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Characterization of Hemadsorption-Negative Mutants of *Mycoplasma pneumoniae*

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Previously isolated mutants of *Mycoplasma pneumoniae* incapable of hemadsorption were characterized with respect to specific protein content, tracheal ring attachment capability, and virulence for both in vitro and in vivo model systems. Two-dimensional gel electrophoresis revealed both quantitative and qualitative differences between the protein complements of two different mutant strains and that of the virulent parent strain. Studies of mycoplasma attachment to hamster tracheal rings in vitro demonstrated that only one of these mutant strains still possessed the ability to attach to the respiratory epithelium via neuraminidase-sensitive receptors. Measurement of [^{3}H]orotic acid uptake in mycoplasma-infected tracheal rings indicated that infection with the hemadsorption-negative mutants resulted in only slight reductions of ribonucleic acid synthesis, similar to levels observed for tracheal rings infected with an avirulent strain of *M. pneumoniae*. The virulence potential of the two mutant strains was further investigated by utilizing the hamster model system. Both mutant strains were rapidly cleared from the lungs of infected animals and produced little or no microscopic pneumonia.

Mycoplasma pneumoniae is a procaryotic respiratory tract pathogen that can produce disease in virtually all segments of the upper and lower respiratory tract and is best known for its ability to cause cold agglutinin-associated primary atypical pneumonia. The mechanisms employed by this organism in the production of disease are just beginning to be elucidated (7, 9, 16a). The ability of this organism to parasitize the respiratory tract is related at least in part to its ability to adhere to respiratory tract epithelium (9). The use of hamster tracheal organ culture has allowed detailed in vitro examination of both this adherence process (2, 9, 15) and the subsequent pathological effects exerted on the respiratory tract epithelium by attached and metabolically active *M. pneumoniae* cells (2, 7, 8). The importance of adherence in the production of *M. pneumoniae*-associated disease has been inferred from the fact that a strain of *M. pneumoniae* which has spontaneously lost the ability to adhere to respiratory tract epithelium is also no longer capable of causing experimental pneumonia in a hamster model system (11, 15). The relevance of this adherence process to human respiratory disease has been established by the observation of attached mycoplasmas on respiratory tract epithelial cells obtained from patients infected with *M. pneumoniae* (3).

In addition to the property of surface attachment, virulent strains of *M. pneumoniae* also form colonies which adsorb erythrocytes in a process called hemadsorption (12). Hemadsorption bears certain similarities to respiratory epithelium attachment by *M. pneumoniae* in that (i) many of the receptor sites for *M. pneumoniae* on both erythrocytes of certain animal species and respiratory epithelial cells appear to contain sialic acid residues (16), and (ii) trypsin treatment of colonies of virulent *M. pneumoniae* eliminates their ability to adsorb either erythrocytes or respiratory epithelial cells (16). These data, which suggested that *M. pneumoniae* might employ the same mechanism(s) for attachment to both respiratory epithelium and erythrocytes, prompted us to isolate mutants of *M. pneumoniae* deficient in hemadsorption (5). Such mutants could be used both to further evaluate the relationship between hemadsorption and respiratory epithelium attachment ability in *M. pneumoniae* and to permit molecular comparisons between wild-type and mutant mycoplasmas.

Hemadsorption-negative mutants produced by chemical mutagenesis could be divided into two different classes (5). One class of mutant, represented by strain HA1, almost completely lost the ability to attach to respiratory epithe-

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lium in vitro, yet apparently possessed the same protein complement as the wild-type parent strain, as determined by standard one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of mycoplasma total cell protein. The other class of nonhemadsorbing mutant, represented by strain HA2, exhibited a substantial reduction in its respiratory epithelium attachment ability and also lost the ability to synthesize at least one high-molecular-weight protein normally found in the wild-type parent strain. The availability of these hemadsorption-negative mutants of *M. pneumoniae* has allowed us to evaluate the specific effect exerted by this loss of hemadsorption capability on the virulence potential of these mutants both in vitro and in vivo. In addition, the use of two-dimensional gel electrophoresis has revealed significant differences in the protein content of these hemadsorption-negative mutant strains.

MATERIALS AND METHODS

Organisms and culture media. The virulent, wild-type M129 strain of *M. pneumoniae* at broth passage level 16 (M129-B16) served as the parent strain. The hemadsorption-negative mutant strains HA1 and HA2 were derived from the M129-B16 strain by chemical mutagenesis, as described (5). The avirulent M129-B181 strain was derived from *M. pneumoniae* strain M129 by 181 consecutive broth passages and does not cause microscopic pneumonia in hamsters after intranasal inoculation (11). This avirulent strain of *M. pneumoniae* is also hemadsorption negative (11). Cultures of these mycoplasma strains were grown at 37°C in 32-oz. (ca. 0.95-liter) glass prescription bottles containing 70 ml of Hayflick medium (9). Cultures were incubated for 48 to 96 h, until the phenol red indicator in the growth medium became orange-yellow in color, approximately pH 6.6. Colony-forming units (CFU) of these strains were quantitated by plating serial dilutions of these organisms on Hayflick medium solidified with 1% (wt/vol) Noble agar (Difco) and lacking both glucose and phenol red. The hemadsorption test (5) was routinely performed on colonies derived from all cultures used in these experiments, to confirm their identity and purity.

