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Hemadsorption and Virulence Are Separable Properties of *Mycoplasma pneumoniae*

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A selective enrichment technique was used to isolate a hemadsorption-positive revertant of a hemadsorption-negative mutant strain of *Mycoplasma pneumoniae*. This hemadsorption-positive revertant was shown to have simultaneously regained both the ability to attach to neuraminidase-sensitive receptors on the tracheal ring respiratory epithelium in vitro and the ability to synthesize three virulent-strain-specific proteins which were not synthesized by the hemadsorption-negative mutant. Despite the persistence of the revertant in hamster lung tissue for 9 to 12 weeks postinfection, no cytopathology was observed. Intranasal inoculation of the revertant provided limited protection against a challenge dose of virulent *M. pneumoniae*.

Mycoplasma pneumoniae is a respiratory tract pathogen which uses attachment to host epithelial cells as a pathogenic mechanism in the production of disease (8, 20, 21, 24, 27, 31). Several *M. pneumoniae* proteins have been associated with mycoplasma attachment to the respiratory epithelium, hemadsorption, and virulence (2, 15, 16, 18, 21, 22). Hemadsorption-negative (HA^-) mutants of *M. pneumoniae* obtained by chemical mutagenesis exhibited significantly reduced abilities to attach to the respiratory epithelium in vitro. These HA^- mutants were also shown to lack one or more proteins normally present in the virulent wild-type parent strain (15, 18). In addition, these mutants did not survive or produce microscopic pneumonia in experimentally infected hamsters.

Determination of the structure-function relationship between *M. pneumoniae* hemadsorption, respiratory epithelium attachment, and virulence was precluded by the possible existence of undetected secondary mutations in the HA^- mutants. To circumvent the effect(s) of possible secondary mutations, hemadsorption-positive (HA^+) revertants of HA^- mutants were sought. Accordingly, we devised a selective enrichment technique for the isolation of spontaneously occurring HA^+ revertants from a population of HA^- mutants. One HA^+ revertant was randomly selected for study of the relationship of hemadsorption to respiratory epithelium attachment and virulence.

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MATERIALS AND METHODS

Organisms and culture conditions. Virulent *M. pneumoniae* strain M129 was originally isolated from a patient with mycoplasma pneumonia (24). The 16th broth passage of this organism (M129-B16) was used as the wild-type, hemadsorbing parent strain. The nonhemadsorbing mutant strain HA1 was derived by nitrosoguanidine mutagenesis, as previously described (15). Glass-adherent cultures were grown in 8-ounce (ca. 240-ml) or 32-ounce (ca. 960-ml) prescription bottles in Hayflick medium (19) at 37°C for 48 to 72 h (until the phenol red pH indicator changed to orange). For some experiments, the monolayers were washed three times with phosphate-buffered saline (PBS; pH 7.2) before collection by centrifugation (9,500 × *g*, 15 min). In other cases, the spent Hayflick medium was decanted, and the monolayers were resuspended in fresh Hayflick medium.

Isolation of hemadsorption-positive revertants. The HA^+ revertant strain HA1-R was isolated from the HA^- mutant strain HA1 by a selective enrichment technique (17). Briefly, 5×10^9 CFU of HA^- strain HA1 were grown in Hayflick medium. The monolayer was suspended in 4 ml of Hayflick medium, and the suspension was passed five times through a 25-gauge needle to disperse clumped organisms. The cell suspension was mixed with 0.05 ml of packed chicken erythrocytes and incubated at 37°C for 1 h. The mycoplasma-erythrocyte suspension was gently agitated periodically to promote adherence of spontaneously occurring HA^+ revertants in the mycoplasma population to erythrocytes. This suspension was then layered onto a 4-ml solution of 0.5% (wt/vol) Methocel (Dow Chemical Co., Midland, Mich.)–20% (vol/vol) Hypaque (Winthrop Laboratories, New York, N.Y.) and centrifuged at 600 × *g* for 20 min at room temperature. The supernatant fluid was removed carefully to avoid disruption of the erythrocyte pellet. The erythrocytes (with adsorbed HA^+ mycoplasmas) were

