37°C for 1 h. Binding of mycoplasmas to specific lipids was visualized by the addition of 4-chloro-1-naphthol. For identification of sulfated glycolipids, plates with separated lipids were overlaid with laminin and developed with anti-laminin antibodies based on the methods described by Roberts et al. (23).

Microtiter plate adherence assay. The microtiter plate adherence

assay was performed as previously reported (28). After extraction of cilia with chloroform-methanol, the precipitate (ciliary proteins) was dissolved in PBS, solubilized with sodium dodecyl sulfate, and coated onto 96-well microtiter plates (10 μg per ml, 100 μl per well). Untreated cilia and gelatin were utilized as positive and negative controls, respectively. Mycoplasmas, rabbit antibodies to *M. hyopneumoniae*, goat anti-rabbit peroxidase conjugate, and substrate (ABTS) were sequentially added to the plates according to standard procedures. Optical density (OD) values at 405 nm representing adherence activity were measured with a microplate reader (Model EL310, Bio-Tek Instruments, Inc, Winooski, VT).

Purification of individual glycolipid receptors. Total ciliary lipids were separated on a TLC plate. A small portion of the plate was sprayed with Bial's reagent to reveal various glycolipid bands. The regions corresponding to the individual bands on the remaining plate, which were known to be receptors of *M. hyopneumoniae,* were scraped from the plate and extracted with chloroform-methanol (2:1). Solvent was separated from the gel by centrifugation at 23,000 x *g* for 20 min. The extraction was conducted three times, and the solvent was pooled and evaporated. The purified receptors were resuspended in chloroform-methanol (2:1). Purity of each preparation was examined by TLC, and the adherence activity of each receptor was detected by overlaying with *M. hyopneumoniae* as described in the standard protocol.

Binding of mycoplasmas to immobilized glycolipids. This assay was performed according to the method reported by Krivan et al. (16) with some

modifications. Briefly, sulfatide, glucocerebroside, and purified ciliary glycolipids were serially diluted in methanol containing auxiliary lipids (0.1 µg/ml cholesterol and 0.1 µg/ml phosphatidylcholine). Various dilutions of glycolipids were added to the wells (100 µl/well) of a 96-well microtiter plate (Immulon®1) and evaporated. Binding of *M. hyopneumoniae* to the immobilized glycolipids was conducted and detected with procedures similar to those described for the microtiter plate adherence assay.

Results

Binding of *M. hyopneumoniae* to standard glycolipids. Various lipid and glycolipid standards were subjected to thin-layer chromatography and tested for the ability to bind *M. hyopneumoniae* (Table 1). *M. hyopneumoniae* bound to sulfatide, GM3, and globoside, but not to GM2, GM1, GD3, GD1a, GD1b, GT1b, asialo-GM1, and other neutral, or non-polar lipids which were tested in this experiment (Table 1). Binding of *M. hyopneumoniae* to sulfatide and GM3 was stronger than to globoside (Fig. 1). Nonspecific binding of antibodies to lipids was not detected. Coating of the TLC plates with polyisobutylmethacrylate was essential to reduce background binding. Unlike the microtiter plate adherence assay in which 90 min of incubation was enough for successful binding of mycoplasmas, it was neccessary to increase the incubation time (5 h at 37°C) of mycoplasmas for optimal binding in the TLC overlay assay.

Fig. 1.   Binding of *M. hyopneumoniae* to standard glycolipids on TLC plates.

Panel A, sprayed with Bial's reagent; panel B, overlaid with *M.*

hyopneumoniae. Lane 1, neutral glycosphingolipids: cerebroside (C,

3 μg), lactosylceramide (L, 3 μg), ceramidetrihexoside (T, 3 μg) and

globoside (G, 3 μg). Lane 2, sulfatide (2.5 μg). Lane 3, gangliosides in

order of their migration (top to bottom): Asialo-GM1 (3 μg), GM1 (3 μg),

GD1a (3 μg), and GD1b (3 μg). Lane 4, gangliosides (top to bottom):
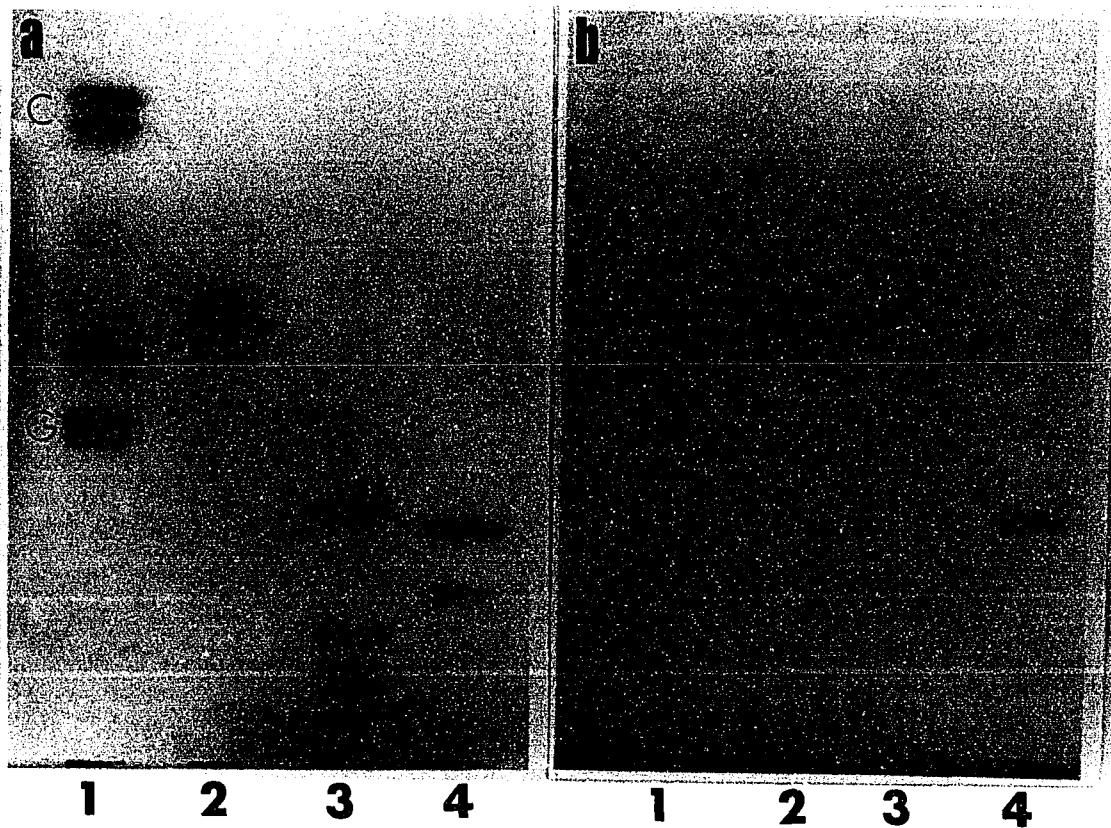
GM3 (3 μg) and GM2 (3 μg).

a					b			
1	**2**	**3**	**4**		**1**	**2**	**3**	**4**

Table 1. Binding of *M. hyopneumoniae* to various lipids and glycolipids

Lipids	Structure	binding[a]
Neutral sphingolipids		
Sphingomyelin		-
Cerebroside	Gal-ß1-1Cer	-
Sulfatide	Gal(3SO$_4$)ß1-1Cer	++
Lactosyl ceramide	Galß1-4Glc1-1Cer	-
Ceramide trihexoside	Galα1-4Galß1-4Glc1-1Cer	-
Globoside	GalNAcß1-3Galα1-4Galß1-4Glc1-1Cer	+
Gangliosides		
GM1	Galß1-3GalNAcß1-4[NeuAcα2-3]Galß1-4Glcß1-1Cer	-
GM2	GalNAcß1-4[NeuAcα2-3]Galß1-4Glcß1-1Cer	-
GM3	NeuAcα2-3Galß1-4Glcß1-1Cer	++
GD1a	NeuAcα2-3 Galß1-3GalNAcß1-4[NeuAcα2-3]Galß1-4Glcß1-1Cer	-
GD1b	Galß1-3GalNAcß1-4[NeuAcα2-8NeuAcα2-3]Galß1-4Glcß1-1Cer	-
GD3	NeuAcα2-8NeuAcα2-3Galß1-4Glcß1-1Cer	-
GT1b	NeuAcα2-3 Galß1-3GalNAcß1-4[NeuAcα2-8NeuAcα2-3]Galß1-4Glcß1-1Cer	-
Asialo-GM1	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer	-
Non-polar lipids		
Cholesteryl palmitate		-
Tripalmitin		-
Palmitic acid		-
Cholesterol		-
Cholesterol sulfate		-

[a] (-) No binding to 5 µg of lipid; (+) positive binding to 3 µg of lipid; (++) positive binding to less than 0.5 µg of lipid.

Binding of mycoplasmas to lipids extracted from porcine cilia. A variety of lipids were extracted from swine cilia. Many of them were detected by using Bial's reagent and therefore regarded as glycolipids. Three of them (La, Lb, and Lc) were bound by *M. hyopneumoniae* in the TLC overlay assay (Fig. 2). Lc had a mobility similar to that of sulfatide, but was barely detectable with Bial's reagent (Panel A of Fig. 2). Binding of mycoplasmas to Lc was stronger than to La and Lb. After Folch partition, Lc along with another lipid (Ld) was distributed to the upper phase, whereas most of the other ciliary lipids were retained in the lower phase (Fig. 2). Lc was also not detected by 2,7-dichlorofluorescein, which detects both saturated and nonsaturated lipids. Ld was detected as a dense band by Bial's reagent and had a mobility similar to that of ganglioside GM2. *M. hyopneumoniae* did not bind to Ld (Fig. 2).

Binding of *M. hyopneumoniae* to purified receptors in microtiter plates. La, Lb, Lc, and Ld were purified to homogeneity from the TLC chromatograms. On TLC plates, preparations of La, Lb, and Ld were detected as single bands by the Bial's reagent; no band was detected with Bial's reagent in the preparation of Lc (data not shown). Purified La, Lb, and Lc still bound *M. hyopneumoniae* in the TLC overlay assay (not shown). This specific binding was further confirmed in microtiter plates in which sensitive and dose-dependent binding of mycoplasmas was detected with immobilized sulfatide, Lc, La, and Lb (Fig. 3). No significant binding was detected to immobilized Ld, glucocerebroside, and the auxiliary lipids.

