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Interaction of *Mycoplasma pneumoniae* with Human Lung Fibroblasts: Role of Receptor Sites

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The biochemical nature of the neuraminidase-sensitive *Mycoplasma pneumoniae* receptor site on human lung fibroblast cells was studied. Purified, mixed sialoglycolipid (ganglioside) preparations from human and bovine tissues did not bind to *M. pneumoniae* organisms and block their subsequent attachment to fibroblasts. Fibroblasts incubated for 24 h in sialoglycolipid solutions to increase the ganglioside content of their membranes did not show increased pathogen attachment when later incubated with mycoplasmas. HeLa cells grown in the presence of sodium butyrate to increase G_{M3} ganglioside levels likewise did not have significantly increased uptake of *M. pneumoniae* organisms. Treatment of fibroblasts with enzymes indicated that the mycoplasma receptor site is trypsin and papain resistant but Pronase sensitive. Pronase digests of fibroblast membranes contained a product(s) which combined with *M. pneumoniae* cells and cosedimented with them during centrifugation. Glycoproteins, purified from fibroblast membranes by a lithium diiodosalicylate solubilization technique, similarly bound to *M. pneumoniae* organisms. Collectively, these data suggest that the major component of the *M. pneumoniae* receptor site is a sialoglycoprotein with little or no lipid.

Mycoplasma pneumoniae is capable of attaching to a wide variety of eucaryotic cells in vitro, ranging from hamster and monkey ciliated epithelial cells (6, 7, 20) to erythrocytes (12), spermatozoa (13), and fibroblasts (11). The organisms apparently attach to cellular receptor sites that contain *N*-acetylneuraminic acid because: (i) pretreatment of host cells with neuraminidase decreases adsorption significantly (6, 13), and (ii) pretreatment of mycoplasmas with substances which contain substantial amounts of neuraminic acid (e.g., mucin, neuraminelactose, or purified sialic acid) lowers subsequent pathogen attachment (20). However, not all mycoplasmas attach to receptors that contain neuraminic acid, since *M. hominis*, *M. salivarium*, and *M. dispar* attach to an unidentified receptor which is not neuraminidase sensitive (10, 12, 13, 20).

Sobeslavsky and colleagues (20) used erythrocytes and isolated tracheal cells in studies of attachment to colonies of *M. pneumoniae*. They concluded that the cell receptor was insensitive to heat and trypsin. However, Manchee and Taylor-Robinson (13) found that the receptor on HeLa cells was sensitive to heat, trypsin, and formalin. Because the precise nature of the *M. pneumoniae* receptor was uncertain, we have attempted to determine the biochemical properties of the receptor by using a new model which consists of human lung fibroblast cells (8). Data suggest that the receptor for *M. pneumoniae* is a trypsin-resistant sialoglycoprotein with little or no glycolipid.

MATERIALS AND METHODS

Mycoplasma-fibroblast interactions. The cultivation of *M. pneumoniae* (strain PI 1428) and the MRC-5 human lung fibroblasts is described in our companion paper (8), along with details of the infection and counting techniques.

Gangliosides. Purified, mixed gangliosides prepared from bovine brain were generously supplied by W. E. Van Heyningen (Oxford University, England). Other bovine brain gangliosides (types II and III) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Gangliosides were also purified from human lung material obtained at autopsy. Tissue from patients who died from nonpneumonic disease or from trauma was minced and was then rinsed extensively (20 to 40 liters/kg) in sterile phosphate-buffered saline (PBS, pH 7.4) at 4°C. Washed lung fragments were stored frozen at −70°C until they were processed to extract gangliosides by the method of Mellanby et al. (15).