resuspended in 4 ml of Hayflick medium, mixed gently by inversion, and layered onto a Methocel-Hypaque solution. The centrifugation and washing procedure described above was performed a total of three times, and the final erythrocyte pellet was inoculated into a 32-ounce (ca. 960-ml) prescription bottle containing 75 ml of Hayflick medium. The erythrocyte-associated mycoplasmas were incubated at 37°C until the phenol red pH indicator in the growth medium became orange (72 to 120 h). The spent medium was removed, the monolayer of mycoplasmas was washed once with 10 ml of PBS, and the cells were scraped into 4 ml of Hayflick medium. The entire procedure described above (incubation with chicken erythrocytes, washing of erythrocytes by centrifugation and resuspension, and growth of erythrocyte-adherent mycoplasmas in Hayflick medium) was repeated three times. The final *M. pneumoniae* monolayer culture was diluted and plated on solidified Hayflick medium (23) and incubated at 37°C until individual colonies appeared (7 to 9 days). Hemadsorption-positive colonies were identified and cloned by standard procedures (15). One HA^+ revertant clone, designated HA1-R, was randomly selected for further experiments. Hamster-passaged strain HA1-Rδ was cloned 3 weeks post-inoculation from lung tissue of a hamster infected with HA1-R.

Two-dimensional gel electrophoresis. Isoelectric focusing–sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and non-equilibrium pH gradient electrophoresis–SDS-PAGE of total mycoplasma proteins were performed as previously described (1, 16, 25, 26).

Assay of mycoplasma attachment to tracheal rings. Tracheal ring attachment of mycoplasmas was monitored by the use of *M. pneumoniae* labeled with [*methyl*-^{3}H]thymidine, as described previously (18, 27). Individual experiments used equivalent numbers of CFU (10^7) for each mycoplasma strain, as determined by plating serial dilutions of radiolabeled mycoplasmas on solidified Hayflick medium. Hamster tracheal rings were prepared as reported previously (9). The effect of neuraminidase treatment of hamster tracheal rings on mycoplasma attachment was determined by incubating tracheal rings with either PBS or PBS containing 10 U of neuraminidase per ml (type VIII; Sigma Chemical Co., St. Louis, Mo.) before use in the standard mycoplasma attachment assay (18, 22, 27).

Virulence studies. The *M. pneumoniae* wild-type and revertant strains were grown in Hayflick medium under standard conditions and were harvested by scraping the organisms into a small volume of fresh Hayflick medium. The suspension was passed four times through a 25-gauge needle to disperse clumps of organisms and was then diluted to the desired concentration for inoculation (4×10^7 CFU/ml). Young adult male Syrian golden hamsters were anesthetized by intraperitoneal injection of sodium pentobarbital. The animals were placed in a supine position in groups of 15, and 100 μl of inoculum was placed in the external nares in sequential 10 to 20-μl doses.

Hamsters were sacrificed (exsanguination after pentobarbital anesthesia) at various times postinfection (10). The thoracic cavity was opened, and the lungs were aseptically removed and blotted on sterile gauze pads. The large lobe of the lung was fixed in 10 ml of 10% Formalin. The remaining lobes were weighed, diluted 1:10 (wt/vol) in Hayflick medium, and aseptically ground with sand. The crude lung suspension was blended in a Vortex mixer for 45 s, and the debris was allowed to settle for 10 min. The supernatant was serially diluted and plated on solidified Hayflick medium. Viable counts obtained were an approximation of the total organisms derived from infected lungs and were expressed as CFU per gram of lung tissue.

Three longitudinal lung sections were randomly selected for examination from the right, middle, and left portions of the Formalin-fixed large lobe. The coded lung sections were stained with hematoxylin-eosin and examined for intrabronchial exudate and peribronchial and perivascular infiltrate (6, 10). Virulence of *M. pneumoniae* was determined by scoring the lung sections for microscopic pneumonia (infiltrate and exudate), with theoretical lung lesion scores ranging from 0 (no damage) to 9 (severe damage). In practice, lung lesion scores reach a maximum of 7 to 8. A representative breakdown of such a score would be: peribronchial infiltrate = 3, intrabronchial exudate = 2, and perivascular infiltrate = 2. A lung lesion score of 4 would typically represent moderate peribronchial infiltrate and intrabronchial exudate with little or no perivascular infiltrate present.