Two-dimensional gel electrophoresis. Isoelectric focusing (IEF) SDS-polyacrylamide gel electrophoresis and non-equilibrium pH gradient (NEPHGE) SDS-polyacrylamide gel electrophoresis of mycoplasma total cell proteins were performed as previously described (6). Each mycoplasma strain was analyzed in four different IEF- and NEPHGE-SDS gel electrophoresis experiments. Only those mutant proteins whose presence, quantity, or position in the gel were consistently altered relative to the proteins of the virulent parent strain were considered to represent true protein differences among these strains.

Studies of mycoplasma attachment to tracheal rings. Quantitation of mycoplasma adherence to hamster tracheal rings in vitro was accomplished by the use of *M. pneumoniae* radiolabeled with [*methyl*-^{3}H]thymidine, as reported previously (9, 15). Determination of the effect of neuraminidase treatment of tracheal rings on the adherence of mycoplasmas required pretreatment of rings with neuraminidase or buffer. Tracheal rings were placed in microtiter wells containing 50 μl of pH 7.2 phosphate-buffered saline or 50 μl of phosphate-buffered saline containing 0.5 U of neuraminidase (type VIII; Sigma Chemical Co., St. Louis, Mo.) and were incubated at 37°C for 30 min. The rings were then rinsed twice in phosphate-buffered saline and immediately used in the standard mycoplasma attachment assay (15).

Measurement of RNA synthesis in mycoplasma-infected tracheal rings. Synthesis of ribonucleic acid (RNA) in infected hamster tracheal rings was quantitated as described previously (7, 8), utilizing [^{3}H]orotic acid (International Chemical and Nuclear Corp., Irvine, Calif.) as the radiolabeled precursor for RNA synthesis. Hamster tracheal rings were exposed to wild-type and mutant strains of *M. pneumoniae* by incubating rings with 10^6 to 10^7 CFU of mycoplasmas suspended in 50 μl of Hayflick medium containing 10% fetal calf serum in place of horse serum, to avoid horse serum toxicity effects on tracheal ring epithelium. This infection step was performed in an air incubator at 35°C for 5 h. At the end of the infection period, rings were transferred (without washing or blotting) to fresh Hayflick medium with 10% fetal calf serum and incubated in a 5% CO_2 atmosphere at 35°C. This medium was changed daily to avoid excess growth of mycoplasmas, which multiplied freely in the growth medium. In addition, the rings were rinsed once in phosphate-buffered saline during the daily medium change to remove loosely associated mycoplasmas. Duplicate pairs of rings infected with each mycoplasma strain were harvested at 24- to 48-h intervals for determination of their RNA synthesis capability.

Infection of hamsters with mycoplasmas. Mycoplasmas used for virulence studies were grown in Hayflick medium under standard conditions and then harvested by scraping the glass-attached organisms into fresh Hayflick medium. Cells of the avirulent M129-B181 strain, which does not adhere to glass, were harvested by centrifugation (12,000 × *g* for 20 min) and then resuspended in fresh medium. Young adult male Syrian golden hamsters were anaesthetized by intraperitoneal injection of sodium pentobarbital and laid in a supine position. A 100-μl volume of mycoplasma inoculum was then instilled into the anterior nares of these animals by sequential addition of 10- to 20-μl portions of the inoculum. The animals remained in the supine position until they regained consciousness and then were returned to their cages.

Quantitation of mycoplasma CFU in the lungs of the infected animals and evaluation of the occurrence and severity of microscopic pneumonia in these animals were accomplished by previously published methods (1, 4). Preliminary experiments established that the lower limit of detectability of *M. pneumoniae* in hamster lung tissue is 10^3 CFU/g of tissue; at least a 10-fold dilution of 10% (wt/vol) lung tissue suspensions before plating is made necessary by the presence in these tissues of substances inhibiting colony growth of *M. pneumoniae* (10). Virulence of these mycoplasma strains was defined as the ability to produce

significant microscopic pneumonia consisting of peribronchial round-cell infiltration with or without intralumenal polymorphonuclear exudation (1, 4). The scoring of pneumonia was performed using coded histological lung sections, and the degree of microscopic pneumonia was rated on a scale of 0 (no evidence of pneumonia) to 9 (severe pneumonia).