Fig. 2.   Binding of *M. hyopneumoniae* to ciliary glycolipids on TLC plates. Panel A, sprayed with Bial's reagent. Panel B, overlaid with *M. hyopneumoniae*. Lane 1, sulfatide (2.5 µg). Lane 2, total lipid extract of cilia from 5 mg wet cilia (5 µl). Lane 3, lower phase of Folch partition of ciliary lipids from 10 mg wet cilia. Lane 4, upper phase of Folch partition of ciliary lipids from 10 mg wet cilia.
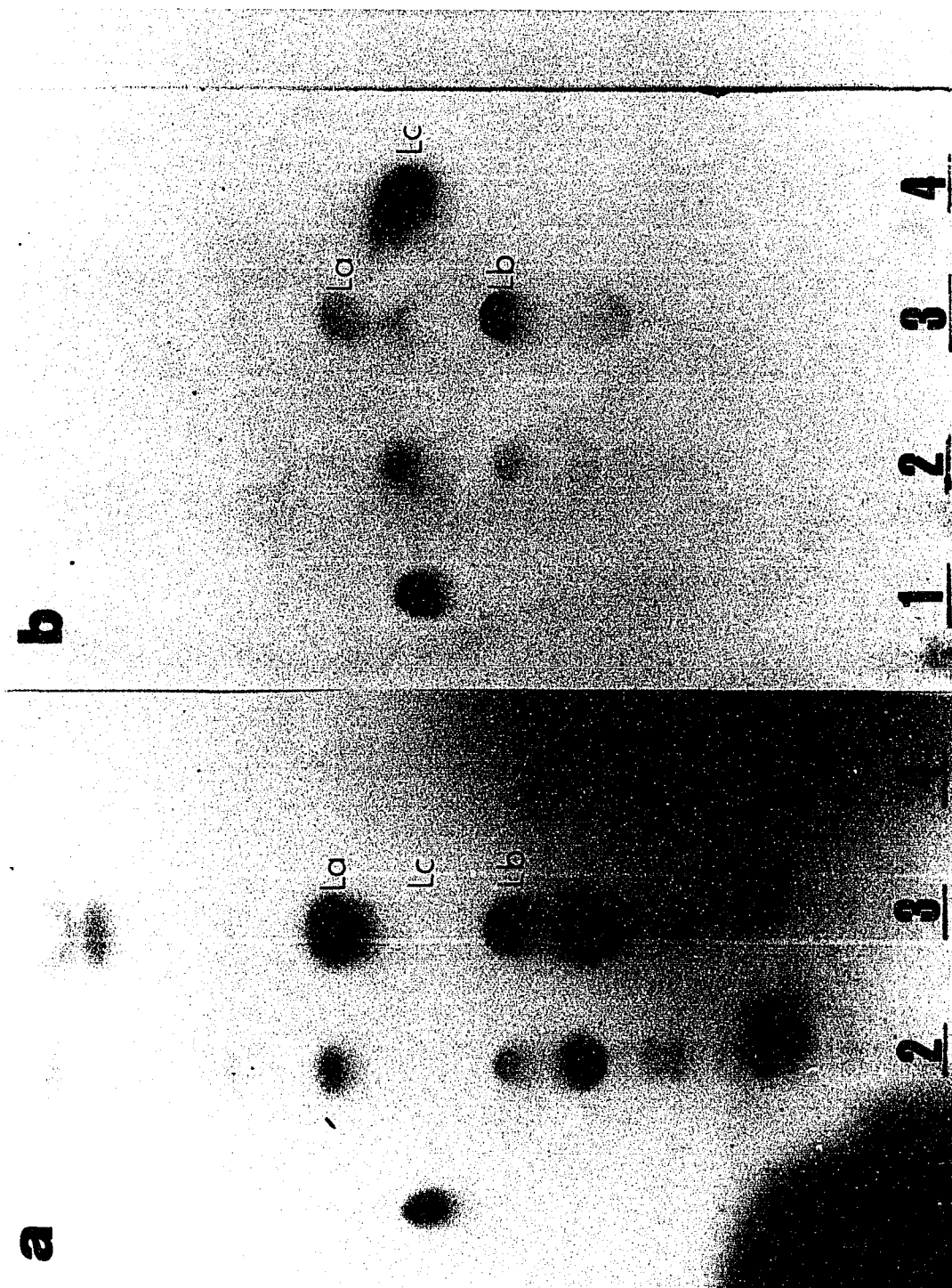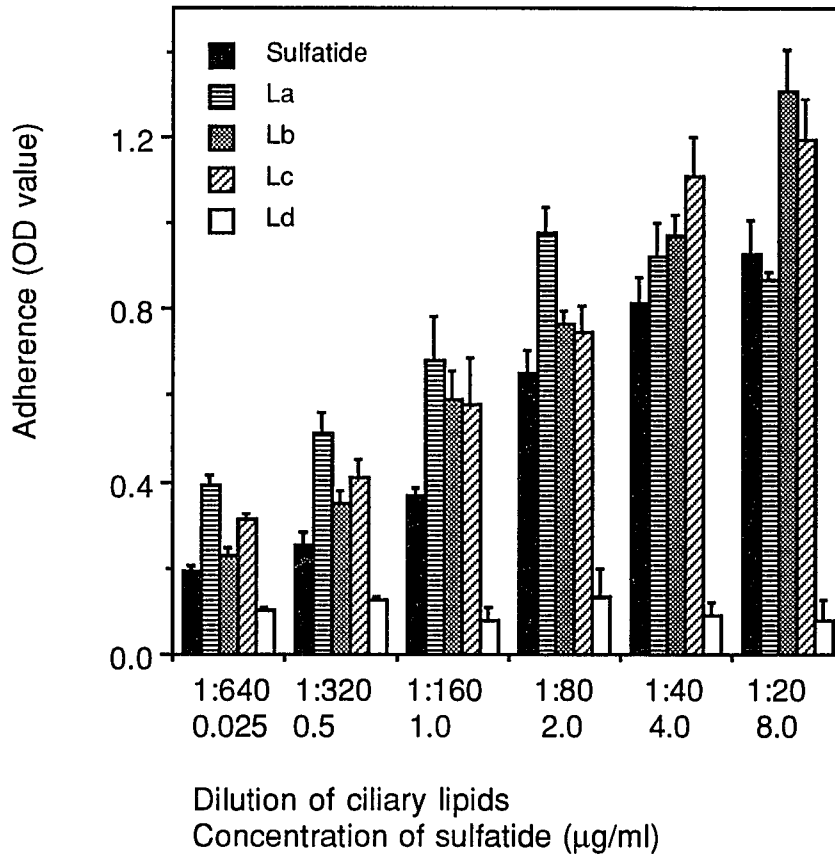
Fig. 3.   Binding of *M. hyopneumoniae* to immobilized glycolipids in microtiter plates. La, Lb, Lc, and Ld purified from 50 mg wet cilia were diluted to 1:640, 1:320, 1:160, 1:80, 1:40, and 1:20 in methanol, and sulfatide was diluted to 0.025, 0.5, 1.0, 2.0, 4.0, and 8.0 μg per ml for coating the microtiter plates as described in the Materials and Methods. *M. hyopneumoniae* (2 x 10^8 CCU per well) was added and incubated for 90 min at 37 °C. Bars represent mean OD ± SD of 4 wells from three experiments.

Binding of laminin to ciliary lipids. To characterize the chemical nature

of the ciliary receptors, ciliary lipids along with standard glycolipids were also

overlaid with laminin, a glycolipid which specifically binds to sulfated glycolipids

but not to non-sulfated glycolipids. As expected, laminin bound to sulfatide, but not

to gangliosides and neutral glycosphingolipids (Fig. 4). Laminin also bound to

three ciliary glycolipids, La, Lb, and Lc (Fig. 4), which were receptors for *M.*

hyopneumoniae. No binding of laminin was detected to other ciliary lipids (Fig. 4).

Adherence activity of lipid-extracted cilia. In order to elucidate the

significance of ciliary glycoproteins in the adherence of *M. hyopneumoniae*, the

proteinaceous precipitate of cilia, derived from choloroform-methanol extraction,

was immobilized to microtiter plates to test for the binding of mycoplasmas. Lipid-

extracted cilia still had adherence activity to the mycoplasma, but the activity was

substantially lower than that obtained with nonextracted cilia (Fig. 5).

Discussion

TLC overlay assay has been widely used for characterization of glycolipid

receptors for microbial pathogens (12, 14). An advantage of the assay is that TLC

plates present glycolipid receptors in a conformation similar to that of the eucaryotic

cell membrane (12). In this study, *M. hyopneumoniae* selectively attached to

several glycolipid standards, including sulfatide, ganglioside GM3, and globoside

(Fig. 1). The specific carbohydrate sequence responsible for binding of *M.*

Fig. 4.   Binding of laminin to standard glycolipids and ciliary lipids on TLC plates. Panel A, sprayed with Bial's reagent. Panel B, overlaid with laminin. Lane 1, neutral glycosphingolipids in order of their migration: cerebroside (C, 3 μg), lactosylceramide (L, 3 μg), ceramidetrihexoside (T, 3 μg) and globoside (G, 3 μg). Lane 2, sulfatide (2.5 μg). Lane 3, total lipid extract of 5 mg wet cilia. Lane 4, gangliosides in order of their migration (top to bottom): Asialo-GM1 (3μg), GM1 (3 μg), and GD1a (3 μg).

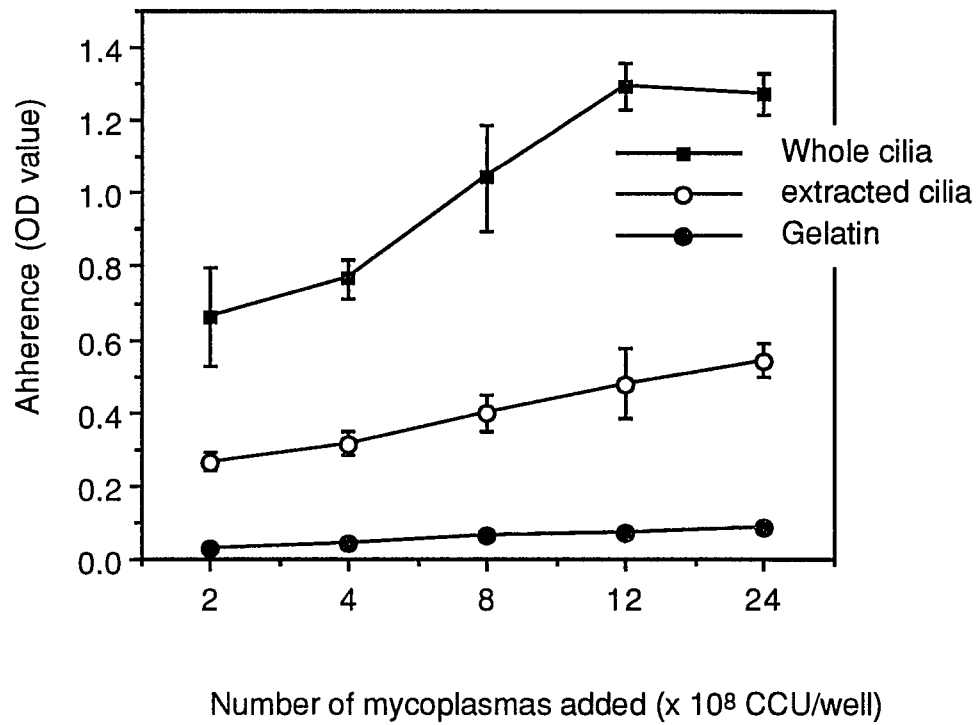Number of mycoplasmas added (x 10^8 CCU/well)
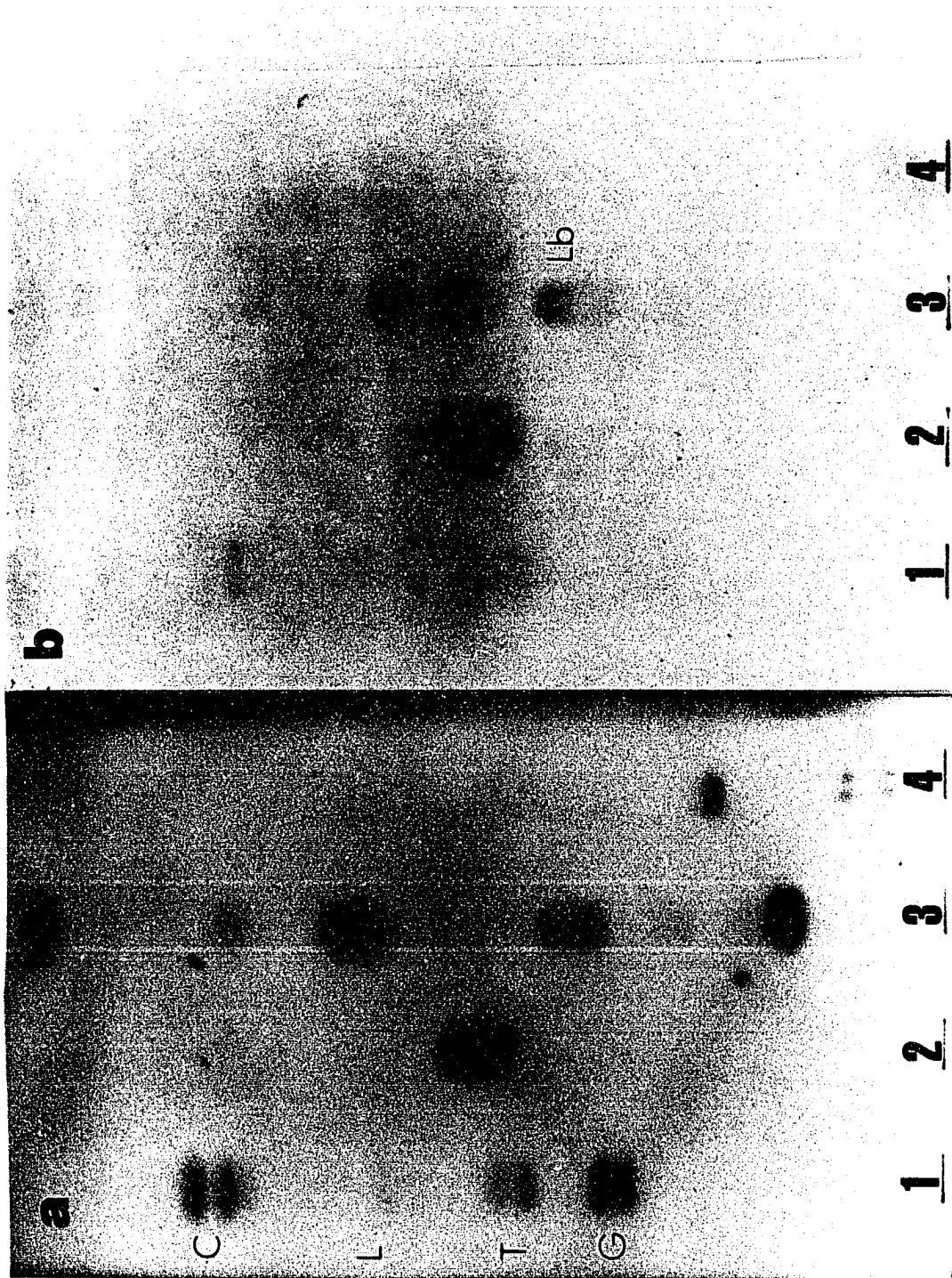
Fig. 5.   Binding of *M. hyopneumoniae* to immobilized whole cilia, lipid-extracted cilia, and gelatin in microtiter plates. Results were presented as mean OD ± SD (n=8).

hyopneumoniae was not identified in the glycolipids. *M. pneumoniae*, a human

pathogen, bound to glycolipids containing the terminal $Gal(3SO_4)\beta 1$- residue (16).

