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Identification of *Mycoplasma pneumoniae* Proteins Associated with Hemadsorption and Virulence

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Twenty-two mutants of *Mycoplasma pneumoniae* spontaneously deficient in hemadsorption were isolated. Examination of mutant protein profiles by one- and two-dimensional polyacrylamide gel electrophoresis permitted the grouping of these mutants into four classes. The largest class of mutants was deficient in four high-molecular-weight proteins (215,000, 210,000, 190,000, and 140,000). A second class of mutants lacked three proteins previously designated A, B, and C (72,000, 85,000, and 37,000, respectively). A single mutant, in addition to lacking proteins A, B, and C, was missing a fourth protein of 165,000 molecular weight. The remaining mutants exhibited protein profiles apparently identical to that of the wild-type strain. All mutant strains attached to the respiratory epithelium of hamster tracheal rings in vitro at reduced levels; however, mutants lacking proteins A, B, and C recognized only neuraminidase-insensitive receptors. None of the mutants tested produced detectable pneumonia in intranasally inoculated hamsters, although one mutant class demonstrated low-level survival in vivo.

Mycoplasma pneumoniae is a pathogen of the human respiratory tract which generally produces a self-limiting, cold agglutinin-associated pneumonia in children and young adults. Studies employing the hamster model system, including hamster tracheal organ culture, have advanced significantly our understanding of *M. pneumoniae* pathogenesis. Utilizing a differentiated tip structure, this procaryote adheres to the ciliated respiratory epithelium (6, 7), where it causes parasitized host cells to undergo changes in metabolism and ultrastructure (5, 19, 20, 33). Strains of *M. pneumoniae* which do not adhere to the respiratory epithelium of hamster tracheal rings in vitro do not significantly alter host cell metabolism, nor do they produce pneumonia in experimentally infected hamsters (24, 29). This demonstrates the importance of mycoplasma attachment to subsequent disease development. The relevance of this model is reinforced by the observation of *M. pneumoniae* adherent to respiratory epithelial cells in sputum samples from patients with mycoplasma pneumonia (8).

Previous studies have suggested that mycoplasma binding sites are proteinaceous in nature (13, 21, 31). To identify mycoplasma proteins associated with virulence, Hansen et al. (15) compared the protein content of virulent *M. pneumoniae* with that of a homologous, nonattaching, avirulent strain, using one- and two-dimensional polyacrylamide gel electrophoresis (PAGE). They identified three proteins consistently present in virulent *M. pneumoniae* and absent in avirulent *M. pneumoniae*. Furthermore, they established the surface location of one of these virulence-specific proteins, suggesting that this protein might mediate trypsin-sensitive attachment of the mycoplasmas to host cells.

A convenient experimental indicator of mycoplasma attachment has been hemadsorption, the adherence of erythrocytes to colonies of *M. pneumoniae*. Although the significance of hemadsorption to pathogenesis remains unclear, and despite apparent differences between hemadsorption and adherence to respiratory epithelium (16, 17), the similarities between these processes (31) justify the use of the hemadsorption model system.

This laboratory has previously isolated nitrosoguanidine (NTG)-derived hemadsorption-negative (HA^-) mutants of *M. pneumoniae* (14). Sodium dodecyl sulfate (SDS)-PAGE and two-dimensional PAGE analyses of total protein from the mutant strains have revealed several protein differences among the mutants and the HA^+ wild-type strain (14, 17). Mutation to nonhemadsorption was accompanied by the loss of specific proteins. Furthermore, loss of the ability to hemadsorb was accompanied by variable

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reductions in the ability of mycoplasmas to adhere to respiratory epithelium, with the degree of reduction dependent upon the nature of the mutation. The most extreme reduction was found with an HA^- mutant which had lost the three virulence-specific proteins described earlier (15). Based on these findings, it was concluded that hemadsorption and adherence to respiratory epithelium are similar, yet distinct, activities. Although the isolation and characterization of an HA^+ revertant of one such HA^- mutant has supported these findings (16), the potential presence of secondary mutations in these strains warranted the isolation and characterization of mutants of *M. pneumoniae* spontaneously deficient in hemadsorption.

The objective of this study was to evaluate further the relationship between HA, adherence to respiratory epithelium, and mycoplasma virulence, using spontaneous nonhemadsorbing mutants. We describe protein profiles and attachment and virulence capabilities of 22 mutants of *M. pneumoniae* expressing an HA^- phenotype. In addition, we present additional evidence linking the presence of three specific mycoplasma proteins with recognition of neuraminidase-sensitive receptors on respiratory epithelium.