Statistical methods. A two-factor analysis of variance was performed on the data involved in Fig. 5. Significant interaction between strain and time was found ($P < 0.01$). Therefore, Newman-Keuls multiple pairwise comparisons were performed to assess the differences among strains at each time period. A three-factor analysis of variance was performed on the data involved in Fig. 1 and 4. In the presence of interaction, differences between strains were assessed by the Newman-Keuls procedure. All Newman-Keuls comparisons were performed at the alpha = 0.05 significance level (17).

RESULTS

General characteristics of hemadsorption-negative mutant strains HA1 and HA2. Mutant strains HA1 and HA2 exhibited generation times in Hayflick medium similar to that of the virulent wild-type parent strain M129-B16 (ca. 7 h; data not shown). Again like the parent strain, both of these hemadsorption-negative mutant strains grew primarily as glass-attached cells in liquid culture, forming a visible film on the glass surface. Therefore, the genetic alteration(s) in these mutant mycoplasmas which resulted in a loss of hemadsorption capability did not grossly affect the ability of these mutants to grow and adhere to glass surfaces.

Figure 1 shows that these two hemadsorption-negative mutants differed from both each other and the virulent wild-type parent strain in their respective abilities to attach to tracheal ring respiratory epithelium. Statistical analysis of these data showed that these three different mycoplasma strains all exhibited tracheal ring attachment frequencies which were significantly different from one another. Furthermore, the two mutant strains also differed in their respective abilities to attach to neuraminidase-treated hamster tracheal rings. Neuraminidase pretreatment of tracheal rings, which drastically reduced the frequency of attachment of the wild-type parent strain, did not affect the relatively low attachment frequency exhibited by mutant strain HA1. In contrast, this enzymatic treatment of tracheal rings caused statistically significant reductions in the attachment frequencies of both mutant strain HA2 and the wild-type parent strain.

Two-dimensional gel electrophoresis of proteins from mutant strains HA1 and HA2. One-dimensional SDS-polyacrylamide gel electrophoresis previously showed that mutant

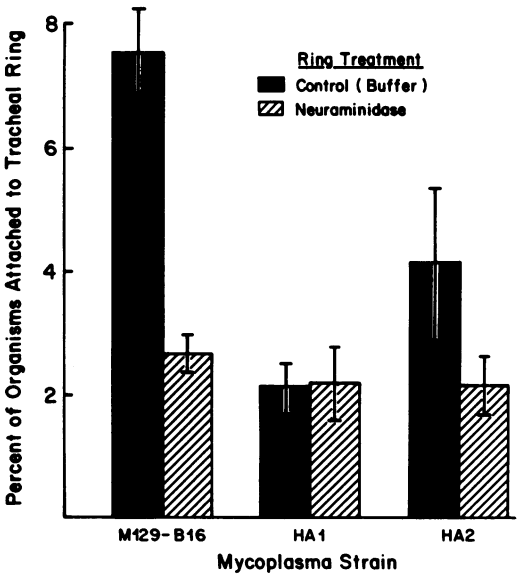

FIG. 1. *Effect of neuraminidase pretreatment of hamster tracheal rings on mycoplasma attachment. Tracheal ring attachment of mycoplasmas was monitored by the use of M. pneumoniae labeled with [methyl-^{3}H]thymidine (9, 15). Equivalent numbers of CFU (10^7) of each mycoplasma strain were used in these experiments. CFU were determined by plating serial dilutions of radiolabeled mycoplasmas on solidified Hayflick medium (4, 11). These data represent the mean from two separate experiments in which three tracheal rings were used for each mycoplasma strain and ring treatment; the standard deviation is indicated accordingly. A three-factor analysis of variance was performed on the actual experimental data, and in the presence of interaction, differences between strains were assessed by using the Newman-Keuls comparisons procedure at the alpha = 0.05 significance level.*

strain HA2 lacked a high-molecular-weight protein possessed by both the virulent wild-type parent strain and mutant strain HA1 (5). Having recently demonstrated that two-dimensional gel electrophoretic analysis of mycoplasma proteins is a highly sensitive method for detecting minor protein differences between mycoplasma strains (6), we utilized this technique to further characterize total cell proteins from the hemadsorption-negative mutant strains HA1 and HA2.