Protection trials. Groups of hamsters were infected intranasally with 10^6 CFU of *M. pneumoniae* strain B16, HA1, and HA1-R or with 10^4 CFU of HA1-R. These inocula will be referred to as immunizing strains. A control group of animals received no inoculum. At 12 weeks post-immunization (day 0 of challenge), four hamsters from each group (control and immunized) were sacrificed for determination of residual, prechallenge titers of *M. pneumoniae* and for evaluation of lung cytopathology. The remaining hamsters were challenged with 3×10^6 CFU of the virulent strain B16. The challenge dose was resuspended in Hayflick medium containing fetal bovine serum, rather than agamma horse serum, to avoid a secondary response in the hamsters to serum components. Three to five hamsters from each group were sacrificed on day 3, 14, and 28 postchallenge, and the lungs were examined for viable organisms and cytopathology.

RESULTS

Isolation of HA^+ revertants. Initial attempts to isolate spontaneously arising HA^+ revertant colonies from plates of HA^- mutant strains were unsuccessful. Since reversion to the HA^+ phenotype apparently occurred at a low frequency, a selective enrichment procedure was developed to increase the probability of isolating HA^+ mycoplasmas from a HA^- population. This technique concentrates HA^+ organisms (by adsorption to erythrocytes) while concomitantly diluting the numbers of nonadsorbed HA^- mycoplasmas. When HA^- mutant strain HA1 was subjected to the selective enrichment procedure, 40% of the resultant colonies had a HA^+ phenotype. The large proportion of HA^- colonies present after enrichment were most likely nonspecifically adsorbed to erythrocytes (membrane-membrane interactions) or were carried along with HA^+ organisms as part of HA^+-HA^-

TABLE 1. Protein differences between wild-type, HA^- mutant, and HA^+ revertant strains of *M. pneumoniae*

Strain	Phenotype	Proteins[a]			
		A	B	C	α-HA1
B16	HA^+	+	+	+	−
HA1	HA^-	−	−	−	+
HA1-R	HA^+	+	+	+	+

[a] +, Present; −, absent.

aggregates of mycoplasmas. In contrast, no HA^+ revertants were obtained from another HA^- mutant strain (HA2) (15, 18) by this method. One HA^+ revertant, designated HA1-R, was randomly chosen for further experiments.

Two-dimensional PAGE. Gel electrophoretic analysis previously demonstrated that HA^- mutant strain HA1 and wild-type strain B16 have identical one-dimensional total protein profiles (15). Two-dimensional PAGE revealed that HA1 lacked three proteins present in the wild-type strain (designated as virulence-specific proteins A, B, and C) and possessed a protein with an altered isoelectric point relative to the virulent parent strain (protein α-HA1) (18).

Two-dimensional PAGE analysis, with isoelectric focusing or non-equilibrium pH gradient electrophoresis in the first dimension and SDS-PAGE in the second dimension, showed that the revertant strain HA1-R possesses virulence-specific proteins A, B, and C which are absent in the HA^- mutant HA1 (Table 1). However, strain HA1-R still synthesizes the altered protein α-HA1 which was observed in the HA^- mutant strain.

Assay of *M. pneumoniae* attachment to tracheal rings. The HA^- strain HA1 was previously shown to attach to tracheal rings in vitro at a frequency much lower than that observed with the wild-type HA^+ parent strain (15, 18). Furthermore, in contrast to the attachment of wild-type *M. pneumoniae*, cytadsorption of HA1 was insensitive to neuraminidase pretreatment of the rings (18). The ability of the HA^+ revertant strain to attach to the respiratory epithelium in vitro is shown in Fig. 1. Strain HA1-R cytadsorbed at a level approaching that exhibited by the wild-type parent strain. In addition, cytadsorption of HA1-R was neuraminidase sensitive to approximately the same degree as was the attachment of the virulent parent strain.

Virulence of HA1-R and HA1-Rδ in hamsters. Since revertant strain HA1-R regained the ability to attach to both erythrocytes and the respiratory epithelium in vitro, the virulence of this strain was examined. Previous experiments indicated that HA1-R persists at intermediate levels in hamster lung tissue at 4 weeks postinfection, while the HA^- strain HA1 is rapidly cleared (17). The survival of HA1-R was therefore examined over an 8-week period (Fig. 2A). The wild-type HA^+ strain produced high titers at week 1, with viable organisms decreasing through week 8. The HA^+ revertant strain was recovered in low numbers at week 1, but persisted at low to intermediate levels in hamster lung tissue throughout the 8-week experiment. The HA^- mutant strain HA1 is not detectable in lung tissue at week 1 or thereafter (17, 18). Infection with virulent B16 produced maximal lung cytopathology at week 2 (Fig. 2B). Histological pneumonia was moderate through week 4, but at week 8 the pneumonia was resolved. At weeks 2 through 4 postinfection, which is the peak period for wild-type cytopathology, HA1-R produced markedly reduced lung lesion scores.

