INFECTION AND IMMUNITY, May 1994, p. 1616–1622
0019-9567/94/$04.00+0

Vol. 62, No. 5

Microtiter Plate Adherence Assay and Receptor Analogs for *Mycoplasma hyopneumoniae*

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Received 13 September 1993/Returned for modification 10 January 1994/Accepted 7 February 1994

A microtiter plate adherence assay for *Mycoplasma hyopneumoniae* was established by use of purified swine tracheal cilia which contained receptors for the mycoplasmas. *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 the binding of the mycoplasmas. The six inhibitors also disrupted the adherence of the mycoplasmas 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 the mycoplasmas, while laminin blocked the receptors in cilia. The basis for the inhibition induced by dextran sulfate was unknown. Treatment of cilia with neuraminidase appeared to promote adherence of the mycoplasmas, whereas treatment of cilia with sodium metaperiodate decreased binding. These results indicate that receptors for *M. hyopneumoniae* in the ciliated epithelium of the respiratory tract of pigs are glycoconjugate in nature.

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 tracheas, 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 and 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 mucociliary system results in inflammation and compromises the defense mechanisms of the porcine respiratory tract, predisposing it 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 erythrocytes (29), cell monolayers (33), and single ciliated cells of the swine respiratory tract (32). Several adherence models have been established on the basis of these findings. However, turkey erythrocytes and cell monolayers may not represent the natural target cells to which *M. hyopneumoniae* adheres. The single ciliated cell adherence assay (SCCAA), which uses natural target cells and mimics the in vivo adherence of *M. hyopneumoniae*, provides a very useful model for the characterization of *M. hyopneumoniae* adherence mechanisms (32). However, the complexity of the procedure and the relatively subjective evaluation of results limit its routine use in the laboratory. Because of the lack of a reliable adherence model, receptors and adhesins involved in the adherence of *M. hyopneumoniae* have not been characterized. In this study, a microtiter plate adherence assay (MPAA) that uses purified cilia which contain receptors for *M. hyopneumoniae* was established. Receptor analogs 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 of MPS and further insight into the development of control measures for MPS.

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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 immunoglobulin G was purchased from Cappel-Organon Teknika Corp. (Durham, N.C.). Peroxidase substrate [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)] was obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Md.). Fluorescein isothiocyanate (FITC)-conjugated antibodies against *M. hyopneumoniae* were supplied by B. Erickson, Iowa State University, Ames. Various sugars and glycoconjugates were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise indicated.

Mycoplasmas. *M. hyopneumoniae* 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 × *g* for 15 min. Color-changing units (CCU), representing the number of mycoplasmas, were determined by serial dilution with 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 of protein per ml.

Preparation of cilia. Specific-pathogen-free pigs free of *M. hyopneumoniae* and 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 previously (32). In brief, 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 the tracheas, and then the tracheas were washed three times by immersion in RPMI 1640 medium before they were cut into small fragments. Ciliated cells were collected by scraping the inner surface of the tracheas with a sterilized stainless steel laboratory spoon. Cilia were extracted from ciliated cells by the methods of Tuomanen et al. (28). In brief, ciliated cells were suspended in 40 ml of buffer containing 20 mM Tris, 10 mM EDTA, and 125 mM sucrose (TES buffer [pH 7.2]). Two washings with TES buffer were conducted by centrifugation at 300 × *g* for 5 min. The cell pellet was resuspended in 6 ml of AES buffer (80 mM acetate, 10 mM EDTA, 125 mM sucrose [pH 6.8]) 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 of TES buffer, and then centrifuged at 500 × *g* for 10 min. The sediment, which contained mainly cell bodies, was saved, and the supernatant, containing cilia, was harvested by centrifugation at 18,000 × *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. The purity of the cilia was ascertained by light microscopy. Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, Ill.) according to the instructions provided with the product. Usually, 6 to 8 mg of ciliary protein was obtained from a trachea collected from a pig weighing 100 to 150 lb (ca. 45 to 68 kg).

