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Glycolipid Receptors for Attachment of *Mycoplasma hyopneumoniae* to Porcine Respiratory Ciliated Cells

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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 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 was detected. Total lipids extracted from cilia of the swine respiratory epithelium, the natural targets of *M. hyopneumoniae* infection, were also 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 believed to be sulfated glycolipids, as determined by laminin binding and staining with azure A. Lc was identified as a putative sulfatide because it had a mobility similar to that of authentic sulfatide and comigrated with sulfatide on TLC plates. Laminin bound to La, Lb, and Lc and produced dose-dependent inhibition of adherence of the mycoplasma to the three ciliary receptors. Binding of the mycoplasma to sulfatide, La, Lb, and Lc was partially inhibited by dextran sulfate, heparin, fucoidan, mucin, and chondroitin sulfate B. These substances blocked the adherence of *M. hyopneumoniae* to cilia and ciliated cells as shown in a previous study (Q. Zhang, T. F. Young, and R. F. Ross, Infect. Immun. 62:1616–1622, 1994). These results indicate that La, Lb, and Lc are the major native receptors for *M. hyopneumoniae* adherence to ciliated cells.

Mycoplasmal pneumonia of swine, caused by *Mycoplasma hyopneumoniae*, is a worldwide, economically important swine disease (28). Lack of knowledge about the pathogenic mechanisms and virulence factors involved in *M. hyopneumoniae* infection is a limiting factor in the development of highly effective vaccines for control of mycoplasmal pneumonia of swine. *M. hyopneumoniae* colonizes the surface of ciliated cells in the tracheas, bronchi, and bronchioles of pigs and does not invade epithelial cells (1, 28). Intimate attachment of this organism to cilia during infection has been well documented (2, 23, 32). This association leads to progressive loss of cilia, desquamation of the epithelium, and development of pneumonia (28). Both in vitro and in vivo studies have shown that *M. hyopneumoniae* adheres only to cilia of swine respiratory ciliated cells but not to nonciliated cells of the porcine respiratory tract (2, 23, 32, 34). These data indicate that cilia of the porcine respiratory epithelium may have receptors for *M. hyopneumoniae.*

Mucosal surfaces are the ports of entry and major sites of many infectious agents. Many pathogens, including viruses (6, 22), bacteria (29), and bacterial toxins (8), bind to specific carbohydrate moieties on the mucosal surfaces, enabling colonization and infection and potentially mediating a toxic effect on the host cells. These carbohydrate moieties may be present in either glycoproteins or glycolipids. Although several in vitro adherence models for *M. hyopneumoniae* have been established (31, 33–35), the adhesin(s) of the mycoplasma and its host receptors have not been identified. In a previous study (33), several carbohydrates and glycoconjugates, including dextran sulfate, heparin, fucoidan, chondroitin sulfate, mucin, and laminin, inhibited adherence of this mycoplasma to porcine respiratory tract 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*. However, the nature of the glycoconjugates has not been defined. In this study, the native glycolipid receptors for *M. hyopneumoniae* were identified by using a thin-layer chromatography (TLC) overlay assay.

MATERIALS AND METHODS

Chemicals and reagents. Neutral glycolipids (cerebroside, sphingomyelin, lactosyl ceramide, ceramide trihexoside, and globoside), sulfatide, gangliosides (GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, and asialo-GM1) and nonpolar 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 obtained 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 (24). Peroxidase-conjugated goat anti-rabbit immunoglobulin G was purchased from Cappel (Durham, N.C.). Peroxidase substrates, ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] and 4-chloro-1-naphthol, were obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Md.).