IEF-SDS gel electrophoretic analysis showed that mutant strain HA2 exhibited a protein spot pattern apparently identical to that of the virulent wild-type parent strain M129-B16, and included the virulent strain-specific protein A (6; Fig. 2b). In contrast, the IEF-SDS gels of mutant strain HA1 differed from those of both the wild-type parent strain and mutant strain HA2 in at

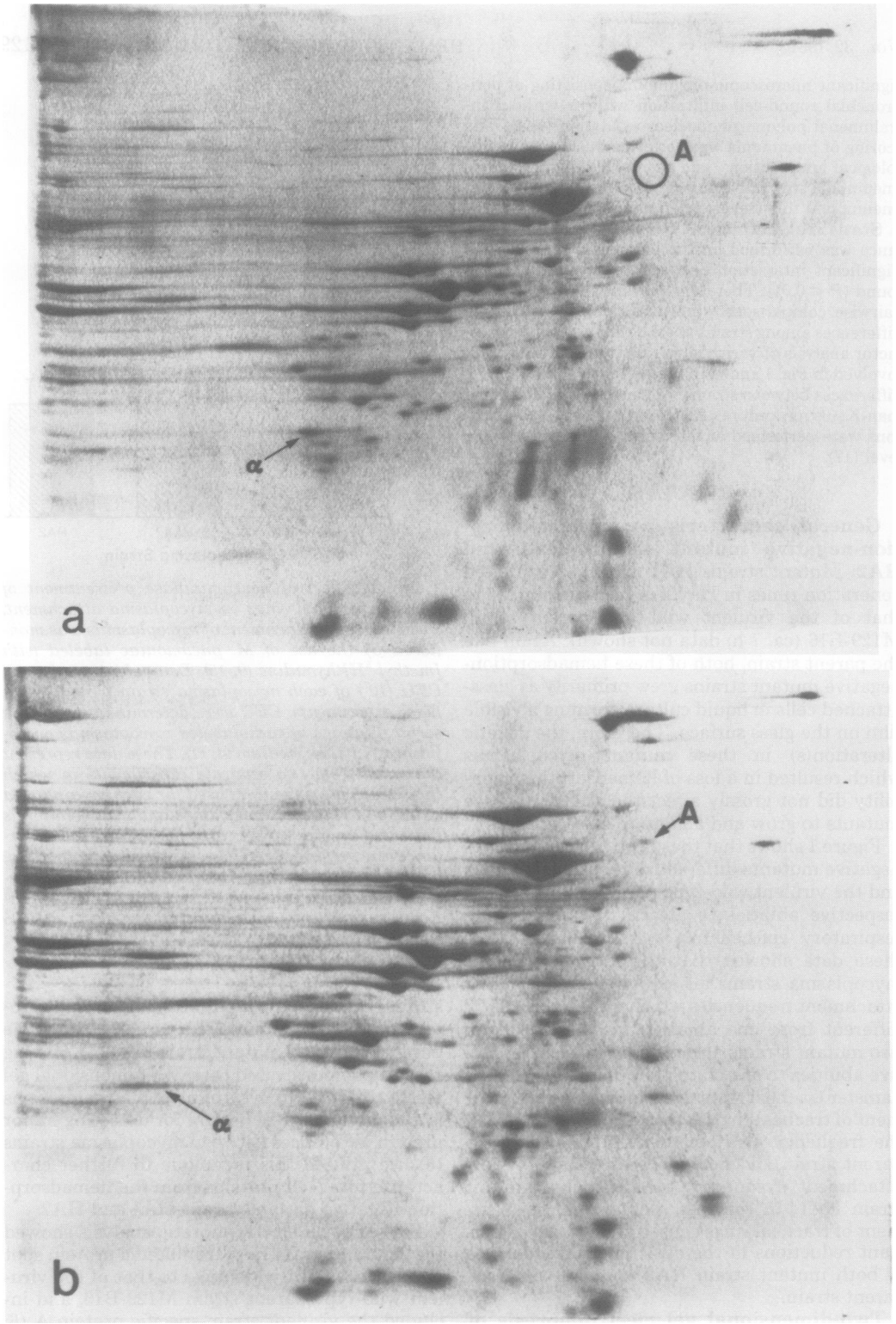

FIG. 2. *IEF-SDS gel electrophoretic analysis of proteins from hemadsorption-negative mutant strains of M. pneumoniae. In accordance with standard convention (13), the basic end of the two-dimensional gel is on the left, the acidic end is on the right, and proteins of decreasing molecular weight run from the top to the bottom of the gel. (a) Mutant strain HA1. The absence of the virulent strain-specific protein spot A is indicated by the circle. The protein spot α-HA1 is indicated by the appropriate arrow. (b) Mutant strain HA2. The virulent strain-specific protein spot A and the protein spot α-HA2 are indicated by appropriate arrows.*

least two respects. First, mutant strain HA1 lacked the virulent strain-specific protein A (Fig. 2a). In addition, mutant strain HA1 possessed a protein (protein spot α-HA1) which had undergone an apparent change in its isoelectric point (Fig. 2a) such that it had become more acidic relative to the corresponding protein (protein spot α-HA2) in the wild-type parent strain (6) and in the mutant strain HA2 (Fig. 2b).

