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Mycoplasma pneumoniae Attachment: Competitive Inhibition by Mycoplasmal Binding Component and by Sialic Acid-Containing Glycoconjugates

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Attachment of *Mycoplasma pneumoniae* to human WiDr cell culture monolayers was examined by using radiolabeled *M. pneumoniae*. The amount of attachment was proportional to the density of the WiDr cells and to the concentration of *M. pneumoniae* in the assay. Saturation of the monolayers was achieved with 40 μg of virulent strain M129 per assay, whereas binding of avirulent strain B176 was 70% less than that of strain M129. A competitive attachment inhibition assay was used to measure specific binding component activity. Attachment was inhibited when WiDr cells were pretreated with unlabeled virulent strain M129, whereas avirulent noncytadsorbing strain B176 did not inhibit attachment as well as the virulent strain. A protein-rich extract prepared from virulent, cytadsorbing strains of *M. pneumoniae* also inhibited attachment. The amount of inhibition was dependent on the amount of extract used, and units for binding component activity in the extract were calculated from the competitive attachment inhibition assays. The competitive attachment inhibition assay was also used to investigate the nature of the receptor site on the WiDr cells. Attachment was inhibited when the radiolabeled *M. pneumoniae* suspensions were pretreated with human sialoglycoproteins, such as orosomucoid and ceruloplasmin, and bovine gangliosides. These findings support the present concept that the mammalian receptor site for *M. pneumoniae* is a sialic acid-containing glycoprotein.

Attachment of *Mycoplasma pneumoniae* to mucosal tissues is an essential step in initiating respiratory disease (5–8), and the available evidence indicates that the host cell receptor site for attachment is composed of neuraminidase-sensitive, sialic acid-containing glycoproteins (2, 5, 9–13, 15, 20, 21). Sialoglycoconjugates are also important for attachment of *M. gallisepticum* (1, 14). The binding component on the surface of *M. pneumoniae* is probably a protein because proteases inhibit attachment (2, 9, 15, 16).

In a previous study, it was reported that radiolabeled *M. pneumoniae* can attach to hamster tracheal organ cultures, hamster tracheal epithelial cell outgrowths, human erythrocytes, and WiDr (human intestinal carcinoma) cell cultures (4). These studies showed that the WiDr cell culture monolayers were the most suitable for quantitating mycoplasma attachment and inhibition of attachment.

A protein-rich extract prepared from virulent, cytadsorbing strains of *M. pneumoniae* (M129 and PI-1428) exhibited several biological activities; it caused ciliostasis and hemagglutination and inhibited attachment of *M. pneumoniae* to mammalian cells (3, 4). In this paper, we show that attachment is inhibited by the mycoplasmal binding component(s) in the extract as a result of competition for receptor sites on the WiDr cells and describe a quantitative assay for measuring mycoplasmal binding component activity. The microattachment assay was also used to measure host cell receptor activity. The results presented show that pretreatment of *M. pneumoniae* with certain select sialic acid-containing glycoproteins also inhibits attachment.

MATERIALS AND METHODS

Growth and labeling of organisms. Virulent *M. pneumoniae* strains M129 and PI-1428 (passages 5 to 12) were grown on glass or plastic surfaces as previously described (3). Mycoplasmas were radiolabeled by adding 1 to 2 mCi of [^{3}H]palmitic acid (12 to 17 Ci/mmol, New England Nuclear Corp., Boston, Mass.) per liter of growth medium. The medium was decanted, and the colonies were washed with phosphate-buffered saline, pH 7.2, scraped into Hanks balanced salt solution (HBSS), and sedimented at 10,000 × *g* for 20 min. The mycoplasmas were then suspended by multiple pipetting in HBSS, portioned, and stored at −70°C. The amount of mycoplasma cell protein was determined by