Mycoplasmas. *M. hyopneumoniae* 232 LI27 was cultured in Friis mycoplasmal medium (10) for 24 h at 37°C. Color-changing units, 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 × *g* for 15 min. The pellet of mycoplasmas was

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resuspended to 1/10 of the original volume in RPMI 1640 medium containing 1% gelatin (adherence buffer). This preparation was further diluted in the adherence buffer to an appropriate number for 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 previously (34), and the cilia were extracted by the methods of Tuomanen et al. (30). Briefly, ciliated cells were harvested from the trachea and suspended in 40 ml of TES buffer (20 mM Tris, 10 mM EDTA, 125 mM sucrose, pH 7.2). The cells were washed twice with TES by centrifugation (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 mixed with 6 ml of 20 mM $CaCl_2$. This mixture was vortexed for 10 min at 25°C, diluted with TES to a volume of 40 ml, and then centrifuged at 500 × *g* for 5 min. The supernate containing extracted cilia was sedimented by centrifugation at 18,000 × *g* for 20 min. Cilia were washed twice with phosphate-buffered saline (PBS) before the extraction of lipids. Extraction of total lipids from cilia was performed as reported by Magnani et al. (21). Briefly, 0.5 g (wet weight) of cilia was homogenized in a mixture of 1.5 ml of water, 5.4 ml of methanol, and 2.7 ml of chloroform. The homogenate was centrifuged at 5,000 × *g* for 20 min. The supernate was collected, and the pellet was extracted twice more with 0.5 ml of water–2.7 ml of methanol–1.35 ml of chloroform. The supernates from three extractions were pooled and evaporated. The residue was dissolved in chloroform-methanol (2:1) and extracted two or three times, until nonsoluble substances (proteins) were totally removed. The total lipid extract was resuspended in 0.5 ml of 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 a mixture of 6 parts chloroform-methanol (2:1) and 1 part water (9). The upper phase was collected, and the lower phase was partitioned five more times with 6 parts chloroform-methanol (2:1) and 1 part water. The upper phases from each partition were pooled, evaporated, dissolved in chloroform-methanol (2:1) and stored at −20°C. In order to observe the distribution of sulfatide in the partition system, authentic sulfatide was also partitioned under conditions similar to those described above.

TLC overlay assay. The TLC overlay assay was performed as described previously (18), with some modifications. Various lipid standards and total lipid extract of cilia were applied to polyester-backed silica gel TLC plates (Sigma Chemical Company). 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 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 dried in a hood. The coated plates were immersed in the adherence buffer at 37°C for 2 h. Then, the plates were overlaid with a suspension of *M. hyopneumoniae* (5×10^8 color-changing units per ml) and incubated at 37°C for 5 h in a humid atmosphere. Unbound mycoplasmas were removed by three washings in PBS, each time for 3 min 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 immunoglobulin G 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.

TABLE 1. Binding of *M. hyopneumoniae* to various lipids and glycolipids on TLC plates

Lipid	Structure	Binding[a]
Sphingolipids		
Sphingomyelin		−
Cerebroside	Gal-β1-1Cer	−
Sulfatide	Gal($3SO_4$)β1-1Cer	++
Lactosyl ceramide	Galβ1-4Glc1-1Cer	−
Ceramide trihexoside	Galα1-4Galβ1-4Glc1-1Cer	−
Globoside	GalNAcβ1-3Galα1-4Galβ1-4Glc1-1Cer	+
Gangliosides		
GM1	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer	−
GM2	GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer	−
GM3	NeuAcα2-3Galβ1-4Glcβ1-1Cer	++
GD1a	NeuAcα2-3 Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer	−
GD1b	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1Cer	−
GD3	NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer	−
GT1b	NeuAcα2-3 Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1Cer	−
Asialo-GM1	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer	−
Nonpolar 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.

For identification of sulfated glycolipids in the ciliary lipid extract, TLC plates with separated lipids were stained with azure A as described previously (14) or overlaid with laminin.

Binding of laminin to ciliary lipids. Binding of laminin to the ciliary glycolipids was performed as described previously (27). In brief, TLC plates with various lipids were developed, coated, and blocked as described for the TLC overlay assay. The plates were overlaid with laminin (10 μg/ml) at 25°C for 2 h. The bound laminin was detected by immunostaining with rabbit anti-laminin antibodies and goat anti-rabbit immunoglobulin G conjugated with peroxidase, followed by color development with 4-chloro-1-naphthol. Laminin was also evaluated for inhibition of *M. hyopneumoniae* binding to ciliary glycolipid receptors. For this purpose, separated glycolipids on TLC plates were first incubated with laminin (1, 10, or 100 μg/ml) for 2 h at 25°C. After three washings with PBS, the plates were further incubated with mycoplasmas, and bound mycoplasmas were detected as described above.