Many pulmonary pathogens were shown to have a binding specificity for the

$GalNAc\beta 1$-4Gal sequence in glycolipids (15). The binding of *M. hyopneumoniae* to

diverse carbohydrate sequences in glycolipids suggests that there might be

several different receptors existing in cilia. Binding of the mycoplasma to

ganglioside GM3 was unexpected because sialic acid did not inhibit adherence of

M. hyopneumoniae, and treatment of cilia with neuraminidase did not affect the

binding of this organism in a previous study (28).

Three ciliary receptors (La, Lb, and Lc) for *M. hyopneumoniae* were

identified with the TLC overlay assay (Fig. 2). Their receptor specificity was further

confirmed by mycoplasmal binding to purified individual receptors immobilized on

microtiter plates (Fig. 3). All three lipids were believed to be glycolipids because

they yielded a positive reaction with Bial's reagent (except for Lc) and were bound

by laminin. Lc had a mobility similar to that of sulfatide on TLC plates, but it was not

detected by either Bial's reagent or 2,7-dichlorofluorescein. Further analysis using

NMR, MS, GLC, or other biochemical means will provide more information on the

chemical nature of Lc. Folch partition separates total lipids into two phases (9, 11);

the upper phase usually contains hydrophilic glycolipids, and the lower phase

contains phospholipids, glycolipids, and neutral lipids (11). Because Lc was

retained in the upper phase and was also bound by laminin, it was believed that Lc

was a hydrophilic sulfated glycolipid. The reason that Lc was not detected by

orcinol ferric chloride and 2,7-dichlorofluorescein is unknown. Lc might be a minor

lipid in the membrane of cilia, and therefore not easily detectable by using the

spray reagents. The strong binding by *M. hyopneumoniae* indicated that Lc might

be a high-affinity receptor for the mycoplasma. It is unlikely that the three glycolipid

receptors are gangliosides because they have different mobilities from those of

standard gangliosides on TLC plates. Ld had a mobility similar to that of GM2, but

was not bound by *M. hyopneumoniae*, suggesting that ciliary gangliosides were

not involved in the adherence although *M. hyopneumoniae* bound to GM3 in the

TLC overlay assay.

Because similar carbohydrate sequences may exist in both glycolipids and

glycoproteins, results from this study did not exclude the possibility that some ciliary

glycoproteins are also involved in attachment of *M. hyopneumoniae*. Two types of

receptors, sialylglycoproteins (22) and sulfated glycolipids (16), were identified for

M. pneumoniae. As shown in Fig. 5, lipid-extracted cilia had substantially lower

adherence activity. The remaining adherence activity could be attributable to lipid

residues or proteinaceous receptors in the extracted cilia. No matter what the

source of the remaining adherence activity is, it can be concluded that ciliary

glycoprotein (s) was not the major receptor (s) for the mycoplasma.

Laminin is a major basement membrane glycoprotein that participates in cell

growth, differentiation and migration (23). In a previous study, we found that laminin

inhibited adherence of *M. hyopneumoniae* to a ciliary extract by interacting with

some components in cilia (28). In this study, laminin bound to La, Lb, and Lc which

were receptors for the mycoplasma. This result provided an explanation for the adherence-interfering mechanisms of laminin. This glycoprotein has a binding specificity to sulfated glycolipids and has been used to identify these lipids (23). Binding by laminin of the three ciliary receptors for *M. hyopneumoniae* indicated that they are sulfated glycolipids in nature. This finding was correlated with the results obtained in a previous study (28) in which several sulfated receptor analogues inhibited adherence of *M. hyopneumoniae* to single ciliated cells and to purified cilia immobilized onto microtiter plates. Several species of mycoplasmas other than *M. hyopneumoniae* also utilized sulfated glycolipids as receptors (16, 17).

The mechanism by which *M. hyopneumoniae* damages ciliated cells is not known. No toxins have been reported for the mycoplasma. DeBey (4) demonstrated that direct contact of *M. hyopneumoniae* with ciliated cells was required for production of cytotoxicity, by separating mycoplasmas from ciliated epithelium with a 0.1 µm membrane. Therefore, it is possible that the adhesin (s) of *M. hyopneumoniae* not only mediates mucosal adherence but also induces pathogenic effects on ciliated cells by unknown means. Enteropathogenic *E. coli* adhere to the intestinal mucosa and cause effacement of microvilli and rearrangement of cytoskeletons in epithelial cells (7). Attachment of enteropathogenic *E. coli* resulted in elevation of intracellular calcium concentration and protein phosphorylation, indicating that the effacing lesion resulted from a subversion of host cell signal transduction by *E. coli* attachment (3, 7). Some

pathogens, such as *Bordetella pertussis* and *Yersinia spp.*, utilize integrins, a family of glycoproteins involved in cell-cell and cell-extracellular matrix recognition, as receptors (13). Binding of the pathogens to integrins triggered signal transduction in host cells and change of cell functions (3). It is not known if *M. hyopneumoniae* can trigger signal transduction in ciliated cells by ligand-receptor mediated adherence. DeBey et al. (5) reported that *M. hyopneumoniae* infection enhanced the level of cytosolic calcium in neutrophils. However, no information pertaining to the change in ciliated cells was available. Analysis of the structures and functions of the ciliary receptors may help to provide more information about the pathogenic mechanisms of *M. hyopneumoniae*.

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IDENTIFICATION AND CHARACTERIZATION OF *MYCOPLASMA HYOPNEUMONIAE* ADHESINS

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Abstract

A major adhesin of *Mycoplasma hyopneumoniae* was identified and characterized in this study. A monoclonal antibody (Mab) F2G5 and its F(ab')$_2$ fragments inhibited the adherence of *M. hyopneumoniae* to porcine cilia, the natural targets to which the mycoplasma binds during infection. Mab F2G5 detected multiple bands, but predominantly recognized a 97 KDa (P97) protein of *M. hyopneumoniae* on immunoblots. Immunolabelling of mycoplasmas with Mab F2G5 under electron microscopy demonstrated that the proteins recognized by Mab F2G5 were located at the surface of the mycoplasma, predominantly on a surface fuzzy layer. Affinity chromatography, conducted with immobilized Mab F2G5, purified mainly P97 and a set of minor proteins with distinct sizes. The purified proteins were able to bind to cilia and blocked the adherence of intact *M. hyopneumoniae* cells to cilia. Surface proteolysis of *M. hyopneumoniae* with trypsin degraded P97 and decreased the adherence activity of the mycoplasma.

The predominant proteins detected by Mab F2G5 were P97 in strains LI27, 232

2A3 and 232 FA1, a 95 KDa protein in strain J, and a doublet (93 KDa and 92 KDa)

in strain 144L, indicating that the antigens bearing the epitope for Mab F2G5

undergo intraspecies size variation. This variation appeared to be related to the

adherence capability of *M. hyopneumoniae* because strains J and 144L had

substantially lower and higher adherence activity, respectively, than the other

strains. Intrastrain size variation of adhesins was manifested as ladders of proteins

on immunoblots stained with Mab F2G5 and as periodic structures produced by

digestion of purified adhesins with trypsin. These results indicate that P97 is a

major adhesin of *M. hyopneumoniae*, which undergoes intraspecies and intrastrain

size variations. Size variation of adhesins may be a pathogenic mechanism utilized

by *M. hyopneumoniae* to evade the porcine immune system.

Introduction

Mycoplasma hyopneumoniae is the etiological agent of mycoplasmal

pneumonia of swine (MPS), an economically important swine disease. It has been

known that *M. hyopneumoniae* colonizes the respiratory epithelium of swine and

produces a chronic pneumonia in pigs (23). During *in vivo* infection, the

mycoplasmas are intimately associated with cilia of ciliated cells and cause an

extensive loss of cilia as observed under an electron microscope (3, 18, 25). There

is a general notion that adherence of *M. hyopneumoniae* to porcine respiratory

ciliated cells is a prerequisite for colonization of the organism on the respiratory epithelium and development of pneumonia. Previous studies have shown that the adherence process is mainly mediated by receptor-ligand interactions (40, 43, 44). Several carbohydrates inhibited the adherence of the mycoplasma to cilia in a microtiter plate adherence assay, indicating that the receptors in cilia were glycoconjugate in nature (40). A subsequent study by use of a thin-layer chromatography overlay assay identified three ciliary glycolipid receptors for *M. hyopneumoniae* in porcine ciliated cells (41).

Virulence factors of *M. hyopneumoniae* have not been well defined. Several proteins of *M. hyopneumoniae*, including a 36 KDa lactate dehydrogenase (11), membrane lipoproteins (30), and a 54 KDa cytotoxic factor (10), have been characterized by different research groups; however, the biological functions of these proteins in the disease process have not been determined. The adhesin(s) of *M. hyopneumoniae* has not been identified. Previous studies showed that treatment of *M. hyopneumoniae* with trypsin decreased the adherence to single ciliated cells and cell monolayers, indicating that the adhesin(s) of *M. hyopneumoniae* might be a trypsin-sensitive protein (43, 44). Identification and characterization of *M. hyopneumoniae* adhesins will improve our understanding of the pathogenic mechanisms utilized by the organism and may lead to the development of highly efficient measures for control of MPS. In this study, monoclonal antibodies, affinity chromatography, surface proteolysis, electron microscopy and amino acid analysis were utilized along with an in vitro adherence

assay to elucidate the adhesins of *M. hyopneumoniae.*

Materials and Methods

Mycoplasmas and culture conditions. *Mycoplasma hyopneumoniae* strain LI27 was originally derived from an experimentally infected pig. Strains 232 2A3 and 232 FA1 were cloned from strain 232. Strain J was originally obtained from the American Type Culture Collection and maintained in Friis mycoplasmal medium (9) in this laboratory. Strain 144L SCI 3AF was isolated in the laboratory from the lung of an infected pig and filter-cloned three times. It was known that strains LI27, 232 and 144L were pathogenic for pigs, whereas strain J was nonvirulent (42). In this study, mycoplasmas were grown in Friis mycoplasmal medium supplemented with 20% acid-adjusted swine serum. The cultures were incubated in a water-bath shaker at 37°C for 24 to 48 h. The number of mycoplasmas was estimated by the determination of color changing units (CCU). Mycoplasmas were harvested by centrifugation at 25,000 x *g* for 15 min for adherence and adherence inhibition assays, the mycoplasmal pellets were resuspended to 1/10 of the original volume and further diluted with adherence buffer (RPMI 1640 containing 1% gelatin) to the appropriate CCU. For production of mycoplasmal proteins required for affinity chromatography, the pellets were further washed three times with phosphate-buffered saline (PBS, pH 7.2) and resuspended in PBS to 1/100 of the original volume.

Monoclonal antibodies and fragmentation. Monoclonal antibodies

(Mab) to various antigens of *M. hyopneumoniae* were supplied by Barbara

Erickson (Veterinary Medical Research Institute, Iowa State University, Ames). For

adherence inhibition, these Mabs in cell culture supernates were concentrated

about 12 times with ammonium sulfate. Mab F2G5, which inhibited the adherence

of *M. hyopneumoniae* to porcine cilia, was purified from ascites with the

ImmunoPure® IgM purification kit (Pierce, Rockford, IL). Purification was conducted

according to the instructions supplied with the kit. The purified Mab F2G5 was

resuspended in PBS and stored at -20°C for affinity chromatography and

fragmentation. Fragmentation of Mab F2G5 was performed with immobilized

pepsin as described (2). This digestion method mainly generates F(ab')$_2$ fragments

from mouse IgM; essentially no (Fc)$_{5\mu}$ can be produced (2). Briefly, 2 mg of Mab

F2G5 was applied to a Centricon™ 100 microconcentrator (Amicon, Danvers, MA)

and spun down at 4,000 *g* for 40 min at 4°C. The retentate containing the Mab was

washed twice with IgM F(ab')$_2$ digestion buffer (10 mM sodium acetate, 150 mM

NaCl, 0.05% NaN$_3$, pH 4.5) and finally resuspended in 1 ml digestion buffer. A

small column packed with 2 ml pepsin-agarose (Pierce, Rockford, IL.) was

equilibrated with 10 ml of the digestion buffer and prewarmed at 37°C for 5 min.

One ml of Mab F2G5 was added to the column and incubated at 37°C for 2 h. The

digest was eluted with 4 ml of the digestion buffer. The eluate was filtered with a

Microsep™ 300 concentrator to remove nondigested IgM. The filtrate was further

concentrated with a Centricon™ 30 microconcentrator. The retentate, mainly

containing F(ab')$_2$ fragments, was washed twice with PBS and resuspended in 0.5 ml PBS. F(ab')$_2$ fragments of Mab 80.1, an antibody against a 64 KDa protein which did not inhibit the adherence of *M. hyopneumoniae* to porcine cilia, were prepared using a similar procedure.

