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Multiphasic Interactions of *Mycoplasma pulmonis* with Erythrocytes Defined by Adherence and Hemagglutination

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The mechanism(s) of interaction between *Mycoplasma pulmonis* and eucaryotic cells was studied by adherence to and hemagglutination of erythrocytes. Simple and complex carbohydrates and glycoproteins were unable to inhibit either adherence or hemagglutination, indicating that neither was a lectin activity. Both interactions appeared to be hydrophobic due to their requirement for salt and their sensitivity to temperature. Hemagglutination, but not adherence, was inhibited by both trypsin and glutaraldehyde treatment of the mycoplasma, suggesting that adherence and hemagglutination are qualitatively different. The erythrocyte receptor sites for the two activities were also separable since hemagglutination, but not adherence, required trypsinization of erythrocytes. The hemagglutinin was shown to be an integral mycoplasma component and not a broth contaminant. Once removed, hemagglutinating activity could not be replenished by incubation in serum or broth at 4°C, but could be regenerated during protein synthesis under nonreplicative conditions. Thus, a mycoplasma membrane protein was detected which was capable of interacting with opposing membrane surfaces through hydrophobic interactions. Consequently, a multiphasic model of *M. pulmonis*-eucaryotic cell interactions was proposed.

Mycoplasma pneumoniae, *Mycoplasma gallisepticum*, and *Mycoplasma pulmonis* are cell wall-less procaryotes which colonize the mucosal surfaces of their specific hosts (humans, poultry, and rodents, respectively) (34). Since their pathogenic potential seems to stem from membrane-membrane interactions with eucaryotic cell surfaces (34), these interactions are of particular interest. *M. pneumoniae* and *M. gallisepticum* host cell receptor sites are neuraminidase sensitive, and the adherence sites on the mycoplasmas are proteolytic sensitive (2, 3, 7, 10, 13). Further, hemadsorption and adherence are related since both are affected by proteolytic and neuraminidase treatments (10, 24, 25). *M. pulmonis*, unlike *M. pneumoniae* (16) and *M. gallisepticum* (2), does not have a readily apparent attachment organelle (4–6). Also, preliminary nonquantitative studies with *M. pulmonis* indicate that its interactions with eucaryotic cells are not neuraminidase sensitive (18, 19, 26) and are thus not amenable to the same type of analysis.

An understanding of the nature of the cell-cell interactions during *M. pulmonis* infections is important for several reasons. Due to the lack of several de novo synthetic capabilities, mycoplasmas must acquire host cell components for growth. Since some of these materials, such as fatty acids, cholesterol, and nucleic acid precursors, are not readily diffusible across the aqueous milieu of the mucosal cell surface or are compartmentalized within the cell, the mycoplasma must maintain an intimate association with the cell membrane to acquire them. Active acquisition of these materials by mycoplasmas is conjectural at this time, but it is certainly possible that *M. pulmonis* has surface structures which can function in vitro or in vivo to acquire host membrane components and other cellular constituents.

M. pulmonis-eucaryotic cell interactions could best be studied by a simple, reproducible model based on a cell which (i) could be probed or modified with a variety of agents while retaining the molecular topography of the eucaryotic cell surface, (ii) is not subject to alterations of its membrane composition and structure by external stimuli, and (iii) is available in essentially a pure state, in large quantities. Erythrocytes (RBCs) were chosen for the present studies because they meet all of these criteria and also because of our detailed knowledge of their membrane structure, including the receptor sites for *M. pneumoniae* and *M. gallisepticum*.

The specific objectives of the present study were to quantitate and characterize adherence (the first step in cell association), and to investigate hemagglutination (HA) as a means of defining possible surface lectin activities which may mediate cellular communication (28). The results give further evidence that the mechanisms for cell association of *M. pulmonis* are fundamentally different from those of *M. pneumoniae* and *M. gallisepticum* and that *M. pulmonis* adherence and HA are mediated through two distinct mycoplasma components. Based on these findings, a multistep model for cell association by *M. pulmonis* is proposed.

MATERIALS AND METHODS

Microorganisms. Strain UAB6510 (fifth artificial passage) of *M. pulmonis* was used throughout this study. It was originally isolated from the lungs of a naturally infected rat and was cloned three times and identified as a pure culture by immunofluorescence (9).

Growth media and culture conditions. Edwards medium was used to grow radiolabeled organisms for the RBC-binding assays (31). For the HA studies, *M. pulmonis* was grown in dialyzed medium to reduce broth component contamination (29). The dialyzed broth base was prepared as described (29) except that brain heart infusion (Difco Laboratories, Detroit, Mich.) was used in lieu of soy peptone. The dialyzed base was supplemented with 10% agamma horse serum (GIBCO Laboratories, Grand Island, N.Y.), 0.5% glucose, and 0.05% thallium acetate (Sargent-Welch Scientific Co., Skokie, Ill.).

For RBC-binding studies, *M. pulmonis* was radiolabeled with [^{35}S]methionine. Early-log-phase cultures (pH 7.6) were centrifuged at 8,000 × *g* for 20 min at room temperature

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and resuspended in a nonreplicative maintenance medium of Hanks balanced salt solution (GIBCO) containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Research Organics, Inc., Cleveland, Ohio), 0.5 mg of glucose per ml, and 10% dialyzed horse serum, pH 7.6 (HBSS). The horse serum was prepared by dialysis against 40 volumes of phosphate-buffered saline (PBS) (0.14 M NaCl, 0.010 M Na_2HPO_4, pH 7.3) overnight at 4°C, followed by sterile filtration. Two μCi of [^{35}S]methionine per ml (Amersham Corp., Arlington Heights, Ill.) was added and incubated for 4 h at 37°C, an equal volume of warm Edwards medium was added, and the suspension was incubated for an additional 2 h. The organisms were then washed twice with PBS containing 0.5 mg of glucose per ml (PBS-glc) by centrifugation at 8,000 × *g* for 20 min and resuspended to about 1 mg of protein per ml (protein dye reagent; Bio-Rad Laboratories, Richmond, Calif.) in the same buffer. Immediately before use in the binding assays, all mycoplasma suspensions were centrifuged at 500 × *g* for 4 min to remove aggregates.