MATERIALS AND METHODS

Organisms and culture conditions. The studies described here were initiated by using strain M129-B16 of *M. pneumoniae*. Originally isolated from an individual with mycoplasma pneumonia (24), this strain has retained its virulence through 16 passages in vitro, as previously established (17). Monolayer cultures of *M. pneumoniae* were grown at 37°C in 32-oz. (ca. 960-ml) or 8-oz. (ca. 240-ml) glass prescription bottles containing 70 or 25 ml, respectively, of Hayflick medium (18). Organisms were harvested in the log phase, when the phenol red indicator in the growth medium became orange in color (48 to 72 h after inoculation). *M. pneumoniae* were washed three times with phosphate-buffered saline (PBS), pH 7.2, and collected by centrifugation at 9,500 × *g* for 15 min. Colony-forming units (CFU) were quantitated as previously described (10). Mycoplasma proteins were radiolabeled intrinsically with [^{35}S]methionine. Log-phase monolayers were washed once with PBS and pulsed for 6 h at 37°C in Hanks balanced salts solution containing 10% dialyzed horse serum and 250 μCi of L-[^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.; specific activity, 1,050 Ci/mmol). After a 1.5-h chase at 37°C in Hayflick medium, labeled monolayers were washed four times with PBS containing 1 mM cold methionine and harvested as before.

Screening and isolation of HA^- mutants. *M. pneumoniae* strain M129-B16 was cloned by means of a single-colony isolation procedure (14) to obtain a homogenous HA^+ parent strain. Briefly, a suspension of strain M129-B16 in Hayflick medium was plated on PPLO agar plates (23) and incubated for 7 days at 37°C. The plates were overlaid with a 0.5% suspension of chicken erythrocytes in saline and incubated for 30 min at 37°C (erythrocytes used in the screening were ≤2 weeks old). After two gentle washes with PBS, the colonies were screened microscopically for hemadsorption. A single HA^+ colony was purified by picking the colony from the plate, suspending it in Hayflick broth, dispersing the organisms by filtration through a 0.45-μm filter, and plating aliquots of the filtrate on PPLO agar plates. This procedure was performed three times before expansion of the clone to produce the HA^+ wild-type strain M129-B25C.

A suspension of *M. pneumoniae* strain B25C in Hayflick medium was serially diluted and plated on PPLO agar plates. The resulting colonies were screened for their hemadsorption properties as described above, and HA^- colonies were identified by their lack of adhering erythrocytes. Cloning and expansion of HA^- colonies were carried out as described above for B25C.

One- and two-dimensional PAGE. One-dimensional SDS-PAGE was performed as described by Laemmli (22), with slab gels 1.5 mm thick and composed of a 3% stacking gel and a 5% separating gel. Whole cell preparations of approximately 150 μg of protein (26) were applied to individual wells. Gels were stained with 0.25% Coomassie brilliant blue or processed for fluorography as described by Bonner and Laskey (4) or both.

Two-dimensional gel electrophoresis was carried out as previously described (15). Briefly, proteins were separated in the first dimension by either isoelectric focusing (27) or nonequilibrium pH gradient electrophoresis (28). Separation was achieved in the second dimension by SDS slab gel electrophoresis by the method of Ames and Nikaido (1). Gels were stained with Coomassie brilliant blue as described by O'Farrell (27). The sources of reagents used for two-dimensional electrophoretic analysis were identical to those used by O'Farrell (27), with the exceptions of acrylamide and *N,N'*-methylene-bisacrylamide (Bio-Rad Laboratories, Richmond, Calif.), and *N,N,N',N'*-tetramethylethylenediamine (Sigma Chemical Co., St. Louis, Mo.).

Organ culture. Hamster tracheal rings were prepared as previously described (9). Excised rings were incubated overnight in Eagle minimal essential medium containing 10% fetal bovine serum and penicillin (1,000 U/ml) at 37°C in 5% CO_2. The following day rings were placed in minimal essential medium in individual wells of 96-well microtiter plates (Falcon Plastics, Oxnard, Calif.), examined microscopically to determine the quality of the sections, and incubated at 37°C in 5% CO_2 before the attachment assays (<2 h).