Adherence and adherence inhibition. A microtiter plate adherence assay (MPAA) was conducted as described previously (40). Briefly, *M. hyopneumoniae* whole cells (2 x 10^8 CCU) or mycoplasmal proteins (10 µg/ml) resuspended in adherence buffer were added into microtiter plates coated with SDS-solubilized porcine tracheal cilia and incubated at 37°C for 90 min. The non-attached mycoplasmas or proteins were washed off the plates with PBS. The mycoplasmas or mycoplasmal proteins attached to the plates were detected by sequential addition of rabbit anti-*M. hyopneumoniae* hyperimmune antibodies and goat anti-rabbit immunoglobulins conjugated with peroxidase. ABTS was finally added, and the optical density (OD) at 405 nm was measured. For inhibition assays, various Mabs or F(ab')$_2$ fragments were preincubated with intact mycoplasmas at 37°C for 60 min. Two washings with PBS were conducted to remove unbound antibodies. The preincubated mycoplasmas were added into cilia-coated plates, and adherence was measured as described above. Percentage of inhibition was calculated as described previously (40). Adhesins purified by Mab F2G5 affinity chromatography were evaluated for inhibition of the adherence of intact mycoplasmal cells. For this purpose, cilia-coated wells of microtiter plates were first incubated with the purified adhesin at 37°C for 90 min. The adherence

buffer was utilized as the noninhibitory control. After 4 washings with PBS, intact *M. hyopneumoniae* cells were added to the wells and incubated at 37°C for 90 min. Four washings with PBS were performed to remove non-attached mycoplasmas. The attached mycoplasmas were sequentially detected with a monospecific rabbit antiserum (R409) against a 64 KDa surface protein of *M. hyopneumoniae* (39) and goat anti-rabbit immunoglobulin conjugated with peroxidase. It was determined in a preliminary experiment that antiserum R409 did not cross-react with the purified adhesins, but reacted with whole cells of the mycoplasmas.

Surface proteolysis of mycoplasmas with enzymes. Proteolytic digestion of intact mycoplasmas was performed as described previously (8). *M. hyopneumoniae* strain LI27 grown in Friis mycoplasmal medium was harvested and washed three times with PBS by centrifugation. The mycoplasmas were resuspended to 0.5 mg/ml (protein concentration) in PBS. One ml of mycoplasmas was incubated with various doses (final concentrations: 0, 0.01, 0.04, 0.1, 0.25, 1, 5, 25, and 100 µg/ml) of trypsin (type XI, DPCC treated, Sigma Chemical Company, St. Louis, MO) at 37°C for 30 min. After digestion, trypsin inhibitor (type I-S, Sigma) was added to the mixture at a final concentration of 200 µg/ml to stop the reaction. The treated mycoplasmas were washed three times in PBS and resuspended in 0.2 ml PBS. Each treated sample was titrated for CCU to determine the viability of treated mycoplasmas, evaluated for adherence activity by use of the MPAA, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with Mab F2G5. Mycoplasmas were also treated with

various doses (see Fig. 3.3) of carboxypeptidase Y under similar conditions. The treated mycoplasmas were analyzed by SDS-PAGE and immunoblotting.

Immunoelectron microscopy. Immunolabelling of mycoplasmas with Mabs was performed as described previously (1). Briefly, *M. hyopneumoniae* cells resuspended in PBS containing 1% gelatin were incubated with 1: 10 diluted Mab F2G5 at 37°C for 1 h. Mycoplasmas incubated with cell culture medium were utilized as negative controls. The mycoplasmas were washed 3 times with PBS and then reacted with 1:10 diluted goat anti-mouse IgM (μ chain specific) conjugated with gold (10 nm, Sigma) at 37°C for 1 h. After three washings with PBS, the mycoplasmas were fixed with 3% glutaraldehyde-cacodylate buffer, dehydrated in acetone, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and observed under a Hitachi H500 electron microscope.

Comparison of different strains for adherence activities. Various strains of *M. hyopneumoniae* were cultured in Friis mycoplasmal medium, harvested by centrifugation, and washed with PBS as described above. The protein concentration of each strain was adjusted to 25 μg/ml for the adherence assay. The protein profiles of different strains were analyzed by SDS-PAGE and by immunoblotting with Mab F2G5.

SDS-PAGE and immunoblotting. SDS-PAGE was performed according to the method of Laemmli with 10% separating gel and 4% stacking gel (16). Mycoplasmas or mycoplasmal proteins were mixed with equal volumes of

treatment buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and heated at 100°C for 3 min prior to electrophoresis. The separated proteins were visualized by staining the gels with Coomassie brilliant blue R-250. For immunoblotting, proteins on the gels were transferred to nitrocellulose membranes as described by Towbin *et al.* (26). The membranes were blocked with 3% BSA , 0.1% Tween 20 in PBS at 25°C for 60 min with gentle shaking. Then, the blots was incubated with 1:100 diluted Mab F2G5 at 25°C for 2 h. After three washings with PBS containing 0.01% tween 20, the blots were further reacted with 1:1000 diluted goat anti-mouse immunoglobulins conjugated with peroxidase at 25°C for 2 h. Three washings were performed prior to color development with 4-chloro-1-naphthol.

Antibody affinity chromatography. Mab F2G5 was coupled onto Affi-Gel®10 (Bio-Rad Laboratories, Melville, New York) according to the instructions supplied with the activated support. In brief, 6 mg of purified Mab F2G5 resuspended in 4.5 ml of coupling buffer (0.1 M HEPES, pH 7.5) was mixed with 3 ml of Affi-Gel® 10. This mixture was incubated at 4°C for 4 h with gentle shaking. Then, 0.5 ml of 1M ethanolamine HCl (pH 8.0) was added and incubated at 4°C for 1 h to stop the coupling. The gel was alternatively washed with 0.1 M Tris-HCl (pH 7.5) and the coupling buffer. After packing into a small column, the gel was washed sequentially with 20 volumes of 10 mM Tris-HCl and 20 volumes of PBS containing 40 mM CHAPS (3-[(3-cholamidopropyl)-dimethyllammonio]-1-propane-sulfonate). Mycoplasmas resuspended in PBS containing 0.2 mM phenylmethylsulfonyl

fluoride (PMSF) were solubilized with 40 mM CHAPS at 4°C for 1 h. The lysate was

ultracentrifuged at 100,000 x *g* for 30 min. The supernate containing solubilized

mycoplasmal proteins was harvested. Preliminary experiments showed that

adhesins of *M. hyopneumoniae* were efficiently solubilized by CHAPS. Three ml of

the supernate containing 12 mg of mycoplasmal proteins were applied to the

column and reacted at 4°C overnight. Unbound proteins were washed off the

column with 50 ml of PBS containing 40 mM CHAPS. The proteins captured by the

Mab were eluted with 0.1 M glycine-HCl (pH 2.5). The eluate was immediately

neutralized to pH 7.5 with 1 M Tris and concentrated to 1 ml with a Centricon™ 10.

The purity of the proteins was determined by SDS-PAGE and immunoblotting; the

adherence activity of the eluted proteins was evaluated in the MPAA; and the

purified proteins were also utilized to block the adherence of intact *M.*

hyopneumoniae cells as described above.

Digestion of purified adhesins with trypsin. The controlled digestion

of purified adhesins was performed as described previously (21). In brief, the

adhesins purified by antibody affinity chromatography were digested with various

concentrations of trypsin (0.08 to 2.0 μg per ml) at 37°C for 30 min. The digested

adhesins were immediately mixed with the treatment buffer and boiled at 100°C for

3 min. The samples from each digestion were pooled and subjected to SDS-PAGE

and immunoblotting with Mab F2G5 as described above.

Protein sequencing and amino acid analysis. The N-terminal

sequence of P97 was determined as described previously (17). The adhesins

purified by Mab F2G5-affinity chromatography were further separated by SDS-PAGE as described above. After electrophoresis was completed, the gel was washed three times with the transfer buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11). The proteins on the gel were electroblotted to polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore) at 50 mA for 60 min. After rinsing with distilled water, the membrane was stained with 0.1% Coomassie brilliant blue R-250 for 5 min. Destaining was conducted with a solution of 50% methanol and 10% acetic acid. Finally, the PVDF membrane was thoroughly washed with distilled water and air-dried. The membrane was submitted to the Protein Facility at Iowa State University where P97 was excised from the membrane and subjected to automatic Edman degradation by using an Applied Biosystems 477A liquid-pulse sequencer. For analysis of total amino acid compositions, P97 was excised and analyzed with an Applied Biosystems 420 PTC amino acid analyzer.

Results

Adherence inhibition by Mabs or Mab fragments. Various Mabs were evaluated for ability to inhibit the adherence of *M. hyopneumoniae* to porcine cilia (Table 1). Two Mabs, F2G5 and F1B6, inhibited up to 67% of the adherence (Table 2). A similar result was obtained when the hyperimmune serum used for detection was replaced with antiserum R409 (a rabbit monospecific antiserum to a

Table 1. Adherence inhibition by various monoclonal antibodies

Mab	Isotype	Specificity[a]	Adherence inhibition
80.1	IgM	64	-
A1B2	IgM	100	-
A2H10	IgG1	100	-
F1B6	IgM	Multiple[b]	+
F2B11	IgG2a	86	-
F2G5	IgM	Multiple[b]	+
F3A6	IgG2b	116	-
F3B8	IgG1	105, 95, 65, 55	-
F3D5	IgG1	105, 95, 65, 55	-
F3D6	IgG1	105, 95, 65, 55	-
I1D8	IgG1	116	-
I2E12	IgG1	86	-
I2E12	IgG1	86	-
I3A6	IgG1	200, 145, 140, 116, 86	-
I4A10	IgG1	70	-
I4A4	IgG1	100, 90, 80, 60, 50	-
I4B1	IgG1	41, 43	-
I4B10	IgG1	41	-
I4B2	IgG1	41	-
I4E3	IgG1	116	-
R1E9	IgG3	40	-
R2E8	IgG1	67	-
R3G7	IgG1	116	-
R4B2	IgG1	40	-
R4B7	IgG2a	116, 105, 90, 80, 65, 60, 55	-
R5F4	IgG1	116	-
R6C10	IgG2a	116, 105, 90, 80, 65, 60, 55	-
R6E1	IgG1	116, 40	-
R6E4	IgG2a	116, 105, 90, 80, 65, 60, 55	-
R6G8	IgG1	46	-

- No inhibition was detected with a 12 fold concentrated culture supernate.
+ Produced dose-dependent inhibition
[a] Sizes of antigens (in kilodaltons) detected with strain Ll27 on immunoblots; some Mabs detected antigens of varied sizes in different strains.
[b] Reacted with a set of proteins with distinct sizes, but predominantly recognized a 97 KDa protein.

Table 2. Adherence inhibition by monoclonal antibodies F2G5, F1B6 and F3A6

Dilution[a]	% inhibition[b] produced by Mabs		
	F3A6	F1B6	F2G5
1:2	-11.9 ± 3.1	67.0 ± 5.1	66.2 ± 4.5
1:10	2.5 ± 2.2	45.9 ± 3.4	58.7 ± 5.0
1:50	-11.1 ± 6.5	-10.8 ± 6.1	29.5 ± 3.7
1:250	-1.1 ± 2.4	-9.7 ± 8.0	13.0 ± 4.1

[a] Mabs were concentrated approximately 12 times from culture supernate and further diluted in the adherence buffer.

[b] Mean ± SD of three independent experiments.

64 kDa surface protein of the mycoplasma), indicating that the two Mabs indeed blocked the adherence of mycoplasmas rather than the detection process. As determined by immunoblotting, both Mabs had identical antigenic specificity, detected multiple bands, and reacted predominantly with P97 (Fig. 1). Several Mabs, such as R6C10, I3A6 and F3D5, which also detected mutiple bands on immunoblots, did not inhibit the adherence (Table 1). The adherence-inhibiting activity was further evaluated with F(ab')$_2$ fragments of F2G5. As compared with the adherence buffer which was a negative control, the F(ab')$_2$ fragments of Mab F2G5 produced 60% inhibition of the adherence of *M. hyopneumoniae*, while no inhibition resulted with the F(ab')2 of Mab 80.1 (data not shown).

Binding of solubilized mycoplasmal proteins to cilia. Preliminary studies showed that CHAPS-solubilized mycoplasmal proteins adhered strongly to swine cilia. *M. hyopneumoniae* proteins solubilized with 40 mM CHAPS were ultracentrifuged at 100,000 *g* for 30 min (at 4°C) to remove nonsolubilized debris. The supernate diluted in adherence buffer was directly added to cilium-coated plates for adherence assay. Mycoplasmal proteins attached to the plates were detected with various Mabs and peroxidase-labelled goat anti-mouse immunoglobulins. The attached mycoplasmal proteins reacted only with Mab F2G5, but not with Mabs against 41 KDa, 64 KDa, 100 KDa and 116 KDa mycoplasmal antigens (Table 3).

