

Mycoplasma pneumoniae Attachment to WiDr Cell Cultures: Competitive Inhibition Assays

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Attachment of radiolabeled *M. pneumoniae* to human WiDr cell culture monolayers was dependent on the WiDr cell density and the concentration of *M. pneumoniae*. Saturation of confluent monolayers grown on 5 mm coverslips was attained with only 40 μg of *M. pneumoniae* protein. Preincubating the WiDr monolayers with unlabeled *M. pneumoniae* or with a protein-rich extract prepared from *M. pneumoniae* inhibited subsequent attachment of radiolabeled organisms. Attachment inhibition by the *M. pneumoniae* extract provided a quantitative assay for mycoplasmal binding components. Treatment of radiolabeled *M. pneumoniae* with orosomucoid, ceruloplasmin, and gangliosides inhibited attachment to WiDr cells. These sialoglycoconjugates may be structural analogues of the target cell receptor.

INTRODUCTION

Attachment of *Mycoplasma pneumoniae* to mucosal tissues is an essential step in initiating respiratory disease and may help to protect the mycoplasma from the defense mechanisms of the host. Stimulation of host immune responses to mycoplasma binding components should inhibit infection and provide an effective means of preventing disease [1]. A protein-rich extract of *M. pneumoniae* which exhibits binding activity [2] is a potential source for the purification and characterization of *M. pneumoniae* binding components. Comparative studies showed that virulent strains of *M. pneumoniae* readily attach to hamster tracheal organ cultures, tracheal outgrowth monolayers, human erythrocytes, and WiDr human carcinoma cell cultures [3]. However, the WiDr cell culture monolayers provided the most useful cell substrate for quantitating attachment inhibition. This report summarizes the WiDr monolayer micro-attachment assay and describes two applications of the procedure: a quantitative assay for mycoplasmal binding components based on inhibition by the protein-rich extract, and characterization of target cell receptor activities based on inhibition by gangliosides and sialic acid-containing glycoproteins.

MATERIALS AND METHODS

M. pneumoniae cultures were grown and labeled with ^3H -palmitic acid as previously described [3,4]. The micro-attachment assay utilized human carcinoma WiDr cell cultures on 5 mm coverslips (10^5 cells per microtiter well) as previously described [4]. The WiDr cell monolayers were incubated at 36°C with 5-80 μg of radiolabeled *M. pneumoniae* cell protein for one hour, the monolayers were washed,

and the washed monolayers with adherent radiolabeled mycoplasmas were counted by liquid scintillation spectrometry.

Inhibition studies were carried out in the following manner: WiDr cells were preincubated with unlabeled mycoplasma suspensions or with the extract prepared by treating *M. pneumoniae* with 2 M NaCl [2]. Suspensions of radiolabeled *M. pneumoniae* were then added and the standard attachment assays were performed. Binding component activity in the extract was calculated by subtracting the attachment value obtained in the presence of extract from the control value obtained without extract (Δ attachment). To determine the receptor site activity of sialic acid-containing glycoconjugates, the labeled *M. pneumoniae* suspension was preincubated with gangliosides or sialic acid-containing glycoproteins for 30 minutes at 36°C. WiDr monolayers were then added, and attachment was determined with the standard procedure [4]. Human ceruloplasmin was from Sigma Chemical Co. (St. Louis, MO), and bovine brain gangliosides and human plasma orosomucoid were from Calbiochem-Behring Corp. (La Jolla, CA).

RESULTS AND DISCUSSION

Micro-Assay for M. pneumoniae Attachment to WiDr Cell Monolayers

Early experiments showed that cells in suspension were unsuitable for standardized, reproducible attachment assays [3], and procedures were developed for using cell culture monolayers in the assays. WiDr cells from a human intestinal carcinoma were selected because they are of human origin and are epithelial-like [5]. A number of cell cultures were screened for *M. pneumoniae* attachment using a DNA staining procedure [6], and comparative studies indicated that the WiDr cell line was superior for *M. pneumoniae* attachment. The time course for the attachment of *M. pneumoniae* to the WiDr cell monolayers at 36°C showed that binding occurred in one hour, while background attachment to control coverslips without cells was less than 10 percent of the attachment to the monolayers [3]. An incubation time of one hour was used in the standard attachment assay.