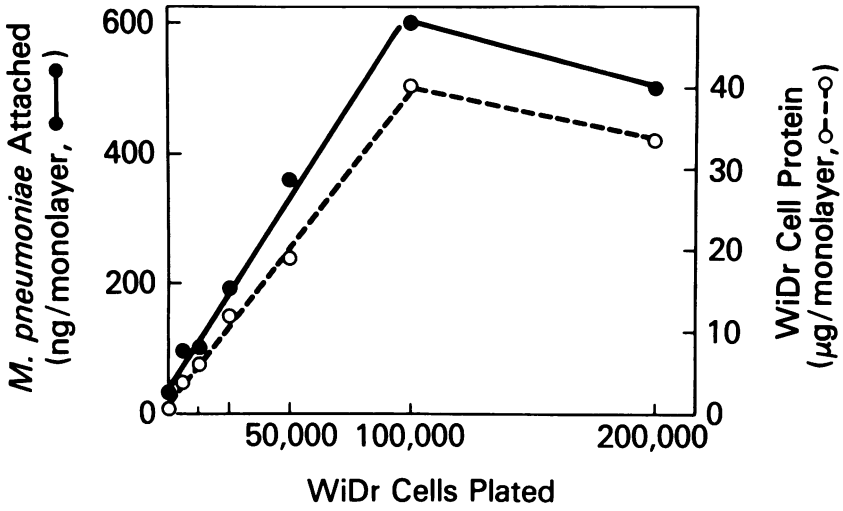

FIG. 1. Effect of WiDr cell density on attachment. Dilutions of WiDr cell suspensions (0.2 ml) were added to microtiter wells containing 5-mm cover slips. After 2 days, attachment of *M. pneumoniae* (strain M129, 13.5 μg, 26,725 cpm/μg) to the monolayers was measured in triplicate with the standard attachment assay. Symbols: ●, ng of mycoplasma attached per monolayer; ○, μg of WiDr cell protein per monolayer.

the Lowry procedure (18) with bovine serum albumin as the standard. Broth cultures of avirulent strain B176 (17) were centrifuged at 10,000 × *g* for 20 min, and the mycoplasma pellet was washed twice with phosphate-buffered saline, suspended in HBSS, and stored as described above.

***M. pneumoniae* extract.** The protein-containing extract was prepared as described previously (3); glass-grown *M. pneumoniae* cultures were washed and then incubated with 2 M NaCl followed by freeze-thaw, ultracentrifugation, and dialysis and lyophilization of the supernatant. The extract was suspended in HBSS for attachment inhibition studies.

WiDr cell culture monolayers. WiDr cells, an epithelium-like cell line from a human intestinal carcinoma (19), were grown as described previously (4) in microtiter wells on 5-mm cover slips in Eagle minimal essential medium containing 10% fetal bovine serum, 2 mM glutamine, and 1,000 U of penicillin G per ml. Confluent WiDr monolayers resulting from adding 1×10^5 to 2×10^5 cells per well were used after 2 to 3 days and contained approximately 30 μg of protein as determined by the Lowry procedure (18).

Attachment assays. Attachment of radiolabeled *M. pneumoniae* to cell culture monolayers was performed as described previously (4) with minor modifications. WiDr cell monolayers were washed with HBSS and transferred to siliconized glass tubes (12 by 75 mm) containing 50 μl of HBSS. Radiolabeled *M. pneumoniae* suspensions (2 to 80 μg in 50 μl) were added, and the tubes were incubated at 36°C with shaking for 60 min. Unattached mycoplasmas were removed by aspiration of fluids, and the monolayers were washed twice with 0.5 ml of HBSS. The washed monolayers bearing attached mycoplasmas were transferred to minivials; 3 ml of Aquasol (New England Nuclear) were added, and the radioactivity was determined by liquid scintillation spectrometry. Assays were performed with 3 or 4 replicates, and the amount of *M. pneumoniae* attached per monolayer was calculated by the specific radioactivity (counts per minute per microgram of protein) of the mycoplasma preparation. Attachment was expressed as nanograms of mycoplasma attached per microgram of WiDr monolayer protein.

Competitive attachment inhibition assays. WiDr cells grown on cover slips were added to tubes containing 50 μl of HBSS (control), unlabeled virulent or avirulent *M. pneumoniae*, mycoplasma extract (3), or other test reagents. The tubes were incubated at 36°C on a shaker for 1 h, radiolabeled *M. pneumoniae* suspensions (10 to 15 μg of protein in 50 μl) were added, and the attachment assay was performed as described above. Mycoplasmal binding component activity (difference in attachment units) was calculated by subtracting the attachment value obtained in the presence of extract from the control value obtained without extract.

The attachment inhibition assay was also used to measure receptor site activity of sialic acid-containing glycoconjugates. In this assay, the labeled *M. pneumoniae* suspension was preincubated with gangliosides or sialic acid-containing glycoproteins for 30 min at 36°C before WiDr monolayers were added. Attachment was determined as described above. The reagents used were bovine serum albumin (fraction V), myoglobin (type I), bovine fetuin (type III), and human ceruloplasmin (type III) purchased from Sigma Chemical Company, St. Louis, Mo., and bovine brain gangliosides and human plasma orosomucoid purchased from Calbiochem-Behring Corporation, La Jolla, Calif.