For HA studies, the cells from an overnight *M. pulmonis* culture were washed once with PBS, pH 7.3, resuspended to 1 to 2 mg of protein per ml in PBS, and sonicated for 15 s on ice with a Biosonik IV sonicator (Bronwill VWR Scientific, San Francisco, Calif.) equipped with a cup tip at 30% output, on high setting. Aggregation of the mycoplasma suspensions was monitored microscopically by adding acridine orange (Allied Chemical, Morristown, N.J.) to the suspension (25 μM final concentration), followed by epifluorescence observation. CFU were determined by plating 10-fold dilutions on agar plates (15) and counting the colonies after 4 to 5 days of incubation.

Preparation of membranes. Organisms grown in Edwards medium were harvested as above, washed twice with 0.25 M NaCl, and resuspended in a minimal volume of PBS (1 to 2 ml/liter of culture). The suspension was injected into water doubled distilled in glass containing 10 μg of phenylmethylsulfonylfluoride per ml (Sigma Chemical Co., St. Louis, Mo.) which was maintained at 37°C. The lysed organisms were cooled immediately to 4°C and centrifuged at 37,000 × *g* for 20 min to separate the cytoplasmic fraction. The membranes were washed once with 0.05 M sodium phosphate–0.05 M NaCl, pH 7.2, and once with distilled water. When examined by transmission electron microscopy, these preparations contained less than 10% intact organisms.

Preparation of RBCs. For the RBC-binding studies, fresh human or rat blood was drawn into citrate anticoagulant, stored at 4°C, and used within 1 week (2). The RBCs were washed three times with PBS-glc by centrifuging at 370 × *g* for 5 min. The final pellet was resuspended in the same buffer to 2% (vol/vol).

RBCs for the HA assays were collected in citrate anticoagulant from humans, rabbits, rats, and mice. Sheep and cow RBCs in citrate anticoagulant were obtained commercially (Sigma, and Flow Laboratories, Inc., McLean, Va.). All RBC suspensions were prepared and stored as described previously (11), with no detectable drop in HA titers over a 12-month period.

Preparation of lymphocytes. Rats were anesthetized with ketamine hydrochloride (Bristol Laboratories, Syracuse, N.Y.) and pentobarbital sodium (The Butler Company, Columbus, Ohio). Spleens were removed aseptically, minced, and passed through a 250-μm mesh nylon screen into RPMI medium (GIBCO) containing 10 mM HEPES and 10% fetal calf serum, pH 7.3. The cells were pelleted by centrifugation at 1,000 × *g* for 7 min and then layered on Ficoll-Paque lymphocyte separation medium (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), after which they were centrifuged at 500 × *g* for 45 min at room temperature. The cells were then washed in RPMI medium, and phagocytic cells were removed by adherence to plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) for 1 h at 37°C. A portion of lymphocytes was resuspended in RPMI medium without serum, treated with trypsin (100 μg/ml) for 15 min at 37°C, washed twice with RPMI medium without serum, and resuspended to 3 × 10^7 to 4 × 10^7 cells per ml in PBS. The viability of the preparations always exceeded 95% by trypan blue exclusion.

Mycoplasma adherence assay. Adherence mixtures consisting of 0.1 ml of PBS-glc, 0.05 ml of a cell suspension (2% RBCs or 3 × 10^7 to 4 × 10^7 lymphocytes per ml), and 0.1 ml of the mycoplasma suspension in PBS-glc were incubated with shaking at 37°C in plastic or siliconized glass test tubes for 30 min (2). Unbound mycoplasmas were separated from the RBCs by layering the reaction mixture on 0.2 ml of 40% sucrose in PBS in a 0.5-ml microfuge tube and centrifuging at 1,000 × *g* for 4 min. The upper two-thirds of the sucrose layer was aspirated, the tube was quickly frozen on dry ice-acetone, and the tip was sliced immediately and placed in a scintillation vial. Protosol (New England Nuclear Corp., Boston, Mass.) was added to each vial (total volume of 0.5 ml) and left overnight at room temperature. Ten milliliters of Econofluor (New England Nuclear) was added, and the samples were counted by liquid scintillation.

HA assay. The HA assay was performed as described previously (12) in V-bottom microtiter plates (CoStar, Cambridge, Mass.). Each test was run in duplicate with a minimum of three replications. An agglutination unit was defined as the reciprocal of the last dilution giving a positive reaction. A partially agglutinated well was considered as 0.5 well.

Adherence and HA inhibition studies. These studies were performed by adding the test substance to the adherence reaction mixture at the indicated concentrations. The effects of preincubation of the mycoplasmas with the test substance was also evaluated. Salt concentrations were tested by washing and resuspending the organisms and RBCs in 0.25 M sucrose containing 10 mM Tris, pH 7.3. Samples of *M. pulmonis* were also treated with 1 mM EDTA (Sigma) or 1 mM ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N'*,*N'*-tetraacetic acid (Sigma) in PBS by diluting the mycoplasmas to 0.1 to 0.2 mg of protein per ml of solution. The cells were incubated for 1 h at 37°C, washed twice with PBS, resuspended to the original volume, sonicated, and then tested for HA activity as described above. Glutaraldehyde-treated mycoplasmas were obtained by incubation with 0.25 or 1% glutaraldehyde (Sigma) in PBS for 30 to 60 min on ice, followed by the addition of glycine (50 mM) and further incubation for 15 to 30 min. The organisms were then pelleted, washed once with PBS, and resuspended in either PBS or PBS-glc.