The micro-attachment assay was used to examine the attachment capacity of radiolabeled strains PI-1428, M129, MAC, FH, and B176 [7]. The binding curves for virulent strains M129 and PI-1428, as well as for the laboratory strains MAC and FH, were nearly identical. Saturation of the monolayers was obtained at approximately 40 μg of mycoplasmal protein per assay. However, attachment of avirulent strain B176 was only 20–30 percent of the level obtained with the other strains.

WiDr cell monolayers of various cell densities were obtained by plating 6×10^3 to 2×10^5 cells per microtiter well on 5 mm coverslips. Attachment was proportional to the number of WiDr cells added, and maximum attachment was achieved using confluent monolayers (approximately 30 μg of protein [8]) obtained with 10^5 cells per well [4]. The monolayer protein content was related to the number of cells added to the wells and closely paralleled the attachment. Hence, standard attachment activity was defined as ng of *M. pneumoniae* protein attached per μg of WiDr monolayer protein, which allowed direct comparison of different experiments. With 10 to 20 μg of *M. pneumoniae* added per monolayer, 4 to 8 percent of the mycoplasmas adhered to the monolayers in one hour. Similar values were observed for attachment to hamster tracheal organ cultures, tracheal outgrowth monolayers, and human erythrocytes [3]. It is possible that only a fraction of the radiolabeled mycoplasmas are capable of attaching to mammalian cells, as shown for the adherence of *M. gallisepticum* to human erythrocytes [9].

Attachment Inhibition: Assay for Mycoplasmal Binding Components

Inhibition studies were performed by preincubating the WiDr monolayers with unlabeled *M. pneumoniae* or the protein-rich extract. Radiolabeled *M. pneumoniae* was then added as described for the standard attachment assay. The capacity of unlabeled *M. pneumoniae* to inhibit attachment of ^3H -*M. pneumoniae* was examined. When the WiDr monolayers were preincubated with equivalent amounts of unlabeled organisms, subsequent attachment of radiolabeled mycoplasmas was inhibited by 50 to 60 percent [4].

We have described a protein-rich extract of *M. pneumoniae* which has the capacity to agglutinate human erythrocytes [2]. The extract was prepared by incubating *M. pneumoniae* with 2 M NaCl followed by freeze-thaw, ultracentrifugation, and dialysis and lyophilization of the supernatant [2]. The hemagglutinating activity of the extract indicates that it contains solubilized mycoplasma binding components which should inhibit attachment. Figure 1 demonstrates that *M. pneumoniae* attachment was progressively inhibited with increasing levels of the protein-rich extract. To calculate binding component activity, the attachment in the presence of extract was subtracted from the control value obtained without extract, as illustrated in Fig. 1. This difference, Δ attachment, can be calculated for each concentration of extract in the assay and provides an estimate of the binding capacity. The resultant hyperbolic dose-response curves show saturation of the monolayers by binding components in the extract and closely resemble the binding curves obtained with radiolabeled *M. pneumoniae* [4,7].

A major advantage of the micro-attachment procedure is its sensitivity for attachment inhibition. Saturation was observed with only 40 μg of *M. pneumoniae* per assay. Saturation at low levels of mycoplasma occurs because of the small number of target cells, and hence limited receptor sites, contained in monolayers on small 5 mm coverslips. The resulting high ratio of mycoplasmas to host target cells permits the solubilized binding components to compete effectively with labeled mycoplasmas for the limited number of receptor sites. This conclusion is supported by experimental data: attachment inhibition by the extract was low or not detectable when saturation was not observed, i.e., when hamster tracheal organ cultures or human erythrocytes were used as the target cells [3]. In contrast, inhibition by the protein-rich extract was readily detectable when WiDr cell culture monolayers or hamster tracheal outgrowth monolayers were used in the micro-attachment assay