Coating of plates. Purified cilia (2 mg/ml in PBS) were solubilized with sodium dodecyl sulfate (SDS; 1 mg/mg of 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 of protein per ml. To each well of flat-bottom microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, Va.) was added 100 μl of this 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 1 year without a loss of adherence ability. Porcine albumin (fatty acid free; 10 μg/ml) and gelatin (10 μg/ml) were used to coat plates as negative controls under the conditions used for cilia. After deciliation, cell bodies of ciliated cells were also solubilized and immobilized on microtiter plates (10 μg of protein per ml).

MPAA. After four washings with PBS (pH 7.4), the cilium-coated plates were blocked with 200 μl of AB per well for 2 h at 37°C. Then, 100 μl of *M. hyopneumoniae* cells resuspended in AB was added to each well, and the plates were incubated at 37°C for 90 min. Nonadherent 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 four times with PBS and then incubated with 100 μl of goat anti-rabbit peroxidase conjugate for 60 min at 37°C. After four washings with PBS, 100 μl of ABTS was added to each well and incubated at 25°C for 10 min. Optical densities (OD) 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 (see Table 1) diluted to appropriate concentrations in AB were mixed with mycoplasmas and added to cilium-coated plates. After incubation and washings, the binding of mycoplasmas to the plates was detected with rabbit anti-*M. hyopneumoniae* antibodies and goat anti-rabbit conjugates as described above. To optimize the sensitivity to interference, 2.5×10^8 CCU of mycoplasmas was used in each well so that the OD of the noninhibitory controls was about 0.5, a value which was in the linear range of the adherence curve (see Fig. 1). Two approaches were used to study competitive inhibition. (i) Competitors were preincubated with mycoplasmas at 37°C for 1 h, unbound competitors were removed by differential centrifugation (25,000 × *g* for 15 min), and incubated mycoplasmas were applied to cilium-coated plates. (ii) Cilium-coated plates were preincubated with competitors at 37°C for 1 h, unbound competitors were removed by four washings, and mycoplasmas were added to the plates. Thereafter, procedures for both approaches were identical, including the sequential addition of antibodies to mycoplasmas, secondary antibody conjugates, and peroxidase substrates. The percent inhibition was calculated as follows: percent inhibition = (OD from AB − OD from an inhibitor)/OD value from AB. The IC_{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.

SCCAA. Substances determined to have inhibitory activity with the MPAA were further evaluated with the SCCAA (32). In brief, single ciliated cells were brushed off the epithelial surface of a trachea from a specific-pathogen-free pig and resuspended in RPMI 1640 medium. Cells were washed, counted, and resuspended in the medium to 10^5 cells per ml. One milliliter of ciliated cells was mixed with 25 μl of mycoplasmas (10^{10} CCU/ml), and 25 μl of an inhibitor was diluted to the appropriate concentration (see Table 4). After incubation in a water bath for 90 min, free mycoplasmas were removed by centrifugation (500 × *g* for 10 min). Cell pellets with associated mycoplasmas were washed twice with PBS and pipetted onto 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 fluorescence score (an estimation of the number of mycoplasmas bound to a ciliated cell) for each sample was calculated as reported previously (32).

Treatment of cilia with neuraminidase and sodium metaperiodate. Purified cilia were incubated with 10 or 100 mM sodium metaperiodate in 10 mM sodium acetate (pH 5.0) at 37°C for 30 min, washed three times with PBS, solubilized with SDS, and used to coat microtiter plates according to methods described above. Cilia treated with 10 mM sodium acetate only were used as controls. The adherence of *M. hyopneumoniae* to treated cilia was evaluated by standard procedures described above. For treatment with neuraminidase, purified cilia were incubated with neuraminidase (1 U/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 used to coat microtiter plates. Adherence was evaluated by standard procedures described above.

Statistics. An analysis of variance with the Tukey contrast at a significance level of 0.05 was used for comparison of multiple treatments in an assay. The Student *t* test was used when only two treatments were evaluated.