Glycoprotein. Purified glycoprotein was prepared from either normal MRC-5 lung fibroblasts or radioactive cells. The latter had been incubated for 72 h in BME, which contained 2 μCi of ^{14}C-amino acids (uni-

formly labeled protein hydrolysate; Amersham) per ml. Cell sheets were washed 3× with PBS, and cells were dispersed with trypsin (0.25% for 10 min at 37°C). The trypsin was inactivated with serum, and the cell pellets were collected by centrifugation at 5,000 × *g* for 15 min. Cells were washed in PBS and were then stored frozen in deionized water at −70°C.

Lysis ensued during five cycles of alternate freezing and thawing. Cell membranes were collected by centrifugation at 50,000 × *g* for 30 min at 4°C and were resuspended in 0.05 M tris(hydroxymethyl)aminomethane, which contained 0.3 M lithium diiodosalicylate (Eastman Chemicals, Rochester, N.Y.). Solubilized glycoprotein was phenol extracted, dialyzed, lyophilized, and washed in ethanol according to the method of Marchesi and Andrews (14).

Crude membrane proteins, including glycoproteins, were prepared from fibroblasts by incubating approximately 50 mg of washed cell pellets in PBS with 50 mg of insolubilized Pronase (*Streptomyces griseus*, bound to carboxymethyl cellulose; Sigma) per 25 ml of cell suspension. The suspension was incubated, with shaking, for 50 min at 37°C. The insolubilized enzyme and cell debris were removed by centrifugation at 10,000 × *g* for 30 min. The supernatant solution was filtered through a 0.22-μm filter. The crude Pronase-sensitive proteins were then stored at 4°C.

Biochemical reagents. Neuraminidase (mucopolysaccharide *N*-acetylneuramyl-hyarolase, EC 3.2.1.18, purified from *Vibrio cholerae*) was obtained from Behringwerke AG (Marburg, Germany). Soluble Pronase (*S. griseus*) was purchased from Koch-Light Laboratories, England. Papain (papaya latex, 2× crystallized), sialic acid (*N*-acetylneuraminic acid, type IV), and purified trypsin (bovine, type XI) were all obtained from Sigma.

RESULTS

Inhibition of mycoplasma binding by sialic acid. To determine the ability of sialic acid to block the *M. pneumoniae*-fibroblast interaction, suspensions of ^{14}C-labeled mycoplasmas were incubated with several concentrations of sialic acid. After 60 min at 37°C, the treated mycoplasmas (approximately 10^7 colony-forming units per ml) were added to cover slip monolayers of MRC-5 human lung fibroblasts. Attachment of mycoplasmas was evaluated after 30 min at 37°C. The results (Fig. 1) indicate that sialic acid does have a blocking effect. At sialic acid concentrations ≤ 0.06%, no difference in attachment relative to PBS controls was detectable. However, from 0.12 to 0.25%, marked differences were noted. At a level of 0.5% sialic acid, attachment was reduced by more than 50% compared to the PBS-treated controls.

Lack of binding inhibition by gangliosides. Gangliosides (sialoglycolipids) were obtained from a variety of sources and were preincubated with *M. pneumoniae* in an attempt to block the subsequent attachment of mycoplasma by the fibroblast cells. Typical results (Table 1) show that gangliosides prepared from both human and bovine tissues with a variety of methods were uniformly negative in such an assay. When tested at levels of 100 μg/ml, mixed gangliosides from bovine brain and human lung did not appreciably alter the uptake of *M. pneumoniae* by lung fibroblasts.

The converse experiment, in which fibroblasts were preincubated with gangliosides to increase their external membrane glycolipid content before the addition of mycoplasmas, was also conducted. The results (Table 2) show that an allowance of 24 h for adsorption of various soluble gangliosides to host cell membranes did not result in increased mycoplasma attachment. In fact, various degrees of decreased mycoplasma uptake were noted after fibroblasts were incu-