The persistence of HA1-R for 8 weeks postinfection suggested two possibilities concerning the virulence potential of the revertant strain in hamsters: (i) hamsters require more than 8 weeks to totally resolve an infection with HA1-R, or (ii) selective pressure in vivo favors survival of a more virulent subpopulation of organisms. To examine these alternatives, one group

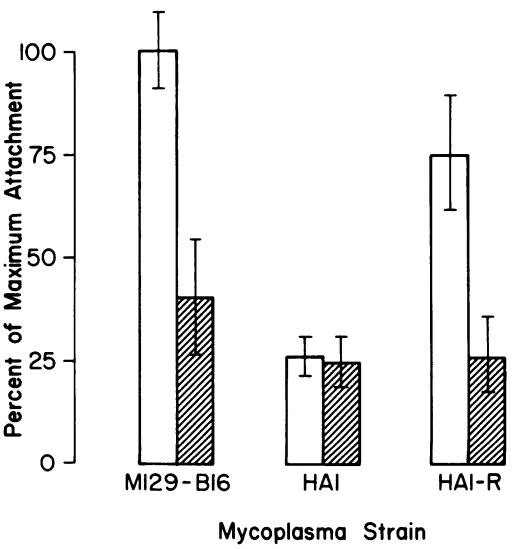

FIG. 1. Effect of neuraminidase pretreatment of hamster tracheal rings on mycoplasma attachment. Tracheal ring attachment of mycoplasmas was monitored by the use of *M. pneumoniae* labeled with [*methyl*-^{3}H]thymidine (18, 27). Equivalent numbers of CFU (10^7) of each mycoplasma strain were used in these experiments. 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. Tracheal rings were treated with either buffer for the control (clear bars) or neuraminidase (hatched bars).

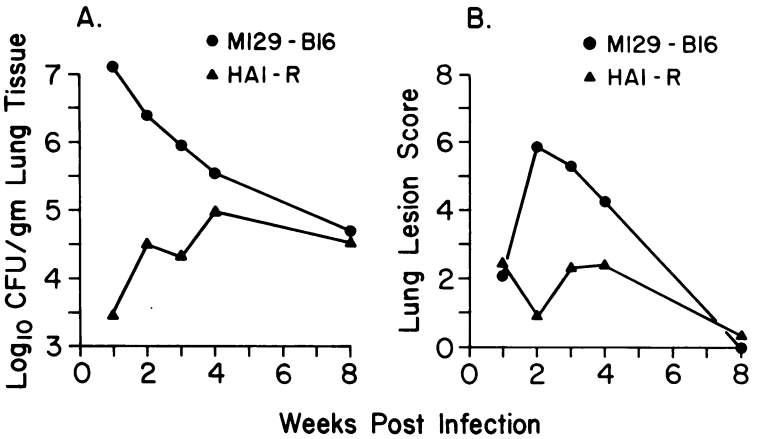

FIG. 2. Virulence of *M. pneumoniae* revertant strain HA1-R and wild-type strain M129-B16 in hamsters. Hamsters were infected intranasally with mycoplasmas (4×10^6 CFU per animal) suspended in 100 μl of Hayflick medium. (A) Viable organisms isolated from lung tissue at various time points postinfection, as indicated. The CFU of mutant strain HA1 are less than 10^3 per g of lung tissue (17, 18). (B) Lung lesion scores determined at various time points postinfection, as indicated. Each point represents a mean value obtained from either three or four individual hamsters. Statistical analyses of the data by the Mann-Whitney U test (28) demonstrate a significant difference ($P < 0.05$) between B16 and HA1-R viable counts at weeks 1 and 2 and between B16 and HA1-R lung cytopathology at weeks 2 and 3.

of hamsters was infected with HA1-R and examined over a 15-week period. Another group of animals was infected with strain HA1-Rδ, which had been isolated from hamster lung tissue after a 3-week passage in vivo.

The comparative survival in hamsters of strains HA1-R and HA1-Rδ was examined (data not shown). Lung titers of HA1-R peaked at week 6 postinfection and then steadily decreased to nondetectable levels ($<10^3$ CFU per g of lung tissue) at week 12. Although titers of HA1-Rδ at week 1 were 1 log higher than those of HA1-R, over a course of 7 weeks the survival of both strains was similar, suggesting that a more virulent subpopulation had not been selected in vivo. No cytopathology was observed during the course of the experiment in either HA1-R- or HA1-Rδ-infected lung tissue.