Enzyme treatments. Human and rat RBCs were treated with *Vibrio cholerae* neuraminidase (Behring-werke AG, Marburg-Lahn, Federal Republic of Germany) as described previously (2) with slight modifications. A 10% RBC suspension in PBS-glc was treated with 0.01 U of enzyme per ml for 30 min at 37°C and washed, and the released sialic acid was determined as described (1). *M. pulmonis* suspensions were treated with trypsin (Sigma) at a concentration of 10 to 100 μg of enzyme per ml of *M. pulmonis* protein for 30 to 60 min at 37°C, except where noted. Pronase (lot 102613; activity, 45,000 PUK/g; Calbiochem-Behring, La Jolla, Calif.) was

used at a concentration of 5 to 50 μg per mg of *M. pulmonis* protein. After treatment, the mycoplasmas were washed three times with PBS by centrifugation (Beckman microfuge, 2 min) with or without the trypsin inhibitor *Nα-p*-tosyl-L-lysine chloromethyl ketone hydrochloride (10^{-4} M; Sigma). All final pellets were resuspended to original volumes and, in the case of the HA assay, were sonicated for 15 s before use. Controls (untreated organisms) were washed and incubated at the specified times simultaneously with the treated organisms.

Glycoproteins and oligosaccharides. Fetuin and ovalbumin were purchased from Sigma. Human glycophorin A was prepared according to the method of Segrest et al. (33). A human breast milk oligosaccharide fraction was prepared by the method of Kobata (20) and contained greater than 300 mg of carbohydrate per ml by the anthrone reaction (35).

Regeneration of the hemagglutinin. Regeneration of the hemagglutinin was tested by incubating trypsin-treated mycoplasmas in HBSS nonreplicative maintenance medium for 5 h at 37°C, washing once with PBS, and then testing for HA activity. Portions of the trypsin-treated organisms were also incubated in the presence of 100 μg of chloramphenicol per ml (Merrell Pharmaceuticals, Inc., Cincinnati, Ohio) or 40 μg of mitomycin C per ml (Sigma).

Statistical analysis. The data from each group of experiments were analyzed by analysis of variance. Variation was considered significant at a P value <0.05.

RESULTS

Mycoplasma adherence. The adherence of *M. pulmonis* to fresh human RBCs was measured as a function of time, temperature, and salt (Fig. 1). Binding was significantly reduced in the absence of salt and at 0°C. Membranes were found to adhere as well as intact cells (data not given). Adherence of *M. pulmonis* to rat RBCs was either comparable with or at the most twice that of *M. pulmonis* to human RBCs (data not given).

Figure 2 compares the effect of trypsin on *M. pulmonis* binding to human RBCs and rat spleen lymphocytes. Trypsin treatment of *M. pulmonis* had no effect on binding to RBCs (Fig. 2). However, trypsin-treated RBCs (tRBCs) bound *M. pulmonis* better than normal RBCs ($P < 0.001$). This increased binding could be removed by trypsin treatment of the mycoplasma. *M. pulmonis* bound more efficiently to lymphocytes than to RBCs ($P = 0.009$), but no difference was observed between tRBCs and lymphocytes. Trypsin treatment of the lymphocyte population had no effect on mycoplasma binding, but trypsin treatment of *M. pulmonis* increased binding to lymphocytes ($P = 0.003$).

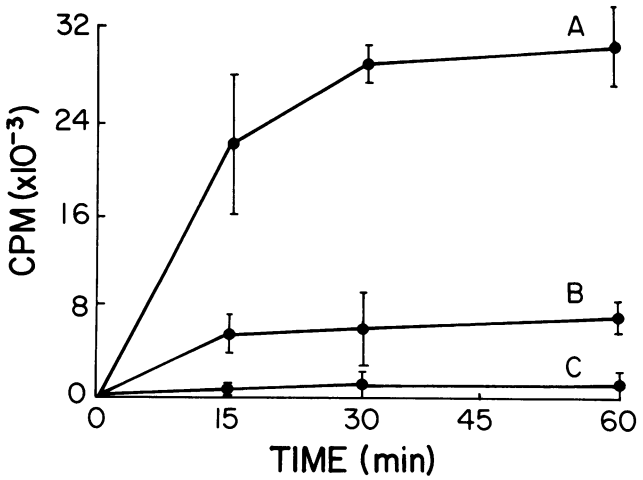

FIG. 1. Attachment of *M. pulmonis* to human RBCs as a function of time, salt, and temperature. Radiolabeled *M. pulmonis* ([^{35}S]methionine) was reacted with human RBCs in PBS at 37°C (A) or 0°C (C). Organisms were also reacted in 0.25 M sucrose–10 mM Tris (pH 7.3) in the absence of salt (B). Data represent the mean ± standard deviation of duplicates.

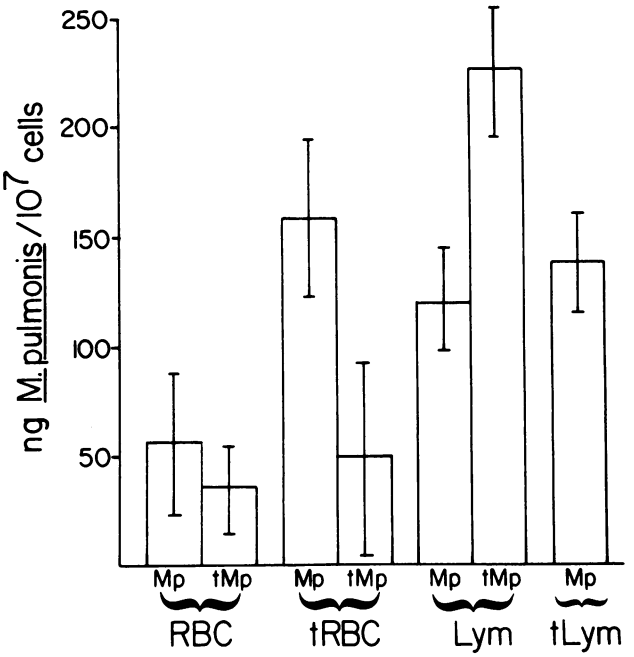

FIG. 2. The effect of trypsin treatment (t) on the binding of *M. pulmonis* (Mp) to human RBCs and rat spleen lymphocytes (Lym). The protein bound was determined from the specific activity (counts per minute per milligram of protein) of [^{35}S]methionine-labeled mycoplasmas. Equal amounts of mycoplasmas were added to both RBCs and lymphocytes. Data represent the mean ± standard deviation of three experiments (RBCs) or two experiments (Lym).

