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Mycoplasma pneumoniae Attachment to Glutaraldehyde-Treated Human WiDr Cell Cultures (42284)

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Abstract. Attachment of *Mycoplasma pneumoniae* to host cells initiates disease, and the attachment components may represent important protective immunogens for preventing disease. We have studied the mechanisms of attachment using *in vitro* cell culture systems and selected pathogenic and nonpathogenic strains of *M. pneumoniae.* Attachment of the pathogenic strains M129 and PI-1428 was several fold greater than attachment of the nonpathogenic strain, and attachment of strains M129 and PI-1428 was reduced by 21 to 63% when human WiDr cell monolayers were exposed to neuraminidase, supporting the concept that *M. pneumoniae* attaches to mammalian cells by a neuraminidase-sensitive glycoconjugate. While attachment of the two pathogenic strains was markedly reduced by treating the WiDr cells with glutaraldehyde, glutaraldehyde treatment produced minimal effects on the attachment of the nonpathogenic strain B176. Glutaraldehyde treatment also altered the temperature dependence of attachment by the pathogenic strains. Because glutaraldehyde-treated WiDr cell monolayers showed little difference in attachment between pathogenic and nonpathogenic strains, glutaraldehyde-treated cells are not appropriate cell substrates for studying *M. pneumoniae* attachment mechanisms or identifying immunogens for vaccine development.

Attachment of *Mycoplasma pneumoniae* to host cells is an important virulence property of this organism, since attachment initiates the course of infection and disease. Thus, preventing attachment should prevent disease and provide an effective approach for immunization. Our studies were designed to identify the mycoplasmal components which mediate attachment, because they provide candidate immunogens to prevent disease.

M. pneumoniae has been shown to attach to human cell cultures, to human and animal erythrocytes, and to hamster and human tracheal organ cultures [Reviewed in Ref. (1)]. A large body of data indicates that *M. pneumoniae* attaches to mammalian cells by neuraminidase-sensitive sialoglycoconjugates. We have previously described a microassay for quantitating attachment to human WiDr cell cultures (2, 3) and have continued to examine the mechanisms by which *M. pneumoniae* attaches to these target cells, including the effect of neuraminidase on attachment. Because neuraminidase treatment may cause cells to detach from the coverslips, we have treated the cells with glutaraldehyde in order to stabilize the monolayers. This report describes the effects of treating WiDr cells with neuraminidase or glutaraldehyde on the attachment of *M. pneumoniae.*

Materials and Methods. *M. pneumoniae cultures.* Medium and growth of *M. pneumoniae* were described previously (2, 5). Pathogenic strains M129 and PI-1428 are low passage isolates from patients with primary atypical pneumonia. Avirulent strain B176 was derived from M129 by passage in broth and has lost virulence for hamsters and ability to cytadsorb (4, 5). Cultures were radiolabeled by adding 1–5 mCi of [^{3}H]palmitate (12–17 Ci/mole) (New England Nuclear Corp., Boston, Mass.) per liter of growth medium as previously described (2). Quantitation of *M. pneumoniae* protein was by the procedure of Lowry *et al.* (6), with bovine serum albumin as the standard.

WiDr cells. WiDr cells, an epithelial-like cell culture line from a human intestinal carcinoma (7), were cultured in flat-bottom microtiter wells on 5 mm coverslips as previously described (2). For glutaraldehyde treatment, the WiDr cell monolayers were washed once with Hanks' balanced salt solution (HBSS) and then incubated with 0.5% glutaraldehyde in

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HBSS at 37°C for 30 min. The monolayers were washed with HBSS supplemented with 50 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.2 (HBSS–Hepes), incubated with 0.05 *M* glycine (37°C, 30 min), and washed again with HBSS–Hepes. In some experiments, the WiDr cell monolayers were treated with neuraminidase and washed prior to attachment. The neuraminidases used were from *Vibrio cholerae* (Calbiochem, La Jolla, Calif.), *Clostridium perfringens,* Type VII (Sigma Chemical Co., St. Louis, Mo.), and influenza virus (Calbiochem.).

Attachment assays. M. pneumoniae attachment to WiDr cells was determined as described previously (2, 3) except that the incubations were carried out in the wells of the microtiter plates. Attachment was performed at pH 7 (optimum pH) with HBSS–Hepes. No evidence was obtained that added divalent cations stimulated adherence. Radiolabeled *M. pneumoniae* in 100 μl HBSS–Hepes was added to each well containing confluent WiDr cell monolayers, and the microtiter plates were incubated for 1 hr at 36°C. The unattached organisms were removed by aspiration, the monolayers were washed, and the coverslips bearing the monolayers and attached mycoplasmas were counted by liquid scintillation spectrometry.