NEPHGE-SDS gel electrophoretic analysis, which permits resolution of proteins with more basic isoelectric points (14), also showed that the protein spot pattern of mutant strain HA2 was apparently identical to that of the virulent wild-type parent strain, and included the virulent strain-specific protein B and C (6; Fig. 3b). The use of NEPHGE-SDS gels also identified new, significant differences between mutant strain HA1 and both the virulent wild-type parent strain and mutant strain HA2, in that mutant strain HA1 lacked the virulent-strain specific proteins B and C (Fig. 3a). In addition, the apparent shift in the isoelectric point of protein spot α-HA1 is readily evident when the NEPHGE-SDS gel of this mutant's proteins (Fig. 3a) is compared to the NEPHGE-SDS gel of mutant strain HA2 proteins (Fig. 3b).

RNA synthesis in mycoplasma-infected tracheal rings. Previous studies from this laboratory have established that attachment of virulent *M. pneumoniae* to tracheal ring epithelium disrupts macromolecular synthetic processes in these tracheal rings, provided the mycoplasmas remain attached and metabolically active (7, 8). To determine whether the loss of hemadsorption capability has in any way altered the ability of these mutants to cause alterations in tracheal ring metabolic processes, RNA synthesis was monitored in tracheal rings infected with the virulent wild-type parent strain M129-B16, the hemadsorption-negative mutant strains, and the avirulent M129-B181 strain. That mycoplasmas were present in the tracheal organ culture systems subsequent to the initial infection phase and throughout the experimental time period was confirmed by the quantitation of mycoplasma CFU in the tracheal organ culture medium during the experiment; between 10^5 and 10^7 free-floating CFU of each mycoplasma strain per ml were detected in the media at both 72 and 144 h postinfection (data not shown). Therefore, the repeated transfer of tracheal rings infected with the mutant strains or the avirulent strain into fresh medium did not totally remove these attachment-deficient strains from the organ culture system, where these organisms multiplied freely. Consequently, the tracheal rings were constantly exposed to large numbers of mycoplasmas of all four strains over the course of the experimental period.

Infection of tracheal rings with the virulent wild-type parent strain M129-B16 resulted in a marked and rapid decline in tracheal ring RNA synthesis ability which was statistically significant at 72, 96, and 144 h postinfection (Fig. 4). In contrast, infection of tracheal rings with the hemadsorption-negative mutant strains resulted in a relatively small decrease in tracheal ring RNA synthesis ability which became significantly different from uninfected control ring values only at 144 h postinfection. Similarly, the avirulent strain exerted a small but statistically significant inhibitory effect on tracheal ring RNA synthesis only at 144 h postinfection.

Virulence of hemadsorption-negative mutants. Animal virulence studies involving the hemadsorption-negative mutant strains HA1 and HA2 were performed to determine what effect, if any, the loss of hemadsorption capability had on the ability of the mutants to cause microscopic pneumonia in hamsters. Hamsters were intranasally challenged with the virulent wild-type parent strain M129-B16, the hemadsorption-negative mutant strains, and the avirulent strain M129-B181, and the development of microscopic pneumonia in these animals was followed with time (Fig. 5). The virulent wild-type parent strain produced statistically significant microscopic pneumonia by 14 days postinfection; the lung lesions in animals infected with this virulent strain were gradually resolved over the remaining 14-day interval. In contrast, like the avirulent strain, neither of the hemadsorption-negative mutant strains caused any significant degree of microscopic pneumonia during the 28-day experimental period.

Comparison of these histological data with the numbers of CFU of the various mycoplasma strains detected in the lungs of infected animals showed that only the virulent, wild-type parent strain persisted at relatively high levels (Table 1). In contrast, CFU of neither hemadsorption-negative mutant strain could be detected at any of the sampling times during the course of the experiment (Table 1). It is noteworthy that whereas CFU of the avirulent strain were not demonstrated in the lungs of most infected animals by 14 days postinfection, avirulent-strain mycoplasmas were present at barely detectable levels in a few individual animals throughout the course of the experiment. Control tests performed with these mycoplasma colonies isolated from animals infected with the avirulent strain confirmed that these organisms were *M. pneumoniae* and were also hemadsorption negative.