Sialic acid-mediated attachment of HA^- mutants to the respiratory epithelium (25, 31) was monitored by preincubating tracheal rings for 30 min at 37°C in 50 μl of either PBS or PBS containing *Clostridium perfringens* neuraminidase (type VIII; Sigma; 10 U/ml). Sialic acid release was evaluated by the thiobarbituric acid procedure of Aminoff (2). The relationship between sialic acid release from neuraminidase-treated tracheal rings and reduction in wild-type mycoplasma attachment was examined by titrating neuraminidase concentrations.

Assay of attachment of HA^- mutants to tracheal rings. The wild-type and mutant strains of *M. pneumoniae* were radiolabeled by incubation in 25 ml of Hayflick medium containing 0.3 mCi of [*methyl-*

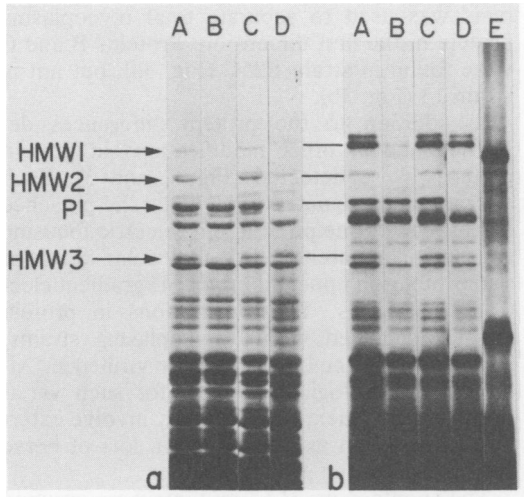

FIG. 1. Electrophoretic analysis of total protein from wild-type and representative spontaneous HA^- mutant strains of *M. pneumoniae*; (a) stained protein profile and (b) equivalent fluorogram. (A) HA^+ M129-B25C; (B) HA^- mutant lacking or markedly deficient in proteins HMW 1, 2, and 3 (the fluorogram reveals the absence of an additional high-molecular-weight protein; see text); (C) HA^- mutant with a one-dimensional protein profile identical to that of B25C; (D) HA^- mutant lacking protein P1; (E) ^{14}C-labeled molecular weight standards: myosin (200,000), phosphorylase b (92,500), and bovine serum albumin (68,000).

^3H]thymidine (Schwarz/Mann, Spring Valley, N.Y.; specific activity, 50 Ci/mmol). At 48 h the labeling medium was removed, the monolayers were washed, and the organisms were harvested in 2.0 ml of Hayflick medium containing 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Sigma) buffered at pH 7.3. Before infection of the tracheal rings, this suspension was passed through a 25-gauge needle four times to assist in dispersing clumped organisms. Preparations generally contained 2×10^4 to 4×10^4 cpm per 10-μl sample, corresponding to 2.5×10^6 to 5×10^6 CFU of *M. pneumoniae*. The infections were initiated by placing neuraminidase-treated or untreated tracheal rings in individual wells of microtiter plates containing 50 μl of radiolabeled mycoplasma suspensions. All samples were tested in triplicate or quadruplicate. After a 3.25-h incubation at 37°C, the rings were removed, blotted once on sterile absorbent paper, rinsed twice with PBS, and prepared for liquid scintillation spectrometry or radioautography as previously described (29). In view of the large number of mutants to be tested, assays were carried out in groups of four to five mutants. Each group included *M. pneumoniae* strain B25C as a control. Background levels of attachment were established by exposing tracheal rings to B25C at 4°C (29).

Virulence studies. The virulence of the spontaneous HA^- mutant strains in the Syrian golden hamster model was determined based upon two parameters: (i) persistence or multiplication (or both) of mycoplasmas in the lungs after intranasal inoculation and (ii) the degree of histological pneumonia produced. Virulence of the mutant strains was compared with that of the wild-type strain B25C. Four hamsters per strain per time point (days 4, 14, and 28 postinfection) were utilized. Four uninfected (untreated) hamsters were included as controls.

Infection of hamsters and processing of lung samples were carried out as described previously (10, 17). Briefly, hamsters were infected intranasally with *M. pneumoniae* suspended in Hayflick medium. The lungs were removed from anesthetized animals at the indicated time points. For each animal, the large lobe was processed for sectioning, and the remaining lobes were ground with a mortar and pestle in Hayflick medium, producing a homogenous suspension which was serially diluted and plated. Quantitation of CFUs was carried out as described elsewhere (10). Stained lung sections were examined microscopically (10), and lung cytopathology was evaluated by scoring lung sections on a scale of 0 to 9 (minimum to maximum cellular infiltration, respectively); this evaluation was carried out in a double-blind fashion.