Surface proteolysis of mycoplasmas. Under the conditions used in this study, treatment of *M. hyopneumoniae* with various doses (0.01 to 100 μg per ml)

Fig. 1.   Antigenic specificity of monoclonal antibodies. Total mycoplasmal

proteins of strain LI27 separated by SDS-PAGE were transferred to a

nitrocellulose membrane and reacted with Mabs F2G5 (lane 1), F1B6

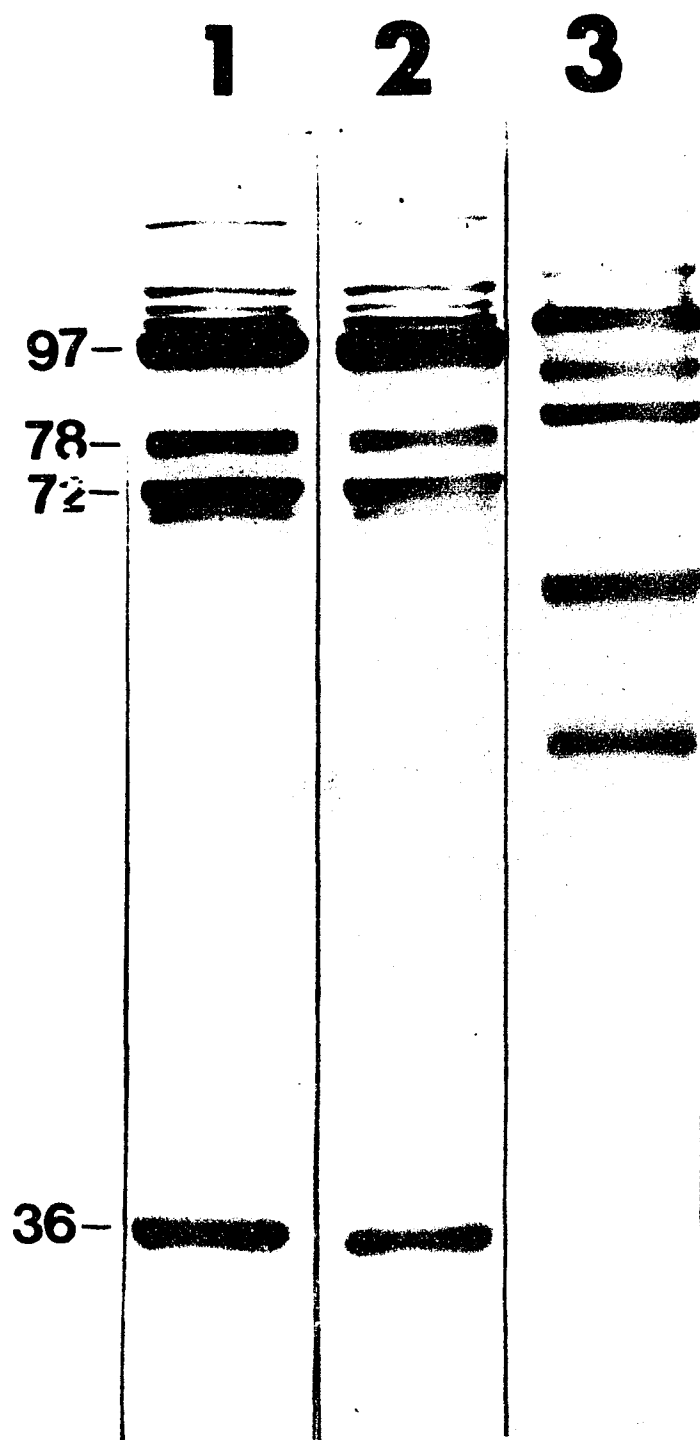(lane 2), and R6C10 (lane 3). Sizes of proteins (in kilodalton) are

shown on the left.

Table 3. Adherence of solubilized mycoplasmal proteins to cilia

Mab	Specificity[a]	OD value[b]
F3A6	116	0.039±0.003
F2G5	Multiple[c]	0.853±0.041
R6C10	105, 90, 80, 65, 60	0.053±0.006
80.1	64	0.038±0.009
I4B2	41	0.010±0.000
A2H10	100	0.026±0.001

[a]Sizes of antigens (in kilodaltons) detected in strain LI27.

[b] Mean ± SD of triplicate experiments

[c]Reacted with multiple bands, but predominantly recognized a 97 K protein.

of trypsin did not significantly affect the viability of mycoplasmas as determined by CCU titration (data not shown). As demonstrated by SDS-PAGE, low concentrations (0.01 to 1 μg/ml) of trypsin selectively degraded three mycoplasmal proteins, including P97, P105 and P46 (Fig. 2). Immunoblotting of trypsin-treated mycoplasmas with Mab F2G5 confirmed that P97 had been gradually digested (Fig. 2). Loss of P97 was accompanied by increased yield of P78 and P72 (Fig. 2). In fact, the P78 region was not a single protein, but contained a group of proteins with sizes ranging from 78 KDa to 82 KDa. P72 and P78 were relatively insensitive to trypsin, but they were also gradually digested at 25 to 100 μg/ml trypsin (data not shown). Mycoplasmas treated with 0.001 to 0.2 μg/ml trypsin had a decreased adherence capability, whereas an increased adherence activity was observed with mycoplasmas treated with 5 and 25 μg/ml trypsin (Table 4). However, solubilized trypsin-treated mycoplasmas showed a linear decrease in adherence to cilia with increased amount of trypsin (data not shown). The changes in protein profiles and adherence activities of *M. hyopneumoniae* caused by trypsin were highly reproducible under the conditions of this study. In contrast to trypsin, carboxypeptidase Y did not digest P97 from the surface of *M. hyopneumoniae* at various concentrations up to 125 μg/ml (Fig. 3).

Strain variation in adherence. Five strains of *M. hyopneumoniae* differed markedly in adherence activity when evaluated using the MPAA (Fig. 4). The adherence activity of strain J was substantially lower than for other strains, whereas strain 144L had the highest adherence activity among the 5 strains. One

Fig. 2.   Surface proteolysis of intact mycoplasmas. *M. hyopneumoniae* (LI27) cells were treated with graded doses of trypsin: 0.0 μg/ml (lane 1), 0.01 μg/ml (lane 2), 0.04 μg/ml (lane 3), 0.1 μg/ml (lane 4), 0.25 μg/ml (lane 5), 0.5 μg/ml (lane 6), 1 μg/ml (lane 7), and 5 μg/ml (lane 8) as described in the text. A, SDS-PAGE profile stained with Coomassie brilliant blue. B, immunostaining with Mab F2G5. Sizes of proteins (in kilodaltons) are shown on the left of each panel.
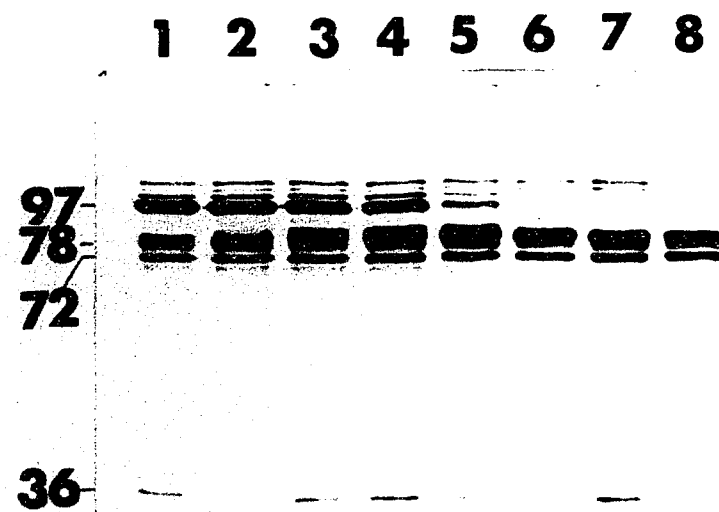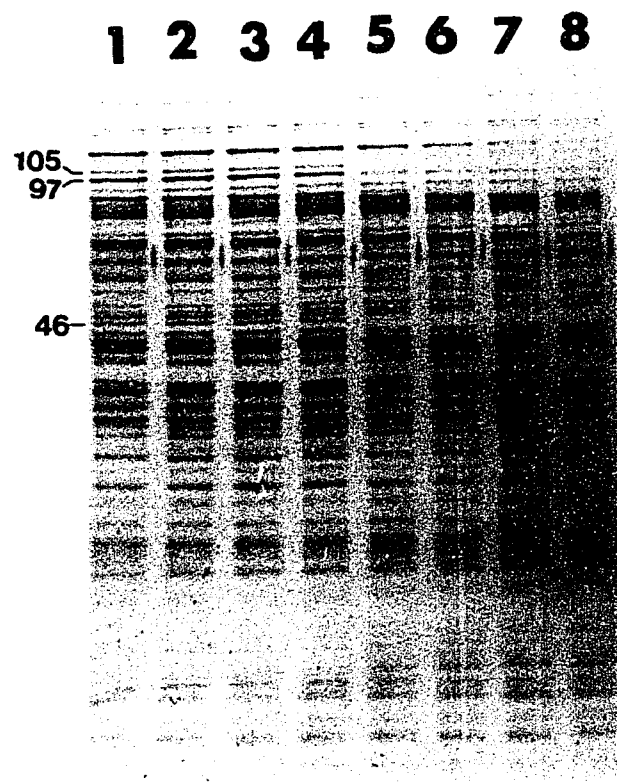
1 2 3 4 5 6 7 8   1 2 3 4 5 6 7 8

105
97

46

97
78
72

36

Table 4. Adherence activities of mycoplasmas treated with trypsin

Concentration of trypsin (μg/ml)	Adherence (OD value)[a]
0.00	0.67 ± 0.01
0.01	0.48 ± 0.02[b]
0.04	0.47 ± 0.02[b]
0.10	0.43 ± 0.01[b]
0.25	0.32 ± 0.01[b]
0.50	0.43 ± 0.03[b]
1.00	0.71 ± 0.01
5.00	1.25 ± 0.03[b]
25.0	0.80 ± 0.02[b]
100	0.60 ± 0.02

[a] Mean $\pm$ SD in triplicate experiments.

[b]Significantly different from the non-treated control as determined by an analysis of variance with the Tukey contrast ($P<0.05$).

Fig. 3.   Digestion of mycoplasmas with carboxypeptidase. *M.*

hyopneumoniae (LI27) cells were treated with various

concentrations of carboxypeptidase Y as described in the text:

lane 1, nontreated control; lane 2, 0.2 μg/ml; lane 3, 1.0 μg/ml;

lane 4, 5 μg/ml; lane 5, 25 μg/ml; lane 6, 125 μg/ml. Mycoplasmas

were analyzed by SDS-PAGE and immunoblotted with Mab F2G5.

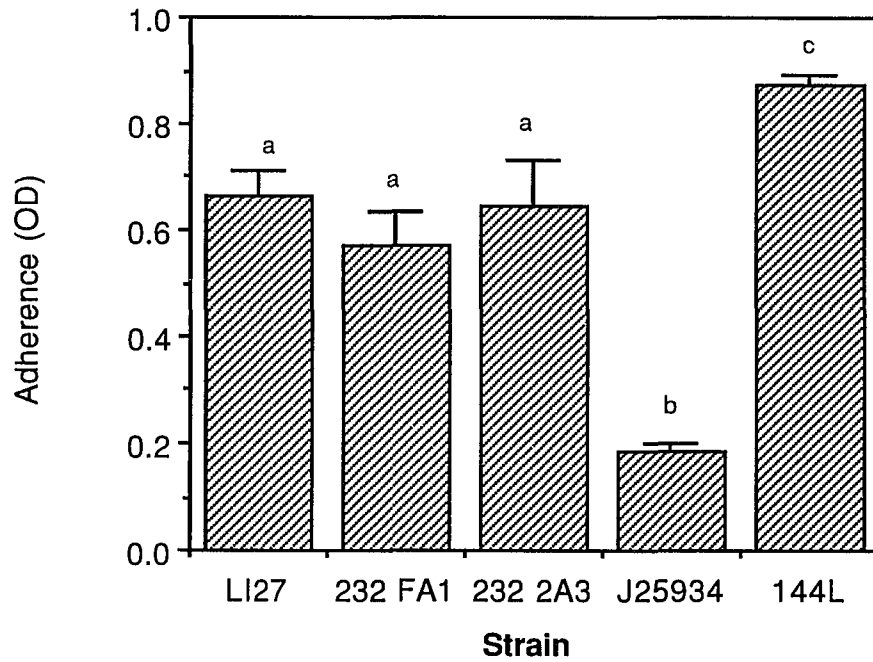Sizes of proteins (in kilodaltons) are shown on the left of each

panel.

Fig. 4.   Adherence activities of various *M. hyopneumoniae* strains.
Mycoplasmas from each strain were adjusted to 25 µg protein/ml and
evaluated for adherence activity in the MPAA. Bars with different
letters were significantly different as determined by analysis of
variance (P<0.01).

of the major differences revealed by SDS-PAGE among the five strains was in the regions detected by Mab F2G5 (Fig. 5). Several bands including P97 and P46 were missing in strains J and I44L. P116 was also absent in strain J. Like P97, P46 was also sensitive to trypsin as show in Fig. 2. Immunoblotting with Mab F2G5 revealed that P97 was missing in strain 144L and strain J, with the concomitant appearance of a 95 KDa protein in strain J and a doublet (93 KDa and 92 KDa) in strain 144L that reacted with Mab F2G5 (Fig. 5). Also, P72 was hardly detectable in strain J and disappeared in strain 144L, but both strains J and 144L had an extra band (approximately 69 KDa) that reacted with the Mab (Fig. 5).

Immunoelectron microscopy. *M. hyopneumoniae* cells were reacted with F2G5 and goat anti-mouse IgM conjugated with gold particles to determine the distribution of the adhesins on mycoplasmas. Mab F2G5 stained the fuzzy surface structures of *M. hyopneumoniae* (Fig. 6). The gold-labeling distributed randomly on the surface of mycoplasmas; it was not polarized at a specific region as demonstrated with the P1 adhesin of *M. pneumoniae*. *Mycoplasma hyopneumoniae* cells reacted with cell culture medium and conjugate were utilized as a negative control and did not display any labeling with the gold particles (Fig. 6).