DISCUSSION

The ability of virulent strains of *M. pneumo-*

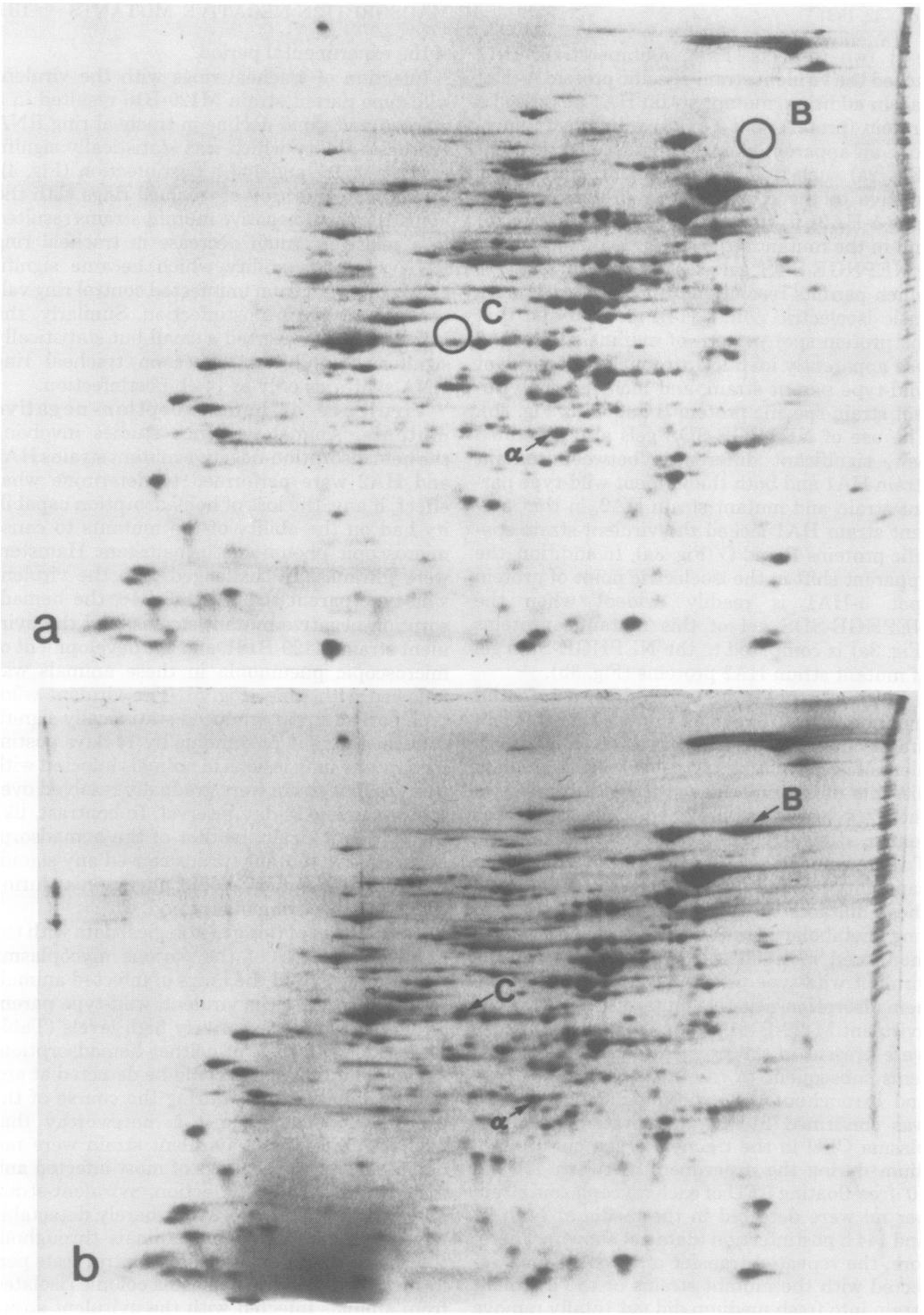

FIG. 3. *NEPHGE-SDS gel electrophoretic analysis of proteins from hemadsorption-negative mutant strains of M. pneumoniae. (a) Mutant strain HA1. The absence of the virulent strain-specific protein spots B and C is indicated by the appropriate circles. Protein spot α-HA1 is indicated by the appropriate arrow. (b) Mutant strain HA2. The virulent strain-specific protein spots B and C, and spot α-HA2, are indicated by arrows. Note that protein spot C is the uppermost of two closely migrating proteins.*