Protection trials with strain HA1-R as an immunizing agent. Since the virulence studies with HA1-R demonstrated that this revertant strain could survive in vivo for an extended period without concomitant production of lung cytopathology, studies were initiated to determine whether prior exposure of hamsters to HA1-R offered protection from subsequent challenge with virulent *M. pneumoniae*.

Groups of hamsters were intranasally inoculated with *M. pneumoniae* strain B16, HA1, or HA1-R. (The latter strain was administered at two different doses, 10^4 or 10^6 CFU per animal.) At 12 weeks post-immunization, the animals were challenged with virulent strain B16. At the time of challenge (day 0), each immunization group had no detectable organisms remaining in the lungs ($<10^3$ CFU per g of lung tissue). At day 3 postchallenge, the B16-immunized animals contained 2 logs less viable counts than the unimmunized control hamsters or the mutant- or revertant-immunized hamsters (data not shown). At day 14, lung tissue mycoplasma titers remained high in the control animals and the HA1- and HA1-R(10^4)-immunized animals and low in the B16-immunized group. The HA1-R(10^6)-immunized group, however, had reduced the number of challenge organisms in the lungs to intermediate levels.

Microscopic examination of lung tissue at day 3 postchallenge revealed low-level cytopathology in control and immunized hamsters. At day 14 postchallenge, lung sections from unimmunized control animals and HA1-immunized and HA1-R(10^4)-immunized animals revealed severe lung cytopathology (lung lesion score equal to or greater than 6; Fig. 3). The reduced histological pneumonia observed at day 14 in the B16-immunized hamsters is only slightly greater than that observed at day 3 in this group. The HA1-R(10^6)-immunized animals demonstrated only moderate cytopathology at day 14 of challenge, correlating with the intermediate numbers of viable *M. pneumoniae* recovered from lung tissue at this time point.

DISCUSSION

Mutant analysis of *M. pneumoniae* has provided useful information concerning the relationship between various biological activities

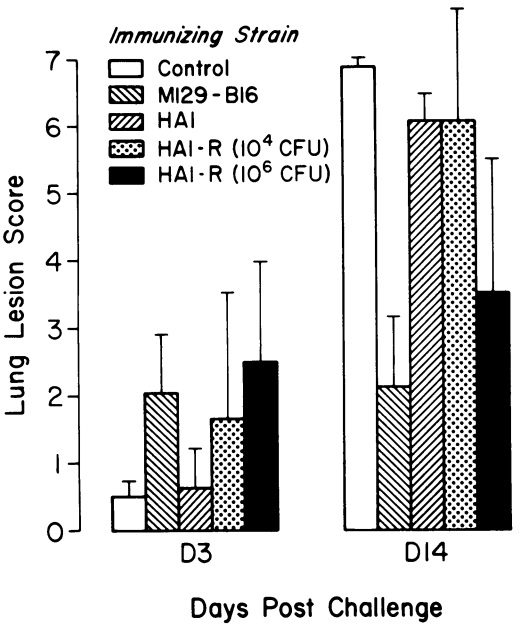

FIG. 3. Effect of prior immunization with wild-type, mutant, or revertant *M. pneumoniae* strains on development of pneumonia subsequent to challenge with virulent organisms. Hamsters were inoculated intranasally with mycoplasmas (10^6 CFU per animal, except one group of revertant-infected hamsters which received 10^4 CFU per animal) in 100 μl of Hayflick medium. At week 12 postimmunization, all animals received a challenge dose of strain M129-B16 (3×10^6 CFU per animal) in Hayflick medium containing fetal bovine serum in place of agamma horse serum. Each column represents the mean lung lesion score obtained from three individual hamsters at the time points indicated. The bars represent positive standard deviations. When the data obtained at day 14 were analyzed by analysis of variance and the Duncan test ($\alpha = 0.05$; reference 29), control, HA1 and HA1-R(10^4) lung lesion scores were significantly different from those of the B16 and HA1-R(10^6) groups, but B16 and HA1-R(10^6) scores were not statistically different from each other.