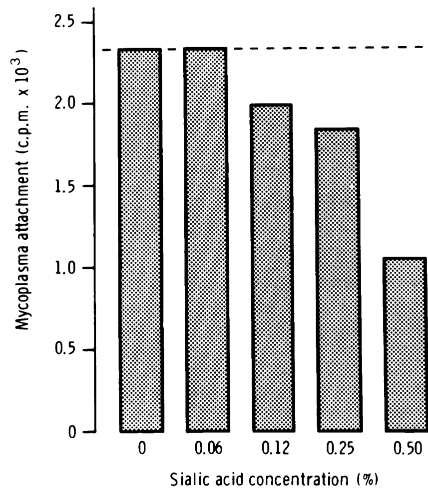

FIG. 1. *Effect of preincubation of ^{14}C-labeled M. pneumoniae cell suspensions with sialic acid on their attachment to fibroblast monolayers on cover slips. Mean data from a typical experiment with each assay run in quadruplicate.*

TABLE 1. *Effect of preincubation of M. pneumoniae organisms with mixed gangliosides (100 μg/ml) on subsequent attachment of the mycoplasmas to MRC-5 fibroblast monolayers*

Ganglioside	Mycoplasma attachment[a]	
	cpm/cover slip	% Control
None (PBS control)	1,753	100
Sigma II	1,909	109
Sigma III	1,955	112
Bovine brain	1,806	103
Human lung	1,908	113

[a] Mean data from six replicates per ganglioside.

TABLE 2. *Effect of ganglioside adsorption (20 μg/ml for 24 h) to MRC-5 fibroblast cells on subsequent attachment of ^{14}C-labeled M. pneumoniae organisms*

Ganglioside	Mycoplasma attachment[a]	
	cpm/cover slip	% Control
None (PBS control)	2,738	100
Sigma II	2,622	96
Sigma III	2,501	91
Bovine brain	2,378	87
Human lung	2,067	75

[a] Mean data from five replicates per ganglioside.

bated in ganglioside solutions and then infected with mycoplasmas.

The suggestion that glycolipid is not a major component of the sialic acid-containing receptor sites in fibroblasts was confirmed in experiments with HeLa cells. Sodium butyrate (5 mM) was added to half of the HeLa cell cultures to increase the level of the GM_3 ganglioside. After an 18- to 30-h butyrate exposure, the morphology of such cells changed from epithelioid to fibroblastic, indicative of a significant membrane ganglioside increase. When both cell types (epithelioid with normal ganglioside levels, and fibroblast-like with increased ganglioside levels) were compared for *M. pneumoniae* uptake, only minor differences could be discerned (Table 3).

Enzyme sensitivity of receptor. To evaluate the role of sialoglycoproteins in the *M. pneumoniae* receptor on fibroblasts, MRC-5 cells were exposed to various proteolytic enzymes prior to infection. To compensate for the loss of cells during the enzyme treatment, fibroblasts were pulse-labeled with tritiated thymidine for 1 h (1 μCi/ml) prior to being washed. They were treated with enzyme for 30 min at 37°C and then infected with ^{14}C-labeled mycoplasmas so that attachment could be standardized. The results (Table 4) show that neuraminidase (100 U/ml) does indeed markedly reduce mycoplasma attachment. The proteolytic enzymes, each evaluated at 0.025%, differed in their effects. The receptor appears to be trypsin resistant and very mildly sensitive to papain. Pronase, however, had a significant effect and reduced *M. pneumoniae* attachment to fibroblasts by 35%.

***M. pneumoniae* interaction with Pronase digests.** Pronase digests were prepared from ^{14}C-labeled MRC-5 fibroblast membranes, and the ability of these soluble proteins to bind to "cold" mycoplasmas was evaluated. After 30 min of incubation at 37°C with Pronase-sensitive proteins, the *M. pneumoniae* organisms were collected by centrifugation, and the pellet was assayed for radioactivity. The data (Table 5)

TABLE 3. *Effect of sodium butyrate treatments on HeLa cells*[a]

Treatment	Morphology	Mycoplasma attachment	
		cpm/cover slip	cpm/10^3 cells
Control	Epithelioid	6,030	379
NaB	Fibroblastic	6,434	460

[a] Cells were treated with 5 mM sodium butyrate (NaB) for 24 h and evaluated by attachment of ^{14}C-labeled *M. pneumoniae* (mean data from six replicates for each treatment).