HA. To test the possibility that *M. pulmonis* could bind to cryptic RBC receptors (32) and cause agglutination, trypsinized as well as nontrypsinized RBCs were tested in a HA microtiter assay. In confirmation of earlier reports (24), no HA was observed with untreated RBCs. After trypsin treatment, however, the RBCs were agglutinated by *M. pulmonis*.

The effect of formaldehyde treatment of the RBC was tested by comparing tRBCs with and without formaldehyde treatment. In addition, formaldehyde-fixed tRBCs were treated with PBS plus 100 mM glycine or 50 mM Tris, pH 7.3, to block any possible chemical reactive sites. Nontrypsinized, formaldehyde-fixed RBCs were also tested. In all cases formaldehyde had no effect on HA.

M. pulmonis agglutinated formaldehyde-fixed tRBCs from all animal species tested (100 to 400 HA units per mg of protein). Sonication of the mycoplasma suspension under mild conditions was necessary to achieve maximum HA activity (at least a fourfold increase), but it had no effect on mycoplasma viability (data not given). This increased activity was due to disruption of mycoplasma aggregates, as determined by acridine orange staining.

Effect of culture conditions on HA. To test the possibility that HA activity is sensitive to medium effects, organisms

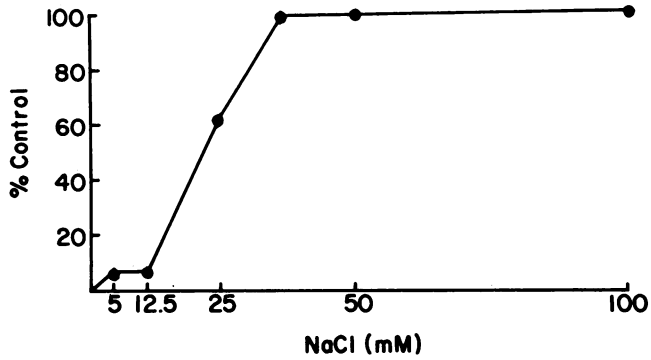

FIG. 3. Effects of NaCl on HA. Organisms and RBCs were washed in 0.25 M sucrose–10 mM Tris (pH 7.3). HA was measured in a standard microtiter assay with trypsin-treated, formaldehyde-fixed human RBCs. NaCl was added at the indicated concentrations. Data represent the mean of duplicates.

were grown in Edwards or dialyzed medium supplemented with various sera and harvested at various points in the growth cycle. Organisms grown in Edwards medium or in dialyzed medium supplemented with various sera and harvested in mid-log phase (pH 7.2) did not differ in HA activity toward human RBCs (data not given). However, culture age did affect HA activity. Maximum activity was always obtained with organisms in late-log phase (pH 6.8 to 7.2) as opposed to those in early-log (pH 7.8 to 8.0) or stationary phase (pH <6.8) (data not given).

Inhibition of adherence and HA. HA is characteristic of lectin-binding activities (28), and to identify possible *M. pulmonis* surface lectin activity, simple carbohydrates were tested for adherence inhibition activity with human RBCs and for HA inhibition activity against both human and rat RBCs (see below). None of the carbohydrates tested had inhibitory activity at the indicated concentrations. Complex oligosaccharide preparations from three different donors of human breast milk also had no inhibitory activity (1 to 1.5 mg of carbohydrate per ml).

All carbohydrates were tested in the HA assay at a 30 mM concentration against human and rat RBCs. These include D-glucose, D-glucosamine, *N*-acetyl glucosamine, D-galactose, D-galactosamine, *N*-acetyl galactosamine, D-mannose, D-mannosamine, D-α-methyl mannoside, D-1-0-methyl-α-D-glucopyranoside, L-fucose, D-fucose, *N*-acetyl neuraminic acid, thiodigalactoside, rhamnose, lactose, sucrose, maltose, raffinose, melezitose, melibionic acid, melibiose, trehalose, salicin, cellobiose, stachyose, and gentiobiose. D-glucosamine, *N*-acetyl glucosamine, D-galactose, D-galactosamine, *N*-acetyl galactosamine, D-α-methyl mannoside, L-fucose, D-fucose, rhamnose, lactose, sucrose, and maltose were also tested in the HA assay at a 60 mM concentration. The carbohydrates tested in the adherence assay at a 100 mM concentration against human RBCs were D-glucosamine, *N*-acetyl glucosamine, D-galactose, D-galactosamine, *N*-acetyl galactosamine, L-fucose, D-fucose, *N*-acetyl neuraminic acid, and thiodigalactoside.

Purified glycophorin, the major RBC surface glycoprotein, had no effect on HA or adherence, although it did aggregate mycoplasmal suspensions when incubated for 20 min at 37°C at a final concentration of 250 μg/ml. Mild sonication disrupted these aggregates and allowed full expression of HA activity.

Other substances found to have no effect on HA were sodium azide (0.02%), 2-mercaptoethanol (0.1%), ovalbumin (10 mg/ml), and fetuin (10 mg/ml). The latter was also tested in the adherence assay (1 mg/ml) and found to have no effect.