Affinity chromatography. From 12 mg of starting material, approximately 150 µg of protein were purified by Mab F2G5 affinity chromatography. SDS-PAGE showed that the predominant protein captured by immobilized F2G5 from LI27 was P97 (Fig. 7). Some faint bands were also present below P97. These minor proteins

Fig. 5.   Antigenic heterogeneity among various strains. A, SDS-PAGE protein
profiles stained with Coomassie brilliant blue. B, immunostaining with
Mab F2G5. Lane 1, LI27, Lane 2, J. Lane 3, 232 FA1. Lane 4, 232 2A3.
Lane 5, 144L. Sizes of proteins (in kilodaltons) are shown on the left and
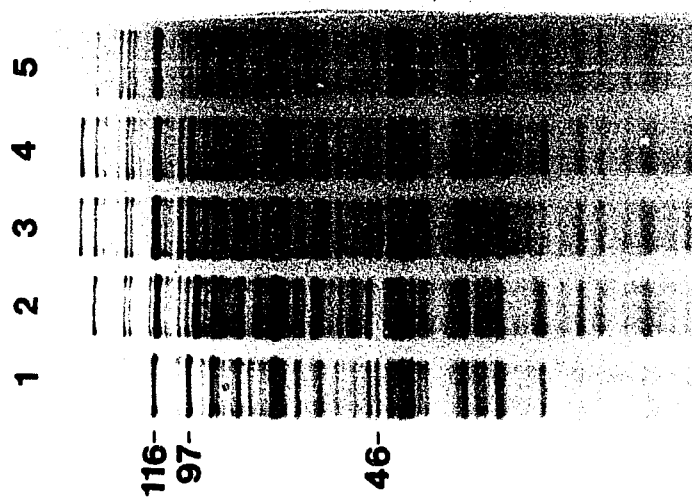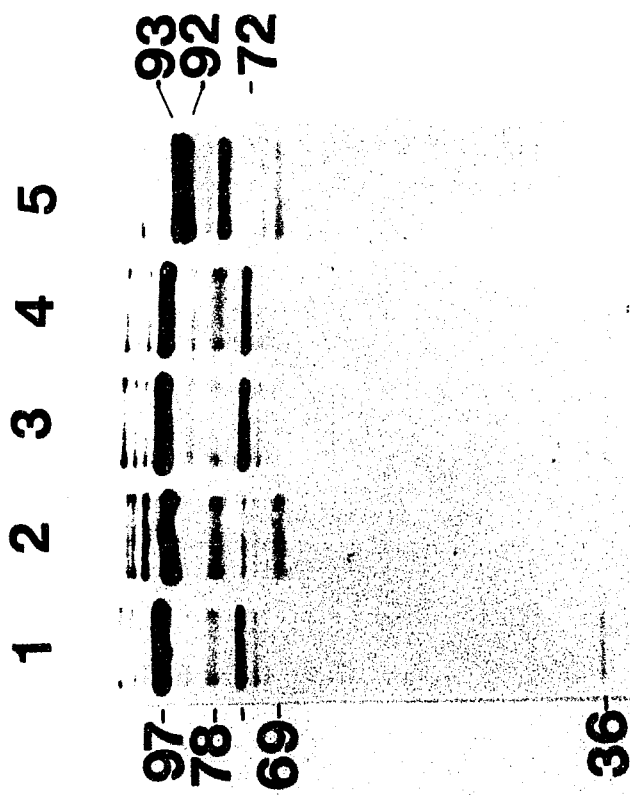right of each panel.

Fig. 6.   Transmission electron micrograph of mycoplamas immunolabelled with
Mab F2G5 and gold particles. A, mycoplasmas incubated with cell culture
medium and gold-conjugates as a negative control. B, mycoplasmas
reacted with Mab F2G5 and gold-conjugates. Arrows indicate the fuzzy
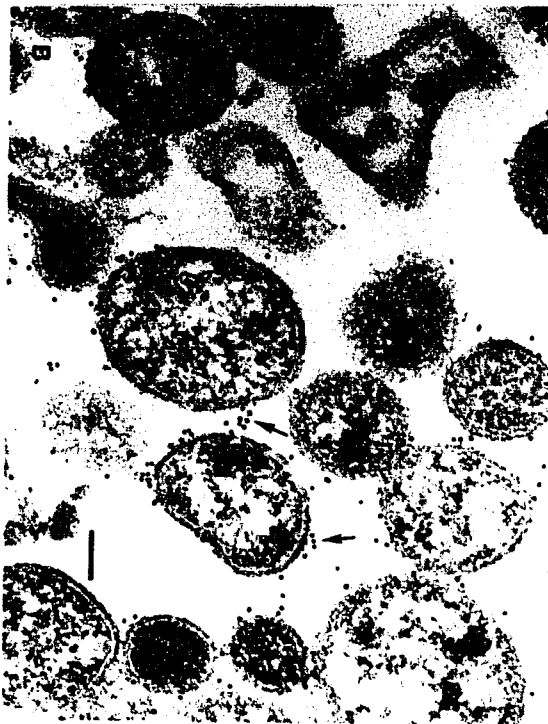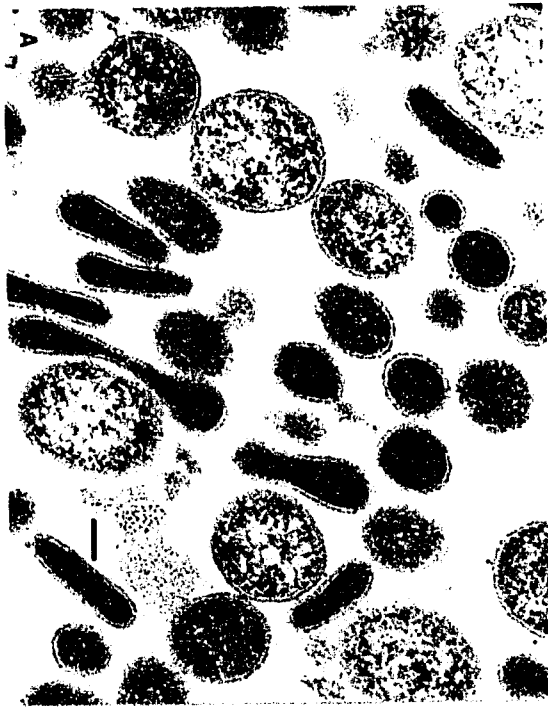structures labelled with gold particles. Bars represent 0.1 µm.

Fig. 7.   Purification of adhesins by antibody (Mab F2G5) affinity

chromatography. Lane 1, total mycoplasmal proteins (LI27); lane 2,

eluate from Affi-Gel 10-Mab F2G5 column (1.5 μg); lane 3, 2 fold

greater load of the eluate. A, SDS-PAGE profile stained with

Coomassie brilliant blue. B, immunostaining with Mab F2G5. Sizes of

proteins (in kilodaltons) are shown on the left and right of each panel.
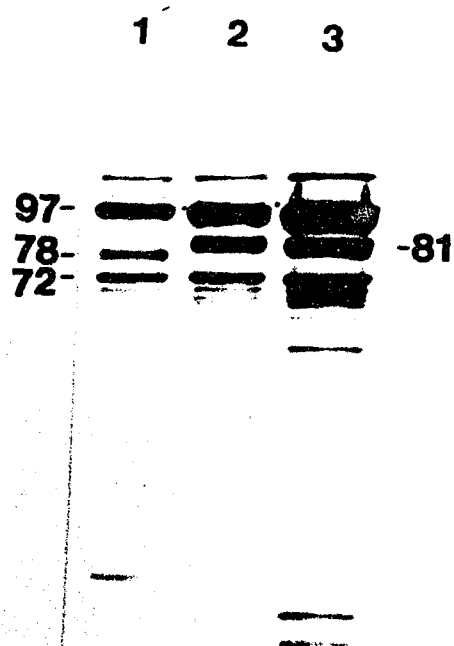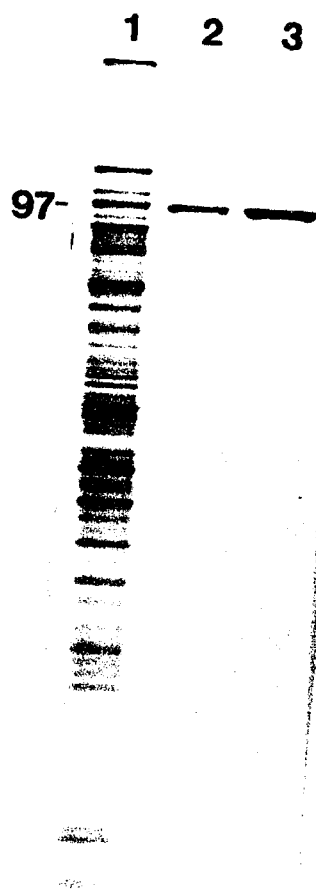
also reacted with Mab F2G5. On immunoblots, the purified proteins reacted

specifically with Mab F2G5 (Fig. 7). A ladder of proteins was detected by F2G5 in

close proximity to P72. These bands had a spacing periodicity of approximately 2

KDa. Interestingly, Mab F2G5 did not detect P78, but did detect a 81 KDa protein in

the purified materials (Fig. 7). The purified proteins adhered strongly to cilium-

coated wells, but not to control wells coated with gelatin or albumin in the microtiter

plate adherence assay (Fig. 8). The purified proteins also produced a dose-

dependent inhibition of the adherence of intact *M. hyopneumoniae* cells to cilia

(Table 5).

Size variation. Since Mab F2G5 reacted with mutiple proteins of *M.*

hyopneumoniae from a single strain, the relationship among these proteins

needed to be determined. One possibility was that these proteins were size

variants. Size variation of epitope-bearing proteins in mycoplasmas has been

illustrated by increasing the population of mycoplasmas loaded for SDS-PAGE or

by treatment of mycoplasmas with proteolytic enzymes (21). In this study, ladders of

bands were demonstrated with Mab F2G5 when a five to ten fold greater load of

LI27 was used for SDS-PAGE (Fig. 9). It seemed that there were different sets of

size variants (brackets in Fig. 9) in a single strain; each one had a predominant

protein. This reactivity of Mab F2G5 to multiple proteins was unchanged when

SDS-PAGE was run under nonreducing conditions. Since size-variable antigens

usually contain repetitive amino acid sequences and display periodic structures

when treated with trypsin, the purified P97 were also digested with trypsin. This

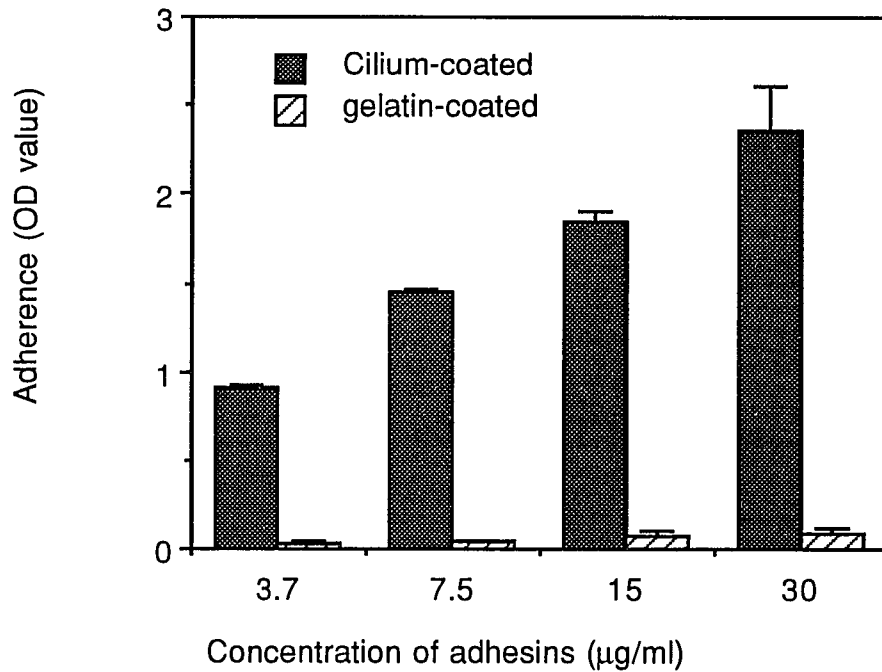Fig. 8.   Dose-dependent binding of purified adhesins to cilia. The adhesins
purified by antibody affinity chromatography were diluted in the
adherence buffer and directly applied to wells of microtiter plates
coated with solubilized cilia or with gelatin (negative control).
Attached proteins were detected with hyperimmune antibodies as
described in the text. Data were expressed as mean OD ± SD of three
independent experiments.

Table 5. Inhibition of *M. hyopneumoniae* adherence by purified adhesins

Mycoplasma	Adherence[a] in the presence of purified adhesins:			
(CCU)	0.0 µg/ml	8 µg/ml	30 µg/ml	60 µg/ml
5×10^8	0.55±0.03	0.47±0.03	0.37±0.03[b]	0.24±0.02[b]
2×10^8	0.40±0.01	0.31±0.02[b]	0.20±0.02[b]	0.11±0.01[b]
8×10^7	0.21±0.01	0.15±0.01[b]	0.12±0.01[b]	0.07±0.01[b]

[a] Detected with a monospecific serum (R409) that did not react with P97 and its size variants. Data were expressed as mean OD ± SD of duplicate experiments.