Statistical analyses. The data in Table 2 were evaluated at the $P < 0.05$ significance level by using the nonparametric Mann-Whitney U test (30) to determine the statistical significance of differences observed among the classes of HA^- mutants. The data in Table 3 and Fig. 4 were evaluated by using a two-factor analysis of variance. Interaction between strains was significant ($P < 0.05$); therefore, differences among the strains were assessed by using the Duncan test (32) at the $\alpha = 0.05$ significance level.

RESULTS

Isolation of HA^- mutants. A total of 10,000 colonies of *M. pneumoniae* originating from the cloned strain B25C were screened for their hemadsorption characteristics. Seventy-three colonies (0.73%) were HA^-. Twenty-two of these HA^- mutants were subsequently isolated and characterized.

One- and two- dimensional PAGE. Examination of the spontaneous HA^- mutants by using one-dimensional SDS-PAGE and Coomassie blue staining established that qualitative and quantitative protein differences exist between the HA^+ strain B25C and certain HA^- strains. Three different protein profiles were found among the 22 mutants (Fig. 1a). Certain mutants (Fig. 1a, track B) lacked (or were markedly deficient in) three proteins (molecular weights, 210,000, 190,000, and 140,000), present in B25C (Fig. 1a, track A). We designated these proteins HMW 1, HMW 2, and HMW 3, respectively. One mutant (Fig. 1a, track D) lacked protein P1 (molecular weight, 165,000), the surface-associated macromolecule previously implicated in attachment (21). The remaining mutants (Fig. 1a, track C) exhibited one-dimensional protein profiles identical to that of B25C.

The more sensitive technique of fluorography of radiolabeled mycoplasma protein profiles (Fig. 1b) confirmed the results obtained by Coomassie blue staining. In addition, this procedure

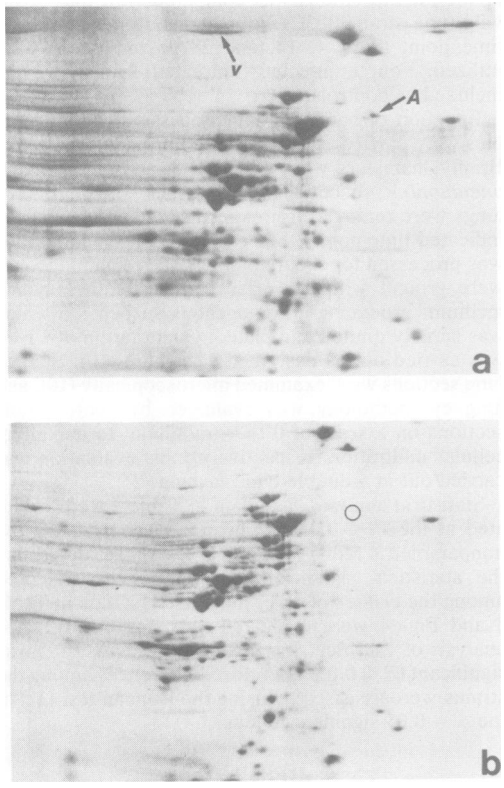

FIG. 2. Isoelectric focusing-SDS electrophoretic analysis of total protein from (a) HA^+ strain B25C, and (b) spontaneous HA^- mutant strain 13. Gels are shown with the acidic end to the right and the basic end to the left. The presence of protein A in B25C and its absence in HA^- strain 13 are indicated by an arrow and open circle, respectively. Spot v is a variable protein whose presence or absence occurs independently of hemadsorption or virulence. HA^- mutant strain 13 was arbitrarily chosen as a representative of those mutants deficient in proteins A, B, and C, as shown here and in Fig. 3.

revealed a protein (molecular weight, 215,000) migrating just above HMW 1 in the wild-type strain which was markedly reduced in mutants deficient in HMW 1, 2, and 3 (Fig. 1b, track B).