(i.e., hemadsorption, respiratory epithelium attachment, virulence) of this microorganism (15, 17, 18, 22). Previous examination of nitrosoguanidine-derived *M. pneumoniae* mutants correlated loss of specific mycoplasma proteins with (i) loss of hemadsorption ability, (ii) reduction in adherence to epithelial cells, and (iii) lack of virulence in experimentally infected hamsters. Recent studies with numerous spontaneous HA^- mutants reinforce the association of specific *M. pneumoniae* proteins with cytadsorption (2, 22). The possible existence of secondary mutations in the nitrosoguanidine-derived HA^- mutants led to the isolation of HA^+ revertants from these strains. A selective enrichment technique permitted isolation of HA^+ revertants from HA^- mutant strain HA1, but did not yield any HA^+ revertants from the HA^- mutant HA2. This observation suggests that either a single, very stable genetic lesion or a multiple mutation is responsible for the HA^- phenotype of HA2.

Two-dimensional gel electrophoresis of total protein from the HA^+ revertant HA1-R revealed that this strain possesses virulence-specific proteins which are present in wild-type *M. pneumoniae* but are absent in the HA^- mutant HA1. However, the HA^+ revertant still synthesizes protein α-HA1 which is found in the HA^- mutant strain HA1 and which exhibits an altered isoelectric point relative to that found in the virulent parent strain (18). Reversion to the HA^+ phenotype was also accompanied by increased in vitro attachment to tracheal rings. The attachment of HA1-R was significantly reduced when tracheal rings were pretreated with neuraminidase, in contrast to the neuraminidase insensitivity of HA1 attachment. Therefore, the genetic event(s) which resulted in the reversion of strain HA1 to the HA^+ phenotype simultaneously conferred upon strain HA1-R the ability to both attach in vitro to neuraminidase-sensitive sites on the respiratory epithelium and synthesize the virulence-specific proteins A, B, and C. These data implicate proteins A, B, and C in both the hemadsorption process and attachment to the respiratory epithelium. It is of interest to note that although the precise structural or functional role of these three proteins in cytadsorption remains undefined, it has been established that protein B is exposed on the cell surface of *M. pneumoniae* (16).

Examination of the virulence of strain HA1-R in vivo showed that the revertant had regained the ability to survive in hamster lung tissue, in contrast to the HA^- mutant HA1 which is rapidly cleared (17, 18). However, the titers of HA1-R were 1 to 3 logs lower than the titers of the virulent wild-type strain B16 over the first 3 weeks postinfection. At 4 to 8 weeks postinfection the virulent and revertant titers were comparable. Infection with HA1-R produced only low-level cytopathology, whereas B16 infection resulted in significant microscopic pneumonia. These data suggest that *M. pneumoniae* properties of both hemadsorption and in vitro attachment to the respiratory epithelium can be separated from virulence in vivo. Apparently a large percentage of the HA1-R inoculum was cleared from hamster lungs at some time during week 1 postinfection. However, the HA^+ revertants that did persist in lung tissue at week 1 postinfection continued to survive and replicate (and actually slightly increase in number) for 8 to 12 weeks. Selection in vivo of a more virulent

subpopulation of HA1-R was ruled out when the hamster-passaged revertant strain HA1-Rδ did not increase in titer above 10^5 CFU per g of lung tissue, but rather persisted at titers of 10^4 to 10^5 CFU per g of lung tissue throughout a 7-week study. The mechanism(s) leading to intermediate-level persistence of HA1-R in hamsters is unclear. We cannot rule out the existence of undetected secondary mutations in this revertant, which may have altered or deleted some additional virulence factors. Lipid or carbohydrate mutations, in addition to any direct effect, could also influence the mycoplasma membrane topography and thus modify the presentation of protein macromolecules on the mycoplasma surface.

It is not known why microscopic pneumonia was not detected in hamsters intranasally inoculated with HA1-R. Perhaps the intermediate number of revertant organisms in the lungs constituted an insufficient antigenic dose for triggering infiltration of host cells into pulmonary tissue adjacent to airways and vasculature. Alternatively, undetected alterations of a virulence factor(s) could result in an effective stimulus for the host cellular response to infection. Similar observations of moderate titers of organisms in lung tissue accompanied by no cytopathology have been described in studies of temperature-sensitive mutants of *M. pneumoniae* (4).