RESULTS

***M. pneumoniae* attachment to WiDr cell cultures.** *M. pneumoniae* attachment to WiDr cell cultures was a function of the number of cells in the monolayer and of the concentration of *M. pneumoniae* in the assay. Maximum attachment

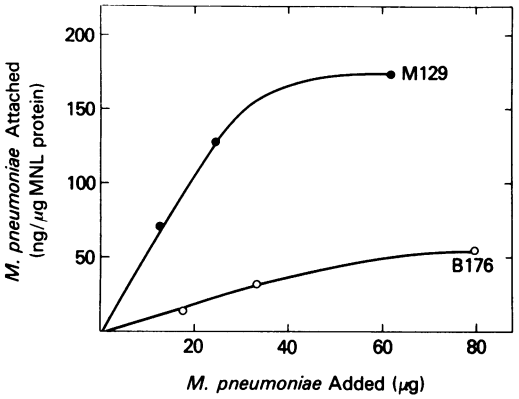

FIG. 2. Attachment as a function of *M. pneumoniae* concentration. Attachment of *M. pneumoniae* (ng mycoplasma attached/μg monolayer [MNL] protein) was determined in quadruplicate with the standard assay. The WiDr monolayers contained 14.0 μg of protein. Symbols: ●, strain M129 (12,280 cpm/μg protein); ○, strain B176 (9,644 cpm/μg protein).

was attained when the monolayers were confluent and contained 100,000 cells per microtiter well (Fig. 1). WiDr cell monolayers incubated with *M. pneumoniae* under the standard conditions of attachment were washed, fixed, and stained with specific fluorescent-labeled anti-*M. pneumoniae* antiserum. This reagent revealed *M. pneumoniae* adherent to the WiDr cell monolayers, thus confirming the attachment results obtained with radiolabeled organisms. Attachment also paralleled the amount of monolayer protein present, and attachment activity was defined as nanograms of *M. pneumoniae* attached per microgram of WiDr monolayer protein. This definition of attachment activity standardized the assay and permitted direct comparison of different experiments. When 10 to 20 μg of *M. pneumoniae* were added to the monolayer assays, 4 to 8% of the added mycoplasmas adhered to the monolayers.

The results on attachment of virulent *M. pneumoniae* strain M129 and avirulent strain B176 to WiDr cell monolayers are shown in Fig. 2. Attachment was proportional to the amount of ^{3}H-*M. pneumoniae* added; there was increased attachment with increased mycoplasma concentrations. Attachment of the virulent strain approached saturation at approximately 40 μg of mycoplasma per assay, whereas attachment of the avirulent strain was only about 30% of that observed with the virulent strain.

Pretreatment of WiDr cell cultures: Inhibition assay for mycoplasmal binding component activity. To examine the specificity of attachment, we preincubated monolayers with unlabeled mycoplasma suspensions. Preincubation with the virulent, cytadsorbing strain M129 markedly inhibited the subsequent attachment of radiolabeled strain M129, whereas the avirulent, noncytadsorbing, nonhemadsorbing strain B176, derived from strain M129 (17), produced much less inhibition. Attachment of radiolabeled strain M129 (12 μg) was inhibited 65% by 9 μg of the homologous unlabeled strain M129 preparation, whereas inhibition by 8 μg of unlabeled strain B176 was only 30%.

We have shown that the WiDr cell monolayers provide a sensitive cell substrate for detecting attachment inhibition by a protein-containing mycoplasma extract (4). To examine the specificity of the mycoplasmal extract, we tested the effect on attachment of several nonmycoplasma proteins as well as the effect of the extract obtained from *M. pneumoniae*. Table 1 shows that 36 μg of the *M. pneumoniae* protein extract inhibited attachment by 55%. However, 100 μg of myoglobin, bovine serum albumin, or fetuin did not inhibit attachment. Additional experiments have shown that the extent of attachment inhibition by mycoplasmal extract was the same (e.g., 41 and 44%) in the absence or presence of excess fetuin.