Results. When WiDr cell monolayers were exposed to neuraminidase, *M. pneumoniae* attachment was reduced by 21 to 63% (Table I). These experiments support the concept that attachment of *M. pneumoniae* to WiDr cells is mediated by a neuraminidase-sensitive component. However, in some experiments neuraminidase treatment would detach the monolayers from the glass coverslips. Thus, monolayers were fixed to the coverslips with 0.5% glutaraldehyde before they were exposed to neuraminidase. The glutaraldehyde-treated WiDr cells had reduced attachment capacity for *M. pneumoniae,* and when these monolayers were further treated with neuraminidase, the attachment was reduced by 5 to 34% (Table I).

The attachment curves of virulent and avirulent *M. pneumoniae* strains to glutaraldehyde-treated (0.5%) or untreated WiDr cell monolayers are shown in Fig. 1. With untreated monolayers, attachment of the virulent strains M129 and PI-1428 was much greater than that of the avirulent strain B176. However, attachment of the virulent strains was markedly reduced when the monolayers were pretreated with glutaraldehyde, while glutaraldehyde treatment had much less effect on the attachment of the avirulent strain. The mean value for reduction of attachment after glutaraldehyde treatment of the monolayers

TABLE I. REDUCTION OF ATTACHMENT BY NEURAMINIDASE TREATMENT OF WiDr CELLS

Neuraminidase treatment[a]		WiDr cells without glutaraldehyde fixation			
		^{3}H-*M. pneumoniae* added		*M. pneumoniae* Attached (μg)	
Source	Units per monolayer	μg	Strain	Treated/Control[b]	Percentage reduction
CP[c]	0.02	15	M129	0.38 ± 0.05/0.67 ± 0.36	43
VC	0.04	24	M129	0.49 ± 0.05/0.62 ± 0.13	21
IV	5	15	PI-1428	0.28 ± 0.08/0.76 ± 0.01	63
VC	0.02	17	PI-1428	0.36 ± 0.02/0.60 ± 0.21	40
		WiDr cells fixed with 0.5% glutaraldehyde			
CP	0.02	17	PI-1428	0.29 ± 0.04/0.44 ± 0.13	34
IV	1	10	M129	0.14 ± 0.01/0.15 ± 0.02	5
IV	1	40	M129	0.29 ± 0.05/0.38 ± 0.08	23
CP	0.5	10	M129	0.10 ± 0.06/0.15 ± 0.08	33

[a] 36°C/1 hr.
[b] μg attached ± SD, control value was for WiDr cells without neuraminidase treatment.
[c] CP = *Cl. perfringens;* VC = *V. cholerae;* IV = influenza virus.

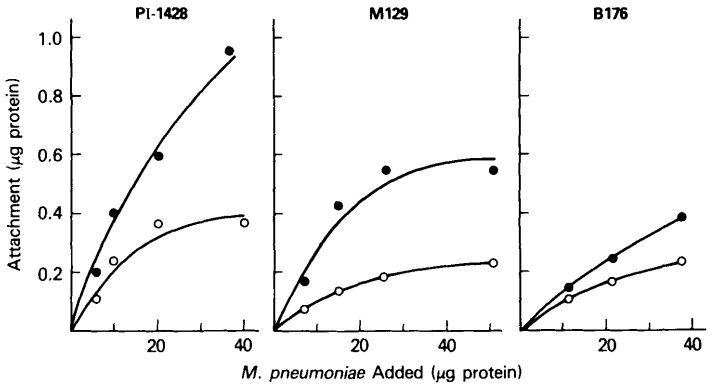

FIG. 1. Attachment of virulent and avirulent *M. pneumoniae* strains to untreated and glutaraldehyde-treated WiDr cell monolayers. *M. pneumoniae* strains PI-1428 (6000 cpm/μg protein), M129 (5800 cpm/μg protein), and B176 (10,300 cpm/μg protein) were incubated with the monolayers for 60 min. Attachment was determined by the standard assay: ●, untreated monolayers; ○, monolayers pretreated with 0.5% glutaraldehyde.

was 53.2 ± 16.4% for the virulent strains (mean ± standard deviation for 19 separate determinations in 6 different experiments) as compared to only 27.2 ± 7.6% for the avirulent strain (mean ± standard deviation for 9 separate determinations in 3 different experiments). After exposure of the monolayers to glutaraldehyde, the amount of attachment of the virulent parent strain M129 was approximately the same as the level of attachment of the avirulent strain B176.

When we compared *M. pneumoniae* attachment to treated and untreated monolayers at different temperatures, attachment to untreated monolayers was maximal at 37°C, which was approximately 10-fold greater than that at 4°C (Fig. 2). On the other hand, attachment to monolayers previously treated with glutaraldehyde was the same at 24 or 37°C, and these values were only threefold greater than the attachment at 4°C. Thus, glutaraldehyde treatment of the WiDr cells altered the temperature dependence of *M. pneumoniae* attachment.