Two-dimensional PAGE of total protein of each of the 22 HA^- mutants revealed additional differences. Certain HA^- mutants lacked the three virulence-specific proteins previously designated A, B, and C (molecular weights, 72,000, 85,000, and 37,000, respectively) (15). These differences are illustrated in Fig. 2 and 3, which represent profiles of B25C and HA^- mutant strain 13 (refer to Table 1 for mutant categorization). In Fig. 2, total cell protein was separated in the first dimension with isoelectric focusing. Protein A was present in strain B25C (Fig. 2a) and absent in strain 13 (Fig. 2b), as indicated. In Fig. 3, nonequilibrium pH gradient electrophoresis was used to separate total mycoplasma protein in the first dimension. Proteins B and C were found in strain B25C (Fig. 3a), but not in strain 13 (Fig. 3b).

In addition to the protein differences described above, other modifications in protein patterns were detected (Fig. 2 and 3). Such alterations (designated v) included the presence or absence of one protein in isoelectric focusing gels and an apparent shift in molecular weight of two proteins in nonequilibrium pH gradient electrophoresis gels. These variations in protein profiles occurred within mycoplasma strains, independent of cell adherence or virulence. Although the biological stimulus for such variations remains unexplained, it may involve external factors such as differences in lots of horse serum used in the growth medium.

The spontaneous HA^- mutants were grouped into four classes based upon the consistent differences between Coomassie blue protein pro-

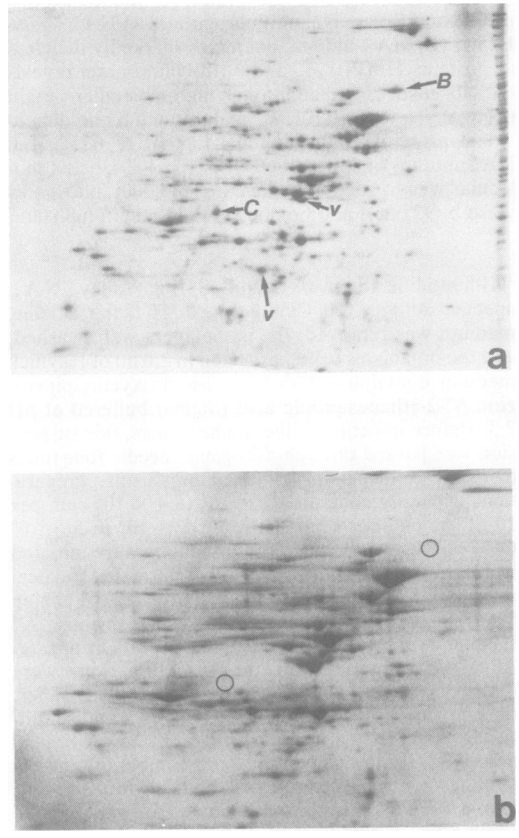

FIG. 3. Nonequilibrium pH gradient gel electrophoretic-SDS analysis of total protein from (a) HA^+ strain B25C and (b) spontaneous HA^- mutant strain 13. The presence of proteins B and C in B25C and their absence in HA^- strain 13 are indicated by arrows and open circles, respectively. Variable proteins (v), whose presence or absence occur independently of hemadsorption, are indicated by arrows.

TABLE 1. Comparison of protein differences between wild-type *M. pneumoniae* strain B25C and the spontaneous HA$^-$ mutants

	Mutants[a]	Proteins						
		HMW 1	HMW 2	HMW 3	A	B	C	P1
B25C		+[b]	+	+	+	+	+	+
Class I	2,5,6,8,9,10,11,12, 14,16,17,18,21	±[c]	−[d]	−	+	+	+	+
Class II	1,3,7,15	+	+	+	+	+	+	+
Class III	4,13,19,20	+	+	+	−	−	−	+
Class IV	22	+	+	+	−	−	−	−

[a] Designation of individual isolates within each class.
[b] Protein present.
[c] Protein markedly deficient.
[d] Protein absent.

files of each mutant and that of B25C (Table 1). Class I mutants were missing or severely deficient in the high-molecular-weight proteins HMW 1, 2, and 3. No protein differences were detected among the mutants in class II and B25C. Proteins A, B, and C, although present in B25C and class I and II mutants, were absent in class III mutants. The lone mutant in class IV, in addition to missing proteins A, B, and C, lacked protein P1.

Assay of HA$^-$ mutant attachment to hamster tracheal rings. We assessed the ability of each mutant relative to that of wild-type B25C to attach to the respiratory epithelium of hamster tracheal rings in vitro. The results were grouped according to mutant class (Table 2). Each class of mutants attached at a level which was significantly lower than that seen with B25C, yet greater than background levels observed when tracheal rings were infected with B25C at 4°C. Whereas the differences seen between attachment of B25C at 37 or 4°C and the attachment of the mutants were statistically significant, differences among the mutant classes were not.