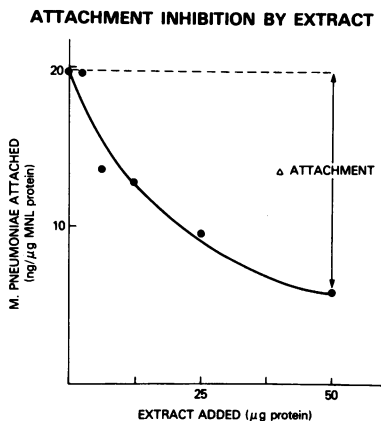


FIG. 1. Attachment inhibition by *M. pneumoniae* protein-rich extract. WiDr monolayers containing 19 μg protein were preincubated with extract at the indicated concentrations for 60 minutes. Radiolabeled *M. pneumoniae* (strain PI-1428, 11 μg , 1636 cpm/ μg protein) was then added and attachment was determined with the standard assay. Δ attachment for each concentration of extract was determined by subtracting the attachment in the presence of extract from the control value without extract.

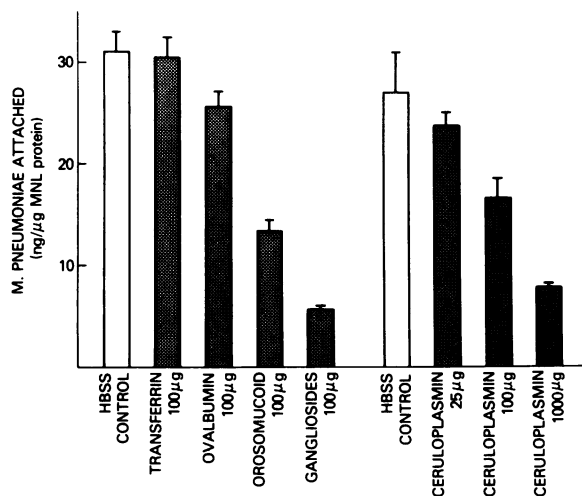


FIG. 2. Effect of glycoproteins and gangliosides on *M. pneumoniae* attachment. Radiolabeled *M. pneumoniae* (Experiment 1, left, 13 μg strain PI-1428, 1636 cpm/μg; Experiment 2, right, 14 μg strain PI-1428, 1720 cpm/μg) was preincubated with glycoproteins or gangliosides as shown for 30 minutes at 36°C. WiDr cell monolayers (Experiment 1, 32 μg protein; Experiment 2, 37 μg protein) were then added and attachment was determined with the standard assay.

[3] Thus, the micro-attachment assay provides the appropriate mycoplasma/receptor site ratio for quantitative inhibition studies.

It is noteworthy that the hyperbolic attachment curves obtained with the mycoplasmal binding components as well as with the intact cells approximate the Langmuir adsorption isotherms expected for receptor-ligand binding [10]. Moreover, the Lineweaver-Burke double reciprocal plots of the binding data from these curves were linear [Chandler DKF: unpublished results]. These observations provide further evidence that the attachment of radiolabeled mycoplasmas and the attachment inhibition by binding components in the extract represent specific interactions with receptor components of the WiDr cells.

Attachment Inhibition: Detection of Target Cell Receptor Analogues

Attachment inhibition was also observed when the radiolabeled *M. pneumoniae* was treated with certain select sialoglycoconjugates. Figure 2 shows the results of two experiments in which we examined attachment inhibition by transferrin, ovalbumin, orosomuroid, gangliosides, and ceruloplasmin. Inhibition by transferrin and ovalbumin was less than 20 percent, while attachment inhibition by orosomuroid and gangliosides was 57 percent and 82 percent, respectively. In the second experiment, the dose response for ceruloplasmin shows progressive inhibition with increasing concentration (data plotted from [4]).

Previous work has indicated that receptor sites on the host cell surface comprise sialic acid-containing glycoconjugates [11-16]. Attachment of *M. pneumoniae* to WiDr cells was inhibited by gangliosides, ceruloplasmin, and orosomuroid, which supports this concept of the host cell receptor. Gangliosides, ceruloplasmin, and orosomuroid are sialoglycoconjugates which may contain structural analogues of the specific cell receptors that bind to the mycoplasmas, thereby blocking attachment to target cells. Hence, the relative binding activities of these and other sialoglycoconjugates may also provide information about the specificity of the receptor sites.