To test the possibility that media contaminants were responsible for HA, complete media (1:1 dilution in PBS), horse serum (1%), bovine serum albumin (1%), and fetal calf serum (1%) were tested and found to have no effect.

The effects of EDTA and ethylene glycol-bis(β-amino ethyl ether)*N*,*N*,*N*′,*N*′-tetraactic acid on HA were also tested at various concentrations with human RBCs. There was no effect at 0.1 mM, but higher concentrations showed reduced activity: 70% of control at 1 mM and 35% of control at 10 mM. When *M. pulmonis* was treated with 1 mM EDTA or ethylene glycol-bis(β-amino ethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid for 1 h at 37°C and then washed and tested, less than 10% of the HA activity remained in the preparations (data not given).

Glutaraldehyde treatment of *M. pulmonis* did not affect adherence to RBC but did eliminate all HA activity (data not given). Heat treatment (56°C, 15 min) inhibited both adherence and HA (data not given).

Effect of salt, temperature, and pH on HA. In addition to lectin activities, bacterial adhesion is also mediated by hydrophobic interactions (22) which are sensitive to salt concentrations, temperature, and pH (8). In the absence of NaCl (Fig. 3), there was no HA, and there appeared to be a linear relationship between HA and NaCl concentrations in the range of 12 to 40 mM. Magnesium and calcium could replace NaCl in the buffer at 1 to 10 mM, but not at 0.1 mM (data not given). HA was also reduced by 85% at 4°C as compared with 23 and 37°C (data not given). The pH of the buffer had no effect on HA in the physiological range of 7.0 to 8.0, but there was a reduction to 35% of control at pH 6.5 (data not given).

Enzymatic treatment effects on HA. In contrast to adherence (Fig. 2), HA was extremely sensitive to proteolytic treatments (Table 1). Trypsin at 1 μg/ml reduced HA activity to 40% of control with only a 7% loss of protein. Other experiments with the trypsin inhibitor *N*α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride showed that neither the inhibitor alone nor trypsin plus inhibitor (10^{-4} M) had an effect on HA. Thus, trypsin was not inhibiting HA activity by binding to the mycoplasma but rather by its proteolytic function. The possibility that trypsin treatment induced aggregation of the mycoplasma suspension was excluded by monitoring trypsin-treated, sonicated preparations by acridine orange staining and epifluorescence for 1.5 h, the duration of the HA assay.

Replacement or regeneration of the trypsin-sensitive hemagglutinin. To show that HA activity was associated with a mycoplasma protein and not derived from the growth media, the replacement or regeneration of the hemagglutinin was attempted. Trypsin-treated mycoplasmas were incubated in broth, 1% horse serum, or 1% bovine serum albumin at 4°C for 1 h. The trypsin-sensitive hemagglutinin could not be replenished in this way.

Regeneration of HA was tested by incubating trypsin-treated organisms in HBSS maintenance medium. This medium was shown to sustain mycoplasma viability without replication by measuring CFU during the experiment (data not given). After a 5-h incubation period, there was regeneration of HA activity (Table 2). The requirement for protein synthesis, and not nucleic acid synthesis, in regeneration was demonstrated by the ability of the organisms to regain HA activity in the presence of mitomycin C (Table 2) but not in the presence of chloramphenicol.

DISCUSSION

The present studies demonstrate the ability of *M. pulmonis* to adhere to and agglutinate tRBCs and to adhere to, but not agglutinate, untreated RBCs. Previous studies have shown that *M. pulmonis* colonies on agar could hemadsorb untreated RBCs, but not agglutinate the same in suspension (24). The abilities to adhere and hemadsorb and the inability to agglutinate untreated RBCs seem contradictory since these phenomena all require interactions with the RBC surface. Several explanations for these discrepancies are possible. Weak binding interactions of individual organisms may be sufficient to cause hemadsorption on colonies, but may not be strong enough to effectively cross-link RBCs, thereby causing agglutination. Adherence, and possibly hemadsorption, may be univalent in nature, or once bound, *M. pulmonis* may lose its affinity for other RBC adherence receptor site(s). Another possibility, which is suggested by the present studies, is that adherence and hemadsorption are mediated by mycoplasma elements other than those involved in HA, and that their respective host cell receptor sites are different.

Adherence and HA have been differentiated in the present studies by proteolytic and glutaraldehyde sensitivities. Failure of RBC adherence to be blocked by proteolytic, neuraminidase, and glutaraldehyde treatments is consistent with the failure of these treatments to block adherence of *M. pulmonis* to mouse macrophages (18). Thus, the similarities of *M. pulmonis* adherence to both RBCs and macrophages suggest that the RBC is a valid model in investigations of *M. pulmonis*-eucaryotic cell interactions at the cellular and molecular levels.

Hydrophobicity plays an important role in bacterial adhesion, as has been shown with *Salmonella* sp., *Escherichia coli*, and *Neisseria gonorrheae* (23). The requirement for salt and the sensitivity to temperature indicate that both *M. pulmonis* adherence to fresh RBCs and agglutination of tRBCs are hydrophobic interactions. Since proteolytic treatment of the mycoplasma was capable of removing HA activity, an *M. pulmonis* protein seemed to be involved. The protein nature was further established by regeneration experiments in which chloramphenicol, but not mitomycin C, blocked regeneration of the HA activity. Thus it appeared that an *M. pulmonis* membrane protein(s) was capable of interacting with the opposing RBC surface through hydrophobic interactions.

To incorporate the separate phenomena of adherence and HA into a working hypothesis, we propose that the interaction of *M. pulmonis* with eucaryotic cells is a multiphasic process involving an initial recognition event, the exposure of additional binding sites by the induction of membrane protein diffusion on the eucaryotic cell surface, and the recognition and binding to these additional receptors by the mycoplasma (Fig. 4). The culmination of these events is an intimate association between the membranes and a possible avenue by which *M. pulmonis* parasitizes host membrane surfaces.