Increasing amounts of *M. pneumoniae* protein extract produced increased inhibition of attachment. For example, 6, 12, 25, and 50 μg of extract inhibited attachment by 29, 37, 46, and 66%, respectively. On the assumption that attachment inhibition results from occupation of the WiDr cell receptor sites by mycoplasmal binding component(s), the binding component activity in the extract was determined by subtracting the value for attachment in the presence of extract from the control value without extract. The dose response for binding component activity, expressed as difference in attachment units, obtained with two different lots of extract is shown in Fig. 3. The hyperbolic dose response curves show saturation of the monolayers by the

TABLE 1. Effect of control proteins on attachment

Treatment[a] (μg)	*M. pneumoniae* attachment[b]
Control	20.5 ± 1.5
Myoglobin (100)	23.4 ± 3.0
Bovine serum albumin (100)	20.8 ± 2.4
Fetuin (100)	24.8 ± 2.1
Extract (36)	9.5 ± 0.5

[a] WiDr monolayers were incubated with Hanks balanced salt solution (control) or protein as indicated for 60 min at 36°C. Radiolabeled *M. pneumoniae* (^{3}H PI-1428, 14.3 μg of protein) was added, and the standard attachment assay was performed.

[b] Expressed as nanograms of *M. pneumoniae* attached per microgram of monolayer protein ± standard error of the mean.

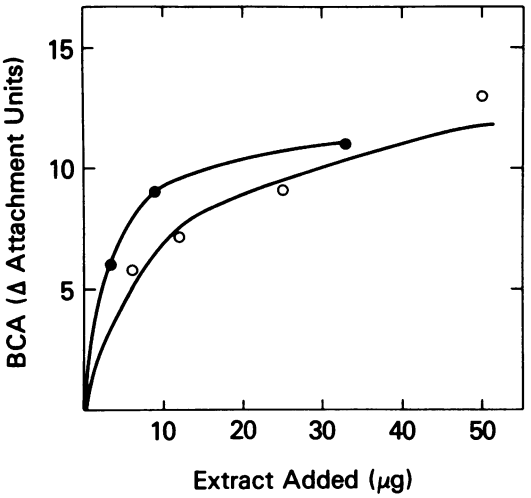

FIG. 3. Binding component activity (BCA) in *M. pneumoniae* extracts. Difference (Δ) in attachment units was calculated by subtracting the value for attachment obtained in the presence of extract from the control value without extract. Symbols: ●, extract of lot 11 (control attachment was 20.6 ng mycoplasma attached per μg of monolayer protein); ○, extract of lot 13 (control attachment was 19.6 ng mycoplasma attached per μg of monolayer protein).

binding component in the extract. The specific binding component activities (difference in attachment units per microgram of extract) for the two extract lots were found to be similar (Table 2).

Pretreatment of labeled mycoplasmas: Inhibition assay for host cell receptor activity. The effect of preincubating the labeled *M. pneumoniae* with sialic acid or sialic acid-containing glycoprotein reagents was examined. At neutral pH, *N*-acetylneuraminic acid (50 to 150 μg per assay) and *N*-acetylneuramin lactose (50 to 250 μg per assay) had no significant effect on attachment. However, a significant reduction of attachment was observed when *M. pneumoniae* suspensions were preincubated with human plasma sialoglycoproteins, orosomucoid and ceruloplasmin, or with bovine brain gangliosides (Table 3). Other sialic acid-containing glycoproteins such as bovine fetuin and human transferrin did not significantly reduce attachment.

DISCUSSION

A protein-rich extract prepared from virulent cytadsorbing strains of *M. pneumoniae* possessed ciliostatic and hemagglutinating activities (3) and inhibited attachment of radiolabeled *M. pneumoniae* to WiDr cell culture monolayers (4). When WiDr cells were pretreated with 5 to 100 μg of the extract, attachment of ^{3}H-*M. pneumoniae* was reduced by 30 to 90%. In the present paper, we show that attachment inhibition was based on competition for available receptor sites; hence, pretreatment of WiDr cells with unlabeled virulent cytadsorbing strain M129 of *M. pneumoniae* inhibited attachment. Pretreatment of cell monolayers with avirulent noncytadsorbing strain B176 produced far less inhibition than did the virulent strain; that is, the capacity to inhibit attachment correlated with the capacity to attach in the micro-attachment assay. Mechanisms of attachment inhibition other than competition must be considered; for example, enzymatic activities such as neuraminidase or protease are possible. However, these are unlikely because inhibition by the extract occurred in the presence of excess fetuin.