Radioautography of cross sections of infected tracheal rings permitted the localization of attached, radiolabeled mycoplasmas (29). Emulsion grains representing HA$^-$ *M. pneumoniae* mutants were less dense but clearly distributed along the luminal surface of the epithelium when compared with the heavily infected rings of HA$^+$ B25C (data not shown).

The effect of neuraminidase pretreatment of the tracheal rings on attachment of the mutants was examined. Neuraminidase treatment was carried out at an enzyme concentration sufficient to remove sialic acids maximally under the conditions described, as determined by titrating enzyme concentration versus sialic acid release and inhibition of mycoplasma adherence (data not shown). Neuraminidase treatment reduced attachment of class I and II mutants, although to a lesser extent than for B25C (Table 2). Inhibition was defined as decreased attachment of [^{3}H]thymidine-labeled mycoplasmas to tracheal rings treated with neuraminidase. Neuraminidase treatment had no effect on the levels of attachment of class III or IV mutants. The neuraminidase-mediated inhibition of attachment of the mutants in class I occurred at two levels. Certain mutants in class I were inhibited approximately 14%, whereas the remaining mutants in class I were inhibited to a greater extent (34%). On this basis, the mutants in class I were divided into two subclasses (Table 2). The differences found between classes I, I′, or II and classes III or IV were statistically significant ($\alpha = 0.05$).

Examination of virulence of HA$^-$ mutants in hamsters. Representatives of each class of spontaneous HA$^-$ mutants were evaluated to determine their ability to survive and multiply in the lungs of hamsters after intranasal inoculation

TABLE 2. Attachment of wild-type and HA$^-$ mutant classes of *M. pneumoniae* to control and neuraminidase-treated hamster tracheal rings in vitro

Class	% Control[a]	% Inhibition[b]
I	32 ± 6	34 ± 6
I′[c]	37 ± 6	14 ± 4
II	26 ± 5	12 ± 4
III	25 ± 7	0.5 ± 1
IV	28	0
M129-B25C (wild-type strain)	100	72
M129-B25C (4°C)[d]	13	

[a] Percent control = (% attachment of mutant)/(% attachment of wild-type strain) × 100%; % attachment = (counts per minute bound per tracheal ring)/(counts per minute added per tracheal ring) × 100%.
[b] Percent inhibition = {1 − [(% attachment to neuraminidase-treated rings)/(% attachment to PBS-treated rings)]} × 100%.
[c] Class I was subdivided on the basis of two observed levels of neuraminidase inhibition of attachment (see text).
[d] Tracheal rings infected with B25C at 4°C served as a negative control.

TABLE 3. Quantitation of viable mycoplasmas in hamster lungs after intranasal inoculation with wild-type or HA$^-$ mutant *M. pneumoniae*

Class	Inoculum[a]	Log_{10} CFU/g of lung tissue on day postinfection[b]:		
		4	14	28
I	6.0	ND	ND	ND
I'	5.9	ND	ND	ND
II	6.2	3.3	3.0	3.9
III	6.3	ND	ND	ND
IV	6.2	ND	ND	ND
Wild-type M129-B25C	6.0	5.3	6.3	4.8

[a] Log_{10} CFU/hamster.
[b] ND, Not detectable ($<10^3$ CFU/g of lung tissue). Each number represents the mean value obtained from three or four hamsters.

(Table 3). Only the virulent strain B25C persisted in the lungs at high levels during the 4-week study. The mutant categorized in class II persisted at titers slightly above the limits of detection (10^3 CFU/g of lung tissue). The titers of the other mutants tested were below detectable levels.

Microscopic examination of lung sections from infected hamsters permitted evaluation of the extent of histological pneumonia (10, 17). Hamsters infected with the virulent strain B25C developed significant pneumonia by day 14 postinfection (Fig. 4). By day 28 postinfection, most of the cellular infiltrate had been resolved. In contrast, hamsters infected with the spontaneous HA$^-$ mutants did not develop pneumonia. Their cytopathology, as rated by lung lesion scores, was not significantly different than baseline cytopathology observed in untreated control hamsters.