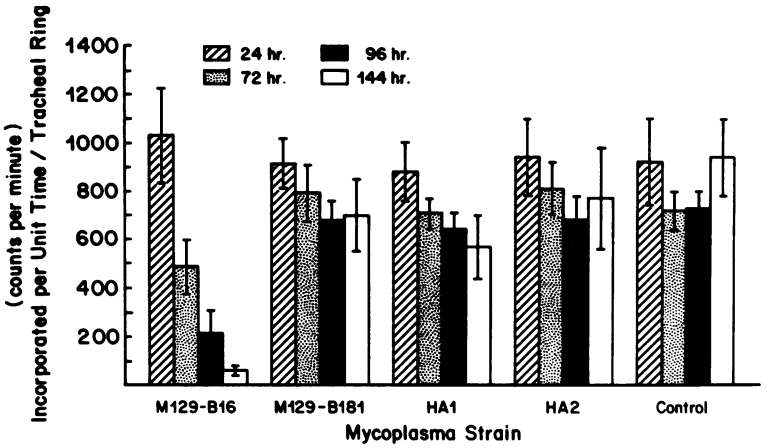

FIG. 4. *RNA synthesis in tracheal rings infected with wild-type, mutant, and avirulent strains of M. pneumoniae. The numbers of mycoplasmas used for infection were: M129-B16, 7.3 × 10⁶ CFU; M129-B181, 1.7 × 10⁷ CFU; HA1, 1.7 × 10⁷ CFU; HA2, 1.6 × 10⁷ CFU. These data represent the mean from three separate experiments in which a pair of tracheal rings infected with each strain was harvested at each time point for analysis; the standard deviation is indicated accordingly. A three-factor analysis of variance was performed on the actual experimental data, and in the presence of interaction, differences between strains were assessed by using the Newman-Keuls comparisons procedure at the alpha = 0.05 significance level.*

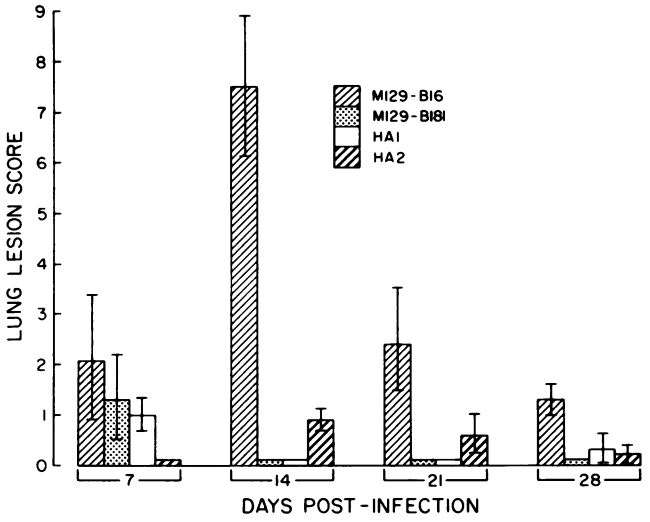

FIG. 5. *Virulence of wild-type, mutant, and avirulent strains of M. pneumoniae in hamsters. Hamsters were infected intranasally with mycoplasmas suspended in 100 μl of Hayflick broth. The inocula employed in this experiment are listed in Table 1. Each column represents the mean lung lesion score from either two or three individual animals; the standard deviation is indicated accordingly. A two-factor analysis of variance was performed on the actual experimental data to assess the effects of mycoplasma strain and time. Significant interaction between strain and time was found to be present (P < 0.01). Therefore, Newman-Keuls multiple pairwise comparisons were performed to assess the differences among strains at each time period. These tests were performed at the alpha = 0.05 significance level.*

niae to form colonies which adsorb erythrocytes is a well-known characteristic of this organism, although the functional and/or structural relationship of this hemadsorption process to both respiratory epithelium attachment and the ability of this respiratory tract pathogen to produce experimental pneumonic disease has not been previously investigated. The recent isolation of mutants of *M. pneumoniae* specifically deficient in hemadsorption, however, has permitted us to

TABLE 1. *Quantitation of viable mycoplasmas in hamster lungs after intranasal challenge*

Strain	Inoculum per animal (CFU)	CFU/g of lung tissue[a] at day postinfection:			
		7	14	21	28
Wild-type M129-B16	3.0×10^6	3.0×10^5 6.1×10^5 4.5×10^5	2.6×10^6 9.6×10^5	7.5×10^5 1.0×10^3	6.9×10^4 9.0×10^4
HA1	4.2×10^6	ND ND ND	ND ND	ND ND	ND ND
HA2	2.0×10^6	ND ND ND	ND ND	ND ND	ND ND
M129-B181	2.3×10^6	1.8×10^4 1.2×10^4 1.2×10^4	ND ND	1.0×10^3 ND	1.0×10^3 ND

[a] Each number represents a single animal. ND, Not detectable, or less than 10^3 CFU/g of lung tissue.

correlate alterations in the physiology and virulence of this organism with the loss of hemadsorption capability.

The hemadsorption-negative mutant strains HA1 and HA2 have been shown to differ markedly in their relative abilities to attach to respiratory epithelium (5; Fig. 1). Similarly, neuraminidase pretreatment of the tracheal rings employed in the attachment assay had significantly different effects on the adherence frequencies of these two mutants (Fig. 1). Mutant strain HA1 is incapable of hemadsorption and exhibits a very limited ability to attach to respiratory epithelium. Consequently, neuraminidase pretreatment of tracheal rings does not alter the very low attachment frequency exhibited by this particular hemadsorption-negative mutant. In contrast, mutant strain HA2 still remains capable of a significant sialic acid-mediated attachment to respiratory epithelium in vitro, while completely losing its hemadsorption ability. Therefore, hemadsorption is not necessarily synonymous with respiratory epithelium attachment ability. Furthermore, these data suggest receptor similarities and differences among these eucaryotic cells and provide evidence for distinct mechanisms of interaction between the mycoplasma ligand(s) and the host cell surface.