[b] Significantly different from the noninhibitory controls as determined with analysis of variance ($P < 0.05$).

Fig. 9.   Demonstration of F2G5-reacting size-variable antigens. Mycoplasmal

proteins (LI27) separated by SDS-PAGE were immunostained with

Mab F2G5. The number of mycoplasmas (CCU) in each of the lanes

were approximately 1×10^8 (lane 1), 2.5×10^8 (lane 2), 5×10^8 (lane

3), and 1×10^9 (lane 4). Sizes of proteins (in kilodaltons) are

indicated on the left. The brackets indicate ladders of proteins

recognized by Mab F2G5.
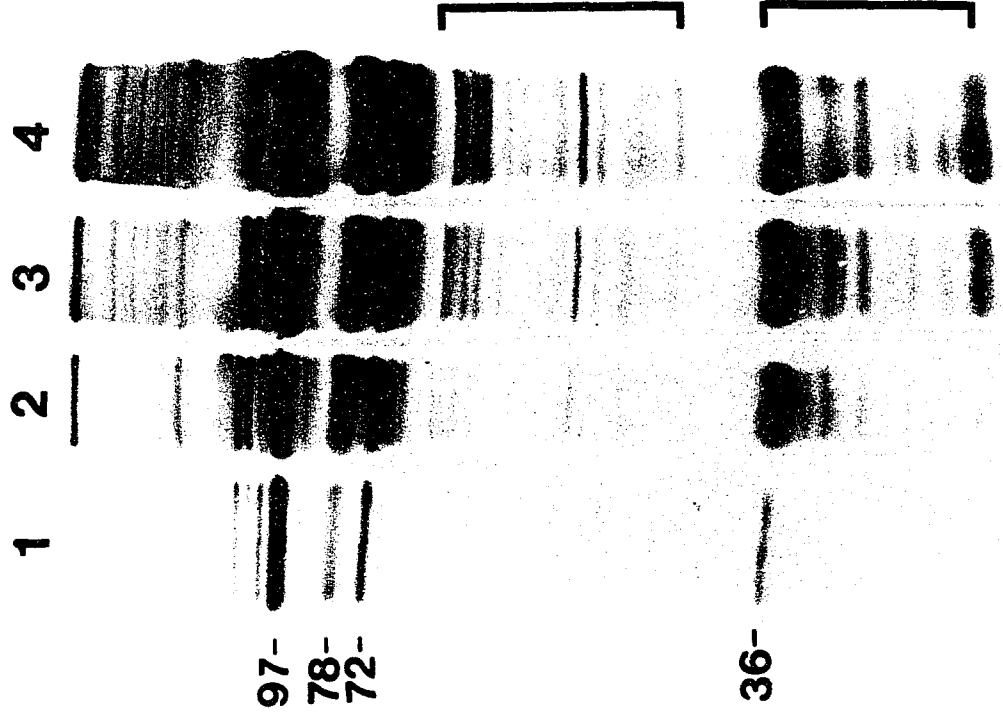
treatment yielded a ladder of peptides that reacted with F2G5 (Fig. 10). The

minimum resolvable fragment of trypsin-digested adhesins was approximately 20.5

KDa. The size difference between the peptides was the multiple of 0.5 KDa.

N-terminal sequence and amino acid composition. Expressed in

single-letter code, the N-terminal sequence of P97 beginning with alanine was

ADXKTDSDKDPSTLRAIDEQ. The X in position 3 indicated an ambiguous residue.

This sequence was compared by GCG with all known protein and DNA sequences

in GenBank, EMBL and Swiss-Prot. No homology was detected between

thissequence and the sequences of known bacterial or mycoplasmal adhesins.

Analysis with Peptidestructure in the GCG sequence analysis software package

predicted that the N-terminus of P97 was hydrophilic and surface-exposed (data

not shown). The total amino acid composition of P97 was also deterrnined,

showing that there was no cysteine in P97 (Table 6).

Discussion

It was found in this study that Mab F2G5, which recognized a predominant

97 KDa protein in several strains of *M. hyopneumoniae*, and its F(ab')$_2$ fragments

inhibited the adherence of *M. hyopneumoniae* to cilia (Table 2); purified P97 and

its size variants adhered to cilia and blocked the adherence of intact *M.*

hyopneumoniae cells (Fig. 8 and Table 5); treatment of mycoplasmas with trypsin

degraded P97 and decreased adherence of *M. hyopneumoniae* (Fig. 3.2 and

Fig. 10.   Proteolysis of purified adhesins. The adhesins purified by antibody

affinity chromatography were digested with graded doses of trypsin

(0.08, 0.2, 0.5, and 2.0 µg/ml) as described in the text. Samples were

pooled, separated by SDS-PAGE, and immunoblotted with Mab

F2G5. Lane 1, mixture of digested adhesins (6 µg); lane 2,

nondigested adhesins (3 µg). Sizes of proteins (in kilodaltons) are

indicated on the right.

1 2

−97

−72
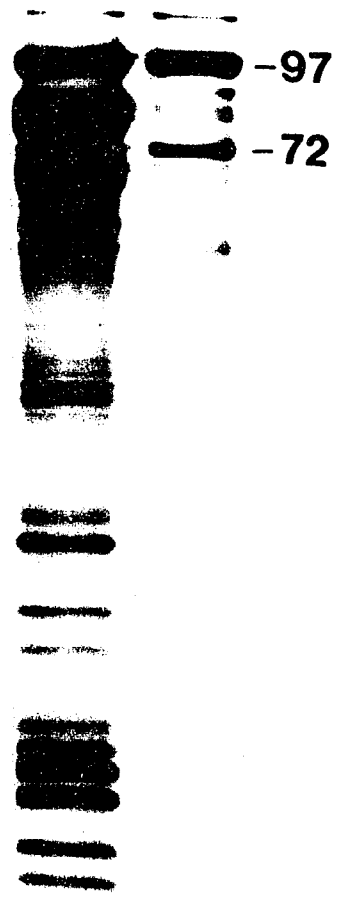
−20.5

Table 6. Amino acid composition of P97

Amino acid	Composition (Mol%)
Asp	17.46
Glu	16.28
Ser	6.18
Gly	5.71
His	0.77
Arg	1.85
Thr	4.54
Ala	8.70
Pro	4.10
Tyr	2.94
Val	3.25
Met	0.31
Ile	4.02
Leu	8.71
Phe	4.29
Lys	10.92

Table 4); and immunoelectron microscopy demonstrated that Mab F2G5-reacting

proteins were located on the surface of *M. hyopneumoniae* (Fig. 6). Based on these

findings, it can be concluded that P97 is a major adhesin of *M. hyopneumoniae*.

Previous studies indicated that P97 was expressed during in vivo infection

and was one of the major immunogens of *M. hyopneumoniae* (37, 38). In fact, P97

elicited a very early immune response in the respiratory tract of swine because IgA

and IgM antibodies to P97 were detected in the air way washings of contact-

exposed pigs 35 to 60 days earlier than to other major immunogens of the

mycoplasma (38). Since P97 was only expressed by virulent *M. hyopneumoniae*

and not by nonvirulent mycoplasmas, it had been proposed that P97 might be one

of the virulence-associated antigens of *M. hyopneumoniae*, although the biological

function of P97 was unknown at that time (37). Results from this study provided new

evidence for the biological function of P97 in *M. hyopneumoniae* infection. These

findings strongly indicate that P97 is an important virulence factor of *M.*

hyopneumoniae.

Sensitivity of P97 to surface proteolysis with trypsin confirmed the

localization of the protein on the surface of the mycoplasma, as demonstrated with

immunoelectron microscopy. Mab F2G5 mainly stained the fuzzy structures on the

mycoplasmas, suggesting that the fuzzy structures may be involved in the

adherence of the mycoplasmas. It was reported previously that the fuzz on *M.*

hyopneumoniae bridges the interaction between mycoplasmas and cilia (3, 25),

and that the fuzzy structures were only observed with a pathogenic strain but not

with a strain that was nonadherent and nonpathogenic in pigs. This evidence,

together with the information generated from this study, strongly suggested that the

fuzzy structures on *M. hyopneumoniae* play an important role in adherence. The

fuzz on the surface of *streptococci*, composed of M proteins and lipoteichoic acid, is

known to mediate the adherence of *streptococci* to eucaryotic cells (6, 7, 27).

It is unlikely that the reactivity of Mab F2G5 to multiple proteins was caused

by the impurity of the Mab. The hybridoma cell line that secretes Mab F2G5 was

subcloned according to standard procedures, and the secreted antibodies were

detected as a homogenous IgM isotype. Also, Mab F1B6 which was produced by

an independent hybridoma had identical antigenic specificity as F2G5. It is also

unlikely that the multiple reactivity was caused by the degradation of P97 during

the process of harvesting or SDS-PAGE, because inclusion of a proteinase

inhibitor (PMSF) during the process did not change the reacting patterns of Mab

F2G5. It is believed that the reactivity of Mab F2G5 to multiple proteins was indeed

due to the presence of the epitope in multiple antigens. In fact, the expression of an

epitope on multiple proteins is very common to many mycoplasmas, including *M.*

hyorhinis (21, 22), *M. pulmonis* (31), *M. hominis* (19), *Ureaplasma urealyticum* (30),

and *M. fermentans* (34).

In this study, we found that Mab F2G5 reacted with ladders of bands on

immunoblots (Fig. 9) and recognized a different predominant protein in different

strains (Fig. 5). Treatment of purified P97 yielded periodic peptides (Fig. 10). These

results suggested that *M. hyopneumoniae* proteins reacted with Mab F2G5

undergo both intraspecies and intrastrain size variation. P97 was a major size variant with adherence activity. Since the population of the mycoplasmas was rather heterogenous, isogenic populations of *M. hyopneumoniae* derived from single clones will be required to thoroughly determine the size variation and its effect on adherence activity of *M. hyopneumoniae*.

The P1 adhesin of *M. pneumoniae* was sensitive to trypsin, while the P30 adhesin of *M. pneumoniae* and the MgPa adhesin of *M. genitalium* were resistant to trypsin (20). Treatment of *M. pneumoniae* with trypsin removed two surface proteins (P1 and P2) and diminished the adherence of *M. pneumoniae* to human respiratory epithelium (14, 15); regeneration of P1 led to the recovery of the adherence activity (14). P97, P105, and P46 were three *M. hyopneumoniae* proteins that were extremely sensitive to surface proteolysis with trypsin (Fig. 2), suggesting that they must be surface-exposed. Besides reactivity with Mab F2G5, P105 also reacted with several other Mabs that did not inhibit the adherence of *M. hyopneumoniae* to cilia (Table 1). P46 was absent in strain J which is a low-adherent and nonpathogenic strain. However, P46 was also missing in strain 144L which is high-adherent and pathogenic in pigs (Fig. 5). A monoclonal antibody to P46 did not inhibit the adherence of *M. hyopneumoniae* (Table 1). The functions of P46 and P105 remain to be defined in future studies. The decrease of adherence produced by trypsin digestion was directly correlated with loss of P97. The increased adherence activity of mycoplasmas, which had been treated with 5 and 25 µg/ml trypsin and had lost P97, can not presently be explained. The

mycoplasmas treated with higher concentrations of trypsin became very sticky and hard to disperse, suggesting that the membrane properties of the mycoplasmas might have been substantially altered. Therefore, the increased adherence might have been due to altered membrane properties rather than to an intrinsic increase in adherence ability. This speculation was supported by the adherence results obtained with solubilized trypsin-treated mycoplasmas, which displayed a linear decrease in adherence to cilia with increased concentration of trypsin.

Degradation of P97 resulted in an increased quantity of P78 (Fig. 2). The region of P78 was not a single protein but contained a set of proteins with only slight difference in size. Increased quantity of P72 was also observed following digestion of P97. Because mycoplasmas were washed after surface proteolysis, P78 and P72 must be membrane-associated. Although higher concentrations of trypsin also resulted in gradual digestion of P78 and P72, they were less sensitive to trypsin than P97. These results suggested that P97, P78 and P72 reacting with mab F2G5 were interrelated in structure. One possibility was that P78 and P72 were the size variants of P97. P97 might have repetitive amino acid sequences as shown with the M protein of *Streptococci* and the variable lipoproteins (Vlps) of *M. hyorhinis* (13, 36). This hypothesis was supported by the production of a ladder of peptides from the digestion of purified P97 (Fig. 10). It is known that the genes encoding for size-variable proteins, such as M proteins and Vlps, usually contain repetitive coding sequences, and that gain or loss of the repeats leads to expression of size-variable proteins (13, 36). A similar mechanism may operate in

P97. Molecular cloning and sequencing of the gene for P97 will be required to confirm this hypothesis. Interestingly, P81 rather than P78 was detected with Mab F2G5 in the eluate obtained from antibody affinity chromatography (Fig 7). The cause for this discrepancy is unknown at this stage.