RESULTS

Development of the MPAA. *M. hyopneumoniae* bound specifically to cilium-coated wells but not to control wells coated with gelatin or porcine albumin (Fig. 1). ODs obtained with the control wells were always less than 0.08. The degree of binding

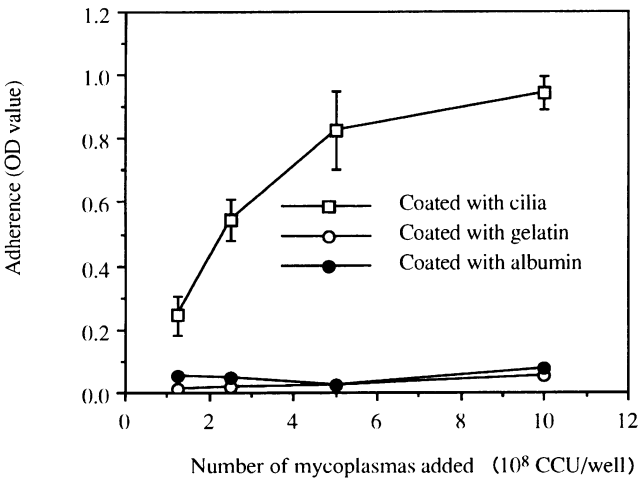

FIG. 1. Dose-dependent binding of mycoplasmas to immobilized cilia in the MPAA. Data represent the mean OD ± standard deviation in three independent experiments. Mycoplasmas bound to cilium-coated wells but not to control wells.

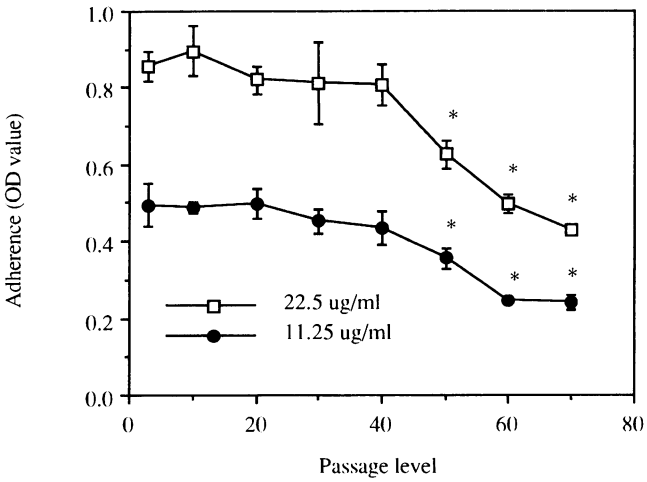

FIG. 2. Effect of passage level on adherence. Mycoplasmal proteins from different passages were adjusted to 11.25 and 22.5 μg/ml. Adherence was tested with cilium-coated plates. Data represent the mean OD ± standard deviation in three experiments. Asterisks indicate the passages at which a significant reduction in adherence was observed, in comparison with passage 3 ($P < 0.01$).

was influenced by both the number of mycoplasmas and the concentration of cilia used to coat plates. The optimum concentration of cilia for coating plates was 1 μg of protein per well. About 2×10^8 CCU of mycoplasmas was required for half-maximal binding at 37°C. Saturated binding was observed with more than 10^9 CCU of mycoplasmas per well. Compared with that at 37°C, the binding activity of *M. hyopneumoniae* was about three times lower at 25°C and minimal at 4°C. Similar adherence patterns were observed with solubilized mycoplasmas at different temperatures (37, 25, and 4°C). 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 differences were negligible (<5%). Mycoplasmas resuspended in PBS or RPMI 1640 medium adhered equally well to cilium-coated plates (data not shown), although the latter were used throughout this study. Mycoplasmas solubilized with SDS or by sonication also had the ability to adhere. In fact, solubilized mycoplasmas showed 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 increasing passage level, *M. hyopneumoniae* gradually showed decreased adherence (Fig. 2), with a significant decrease starting at passage 50 ($P < 0.01$). Solubilized mycoplasmas displayed a similar trend of decreased adherence with increasing passage level (data not shown). Mycoplasmas bound equally well to cell body-coated wells and to cilium-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 mycoplasmas in the presence of various sugars and glycoproteins. To facilitate screening, competitors and mycoplasmas were added at the same time to cilium-coated plates. Among the carbohydrates and glycoconjugates evaluated in this experiment, dextran sulfate, heparin, fucoidin, chondroitin sulfate, mucin, and laminin inhibited the adherence of *M. hyopneumoniae* (Table 1). Three different molecular weights of dextran sulfate produced similar inhibition kinetics and inhibited adherence by as much as 90%, but dextran was not an efficient competitor. Other sulfated compounds, including D-galactose 6-sulfate, D-glucose 6-sulfate, *N*-acetylglucosamine 3-sulfate, and *N*-acetylglucosamine 6-sulfate, had no effect on the 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 levels of 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 with chondroitin sulfate A, B, or C. Chondroitin sulfate B and chondroitin sulfate A had IC_{50}s of 20 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 activities than fetuin and mucin (Table 2).