DISCUSSION

Recent studies with NTG-derived HA-deficient mutant and homologous HA$^+$ revertant strains of *M. pneumoniae* have advanced our understanding of the relationship between hemadsorption and adherence of *M. pneumoniae* to respiratory epithelial cells (16, 17). Definitive conclusions which might be drawn from such studies concerning structure-function relationships and cell adherence are precluded somewhat by the possible existence of secondary mutations in NTG-treated mycoplasmas. The isolation of spontaneously arising HA$^-$ mutants has minimized the possibility of secondary mutations and allowed us to more directly evaluate the molecular basis for mycoplasma-host cell adherence.

Spontaneous loss of hemadsorption occurred at a rate (7×10^{-3}) which was unexpectedly high. Whether the change to an HA$^-$ phenotype was the result of a chromosomal alteration or a modification in gene expression is unknown. Although the loss of hemadsorption occurred at a high frequency, the nonhemadsorbing clones retained the HA$^-$ phenotype with passage. Attempts to isolate HA$^+$ revertants were successful only after a series of enrichments (data not shown; procedure described in reference 16). In addition, many similarities were seen in the protein profiles and activities of the spontaneous HA$^-$ mutants and those of previously described NTG-derived HA$^-$ mutants (14, 17). This evidence supports the likelihood that the loss of hemadsorption was the result of a relatively stable genetic event.

One- and two-dimensional electrophoretic analysis of total protein from HA$^+$ strain B25C and the spontaneous HA$^-$ mutants permitted the identification of protein differences in these strains. On the basis of their Coomassie blue protein profiles the HA$^-$ mutants were grouped into four classes. The differences observed generally did not involve a single protein or polypeptide, but rather, groups of proteins. The mutants in class I, the predominant nonhemadsorbing class, were deficient in three high-molecular-weight proteins (HMW 1, 2, and 3; Fig. 1). Protein HMW 2 appeared to correspond to the high-molecular-weight protein missing in the NTG-derived HA$^-$ mutant HA2 (14). Class III mutants contained normal levels of HMW 1, 2, and 3, but lacked the virulence-specific proteins A, B, and C (15). In this respect, they were similar to the NTG-derived HA$^-$ mutant HA1

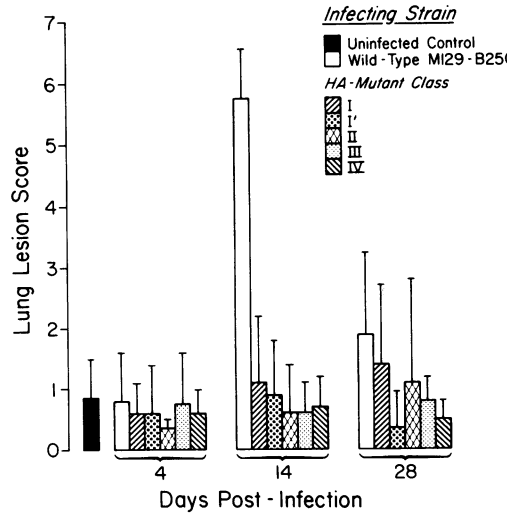

FIG. 4. Lung histopathology in hamsters after intranasal inoculation with wild-type and mutant *M. pneumoniae*. Each bar represents the mean value of lung lesion scores from three or four hamsters. Positive standard deviations are given.

(16). A single mutant (class IV), in addition to lacking proteins A, B, and C, was missing protein P1. Certain mutants (class II) exhibited no apparent differences in protein content as compared with B25C. These mutants will be discussed in more detail below.

Fluorography of ^{35}S-labeled one-dimensional protein profiles verified the differences among mutant classes found by using Coomassie blue staining. Furthermore, intrinsic radiolabeling established that the alterations involved mycoplasma proteins and did not reflect differences in binding of proteins from the growth medium. In addition, fluorography revealed a reduction in an additional band in class I mutants, further illustrating the complexity of these mutations.

The loss of the ability to adsorb erythrocytes was accompanied by a reduction (though not complete loss) in the capacity to attach to the respiratory epithelium of hamster tracheal rings. This suggests that the mechanisms employed for hemadsorption and attachment to respiratory epithelium are similar, but not identical, confirming the conclusion reached by Hansen et al. (17).