CONCLUSIONS

The WiDr cell monolayer micro-attachment assay provides the appropriate mycoplasma/receptor site ratio for quantitative inhibition studies and binding

analyses. Mycoplasmal binding components in a protein-rich extract of *M. pneumoniae* can be quantitated using the attachment inhibition assay, and the extract presents a suitable source for the characterization and purification of the binding components. The WiDr cell monolayer attachment inhibition assay can also identify host cell receptor activities. Thus, the WiDr cell culture monolayers provide an important *in vitro* model to examine *M. pneumoniae* attachment mechanisms.

REFERENCES

1. Barile MF, Chandler DKF, Yoshida H, et al: Hamster challenge potency assay for evaluation of *Mycoplasma pneumoniae* vaccines. *Israel J Med Sci* 17:682-686, 1981
2. Chandler DKF, Barile MF: Ciliostatic, hemagglutinating, and proteolytic activities in a cell extract of *Mycoplasma pneumoniae*. *Infect Immun* 29:1111-1116, 1980
3. Chandler DKF, Collier AM, Barile MF: Attachment of *Mycoplasma pneumoniae* to hamster tracheal organ cultures, tracheal outgrowth monolayers, human erythrocytes, and WiDr human tissue culture cells. *Infect Immun* 35:937-942, 1982
4. Chandler DKF, Grabowski MW, Barile MF: *Mycoplasma pneumoniae* attachment: competitive inhibition by mycoplasmal binding component and by sialic acid-containing glycoconjugates. *Infect Immun* 38:598-603, 1982
5. Noguchi P, Wallace R, Johnson J, et al: Characterization of WiDr: a human colon carcinoma cell line. *In Vitro* 15:401-408, 1979
6. Barile MF, Grabowski MW: Mycoplasma-cell culture-virus interactions: a brief review. In *Mycoplasma Infection of Cell Cultures*. Edited by GJ McGarrity, DG Murphy, WW Nichols. New York, Plenum Publishing Corp, 1978, pp 135-150
7. Chandler DKF, Razin S, Stephens EB: Genomic and phenotypic analyses of *Mycoplasma pneumoniae* strains. *Infect Immun* 38:604-609, 1982
8. Lowry OH, Rosebrough NJ, Farr AL, et al: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275, 1951
9. Banai M, Kahane I, Razin S, et al: Adherence of *Mycoplasma gallisepticum* to human erythrocytes. *Infect Immun* 21:365-372, 1978
10. DeMeyts P: Insulin and growth hormone receptors. In *Methods in receptor research*, Vol 9, Part 1. Edited by M Blecher. New York, Marcel Dekker, 1976, pp 343-356
11. Banai M, Razin S, Bredt W, et al: Isolation of binding sites to glycophorin from *Mycoplasma pneumoniae* membranes. *Infect Immun* 30:628-634, 1980
12. Feldner J, Bredt W, Kahane I: Adherence of erythrocytes to *Mycoplasma pneumoniae*. *Infect Immun* 25:60-67, 1979
13. Gabridge MG, Taylor-Robinson D: Interaction of *Mycoplasma pneumoniae* with human lung fibroblasts: role of receptor sites. *Infect Immun* 25:455-459, 1979
14. Hansen EJ, Wilson RW, Clyde WA Jr, et al: Characterization of hemadsorption-negative mutants of *Mycoplasma pneumoniae*. *Infect Immun* 32:127-136, 1981
15. Powell DA, Hu PC, Wilson M, et al: Attachment of *Mycoplasma pneumoniae* to respiratory epithelium. *Infect Immun* 13:959-966, 1976
16. Sobeslavsky O, Prescott B, Chanock RM: Adsorption of *Mycoplasma pneumoniae* to neuraminic acid receptors of various cells and possible role in virulence. *J Bacteriol* 96:695-705, 1968