Inhibitory mechanisms. It was speculated that the positive results obtained in the adherence assay with various carbohydrates and glycoconjugates could have resulted either from interference with adhesins of mycoplasmas or from 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 mycoplasmas; laminin (6%) and dextran sulfate (16%) resulted in limited inhibition (Table 3). When the six competitors were preincubated with cilia, laminin produced 75% inhibition, whereas fucoidan produced 27%, a level much lower than that resulting from preincubation with mycoplasmas; the other four competitors had no inhibitory effect. The six competitors also significantly reduced the attachment of *M. hyopneumoniae* to intact ciliated cells in the SCCAA (Table 4). The effect of the six competitors on the

TABLE 1. Carbohydrates and glycoconjugates evaluated for the inhibition of adherence

Carbohydrate or glycoconjugate	IC_{50}[a]
Asialofetuin	p
Asialomucin (bovine submaxillary glands)	p
Chondroitin sulfate A	80
Chondroitin sulfate B	20
Chondroitin sulfate 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)[b]	<1
Dextran sulfate (MW, 8,000)	5
Dextran sulfate (MW, 500,000)	<1
Diacetylchitobiose	—
Fetuin	p
Fucoidan	<1
Galactopyranosyl-galactopyranose	—
Heparin	<1 U/ml
Hyaluronic acid	—
L-Fucose	—
Lactose	—
Laminin	50
Lactoferrin	—
Melibiose	—
Mucin (bovine submaxillary glands)	p
Mucin (porcine intestine)	40
N-Acetylgalactosamine	—
N-Acetylglucosamine	—
N-Acetylneuraminlactose	—
N-Acetylmannosamine	—
N-Acetyllactosamine	—
N-Acetylglucosamine 3-sulfate	—
N-Acetylglucosamine 6-sulfate	—
Sialic acid	—
$MgSO_4$	—
Methionine	—
Tetramethyl urea	—

[a] Given in micrograms per milliliter unless indicated otherwise. p, partial inhibition was observed, but it was not more than 50% at the maximum concentration (2 mg/ml) tested. —, no inhibition was detected at concentrations of up to 2 mg/ml.
[b] MW, molecular weight.

detection of *M. hyopneumoniae* with antibodies was evaluated by an enzyme-linked immunosorbent assay. 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 ODs between the wells with the competitors and the control wells without the competitors, indicating that these substances had an impact on the mycoplasma-cilium interaction rather than on the detection process.

TABLE 2. Effect of sialylation on adherence

Concn (μg/ml)	% Inhibition[a] obtained with:			
	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 ± standard deviation in three experiments. ND, not determined.
[b] $P < 0.01$ versus fetuin, as determined by the Student t test.
[c] $P < 0.05$ versus mucin, as determined by the Student t test.

TABLE 3. Inhibitory mechanisms of different competitors

Inhibitor[a]	% Inhibition[b] produced by preincubation with:	
	Cilia	Mycoplasmas
Laminin (200)	74.8 ± 0.6	6.2 ± 5.8
Mucin (200)	−6.6 ± 6.6	78.3 ± 3.1
Dextran sulfate[c] (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 U/ml)	−2.2 ± 6.2	93.4 ± 11.5

[a] Concentrations are given in micrograms per milliliter unless indicated otherwise.
[b] Relative to that in AB and reported as mean ± standard deviation in triplicate assays.
[c] Molecular weight, 500,000.

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

DISCUSSION

The results of this study indicated that the MPAA is specific and reproducible. Compared with other assays, this assay is more objective and sensitive. The MPAA provides a convenient method for the 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 by the MPAA, resulting in the identification of receptor analogs. Furthermore, the nature of receptors may also be determined with this assay.