TABLE 1. Effect of proteolytic treatment on HA

Enzyme[a]	Concn[b]	% Control	% Protein lost
Control		100	ND[c]
Trypsin	100	0	25
Trypsin + TLCK	100	100	0
Trypsin	10	0	15
Trypsin	1	40	7
Pronase	50	0	40

[a] Enzyme treatments were for 30 min at 37°C. Organisms were then washed and tested for HA activity. TLCK, Nα-*P*-tosyl-L-lysine chloromethyl ketone hydrochloride.

[b] Micrograms per milligram of *M. pulmonis* protein.

[c] ND, Not done.

TABLE 2. Regeneration of *M. pulmonis* HA activity

Treatment[a]	HA (U/mg of protein)[b]
None	134 ± 45
Trypsin treated only	0
Trypsin treated, incubated in HBSS[c]	44 ± 11[d]
Trypsin treated, incubated in HBSS + mitomycin C	27 ± 10[e]
Trypsin treated, incubated in HBSS + chloramphenicol	0

[a] Organisms were treated with trypsin (100 μg/ml of *M. pulmonis* protein) for 1 h at 37°C, washed with PBS, and placed in regeneration media or tested directly for HA activity with human RBCs. Controls consisted of nontrypsinized organisms. Mitomycin C concentration was 40 μg/ml; chloramphenicol concentration was 100 μg/ml.

[b] Data represent the mean ± standard deviation of three experiments. Each treatment was tested in duplicate.

[c] HBSS, Hanks balanced salt solution with 0.5% glucose, 10% dialyzed horse serum, and 10 mM HEPES, pH 7.5. Incubation time was 5 h at 37°C. The organisms were then washed and tested for HA activity.

[d] Significantly different from the trypsin-treated only group ($P < 0.001$).

[e] Significantly different from the trypsin-treated only group ($P < 0.001$) and HBSS + chloramphenicol group ($P < 0.001$).

The initial recognition event in *M. pulmonis*-eucaryotic cell interactions very likely involves long-range, attractive, hydrophobic forces because of (i) the large number of cell types to which *M. pulmonis* can bind (4–6), (ii) the seeming inability of the hydrophilic mucosal secretions to block attachment, and (iii) the results of the present studies which indicate a salt-dependent, temperature-sensitive interaction during adherence (22). Once this initial contact has been made, repulsive forces (electrostatic pressure) could induce molecular rearrangements in the host cell surface topography through diffusional movements of highly charged membrane proteins, thereby exposing additional interaction sites on the cell surface and reducing the electrostatic pressure in the immediate vicinity (30). This type of rearrangement, for instance, is a general characteristic of contact and fusion events between opposing membranes (21). Finally, an *M. pulmonis* specific protein(s), now influenced by a reduced electrostatic pressure, could recognize these additional binding sites, and the membranes could become firmly anchored in intimate association. In this model, the initial binding interactions are represented by the adherence of *M. pulmonis* to untreated RBCs. The second phase of interaction is represented by the HA of tRBCs.

The observation that trypsin treatment of the RBC is required for HA can be explained by the proposed model. Under normal conditions, a rearrangement of surface macromolecules could be required for exposing the second phase or HA receptor sites. The surface glycoproteins on RBCs, however, are firmly anchored in the membrane through the cytoskeletal network (14) and are unable to diffuse in response to the approaching mycoplasma surface, unlike other eucaryotic cell types. Trypsin treatment is required to remove these overlying glycoproteins, primarily glycophorin (17), and to expose the underlying HA receptor sites.

The effect of trypsin treatment on *M. pulmonis* binding to

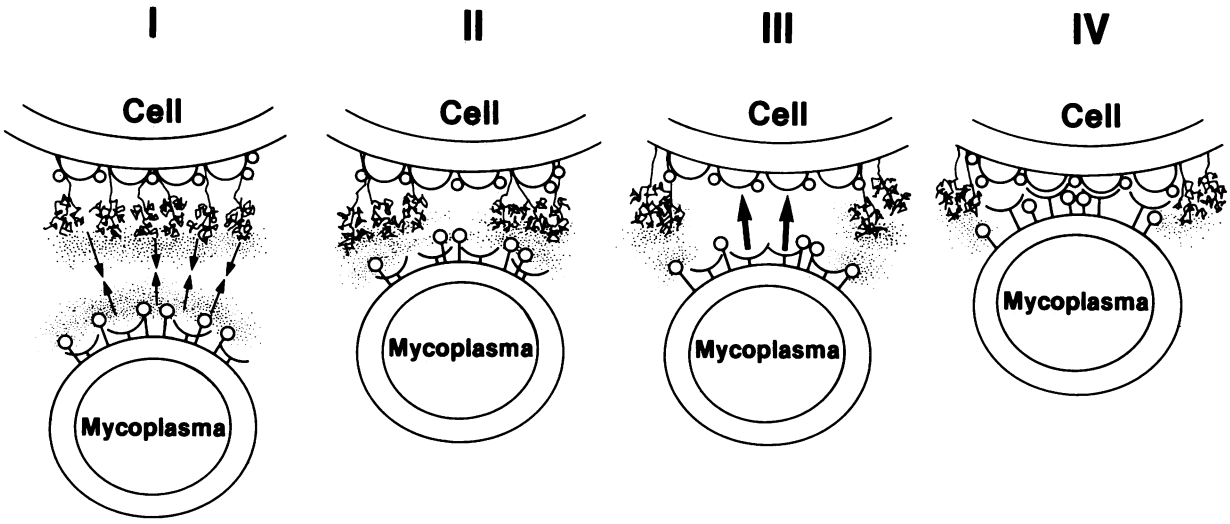

FIG. 4. Proposed model of *M. pulmonis*-eucaryotic cell interactions. (I) Nonspecific interactions dominate the initial recognition phase of the interactions. (II) As the two bilayers approach, repulsive forces induce molecular rearrangements in the cell surface. (III) Diffusional movement of surface proteins uncovers cryptic binding sites. (IV) These sites are recognized and bound by the mycoplasma hemagglutinin, resulting in an intimate association.