Microtiter plate adherence assay. The microtiter plate adherence assay was performed as previously reported (33). 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/ml and 100 μl per well). Untreated cilia and gelatin were utilized as positive and negative controls, respectively. Mycoplasmas, rabbit antibodies to *M. hyopneumoniae*,

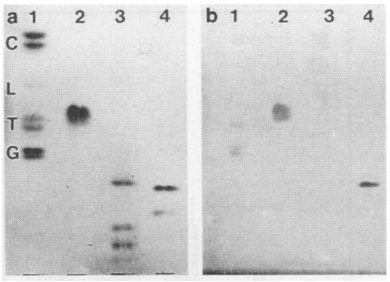

FIG. 1. Binding of *M. hyopneumoniae* to standard glycolipids on TLC plates. Panel a was sprayed with Bial's reagent; panel b was overlaid with *M. hyopneumoniae*. Lanes 1, neutral glycosphingolipids: cerebroside (C; 3 μg), lactosyl ceramide (L; 3 μg), ceramide trihexoside (T; 3 μg), and globoside (G; 3 μg). Lanes 2, sulfatide (2.5 μg). Lanes 3, gangliosides in order of their migration (top to bottom): asialo-GM1 (3 μg), GM1 (3 μg), GD1a (3 μg), and GD1b (3 μg). Lanes 4, gangliosides (top to bottom): GM3 (3 μg) and GM2 (3 μg).

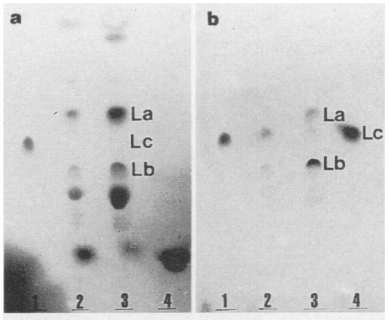

FIG. 2. Binding of *M. hyopneumoniae* to ciliary glycolipids on TLC plates. Panel a was sprayed with Bial's reagent. Panel b was overlaid with *M. hyopneumoniae*. Lanes 1, sulfatide (2.5 μg). Lanes 2, total lipid extract of cilia from 5 mg of wet cilia. Lanes 3, lower phase of Folch partition of ciliary lipids from 10 mg of wet cilia. Lanes 4, upper phase of Folch partition of ciliary lipids from 10 mg of wet cilia.

goat anti-rabbit peroxidase conjugates, and substrates (ABTS) were sequentially added to the plates according to standard procedures (33). Optical density 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 × *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). The 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. Immobilization of glycolipids onto microtiter plates was performed by the method reported by Krivan et al. (18). Briefly, sulfatide, glucocerebroside, and purified ciliary glycolipids were serially diluted in methanol containing auxiliary lipids (cholesterol [0.1 μg/ml] and phosphatidylcholine [0.1 μg/ml]). Various dilutions of glycolipids were added to the wells (100 μl per well) of a 96-well microtiter plate (Immulon 1) and evaporated. Binding of *M. hyopneumoniae* to the immobilized glycolipids was conducted and detected by procedures similar to those described for the microtiter plate adherence assay (33). Several receptor analogs (dextran sulfate, heparin, mucin, fucoidan, and chondroitin sulfate) that were known to inhibit adherence of *M. hyopneumoniae* to cilia were utilized to block binding of the mycoplasma to sulfatide, La, Lb, and Lc as described previously (33).