The use of two-dimensional gel electrophoresis has established that these two mutant strains are distinct from one another in several respects. The mutant strain HA1 differs from mutant strain HA2 and the wild-type parent strain in its lack of the virulent strain-specific proteins A, B, and C and in its possession of a protein (α-HA1) with an altered isoelectric point (Fig. 2 and 3). With the exception of this latter protein, the protein profile of mutant strain HA1 on IEF-SDS and NEPHGE-SDS gels is apparently identical to that of the avirulent M129-B181 strain, which also fails to hemabsorb (6). In contrast, the two-dimensional gel patterns of total cell proteins from mutant strain HA2 are apparently identical to that of the wild-type parent strain. The high-molecular-weight protein which distinguishes the wild-type parent strain from mutant strain HA2 in one-dimensional SDS-polyacrylamide gels (5) was not resolved in two-dimensional gels of wild-type strain proteins under these experimental conditions. Assessment of the possible involvement of either the latter protein or the virulent strain-specific proteins in the hemadsorption process will require the isolation of hemadsorption-positive revertants of these nonhemadsorbing mutants and subsequent one- and two-dimensional gel electrophoretic analyses of the total cell proteins of these revertants.

The loss of hemadsorption capability was shown to exert a considerable effect on the virulence of these mutant mycoplasmas for both in vitro and in vivo systems. In contrast to the virulent wild-type parent strain, both hemadsorption-negative mutants exert only relatively small inhibitory effects on tracheal ring RNA synthesis in vitro (Fig. 4). These data indicate that mutant strain HA2, despite its respiratory epithelium attachment capability, is unable to readily disrupt macromolecular biosynthetic processes in respiratory epithelium. The similar results obtained with these mutants and the avirulent strain in this assay system suggested that this low-level inhibition of tracheal ring RNA synthesis may be independent of the attachment ability of these mycoplasma strains and may be related to other effector molecules, such as mycoplasma-derived metabolic products accumulating in the epithelial cells of the tra-

cheal ring.

The hemadsorption-negative mutants were also shown to be incapable of causing significant microscopic pneumonia in the hamster model system (Fig. 5). That these mutants cannot produce pneumonic disease in hamsters is undoubtedly due to the apparent inability of these nonhemadsorbing mutants to survive or replicate in vivo (Table 1). The importance of adherence in the pathogenesis of *M. pneumoniae* pneumonia makes the lack of virulence of mutant strain HA1 not surprising, since this mutant possesses very little respiratory epithelium attachment ability. The inability of mutant strain HA2 to produce microscopic pneumonia in hamsters is more difficult to reconcile with the apparent in vitro attachment ability exhibited by this mutant. However, the fact that mutant strain HA2 also cannot inhibit tracheal ring RNA synthesis more readily than the attachment-deficient strains HA1 and M129-B181 suggests that the mutation which altered the hemadsorption ability of mutant strain HA2 also simultaneously affected both its virulence for tracheal rings in vitro and its ability to survive, reproduce, and cause microscopic pneumonia in hamsters. This explanation and the alternative possibility, that an undetected secondary mutation in this strain is responsible for its lack of virulence in these experimental systems, can only be evaluated by the isolation and study of a hemadsorption-positive revertant of mutant strain HA2.

The inability of the attachment-deficient avirulent strain M129-B181 to cause pneumonic disease in hamsters reaffirms the importance of epithelial cell adherence in the production of experimental disease by *M. pneumoniae* in this model system. Similarly, this avirulent strain apparently could survive only at barely detectable levels in hamster lungs, again indicating the critical relationship between mycoplasma attachment and the survival and extensive growth of this organism in the hamster lung. That the avirulent strain could apparently survive better in vivo than either of the hemadsorption-negative mutants may be unrelated to attachment factors and might involve alteration of antiphagocytic mechanisms or metabolic properties of these mutants by the same genetic event(s) which eliminated their hemadsorption ability.

In conclusion, the use of these hemadsorption-negative mutants has shown that the loss of hemadsorption capability can be correlated with several significant changes in the biochemistry and virulence of this organism. Indeed, the apparent lack of pathogenicity of these hemadsorption-negative mutants in the hamster model system warrants experimental investigation of the possibility of employing these mutants as vaccinogens. Precise determination of the structural and functional relationship of the hemadsorption process to the survival, growth, and virulence of *M. pneumoniae* in vivo must necessarily await the isolation of hemadsorption-positive revertants of these mutant strains. Experiments designed to isolate these revertants are currently in progress.

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