human RBCs and rat spleen lymphocytes lends support to this model. If overlying glycoproteins on the RBC surface shield HA binding sites, removal of these proteins should be accompanied by an increase in binding. This was shown to be the case. Further, additional binding activity was removed by trypsin treatment of the mycoplasma, demonstrating that trypsin-sensitive proteins can be involved in cell association once their receptor sites are exposed. If these receptors are present on other cell types which exhibit freely diffusible membrane glycoproteins, then *M. pulmonis* should be able to recognize these receptors without the need for trypsin treatment. Lymphocytes showed increased binding of *M. pulmonis* when compared with RBCs but not with tRBCs, which bound equal amounts of mycoplasmas. Trypsin treatment of the lymphocyte had no effect on binding and thus additional receptor sites were not uncovered by proteolytic treatment. This suggests that receptor sites comparable to those exposed on tRBCs are accessible to the mycoplasma on the lymphocyte cell surface. It should be noted that trypsin-treated *M. pulmonis* appeared to bind better to lymphocytes than untreated mycoplasmas. This could be due to the inherent differences in the membrane composition and structure between the cell types. More likely though, the increase could be due to residual macrophages in the lymphocyte preparations, since they have been shown to actively phagocytize protease-treated *M. pulmonis* but not untreated mycoplasmas (18). The 2 to 5% macrophage contamination in the preparations could account for the apparent increase in binding.

Previous observations of *M. pulmonis* interactions with mucosal epithelial surfaces suggested that its interactions were different than those described with *M. pneumoniae* and *M. gallisepticum* (4–6). Adhesion to host cells was mediated by a generalized interaction with the mycoplasma membrane (which conformed to the shape of the cell) rather than by a specialized attachment tip. In addition, electron micrographs indicated alterations of the host membrane at the site of *M. pulmonis* attachment. These alterations seemed to resemble an intercellular junction, i.e., a nexus. In some instances, membrane fusion appeared to occur between *M. pulmonis* and the host cell; thus, the morphological evidence of *M. pulmonis*-host cell interactions in vivo suggests that adhesion is not a simple attachment event.

This model is speculative, and other possibilities exist for interpretations of this data. It is possible that lymphocyte receptors differ categorically from RBC receptors and that lymphocytes may simply express additional *M. pulmonis* receptors or the same receptor in greater numbers on their surface. Alternatively, the RBC receptors may be unique, and similar receptors may not be present on other cell types. If so, a comparison between RBCs and other mammalian cells may not be useful. It should be noted, however, that both the lymphocyte receptor and the high-affinity RBC receptor are trypsin resistant. This lends credence to the idea that similar receptor sites for *M. pulmonis* exist on many, if not all, mammalian cells, including RBCs. Thus the RBC surface would adequately represent the mammalian cell surface seen by the mycoplasma during in vivo colonization.

In summary, the present studies indicate a multiphasic mode of cell association by *M. pulmonis*. One phase is expressed as HA of tRBCs. This activity is sensitive to treatments which have been shown to remove or alter mycoplasmal membrane proteins, i.e., trypsin and EDTA, and is hydrophobic in nature. It can be regenerated after trypsin removal by incubation in nonpermissive media. The role of the hemagglutinin in vivo is unclear, but it could possibly be the mechanism by which acquisition of host cellular components is achieved. This type of activity could induce severe perturbations in a host cell membrane and cause cellular alterations, cell death, and possibly nonspecific lymphocyte activation (27). The other phase of *M. pulmonis*-cell association, adherence, although it is also hydrophobic in nature, utilizes different mycoplasma elements than does HA since it is not trypsin sensitive and occurs with untreated RBCs. The use of the RBC as a model for cell interactions has provided insight into the molecular events which occur between *M. pulmonis* and host cell surfaces.