RESULTS

Binding of *M. hyopneumoniae* to standard glycolipids. Various lipid and glycolipid standards were subjected to TLC 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, asialo-GM1, and other neutral or nonpolar lipids which were tested in this study (Table 1). Binding of *M. hyopneumoniae* to sulfatide and GM3 was stronger than binding 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 necessary to increase the incubation time (to 5 h at 37°C) of mycoplasmas for optimal binding in the TLC overlay assay.

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 were therefore regarded as glycolipids. Three of them (La, Lb, and Lc) were bound by *M. hyopneumoniae* in the TLC overlay assay (Fig. 2). Binding of mycoplasmas to Lc was stronger than binding to La and Lb. Lc was barely detectable with Bial's reagent (Fig. 2a) and 2,7-dichlorofluorescein (data not shown), which detects both saturated and nonsaturated lipids. However, azure A, which detects sulfolipids, detected Lc as well as La and Lb (Fig. 3). After Folch partition, Lc along with another lipid (Ld) was distributed to the upper phase, while most of the other ciliary lipids, including La and Lb, were retained in the lower phase (Fig. 2). Authentic sulfatide was partitioned under similar conditions. As shown in Fig. 3, a large portion of sulfatide was distributed to the upper phase. Ld was detected as a dense band by Bial's reagent and had a

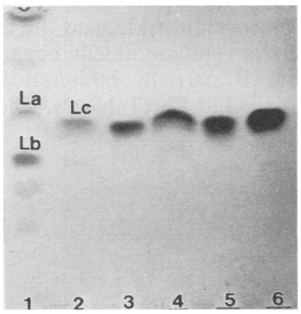

FIG. 3. Staining of glycolipids with azure A. Lane 1, lower phase of Folch partition of ciliary lipids from 10 mg of wet cilia. Lane 2, upper phase of Folch partition of ciliary lipids from 10 mg of wet cilia. Lane 3, sulfatide (1 μg). Lane 4, sulfatide (1 μg) and the upper phase of Folch partition of ciliary lipids from 10 mg of wet cilia. Lane 5, the lower phase of Folch partition of sulfatide (5 μl). Lane 6, the upper phase of Folch partition of sulfatide (5 μl).

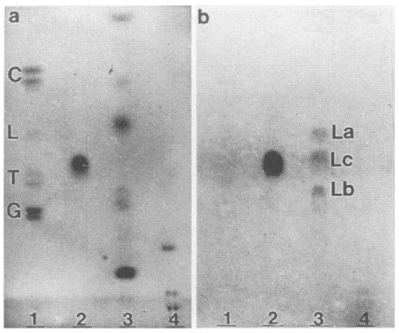

FIG. 4. Binding of laminin to standard glycolipids and ciliary lipids on TLC plates. Panel a was sprayed with Bial's reagent. Panel b was overlaid with laminin (10 μg/ml). Lanes 1, neutral glycosphingolipids in order of their migration: cerebroside (C; 3 μg), lactosyl ceramide (L; 3 μg), ceramide trihexoside (T; 3 μg), and globoside (G; 3 μg). Lanes 2, sulfatide (2.5 μg). Lanes 3, total lipid extract of 5 mg of wet cilia. Lanes 4, gangliosides in order of their migration (top to bottom): asialo-GM1 (3 μg), GM1 (3 μg), and GD1a (3 μg).

mobility similar to that of ganglioside GM2. *M. hyopneumoniae* did not bind to Ld (Fig. 2).

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 glycoprotein which specifically binds to sulfated glycolipids but not to nonsulfated 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 bound by *M. hyopneumoniae* (Fig. 2). No binding of laminin to other ciliary lipids (Fig. 4) was detected. Laminin also produced a dose-dependent inhibition of adherence of *M. hyopneumoniae* to La, Lb, and Lc (Fig. 5). In the presence of 100 μg of laminin per ml, adherence of the mycoplasma to the three ciliary glycolipids was almost abolished (Fig. 5).