Discussion. These studies show that neuraminidase treatment reduced attachment of *M. pneumoniae* to WiDr cells, and they support the concept that *M. pneumoniae* attaches to mammalian cells by a neuraminidase-sensitive sialoglycoconjugate. Neuraminidase treatment was shown previously to reduce attachment of *M. pneumoniae* to hamster tracheal organ cultures (10, 11), human and animal erythrocytes (12–16), and human fibroblast cell cultures (17). Pretreatment with gangliosides and sialoglycoproteins inhibited *M. pneumoniae* attachment to human WiDr cell cultures (3) and also to human erythrocytes (15, 16). While our results indicate that attachment to the WiDr cells also occurs

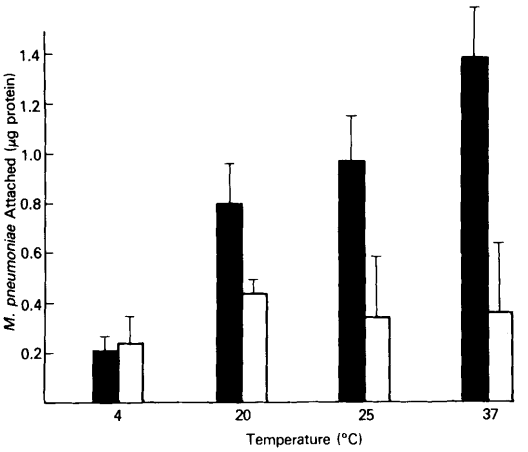

FIG. 2. Effect of temperature on attachment of *M. pneumoniae* to WiDr cell monolayers. Untreated or glutaraldehyde-treated monolayers were incubated for 60 min with 12.5 μg of *M. pneumoniae* strain PI-1428 (7100 cpm/μg protein) at the temperatures indicated. Attachment was determined by the standard assay: ■, untreated monolayers; □, monolayers pretreated with 0.5% glutaraldehyde. Error bars represent standard deviation of quadruplicate determinations.

through a sialic acid-containing receptor, we cannot rule out the possibility that neuraminidase activity could affect other receptor molecules as well.

The data indicate that in addition to neuraminidase-sensitive sites, the WiDr cells contain glutaraldehyde-sensitive and glutaraldehyde-resistant receptor sites for virulent and avirulent strains of *M. pneumoniae,* respectively. Loss of receptor sites for reovirus hemagglutination of human erythrocytes after glutaraldehyde treatment has been described by Dalen (18), although the receptors for influenza virus on hen erythrocytes were resistant to glutaraldehyde. The WiDr cell monolayers treated with 0.5% glutaraldehyde differed from untreated monolayers in *M. pneumoniae* attachment assays: (a) glutaraldehyde-treated cells showed less reduction of attachment than untreated cells following neuraminidase treatment; (b) glutaraldehyde-treated WiDr cells showed little difference in the attachment capacities between the pathogenic and nonpathogenic strains, while untreated WiDr cells showed a three- to fourfold difference in attachment between the pathogenic and nonpathogenic strains; and (c) the effects of temperature on attachment were much less dramatic with glutaraldehyde-treated monolayers than with untreated monolayers.

Maximal attachment of *M. pneumoniae* to untreated WiDr cells at 37°C differs from the results obtained for the binding of tetanus toxin to neuronal cell cultures, which is greater at 4°C than at 37°C (8), but is consistent with the maximal attachment of *Entamoeba histolytica* trophozoites to human erythrocytes at 37°C (9). The significance of this difference in optimal temperature has not been established, but it may suggest that the mechanisms of attachment of microorganisms differ from the binding of small molecules to mammalian cells.

The presence of glutaraldehyde- and neuraminidase-sensitive and -resistant receptor sites on the WiDr cells is consistent with proposals that mycoplasmas may attach to mammalian cells by multistep mechanisms (19, 20). It is possible that the virulent strains M129 and PI-1428 utilize both glutaraldehyde-sensitive and glutaraldehyde-resistant sites during attachment, while the avirulent strain B176 has lost the capacity to attach to the glutaraldehyde-sensitive sites but retained the capacity for low level attachment to glutaraldehyde-resistant sites. Since the untreated WiDr cells were more sensitive to neuraminidase treatment than the glutaraldehyde-treated cells, the sialic acid-containing site may also be sensitive to glutaraldehyde. Additional studies on the neuraminidase sensitivity as well as the temperature dependence for attachment of the avirulent strain B176 should provide further information on *M. pneumoniae* attachment mechanisms.

Other studies have utilized glutaraldehyde-treated erythrocytes (21) and hamster tracheal epithelial cells (22) to investigate attachment mechanisms of *M. pneumoniae.* Our results indicate that the use of glutaraldehyde-treated target cells to study attachment mechanisms must be examined with caution. Because the glutaraldehyde-resistant sites showed altered temperature dependence and did not show differences in the binding capacity of the virulent and avirulent strains, which were evident with untreated cells, glutaraldehyde-treated cells may not provide the most appropriate models to study *M. pneumoniae* pathogenicity or to identify immunogens for vaccine development.

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