The effect of the concentration of the reactants on attachment was also examined, and the results indicated that attachment is proportional to the WiDr cell density and to the number of radiolabeled mycoplasmas used. Attachment of the virulent strain M129 was three times that of the avirulent strain B176. It is improbable that this difference was due to the growth conditions of the cultures (glass adherent versus suspension), because similar levels of attachment have been obtained with both glass adherent and suspension cultures of strains M129 and PI-1428. Since inhibition was dependent on saturation of the available receptor sites, additional studies were performed to increase the sensitivity of the attachment inhibition assay. Saturation was readily achieved by using a small number of WiDr cells (with a limited number of receptor sites) and a relatively larger number of mycoplasmas. This high *M. pneumoniae* and low

TABLE 2. Determination of binding component activity in *M. pneumoniae* extracts

Extract	*M. pneumoniae* attachment[a]	% Inhibition by extract (binding component)	Binding component units (Δ attachment)[b]	Binding component units per μg of extract
0	20.5			
3.6 μg (lot 11)	14.5	29	6.0	1.7
0	19.6			
6.0 μg (lot 13)	13.8	30	5.8	1.0

[a] Expressed as nanograms of *M. pneumoniae* attached per microgram of monolayer protein.
[b] Calculated by subtracting the attachment in the presence of extract from the control value without extract.

TABLE 3. Attachment inhibition of sialoglycoproteins and gangliosides

Treatment[a] (μg)	*M. pneumoniae* attached[b]	% Inhibition
HBSS Control	19.6 ± 4.1	
Orosomucoid (30)	16.7 ± 2.5	15
Orosomucoid (100)	11.8 ± 0.6	40
HBSS Control	26.9 ± 4.0	
Ceruloplasmin (25)	23.6 ± 1.3	12
Ceruloplasmin (100)	16.5 ± 3.0	39
Ceruloplasmin (1,000)	7.7 ± 0.2	71
Gangliosides (100)	4.2 ± 0.3	84

[a] Radiolabeled *M. pneumoniae* strain PI-1428 (13 to 14 μg) was preincubated with orosomucoid, ceruloplasmin, gangliosides, or HBSS, and attachment was determined as described in the text.

[b] Expressed as nanograms of *M. pneumoniae* attached per microgram of monolayer protein ± standard error of the mean.

receptor site ratio was obtained by growing the monolayers on very small 5-mm cover slips, providing a total cell population of 10^5 cells per cover slip per assay.

The micro-attachment assay was also used to examine the nature of the receptor sites on WiDr cells. Earlier reports have shown that the receptor site for *M. pneumoniae* attachment is composed of a neuraminidase-sensitive sialic acid-containing glycoprotein. In our study, pretreating radiolabeled *M. pneumoniae* with selected sialoglycoproteins (such as human plasma orosomucoid and ceruloplasmin) and with bovine brain gangliosides readily inhibited attachment, supporting the concept that the receptor for *M. pneumoniae* is a sialic acid-containing glycoconjugate (2, 9–13, 15, 20, 21). The glycoprotein reagents interact with the binding component of the mycoplasma, and block their ability to attach to the WiDr cells. These findings suggest that orosomucoid, ceruloplasmin, and bovine gangliosides contain structural analogs of the specific cell receptors. Thus, such glycoproteins may be useful as affinity chromatography reagents for isolation and purification of the mycoplasmal binding component in the protein extract. Our findings which show that bovine gangliosides can inhibit attachment of *M. pneumoniae* are in contrast with those reported by Gabridge, et al. (12) who used MRC-5 human lung fibroblast cell cultures. The differences observed may be due to variations in the assay, in the amount of saturation achieved, or in the nature of the receptor sites on the fibroblastic (MRC-5) and epithelium-like (WiDr) cell lines.

The WiDr cell culture micro-attachment assay presented provides a powerful tool for investigating host-parasite adherence. The virulent strain M129, which strongly attaches to WiDr cells, can be compared to the avirulent, nonhemadsorbing strain B176, which exhibits markedly reduced attachment. The competitive inhibition assay is being used to characterize mycoplasmal binding component and host cell receptor activities. The inhibition assay is also being used to quantitate extraction and purification of the binding components of *M. pneumoniae* and the receptor sites on the WiDr cells. Thus, the micro-attachment assay provides an important in vitro model to examine attachment mechanisms.

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