TABLE 4. *Enzyme sensitivity*[a] *of the M. pneumoniae receptor site on MRC-5 lung fibroblasts*

Treatment	Mycoplasma attachment[b]	
	^{14}C/^{3}H ratio	% Control
Control	1.23	100
Neuraminidase	0.30	24
Trypsin	1.41	115
Papain	1.12	91
Pronase	0.80	65

[a] Mean data from five replicates, using a PBS control; 100 units of neuraminidase per ml, or 0.025% enzyme solutions in PBS.

[b] Mean data from five replicates; each consisted of a 30-min, 37°C enzyme treatment of ^{3}H-labeled fibroblast monolayers on cover slips, followed by infection with ^{14}C-labeled *M. pneumoniae* organisms for 30 min at 37°C.

TABLE 5. *Binding of ^{14}C-labeled Pronase digests from fibroblast membranes to M. pneumoniae*

Components		Binding[a]	
Pronase digest	Mycoplasmas	cpm	% Control
−	+	19	12
+	−	153	(100)
+	+	723	473

[a] Binding is expressed as cpm which pelleted with the mycoplasmas during centrifugation.

indicate that the presence of mycoplasmas in the system would result in the sedimentation of almost fivefold more ^{14}C-labeled fibroblast protein. Thus, proteins from MRC-5 cells attached to *M. pneumoniae.*

Interaction of *M. pneumoniae* with fibroblast glycoprotein. Fibroblast membranes were used to prepare purified glycoproteins, and this material bound to *M. pneumoniae* (Table 6). Glycoproteins from several milligrams of ^{14}C-labeled fibroblast membranes were prepared by the lithium diiodosalicylate method; when incubated with "cold" mycoplasmas, soluble glycoprotein cosedimented with the *M. pneumo-*

TABLE 6. *Binding of ^{14}C-labeled glycoprotein from MRC-5 fibroblast membranes to M. pneumoniae organisms*

M. pneumoniae	Glycoprotein binding[a] (cpm in pellet)
−	0
+	2,184
Heated[b]	1,266

[a] Binding measured by cpm to sediment with mycoplasmas during centrifugation after 30 min at 37°C.
[b] Inactivated, *M. pneumoniae* previously heated to 56°C for 30 min.

niae cells. This interaction was related to the mycoplasma viability, as indicated by heat-inactivating (56°C, 30 min) a sample of the mycoplasmas prior to mixing them with the glycoprotein. Such a treatment, known to partially inactivate the *M. pneumoniae* binding site, markedly reduced the degree of mycoplasma-glycoprotein binding. The stock glycoprotein preparation had an activity of 9.94×10^3 counts per minute (cpm)/100 μl. Normal *M. pneumoniae* (approximately 10^7 colony-forming units per ml) bound approximately 7.4% of the radioactivity available, whereas partially heat-inactivated *M. pneumoniae* bound only 4.3% of available glycoprotein.

DISCUSSION

The receptor site to which *M. pneumoniae* binds is known to contain neuraminic acid because of its sensitivity to inactivation by neuraminidase (6, 13) and its tendency to display reduced mycoplasma uptake in the presence of compounds containing sialic acid (20). However, the biochemical nature of the receptor has not been definitively established, due in part to difficulties in the model systems.

Much of the previous work on preliminary characterization of the site has been conducted with erythrocyte or tissue culture cell adsorption to colonies of mycoplasma. This presents a rather unnatural system with large numbers of microbes and proportionately few host cells. Quantitation of the mycoplasma/host cell ratio is difficult, and the degree of binding can only be approximated. In addition, the use of erythrocytes presents difficulties due to the wide interspecies variation in membrane composition (2) and the differences in mycoplasma hemagglutination and hemadsorption reactions that are seen when different animal erythrocytes are compared (10, 20).