Binding of *M. hyopneumoniae* to purified receptors in microtiter plates. La, Lb, Lc, and Ld were purified to homogeneity from the thin-layer chromatograms. On TLC plates, preparations of La, Lb, Lc, and Ld were detected as single bands by the Bial's reagent and/or azure A (data not shown). Purified La, Lb, and Lc still bound *M. hyopneumoniae* in the TLC overlay assay (Fig. 5). This specific binding was further confirmed in microtiter plates in which sensitive and dose-dependent binding of mycoplasmas to immobilized sulfatide, Lc, La, and Lb was detected (Fig. 6). No significant binding was detected to immobilized Ld, glucocerebroside, and the auxiliary lipids for coating plates. Dextran sulfate, mucin, heparin, fucoidan, and chondroitin sulfate B partially inhibited adherence of *M. hyopneumoniae* to La and Lb (Table 2), whereas chondroitin

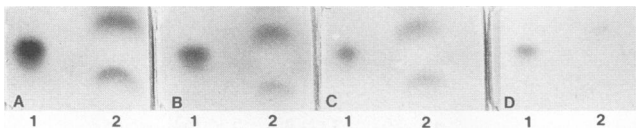

FIG. 5. Inhibition of *M. hyopneumoniae* binding to La, Lb, and Lc by laminin. TLC plates with separated lipids were preincubated with 0.0 (A), 1 (B), 10 (C), or 100 (D) μg of laminin per ml prior to being overlaid with mycoplasmas. Attached mycoplasmas were detected by immunostaining as described in the Materials and Methods. Lanes 1, Lc purified from 20 mg of wet cilia. Lanes 2, La (upper band) and Lb (lower band) purified from 20 mg of wet cilia.

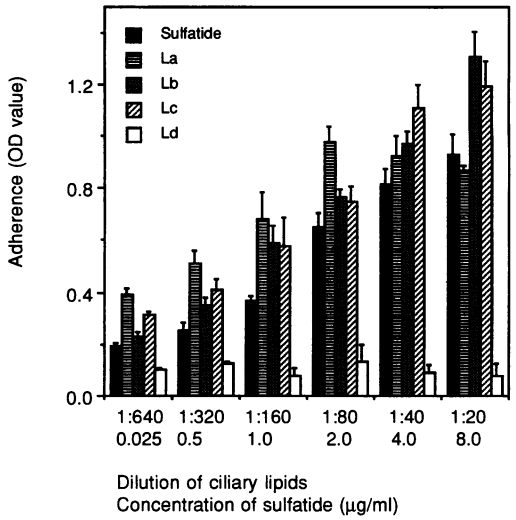

FIG. 6. Binding of *M. hyopneumoniae* to immobilized glycolipids in microtiter plates. La, Lb, Lc, and Ld purified from 50 mg of wet cilia were diluted to 1:640, 1:320, 1:160, 1:80, 1:40, and 1:20 in methanol, and sulfatide was diluted to 0.025, 0.5, 1.0, 2.0, 4.0, and 8.0 μg per ml for coating the microtiter plates as described in Materials and Methods. *M. hyopneumoniae* (2×10^8 color-changing units per well) was added and incubated for 90 min at 37°C. The bars represent mean optical densities (OD) ± standard deviations (SD) for four wells from three experiments.

sulfate C had no effect on the adherence. Binding of the mycoplasmas to sulfatide was also inhibited by these receptor analogs (Table 3). For binding to sulfatide, fucoidan and heparin were the most potent inhibitors. High-molecular-weight dextran sulfate, which strongly inhibited binding of *M. pneumoniae* to sulfatide (18), produced only a partial inhibition of adherence of *M. hyopneumoniae* to sulfatide at certain concentrations (Table 3). These receptor analogs also produced an inhibition of binding of *M. hyopneumoniae* to Lc, as demonstrated by the inhibition of binding to sulfatide (data not shown).