Data from this study demonstrated that the adherence of *M. hyopneumoniae* is temperature and dose dependent. Lysed mycoplasmas had higher adherence activity than intact mycoplasmas, indicating that the viability of *M. hyopneumoniae* is not required for successful binding and that solubilization of mycoplasmal membranes may expose more binding sites.

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

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

[a] Adherence was tested with the SCCAA in the presence of the inhibitors.
[b] See Table 3, footnote *a*.
[c] Mean ± standard deviation in duplicate experiments.
[d] See Table 3, footnote *c*.
[e] Significantly different from the RPMI 1640 medium value, as determined by an analysis of variance with the Tukey contrast ($P < 0.05$).

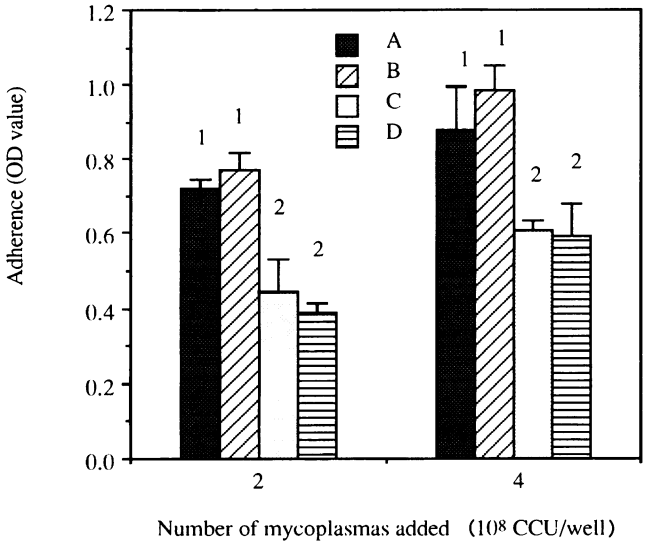

FIG. 3. Effect of treatment of cilia with neuraminidase and sodium metaperiodate on adherence. Cilia were treated with sodium acetate buffer (A), neuraminidase (B), 0.01 M sodium metaperiodate (C), or 0.1 M sodium metaperiodate (D). Bars represent the mean OD ± standard deviation in triplicate assays.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

Similar findings were reported for *Mycoplasma gallisepticum* (12). Unlike *Mycoplasma pneumoniae*, which did not adhere well in nutrient-deficient medium (14), *M. hyopneumoniae* did not require glucose for binding because mycoplasmas resuspended in RPMI 1640 medium and PBS bound equally well. This observation was consistent with the previous finding that metabolically active *M. hyopneumoniae* was not required for binding in the SCCAA (32). Heating (56°C for 30 min) mycoplasmas before adherence testing remarkably decreased binding, indicating that an adhesin(s) of *M. hyopneumoniae* was sensitive to heat. This sensitivity to heat could have resulted from conformational changes in the binding sites for the mycoplasmal adhesins rather than from an impact on metabolic activity because *M. hyopneumoniae* need not be metabolically active for successful binding. Reduced adherence at low temperatures (4 and 25°C) could have been caused by a 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 (31). Attenuation of virulence through successive in vitro passages was also obtained for other mycoplasmas (4, 27). In this study, the capability of *M. hyopneumoniae* to adhere to SDS-solubilized cilia was gradually decreased by passage in Friis medium (Fig. 2). This change in adherence appeared to parallel the decrease in pathogenicity of the mycoplasmas (31). We speculated that selective variations in mycoplasmal adhesins might have occurred with the increase in passage level, therefore leading to a reduced capability for 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 analogs. They are all sulfated glycoconjugates, indicating 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*-acetylglucosamine 3-sulfate, *N*-acetylglucosamine 6-sulfate, and SO_4^{2-}, had no effect on attachment. The inhibitors are all polyanionic molecules. The only structural difference between chondroitin sulfates A and C is the position of sulfate (24). Chondroitin sulfate A inhibited the adherence of the mycoplasmas, whereas chondroitin sulfate C did not. This finding indicates that the inhibition is probably not due to an ionic interaction, since chondroitin sulfate A and chondroitin sulfate C have similar charge properties. Chondroitin sulfate B, which contains iduronic acid instead of the glucuronic acid found in chondroitin sulfate A (24), caused significantly higher levels of inhibition than chondroitin sulfate A. This result suggests that the binding affinity of *M. hyopneumoniae* for cilia is greatly affected by the nature of the carbohydrates and the positions of sulfate on the 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; attachment is an essential process for the establishment of colonization and the production of toxic effects. In this study, the inhibition of adherence by several carbohydrates and the decreased binding caused by the 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 for rat (26) and dog and human (25) respiratory epithelium and for bronchial goblet cells of pigs (6). These data indicate that sulfated carbohydrates are available in vivo for the attachment of pathogens that colonize respiratory epithelium.