Hamsters were immunized with HA1-R at two different doses, since any protection offered against challenge with virulent *M. pneumoniae* may be dose-dependent. Because the HA^+ revertant remains in hamster lung tissue for 9 to 12 weeks postinfection, the animals were rested for 12 weeks between the immunizing dose and the B16 challenge dose. Control and HA1-immunized hamsters demonstrated no protective effect when subsequently infected with virulent strain B16. Prior infection with B16 at a dose of 10^6 CFU per animal resulted in low titers of viable organisms and minimal cytopathology postchallenge, as expected (6, 12). Although immunization with HA1-R at a dose of 10^4 CFU per animal had no detectable protective effect, a dose of 10^6 CFU of HA1-R per animal provided intermediate levels of protection from virulent *M. pneumoniae* challenge. Similarly, examination of histological pneumonia at day 14 postchallenge (Fig. 3) revealed a moderating effect of prior immunization with HA1-R(10^6) on development of lung lesions in B16-challenged animals.

The mechanism of partial protection provided by HA1-R(10^6) immunization against virulent *M. pneumoniae* challenge is undefined. Intranasal inoculation of the viable HA^+ revertant strain may stimulate cellular and humoral immune responses or secretory immune responses or all three in the host; such immune responses have been implicated in the pathogenesis of *M. pneumoniae* disease (3, 5, 7, 11, 13, 30). The paradoxical observation of reduced lung cytopathology accompanied by growth of challenge organisms in lung tissue has been reported in previous studies with temperature-sensitive and inactivated mycoplasma vaccines (4, 14). Perhaps the milieu of the host respiratory tract can be altered by persistence of replicating, attenuated *M. pneumoniae*, so as to inhibit development of pulmonary cellular infiltration and exudation without total suppression of growth of virulent mycoplasmas. Regardless of the incomplete protection provided by immunization with HA1-R, characterization of this revertant has revealed the separability of the biological properties of cytadsorption and virulence. Further characterization and molecular dissection of revertant strain HA1-R will enhance our understanding of pathogenic mechanisms of *M. pneumoniae*.

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LITERATURE CITED

1. **Ames, G. F.-L., and K. Nikaido.** 1976. Two-dimensional gel electrophoresis of membrane proteins. Biochemistry **15**:616–623.
2. **Baseman, J. B., R. M. Cole, D. C. Krause, and D. K. Leith.** 1982. Molecular basis for cytadsorption of *Mycoplasma pneumoniae*. J. Bacteriol. **151**:1514–1522.
3. **Biberfeld, G., and G. Sterner.** 1971. Antibodies in bronchial secretions following natural infection with *Mycoplasma pneumoniae*. Acta Pathol. Microbiol. Scand. **79**:599–605.
4. **Brunner, H., H. Greenberg, W. D. James, R. L. Horswood, and R. M. Chanock.** 1973. Decreased virulence and protective effect of genetically stable temperature-sensitive mutants of *Mycoplasma pneumoniae*. Ann. N.Y. Acad. Sci. **225**:436–452.
5. **Brunner, H., H. B. Greenberg, W. D. James, R. L. Horswood, R. B. Couch, and R. M. Chanock.** 1973. Antibody to *Mycoplasma pneumoniae* in nasal secretions and sputa of experimentally infected human volunteers. Infect. Immun. **8**:612–620.
6. **Clyde, W. A., Jr.** 1971. Immunopathology of experimental *Mycoplasma pneumoniae* disease. Infect. Immun. **4**:757–763.
7. **Clyde, W. A., Jr.** 1979. *Mycoplasma pneumoniae* infections of man, p. 275–306. *In* J. G. Tully and R. F. Whitcomb (ed.), The mycoplasmas, vol. II. Academic Press, Inc., New York.
8. **Collier, A. M., and J. B. Baseman.** 1973. Organ culture techniques with mycoplasmas. Ann. N.Y. Acad. Sci. **225**:277–289.
9. **Collier, A. M., W. A. Clyde, Jr., and F. W. Denny.** 1969. Biologic effects of *Mycoplasma pneumoniae* and other mycoplasmas from man on hamster tracheal organ culture. Proc. Soc. Exp. Biol. Med. **132**:1153–1158.