Adherence activities of lipid-extracted cilia. In order to evaluate the significance of ciliary glycoproteins in the adherence of *M. hyopneumoniae*, the proteinaceous precipitate of cilia, derived from chloroform-methanol extraction, was immo-

TABLE 2. Inhibition of mycoplasmal binding to La and Lb[a]

Inhibitor	Inhibition (% ± SD) for concn (μg/ml) of:		
	1	10	100
Heparin[b]	10.5 ± 6.7	47.4 ± 3.4	64.9 ± 3.2
Mucin	−2.4 ± 4.8	7.9 ± 6.5	46.3 ± 6.0
Dextran sulfate[c]	10.3 ± 7.1	52.7 ± 4.7	53.2 ± 2.5
Fucoidan	37.6 ± 5.4	62.1 ± 2.1	83.7 ± 1.6
Chondroitin sulfate B	3.9 ± 6.3	15.6 ± 4.6	37.3 ± 5.1
Chondroitin sulfate C	1.8 ± 3.5	−5.8 ± 7.5	2.6 ± 6.6

[a] The adherence assay was conducted in microtiter plates with immobilized La and Lb. Each well of the plates was coated with a mixture of La and Lb purified from approximately 50 μg of wet cilia and resuspended in 100 μl of methanol. Mycoplasmas (2×10^8 color-changing units per well) along with various inhibitors were added and incubated at 37°C for 90 min. Data are expressed as mean percent inhibition ± SD of triplicate experiments.

[b] Concentrations shown are in units per milliliter instead of micrograms per milliliter.

[c] Molecular weight, 500,000.

TABLE 3. Inhibition of mycoplasmal binding to sulfatide[a]

Inhibitor	Inhibition (% ± SD) for concn (μg/ml) of:				
	0.01	0.1	1	10	100
Fucoidan	ND	27.1 ± 8.4	55.2 ± 5.0	70.2 ± 6.7	78.4 ± 3.9
Heparin[b]	ND	48.3 ± 7.3	84.7 ± 5.8	72.5 ± 8.4	78.4 ± 5.0
Mucin	ND	−15.2 ± 6.7	15.6 ± 7.2	37.2 ± 1.9	68.6 ± 10.6
Dextran sulfate[c]	−10.2 ± 15.6	56.7 ± 8.4	16.9 ± 9.5	−15.1 ± 10.4	−1.4 ± 7.6
Chon B[d]	ND	−1.8 ± 5.8	10.3 ± 6.9	54.2 ± 7.0	−9.8 ± 0.2
Chon C[e]	ND	−8.9 ± 7.4	1.8 ± 9.5	−8.4 ± 20.0	−18.6 ± 3.8
Dextran	ND	3.6 ± 4.1	−2.7 ± 6.3	5.5 ± 2.6	1.5 ± 3.4

[a] The adherence assay was conducted in microtiter plates with immobilized sulfatide. Each well of the plates was coated with 0.1 μg of sulfatide resuspended in 100 μl of methanol. Mycoplasmas (2×10^8 color-changing units per well) along with various inhibitors were added and incubated at 37°C for 90 min. Data are expressed as mean percent inhibition ± SD of triplicate experiments. ND, not determined.

[b] Concentrations shown are in units per milliliter instead of micrograms per milliliter.

[c] Molecular weight, 500,000.

[d] Chondroitin sulfate B.

[e] Chondroitin sulfate C.

bilized in microtiter plates to test for the binding of mycoplasmas. Lipid-extracted cilia still exhibited adherence activity to the mycoplasma, but the activity level was substantially lower than that obtained with nonextracted cilia (Fig. 7).

DISCUSSION

TLC overlay assay has been widely used for characterization of glycolipid receptors for microbial pathogens (12, 15). 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. hyopneumoniae* was not identified in the glycolipids. *M. pneumoniae*, a human pathogen, bound to glycolipids containing the terminal Gal(3SO_4)β1- residue (18). Many pulmonary pathogens were shown to have a binding specificity for the GalNAcβ1-4Gal sequence in glycolipids (19). The binding of *M. hyopneumoniae* to diverse carbohydrate sequences in glycolipids suggests that there are several different receptors existing in nature.