It is known that different strains of *M. hyopneumoniae* vary in pathogenicity for pigs (42). Strains LI27 and 144L were pathogenic, while strain J was incapable of inducing pneumonia in pigs. Also, no mycoplasmas were isolated from lungs of pigs inoculated with strain J, indicating that this strain had lost the ability to colonize the respiratory epithelium (42). Strains 232 2A3 and 232 FA1 were both pathogenic for pigs (38, 42), although the experimental infection with strain 232 FA1 had a prolonged incubation period and induced only a mild clinical disease (38). It was found in this study that strain J had substantially lower adherence activity than the other strains, whereas strain 144L had the highest adherence activity among the five strains evaluated in this study. As demonstrated in Fig. 5, the predominant proteins stained by Mab F2G5 in strains J and 144L differed in size from those in other strains. It appeared that the size variation of P97 was related to the change in adherence capability. Also, the adherence ability of *M. hyopneumoniae* appeared to be correlated with pathogenicity in pigs. However, this interpretation should be made cautiously because these strains may also vary in virulence traits other than adherence activity. Isogenic strains with only a difference in adherence should be utilized to determine the correlation between adherence and virulence of *M. hyopneumoniae*.

Carboxypeptidase cleaves a protein from the C-terminus (12). Resistance of P97 to digestion with carboxypeptidase Y suggested that the C-terminus of the protein was not accessible to the enzyme, and therefore was not at the external surface of *M. hyopneumoniae*. These results also implied that P97 might be anchored in the membrane by the C-terminus, rather than the N-terminus as shown with the Vlp of *M. hyorhinis* (36). It is unknown if P97 is a lipoprotein. It appeared unlikely because P97 was nonextractable with Triton X-114 (data not shown). And also, there was no cysteine in P97 (Table 6), which usually is the site for acylation of a bacterial or mycoplasmal protein (35, 24). Although a large number of lipid-modified proteins were found in mollicutes (32, 35), *M. hyopneumoniae* only had 4 lipoproteins with molecular weights ranging from 44 to 65 KDa as demonstrated in a previous study (33). Cysteine was also absent in the two *M. pneumoniae* adhesins, P1 and P30 (20). These results suggested P97 differs in some features from the Vlps of *M. hyorhinis*, but is similar to M protein of *streptococci* which is not acylated and is anchored to the cell wall by the C-terminus (13).

Several mycoplasmal adhesins, such as P1 and P30 of *M. pneumoniae*, and P140 of *M. genitalium*, share considerable homology at both the protein and DNA levels (4, 5, 20). However, the N-terminal sequence of P97 did not have any homology with known mycoplasmal adhesins. This result suggested that P97 of *M. hyopneumoniae* might be quite distinct from other mycoplasmal adhesins. However, it is premature to make this conclusion because the N-terminal sequence obtained from this study represented only a small portion of the amino acid

sequence of P97, and the internal sequence was not available for comparison in this study. Analysis of the amino acid composition of P97 indicated that there were no cysteine and tryptophan in P97 (Table 6). However, this does not mean that P97 does not contain tryptophan because this residue is easily destroyed at the acid hydrolysis step required for amino acid analysis (29). Since asparagine and glutamine were deaminated to the corresponding carboxylic acids (Asp and Glu, respectively) as a consequence of the acid hydrolysis step (29), Asp and Glu (Table 6) represented a mixture of asparagine and aspartic acid and a mixture of glutamine and glutamic acid, respectively. It will not be possible to know the relative amount of each in the mixtures until the full amino acid sequence of P97 has been determined. These data will be helpful for cloning and sequencing of the gene for P97 in future studies.

P97 may not represent all of the adhesins of *M. hyopneumoniae* since mab F2G5 did not completely inhibit the adherence, and removal of P97 by trypsin did not abolish the adherence of *M. hyopneumoniae* to cilia. This is not surprising since pathogenic bacteria and mycoplasmas usually have more than one adhesin. For example, *M. pneumoniae* has 2 defined adhesins, P1, P30 (20); a recent study suggested that another surface protein (P90) was also an adhesin of *M. pneumoniae* (8); *Bordetella pertussis* possesses 4 potential adhesins: filamentous hemagglutinin, pertussis toxin, pertactin and fimbriae (25). These adhesins mediate the adherence of *B. pertussis* either to respiratory ciliated cells or to macrophages (25). The interactions between multiple adhesins of an organism and

the receptors on host cells provide for increased adherence specificity and strength. It is expected that more adhesins, other than those recognized by Mab F2G5, of *M. hyopneumoniae* may be discovered in future studies.

Acknowledgments

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GENERAL DISCUSSION

Mycoplasmas are known to be the smallest self-replicating organisms (109). A striking characteristic of these agents is their inability to synthesize typical eubacterial cell walls (110). Although simple in structure, mycoplasmas possess complex traits to evade host immune systems and to produce persistent infections in humans and animals (143). One of the tactics utilized by mycoplasmas is the firm adherence to target cells during infection, which is especially true for those mycoplasmas that colonize the epithelial linings of the respiratory and urogenital tracts (110), such as *M. pneumoniae, M. genitalium, M. gallisepticum*, and *M. hyopneumoniae*. Because of its importance in infection, adherence of mycoplasmas to host cells has recently received widespread interest from mycoplasmologists.

Based on the results obtained from this project, it can be concluded that 1) the adherence of *M. hyopneumoniae* to porcine ciliated cells is mainly mediated by specific receptor-ligand interactions; 2) P97, a size-variable surface protein, is a major adhesin of the mycoplasma; and 3) the major ciliary receptors for the mycoplasma are glycolipids La, Lb, and Lc. In contrast to a previous finding (159), hydrophobic interactions play a minimal role in the microtiter plate adherence assay. Sensitivity of P97 to trypsin is consistent with previous findings that adherence of *M. hyopneumoniae* to cell monolayers and single ciliated cells was diminished by trypsin (157, 160). However, the hemagglutinins of the mycoplasma

were not affected by trypsin (154), indicating that the hemagglutinins are different from the P97 adhesin and may represent a different class of adhesins.

Compared with other known mycoplasmal adhesins, P97 shares similarities with the P1 and P30 adhesins of *M. pneumoniae* and the MgPa adhesin of *M. genitalium* in that they are all immunodominant antigens and are devoid of cysteine (110). However, the three human mycoplasmal adhesins are clustered at tip organelles of the organisms rather than randomly distributed on the surfaces like P97. In addition, the N-terminal sequence of P97 is distinct from those of P1, P30 and MgPa. Since P97 is exposed on the surface of mycoplasmal cells, it must have a signal sequence that leads to the translocation of P97 from cytoplasm to cell membrane. The first N-terminal amino acid of mature P97 is alanine instead of methionine, indicating the existence of a leader (signal sequence) which is cleaved and removed during the maturation and translocation of P97. P1 and MgPa adhesins are known to have signal sequences of 59 amino acids and 58 amino acids, respectively, which are longer than other bacterial signal peptides (110).

Inhibition of adherence by the receptor analogs and identification of three native ciliary glycolipid receptors suggested that the carbohydrate moieties in the receptors determine the specificity for mycoplasmal adhesins. These results were consistent with previous findings that the carbohydrate structures on eucaryotic cells determine the adherence specificities of pathogenic bacteria and mycoplasmas (70). The carbohydrate structures in the three glycolipids (La, Lb, and Lc) are unknown at present. The only clue was that the polysaccharide chains

must be sulfated as confirmed by laminin binding. This finding was correlated with the result obtained in the microtiter plate adherence assay in which the identified receptor analogs were all sulfated compounds. Lc may be a sulfatide, but further analysis is required to confirm this speculation. Since the receptor analogs were still relatively large molecular structures, the smallest structural unit for the receptor epitope (s) was not established. To solve this puzzle, the basic structural units of the receptor analogs may be utilized in future studies to block the adherence. Also, analysis of the carbohydrate chains in the ciliary glycolipid receptors, by use of various modern analytical techniques, will generate valuable information about the essential structures of the receptor moieties.

In vivo studies (4, 99, 140, 156) and an *in vitro* study (160) have indicated that *M. hyopneumoniae* cells are predominantly associated with cilia of swine respiratory tract ciliated cells, suggesting that the receptors for *M. hyopneumoniae* may be polarized at the ciliary membrane. However, results from this project did not support this speculation. It was found that *M. hyopneumoniae* adhered as well to cell bodies of ciliated cells as to cilia in the microtiter plate adherence assay. The adherence of *M. hyopneumoniae* to cell bodies was also inhibited by the receptor analogs that inhibited the adherence of the mycoplasmas to cilia. In addition, the three native glycolipid receptors identified in cilia were also present in the cell bodies (data not shown). These results suggested that the receptors for *M. hyopneumoniae* were present in both cilia and cell bodies of ciliated cells, and that the polarized adherence to cilia might be caused by other factors. For example, the

mode of presentation on cell surfaces and the neighboring molecules may affect the accessibility or specificity of receptors for pathogens (70).

It is known that different strains or passage levels of *M. hyopneumoniae* have varied pathogenicity in pigs (158). This change in pathogenicity seems to be correlated with the adherence of the mycoplasma because 1) the adherence activity of *M. hyopneumoniae* decreases with increase of in vitro passage levels; and 2) strain J, which is nonpathogenic in pigs, had substantially lower adherence activity than other strains evaluated in this study. It is possible that the decreased adherence by in vitro passage is due to the loss or change of P97. The variation of Mab F2G5-reacting proteins caused by in vitro passage remains to be defined in future studies.

The mechanisms utilized by *M. hyopneumoniae* to damage cilia are unknown. A previous study has indicated that direct contact between mycoplasmas and cilia is required for induction of cytotoxicity (28). This implies that the loss of cilia may be caused in one of two possible ways. *M. hyopneumoniae* may have membrane-associated toxins; the adhesin-receptor mediated attachment brings the toxins close to cilia and produces cytotoxicity. This hypothesis needs to be confirmed by attempts to isolate and characterize membrane-associated toxins from *M. hyopneumoniae*. Alternatively, the binding of *M. hyopneumoniae* adhesins to ciliary receptors may trigger a signal transduction cascade across the ciliary membrane, which somehow results in degradation of ciliary structural proteins and loss of cilia. This theory is possible considering the effacing of microvilli caused by

EPEC adherence to intestinal epithelium (9, 30, 97, 112). This hypothesis for adherence-mediated loss of cilia may be verified by use of purified P97 along with the established tracheal ring organ culture model (28). Inoculation of P97 into the model and evaluation of its effect on ciliary structure and function may provide valuable information on the pathogenesis of MPS.

Unlike *M. pneumoniae, M. gallisepticum, and M. genitalium, M. hyopneumoniae* does not have a specialized attachment organelle. However, *M. hyopneumoniae* has an undefined layer of materials outside the limiting membrane (140, 155). Also, fuzzy structures protruding from the surfaces were observed with mycoplasmas grown *in vivo* and *in vitro* (15, 140). The nature of these structures has not been clarified. Since they are on the outermost surface of *M. hyopneumoniae*, it is plausible to speculate that they are involved in the interaction between mycoplasmas and ciliated cells. Identification of P97 on the fuzzy structures of *M. hyopneumoniae* supports this speculation. Isolation and purification of the fuzzy structures will be required to fully understand the chemical structure and the biological function of the fuzz. Such experiments may be attempted by shearing mycoplasmas by physiological means, followed by precipitation of the sheared fuzz with ammonium sulfate.

Because of the economical importance of MPS, developing highly efficient measures for control of the disease has been and still will be the focus of many research laboratories. The finding of receptor analogs and adhesins of *M. hyopneumoniae* provided potential candidates for prevention of MPS. The roles of

mucin, heparin, fucoidan, and chondroitin sulfate in interfering with the infection of

M. hyopneumoniae need to be evaluated in future studies. As a first step, these

receptor analogs may be evaluated in the swine tracheal ring culture model (28) for

blockage of colonization and cytotoxicity of *M. hyopneumoniae*. The full potential of

the receptor analogs in prophylaxis of MPS may only be understood by conducting

in vivo studies.

Adhesins as vaccines have been proven successful for control of bacterial

diseases (2, 68, 103, 119). It is well known that fimbrial vaccines produced from *E.*

coli induce protective immunity to diarrhea in animals and humans (2, 68, 75, 118,

128). However, immunization of guinea pigs with the P1 adhesin of *M. pneumoniae*

failed to provide protection to subsequent challenge with virulent *M. pneumoniae* in

an experimental model (63, 64). One explanation for the failure was that the

receptor-recognizing domain of the P1 adhesin was not immunogenic (110). The

imunogenicity of P97 in pigs remains to be evaluated in future studies. P97 purified

by antibody affinity chromatography may be utilized to immunize pigs and to study

the immune responses to P97. For production of P97 on a large scale, DNA

recombinant technology should be exploited. This goal can be achieved by cloning

and sequencing the gene for P97. Since UGA codon (a stop codon in *E. coli*) is

used for tryptophan in mycoplasmas, an optimized expression system will be

required for production of recombinant P97 in full size. Molecular genetic

characterization of the gene for P97 will not only facilitate the production of P97 for

vaccination, but also improve our understanding of the mechanisms involved in the

size variation of P97, and therefore shed light on the pathogenic mechanisms

utilized by *M. hyopneumoniae* to produce infection in pigs.

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