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LITERATURE CITED

1. **Aminoff, D.** 1961. Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. **81**:384–391.
2. **Banai, M., I. Kahane, S. Razin, and W. Bredt.** 1978. Adherence of *Mycoplasma gallisepticum* to human erythrocytes. Infect. Immun. **21**:365–372.
3. **Banai, M., S. Razin, W. Bredt, and I. Kahane.** 1980. Isolation of binding sites to glycophorin from *Mycoplasma pneumoniae* membranes. Infect. Immun. **30**:628–634.
4. **Cassell, G. H., J. K. Davis, W. H. Wilborn, and K. S. Wise.** 1978. Pathobiology of mycoplasmas, p. 399–403. *In* D. Schlessinger (ed.), Microbiology—1978. American Society for Microbiology, Washington, D.C.
5. **Cassell, G. H., and A. Hill.** 1979. Murine and other small-animal mycoplasmas, p. 235–273. *In* J. G. Tully and R. F. Whitcomb (ed.), The mycoplasmas. II. Human and animal mycoplasmas. Academic Press, Inc., New York.
6. **Cassell, G. H., W. H. Wilborn, S. H. Silvers, and F. C. Minion.** 1982. Adherence and colonization of *Mycoplasma pulmonis* to genital epithelium and spermatozoa in rats. Isr. J. Med. Sci. **17**:593–598.
7. **Chandler, D. K., A. M. Collier, and M. F. Barile.** 1982. 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.
8. **Chapman, D.** 1982. Protein-lipid interactions in model and natural biomembranes, p. 179–229. *In* D. Chapman (ed.), Biological membranes, vol. 4. Academic Press, Inc., New York.
9. **Davis, J. K., K. M. Delozier, D. K. Asa, F. C. Minion, and G. H. Cassell.** 1980. Interactions between murine alveolar macrophages and *Mycoplasma pulmonis* in vitro. Infect. Immun. **29**:590–599.
10. **Gabridge, M. G., and D. Taylor-Robinson.** 1979. Interaction of *Mycoplasma pneumoniae* with human lung fibroblasts: role of receptor sites. Infect. Immun. **25**:455–459.
11. **Gartner, T. K., D. C. Williams, F. C. Minion, and D. R. Phillips.** 1978. Thrombin-induced platelet aggregation is mediated by a platelet plasma membrane-bound lectin. Science **200**:1281–1283.
12. **Gartner, T. K., D. C. Williams, and D. R. Phillips.** 1977. Platelet plasma membrane lectin activity. Biochem. Biophys. Res. Commun. **79**:592–599.
13. **Glasgow, L. R., and R. L. Hill.** 1980. Interaction of *Mycoplasma gallisepticum* with sialyl glycoproteins. Infect. Immun. **30**:353–361.
14. **Gratzer, W. B.** 1981. The red cell membrane and its cytoskeleton. Biochem. J. **198**:1–8.
15. **Hayflick, L.** 1965. Tissue cultures and mycoplasmas. Tex. Rep. Biol. Med. **23**(Suppl. 1):285–303.
16. **Hu, P. C., R. M. Cole, Y. S. Huang, J. A. Graham, D. E. Gardner, A. M. Collier, and W. A. Clyde, Jr.** 1982. *Mycoplasma pneumoniae* infection: role of a surface protein in the attachment organelle. Science **216**:313–315.
17. **Jackson, R. L., J. P. Segrest, I. Kahane, and V. T. Marchesi.** 1973. Studies on the major sialoglycoprotein of the human red cell membrane. Isolation and characterization of tryptic glycopeptides. Biochemistry **12**:3131–3138.
18. **Jones, T. C., S. Yeh, and J. G. Hirsch.** 1972. Studies on the attachment and ingestion phases of phagocytosis of *Mycoplasma pulmonis* by mouse peritoneal macrophages. Proc. Soc. Exp. Biol. Med. **139**:464–470.
19. **Kahane, I., S. Pnini, M. Banai, J. B. Baseman, G. H. Cassell, and W. Bredt.** 1982. Attachment of mycoplasmas to erythrocytes: a model to study mycoplasma attachment to the epithelium of the host respiratory tract. Isr. J. Med. Sci. **17**:589–592.
20. **Kobata, A., and V. Ginsburg.** 1969. Oligosaccharides of human milk. II. Isolation and characterization of a new pentasaccharide, lacto-*N*-fucopenta-ose III. J. Biol. Chem. **244**:5496–5502.
21. **Lucy, J. A.** 1982. Biomembrane fusion, p. 367–415. *In* D. Chapman (ed.), Biological membranes, vol. 4. Academic Press, Inc., New York.
22. **Magnusson, K.-E.** 1982. Hydrophobic interaction—a mechanism of bacterial binding. Scand. J. Infect. Dis. Suppl. **33**:32–36.
23. **Magnusson, K.-E., J. Davies, T. Grunstrom, E. Kihlstrom, and S. Normack.** 1980. Surface charge and hydrophobicity of *Salmonella*, *E. coli*, and *Gonococci* in relation to their tendency to associate with animal cells. Scand. J. Infect. Dis. Suppl. **24**:135–140.
24. **Manchee, R. J., and D. Taylor-Robinson.** 1968. Haemadsorption and haemagglutination by mycoplasmas. J. Gen. Microbiol. **50**:465–478.
25. **Manchee, R. J., and D. Taylor-Robinson.** 1969a. Studies on the nature of the receptors involved in attachment of tissue culture cells to mycoplasmas. Br. J. Exp. Pathol. **50**:66–75.
26. **Manchee, R. J., and D. Taylor-Robinson.** 1969. Utilization of neuraminic acid receptors by mycoplasmas. J. Bacteriol. **98**:914–919.
27. **Naot, Y., J. G. Tully, and H. Ginsburg.** 1977. Lymphocyte activation by various *Mycoplasma* strains and species. Infect. Immun. **18**:310–317.
28. **Phillips, D. R., and T. K. Gartner.** 1980. Cell recognition systems in eukaryotic cells, p. 401–438. *In* E. H. Beachey (ed.), Bacterial adherence, series B, vol. 6. Chapman and Hall, New York.
29. **Pollack, M. E., and S. V. Bonner.** 1969. Comparison of undefined medium and its dialyzable fraction for growth of *Mycoplasma*. J. Bacteriol. **97**:522–525.
30. **Rand, R. P.** 1981. Interacting phospholipid bilayers: measured forces and induced structural changes. Annu. Rev. Biophys. Bioeng. **10**:277–314.
31. **Razin, S., and S. Rottem.** 1976. Techniques for the manipulation of mycoplasma membranes, p. 3–26. *In* H. Maddy (ed.), Biochemical analysis of membranes. Chapman and Hall, Ltd., London.
32. **Reitherman, R. W., S. D. Rosen, and S. H. Barondes.** 1974. Lectin purification using formalinised erythrocytes as a general affinity adsorbent. Nature (London) **248**:599–600.
33. **Segrest, J. P., T. M. Wilkinson, and L. Sheng.** 1979. Isolation of glycophorin with deoxycholate. Biochim. Biophys. Acta **554**:533–537.
34. **Tully, J. G., and R. F. Whitcomb.** 1979. The mycoplasmas. II. Human and animal mycoplasmas. Academic Press, Inc., New York.
35. **Williams, C. A., and M. W. Chase.** 1969. Chemical analysis, carbohydrate analysis, p. 282–305. *In* C. A. Williams and M. W. Chase (ed.), Methods in immunology and immunochemistry, vol. 2. Academic Press, Inc., New York.