Binding of the mycoplasma to ganglioside GM3 was unexpected because sialic acid did not inhibit adherence of *M. hyopneumoniae* to cilia; and treatment of cilia with neuraminidase did not reduce the binding of this organism in a previous study (33). Although ganglioside GM3 may not be involved in the adherence of *M. hyopneumoniae* to cilia, it may play a role in the interaction of the mycoplasma with other cells known to be bound by the mycoplasma, such as turkey erythrocytes (31), human lung fibroblasts, and porcine kidney cells (35). The function of globoside in the adherence of *M. hyopneumoniae* to ciliated cells is unknown. Although the mycoplasma bound weakly to globoside standard, binding to ciliary globoside was not demonstrated in this study.

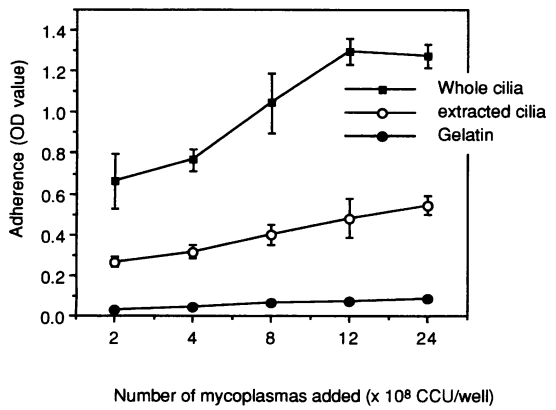

FIG. 7. Binding of *M. hyopneumoniae* to immobilized whole cilia, lipid-extracted cilia, and gelatin in microtiter plates. The adherence assay was conducted in microtiter plates as described in Materials and Methods. Results were presented as means optical densities (OD) ± SD of three independent experiments. CCU, color-changing units.

Sulfatide is distributed in many animal tissues, including lungs (18, 25). Multiple functions, including a possible role in cell adhesion, have been proposed for this sulfated glycolipid (25). Several species of mycoplasmas have exploited sulfatide, a membrane constituent of eucaryotic cells, for attachment (18, 20). It was found in this study that *M. hyopneumoniae* bound to sulfatide on TLC plates (Fig. 1) and in microtiter plates (Fig. 6). Also, a sulfatide-like glycolipid (Lc) was identified in the total lipid extract of cilia and was bound by the mycoplasmas (Fig. 2). In addition, binding of *M. hyopneumoniae* to sulfatide was partially inhibited by heparin, fucoidan, mucin, chondroitin sulfate B, and dextran sulfate, which blocked adherence of the organism to cilia, as shown in a previous study (33). These results strongly suggest that sulfatide is involved in adherence of the mycoplasmas to swine respiratory tract ciliated cells. Dextran sulfate, a potent inhibitor of *Mycoplasma pneumoniae* binding to sulfatide (18), had only a limited effect on *M. hyopneumoniae* binding to this glycolipid. In contrast, heparin, which did not interfere with *M. pneumoniae* adherence to sulfatide (18), strongly inhibited the binding of *M. hyopneumoniae* to this lipid. These findings imply that the two mycoplasmas may not have identical binding specificities for sulfatide, although they both bind to this lipid.

It was noticed that reactions of various glycolipids to Bial's reagent were not equal, even though the same amounts of lipids were applied to TLC plates. A poor reaction to Bial's reagent was always observed with lactosyl ceramide under the conditions utilized in this study. Therefore, the faint lactosyl ceramide band (Fig. 1a) should not be taken as an indication that the amount of this lipid loaded onto the plate was inadequate. Since lactosyl ceramide was bound by *M. pneumoniae* (18), further experiments were conducted to evaluate the possible binding of this lipid by *M. hyopneumoniae* in the TLC overlay assay. TLC plates loaded with various amounts of lactosyl ceramide (1 to 10 μg) did not display any binding by *M.*

hyopneumoniae (data not shown), suggesting that this glycolipid is not involved in the adherence of *M. hyopneumoniae.*