We have employed a new model (8), which consists of lung fibroblast cells and radioactive *M. pneumoniae* organisms, to study binding to host cell membrane receptors. Our data suggest that sialoglycolipid (ganglioside) is apparently not involved to a great extent because mycoplasmas will not bind to soluble gangliosides isolated from several sources, including human lung. In addition, when fibroblasts were incubated with ganglioside (a technique previously shown to increase the membrane ganglioside content of fibroblasts by adsorption [4, 16]), no increased uptake of mycoplasmas could be detected. Further negative evidence on the potential role of gangliosides was given by HeLa cell experiments. HeLa cells have two predominant membrane gangliosides, G_{M3} and G_{D3}. The level of G_{M3} can be increased approximately 400% by incubation of cells in sodium butyrate (5). The major increase in ganglioside, mediated by a specific activation of cytidine 5′-monophosphate–sialic acid:lactosylceramide transferase (3), can be monitored by following the change in morphology from the typical epithelioid HeLa cell shape to a fibroblastic one (5, 19). In our study, this type of change was not correlated with a significant increase in mycoplasma binding, in spite of the fact that HeLa cells do carry a neuraminidase-sensitive receptor for *M. pneumoniae* (12, 13).

These results strongly suggest that glycolipid is not a major component of the *M. pneumoniae* receptor. However, they are not conclusive because a unique sialoglycolipid (distinct from the common gangliosides which we examined) could play a minor role in *M. pneumoniae* attachment. In addition, the spatial configuration of glycolipid in the membrane may be critical, and we may not have maintained it in our procedures. The potential role of lipids could be evaluated with further studies of sialoglycolipid incorporated into artificial membranes or liposomes with the use of techniques recently developed (1, 9).

Our data do indicate that sialoglycoproteins are a major component in the *M. pneumoniae* receptor site. The receptor site is trypsin resistant, in contrast to a published study (13). The discrepancy is undoubtedly due to the use of a crude trypsin preparation and a different model system (HeLa cells added to mycoplasma sheets) in the earlier work. In the current study, the receptor was quite sensitive to Pronase-induced proteolysis. Pronase digests were prepared with insolubilized enzyme to moderate proteolysis and promote rapid enzyme removal (so as to preserve the *M. pneumoniae* proteinaceous attachment tip). These Pronase digests bound to *M. pneumoniae* organisms. Efficiency of binding was low, however, due to the wide variety of sizes and types of proteins released by the Pronase, in addition to sialoglycoproteins (17). Specific binding to *M. pneumoniae* in-

creased markedly when purified glycoproteins of fibroblast membranes were used. This attachment was sharply reduced by heat-inactivating the mycoplasmas, thereby indicating that the binding was not simple surface adsorption.

These results indicate that procedures which enzymatically perturb glycoproteins can reduce *M. pneumoniae* attachment. Similarly, procedures which extract glycoprotein and exclude major glycolipids yield a product which binds to viable *M. pneumoniae* cells. Work is currently underway to isolate and characterize the specific sialoglycoprotein(s) to which *M. pneumoniae* binds. Recent evidence (18; J. Feldner et al., Abstr. 2nd Conf. Int. Org. for Mycoplasmology, Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I. Orig. Reihe A **241**:206, 1978; M. Banai et al., Abstr. 2nd Conf. Int. Org. for Mycoplasmology, Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig. Reihe A **241**:207, 1978) suggests that the neuraminidase-sensitive proteins which act as membrane receptors for mycoplasmas are not the same. *M. gallisepticum* binds to erythrocyte glycophorin, whereas *M. pneumoniae* does not. The ultimate, definitive characterization of these unique receptor sites will aid in our understanding of the initial events in pathogenesis and may also help to explain species and organ specificity for the pathogenic mycoplasmas.

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