Many types of eucaryotic cells contain membrane-associated heparin or heparin-like polysaccharides which facilitate cell-cell interactions and cell-extracellular matrix interactions (5, 21). It is not known whether ciliated cells contain surface-exposed heparin or heparin-like polysaccharides. *M. hyopneumoniae* may utilize such a 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 have been 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 high molecular weight, did not interact with molecules on mycoplasmas. It has been 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 the adherence of *M. hyopneumoniae*. Sialic acid has been reported to be a receptor site for bacteria (11), viruses (16), and mycoplasmas (10, 18). The results of this study demonstrated that sialic acid is not involved in the adherence of *M. hyopneumoniae*. In fact, the removal of sialic acid from cilia appeared to promote the adherence of *M. hyopneumoniae* (Fig. 3). This trend was correlated with increased inhibition by asialomucin and asialofetuin (Table 2). Sialic acid is usually at the terminal position in oligosaccharide chains. Its removal may either change the charge properties on cilia or uncover more receptor epitopes for mycoplasmas. It is not known whether *M. hyopneumoniae* produces neuraminidase during infection.

The secretion of large amounts of mucus during MPS seems

to be a host defense mechanism for trapping *M. hyopneumoniae*. However, this mycoplasma causes extensive damage to cilia (3, 17, 30) by unknown means, compromising the major clearance system in the swine respiratory tract. Thus, adherence to mucin may promote *M. hyopneumoniae* infection, since secretions cannot be efficiently excluded from the respiratory tract because of a malfunction of the mucus transportation system. DeBey et al. (6) quantitated bronchial goblet cell secretions of pigs by image analysis of sections stained with diamine-Alcian blue containing a high iron concentration. 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 the production of certain mucosubstances in response to infection with the mycoplasma.

The adherence of *M. hyopneumoniae* to swine ciliated cells is a complicated process. To reach ciliated cells, the mycoplasma must overcome the sweeping force and penetrate the mucus gel which covers the surface of the epithelium. Receptor-ligand interactions as well as hydrophobic interactions may contribute to this process (32). It was speculated (32) that hydrophobic interactions initiated the first phase of adherence (nonspecific and low affinity) and that the adhesin-receptor interactions mediated the second phase (specific and high affinity). Tetramethyl urea, which disrupts hydrophobic interactions, partially inhibited adherence in the SCCAA (32), but it did not inhibit adherence in the MPAA (Table 1). This discrepancy might suggest that in the MPAA, in which the ciliary membranes were solubilized and the receptor(s) was directly exposed to mycoplasmas, hydrophobic interactions were no longer necessary for adherence. Under the conditions utilized with the MPAA, adherence was mainly adhesin-ligand mediated, since (i) *M. hyopneumoniae* did not bind significantly to microtiter plate wells without coated cilia (Fig. 1), (ii) receptor analogs inhibited adherence by as much as 90%, and (iii) tetramethyl urea did not interfere with adherence (Table 1).

M. hyopneumoniae also attached to cell bodies of ciliated cells in the MPAA, indicating the 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 in the SCCAA, in which this mycoplasma bound predominantly to the ciliary tuft and only occasionally to cell bodies (32). This contradiction may be explained by the varied accessibility of receptors in different locations of ciliated cells. These findings suggest that host receptors that interact with the adhesins of a pathogen are not the sole factor required for tissue tropism.

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

ACKNOWLEDGMENTS

This research was supported in part by SmithKline Beecham Animal Health, Inc., USDA grant 91-37204-6488, and the Iowa Livestock Health Advisory Council.

We thank Barbara Erickson for editorial assistance and Nancy Upchurch for technical assistance.

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