M. hyopneumoniae bound to three ciliary glycolipids (La, Lb, and Lc) in the TLC overlay assay (Fig. 2) and in the microtiter plate (Fig. 6); the receptor analogs that blocked adherence of *M. hyopneumoniae* to ciliated cells (33) also inhibited binding of the mycoplasma to La, Lb, and Lc. These results indicate that La, Lb, and Lc are the native receptors for *M. hyopneumoniae* adherence to swine ciliated cells. La, Lb, and Lc were believed to be sulfated glycolipids, because they yielded a positive reaction with azure A, which is a cationic dye that stains anionic sulfolipids (16), and were bound by laminin, a glycoprotein that has a binding specificity for sulfated glycolipids and has been used to identify these lipids (27). Lc had a mobility similar to that of sulfatide and comigrated with sulfatide on TLC plates, suggesting that it was putative sulfatide. However, Lc was not detected by Bial's reagent which displayed La, Lb, and authentic sulfatide. This discrepancy might not be caused by the low levels of Lc, because the amount of Lc in cilia was comparable to the amounts of La and Lb (Fig. 3). The reason for the nonreactivity to Bial's reagent of Lc is unknown. It is unlikely that the three ciliary 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. Further analysis using nuclear magnetic resonance, mass spectrometry, gas-liquid chromatography, or other biochemical means will be required for detailed characterization of La, Lb and Lc.

Folch partition separates total lipids into two phases, the upper phase usually containing acidic glycolipids and the lower phase containing phospholipids, glycolipids, and neutral lipids (9, 11). Lc was the only ciliary receptor that was partitioned into the upper phase (Fig. 2). This unique distribution made it easy to distinguish Lc from La and Lb. Under similar partition conditions, most of the authentic sulfatide was also distributed to the upper phase (Fig. 3), indicating that sulfated glycolipids can be partitioned into the upper phase under the conditions utilized in this study. It should be mentioned that no salts were added in the upper phase during the partition process conducted in this study. As reported by Folch et al. (9), the presence of salts, such as NaCl, KCl, or $CaCl_2$, in the partition system affects the distribution of lipids; substantial amounts of acidic lipids (gangliosides and sulfolipids) are present in the upper phase when no salts are added in the upper phase. This may provide an explanation for distribution of Lc to the upper phase. Although La and Lb are also sulfated lipids, they are partitioned into the lower phase, indicating that sulfate on a glycolipid is not the only factor that affects the distribution of a lipid in the Folch partition system.

Laminin is a major basement membrane glycoprotein which participates in cell growth, differentiation, and migration (17, 27). In a previous study, we found that laminin inhibited adherence of *M. hyopneumoniae* to ciliated cells by interacting with some components in cilia (33). In the present study, it was demonstrated that laminin bound to La, Lb, and Lc and blocked adherence of *M. hyopneumoniae* to the three ciliary receptors. These findings provided an explanation for the adherence-interfering mechanisms of laminin. It has been known (17) that laminin contains a carbohydrate sequence (NeuAcα2-3Galβ1-4GlcNAcβ1) similar to that of GM3, which was bound by *M. hyopneumoniae*. However, this carbohydrate sequence may not be directly accessible to mycoplasmas, because laminin is highly glycosylated by various oligosaccharides (17). This speculation is supported by a previous finding (33) that laminin itself does not interact with the mycoplasma but with the ciliary receptors. Therefore, it is believed that the carbohydrate sequence of laminin is irrelevant to the adherence-inhibiting activity of this glycoprotein.

Since 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 (26) and sulfated glycolipids (18), were identified for *M. pneumoniae*. As shown in Fig. 7, lipid-extracted cilia had substantially lower adherence activity. The remaining adherence activity could be attributable to lipid residues or proteinaceous receptors in the lipid-extracted cilia. No matter what the source of the remaining adherence activity is, it can be concluded that ciliary glycoprotein(s) was not a major factor(s) for adherence of *M. hyopneumoniae.*

The mechanism(s) 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 *Escherichia coli* adhere to the intestinal mucosa and cause effacement of microvilli and rearrangement of cytoskeleton 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 a change of cell functions (3). It is not known whether *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 was available about the change in ciliated cells. 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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