Virulent *M. pneumoniae* adhere to neuraminidase-sensitive receptors on respiratory epithelial cells and fibroblasts (12, 25, 31). However, sialic acid-mediated attachment may not account for all mycoplasma adherence. Pretreatment of tracheal rings with neuraminidase reduces adherence by approximately 70%. The mechanism responsible for the remaining mycoplasma-host cell interaction remains unexplained. Examination of HA^- mutant attachment to neuraminidase-treated tracheal rings revealed that only the mutants in classes I and II demonstrated reduced adherence. Attachment of class III and IV mutants was insensitive to neuraminidase. It is conceivable that neuraminidase-insensitive attachment is due to mycoplasma recognition of sialic acid-containing receptors inaccessible to neuraminidase. It seems unlikely, however, that such receptors would be accessible to mycoplasma recognition but not neuraminidase activity. Alternatively, certain sialic acids on the respiratory epithelium may be insensitive to hydrolysis by neuraminidase under the conditions described, perhaps due to steric hindrance from complex substituents associated with these sites. *M. pneumoniae* may also recognize nonsialic acid receptors on host cells. The latter possibility is supported by the observation that *M. pneumoniae* exhibits neuraminidase-insensitive adsorption of rabbit erythrocytes (11), which are relatively deficient in surface-associated sialic acid (3).

These data suggest a pattern in structure-function relationships. The loss of either of the two groups of mycoplasma proteins discussed above accompanied a loss of the ability to hemadsorb as well as a reduction in respiratory cell adherence. In addition, the loss of proteins A, B, and C correlated with the loss of recognition of neuraminidase-sensitive host cell receptors on respiratory epithelium. This confirms the finding of Hansen et al., who reported that the NTG-derived HA^- mutant HA1, which also lacks proteins A, B, and C, does not exhibit neuraminidase-sensitive attachment (17). It has been shown previously that protein B is accessible to lactoperoxidase-catalyzed radioiodination and trypsin treatment of intact mycoplasmas, establishing its location on the outer surface of *M. pneumoniae* (15). Additional studies are necessary to more clearly define the function of proteins, A, B, and C, as well as external protein P1 and HMW 1, 2, and 3, in mycoplasma adherence.

The molecular events leading to the loss of hemadsorption by *M. pneumoniae* resulted in their apparent loss of virulence in experimentally infected hamsters. Whereas the wild-type strain B25C persisted at high levels in the lungs of animals, none of the spontaneous HA^- mutants persisted at detectable levels after infection, with the exception of the mutant from class II. As stated above, class II mutants possessed protein profiles apparently identical to that of B25C. Despite its low-level persistence in infected animals, this mutant did not produce lung cytopathology significantly greater than that associated with the other HA^- mutants tested or with baseline levels found in uninfected controls. Thus, the reduced capacity of the HA^- mutants to attach to host cells in vitro correlates in each case with diminished in vivo survival.

The existence of HA^- mutant strains exhibiting protein profiles apparently identical to that of the wild-type strain warrants additional discussion. Minor amino acid substitutions might alter the conformation or biological activity (or both) of a protein and yet not produce a significant change in its migratory pattern in one- and two-dimensional PAGE. Thus, the similarities in protein profiles between the wild-type strain and class II mutants may be due to inherent limitations of the procedures employed to evaluate slight protein differences in these strains. Alternatively, modified carbohydrate or lipid moieties could be responsible for loss of hemadsorption by affecting the disposition of attachment proteins in the mycoplasma membrane. In any case, the data suggest that mycoplasma adherence to host cells is a complex, multicomponent process.

A total of eight mycoplasma proteins have been indirectly associated with adherence. Determination of whether all of these proteins actually play a role in attachment requires fur-

ther investigation, although the recent isolation of a homologous HA^+ spontaneous revertant from an NTG-derived HA^- mutant (16) reinforces the apparent correlation between specific *M. pneumoniae* proteins and adherence. However, certain proteins may be unrelated structurally or functionally, although they appear to be coexpressed. Alternatively, some of the proteins may be precursors or cleavage products of others, or may simply represent polypeptides of parent molecules dissociated by SDS-reducing conditions. Possibly attachment involves multiprotein complexes in the mycoplasma membrane. The loss or deficiency of one or more proteins from such a complex could affect hemadsorption and attachment to respiratory epithelium by differing mechanisms, and yet the possible lack of protein alterations in class II mutants accompanied by a reduction in attachment in vitro and survival in vivo suggests that nonproteinaceous factors may also be important. Thus, the availability of classes of spontaneous mycoplasma mutants permits further dissection of the pathogenicity of *M. pneumoniae* and assessment of the potential of these mutants as vaccine candidates.

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