10. **Dajani, A. S., W. A. Clyde, Jr., and F. W. Denny.** 1965. Experimental infection with *Mycoplasma pneumoniae* (Eaton's agent). J. Exp. Med. **121**:1071–1084.
11. **Fernald, G. W.** 1979. Humoral and cellular immune responses to mycoplasmas, p. 399–423. *In* J. G. Tully and R. F. Whitcomb (ed.), The mycoplasmas, vol. I. Academic Press, Inc., New York.
12. **Fernald, G. W., and W. A. Clyde, Jr.** 1970. Protective effect of vaccines in experimental *Mycoplasma pneumoniae* disease. Infect. Immun. **1**:559–565.
13. **Fernald, G. W., and W. A. Clyde, Jr.** 1970. Pulmonary immune mechanisms in *Mycoplasma pneumoniae* disease, p. 101–130. *In* C. H. Kirkpatrick and H. Y. Reynolds (ed.), Immunologic and infectious reactions in the lung. Marcel Dekker, New York.
14. **Greenberg, H., C. M. Helms, M. B. Grizzard, W. D. James, R. L. Horswood, and R. M. Chanock.** 1977. Immunoprophylaxis of experimental *Mycoplasma pneumoniae* disease: effect of route of administration on the immunogenicity and protective effect of inactivated *M. pneumoniae* vaccine. Infect. Immun. **16**:88–92.
15. **Hansen, E. J., R. M. Wilson, and J. B. Baseman.** 1979. Isolation of mutants of *Mycoplasma pneumoniae* defective in hemadsorption. Infect. Immun. **23**:903–906.
16. **Hansen, E. J., R. M. Wilson, and J. B. Baseman.** 1979. Two-dimensional gel electrophoretic comparison of proteins from virulent and avirulent strains of *Mycoplasma pneumoniae*: Infect. Immun. **24**:468–475.
17. **Hansen, E. J., R. M. Wilson, and J. B. Baseman.** 1981. Hemadsorption and virulence of *Mycoplasma pneumoniae*, p. 241–251. *In* J. W. Streilein, D. A. Hart, J. Stein-Streilein, W. R. Duncan, and R. E. Billingham (ed.), Hamster immune responses in infectious and oncologic diseases. Plenum Publishing Corp., New York.
18. **Hansen, E. J., R. M. Wilson, W. A. Clyde, Jr., and J. B. Baseman.** 1981. Characterization of hemadsorption-negative mutants of *Mycoplasma pneumoniae*. Infect. Immun. **32**:127–136.
19. **Hayflick, L.** 1965. Tissue cultures and mycoplasmas. Tex. Rep. Biol. Med. **23**(Suppl. 1):285–303.
20. **Hu, P. C., A. M. Collier, and J. B. Baseman.** 1976. Interaction of virulent *Mycoplasma pneumoniae* with hamster tracheal organ cultures. Infect. Immun. **14**:217–224.
21. **Hu, P. C., A. M. Collier, and J. B. Baseman.** 1977. Surface parasitism by *Mycoplasma pneumoniae* of respiratory epithelium. J. Exp. Med. **145**:1328–1343.
22. **Krause, D. C., D. K. Leith, R. M. Wilson, and J. B. Baseman.** 1982. Identification of *Mycoplasma pneumoniae* proteins associated with hemadsorption and virulence. Infect. Immun. **35**:809–817.
23. **Lipman, R. P., and W. A. Clyde, Jr.** 1969. The interrelationship of virulence, cytadsorption, and peroxide formation in *Mycoplasma pneumoniae*. Proc. Soc. Exp. Biol. Med. **131**:1163–1167.
24. **Lipman, R. P., W. A. Clyde, Jr., and F. W. Denny.** 1969. Characteristics of virulent, attenuated, and avirulent *Mycoplasma pneumoniae* strains. J. Bacteriol. **100**:1037–1043.
25. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
26. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12**:1133–1142.
27. **Powell, D. A., P. C. Hu, M. Wilson, A. M. Collier, and J. B. Baseman.** 1976. Attachment of *Mycoplasma pneumoniae* to respiratory epithelium. Infect. Immun. **13**:959–966.
28. **Siegel, S.** 1956. Nonparametric statistics for behavioral sciences. McGraw-Hill Book Co., New York.
29. **Steel, R. G. D., and J. H. Torrie.** 1960. Principles and procedures of statistics. McGraw-Hill Book Co., New York.
30. **Taylor, G., D. Taylor-Robinson, and G. W. Fernald.** 1974. Reduction in the severity of *Mycoplasma pneumoniae*-induced pneumoniae in hamsters by immunosuppressive treatment with anti-thymocyte sera. J. Med. Microbiol. **7**:343–348.
31. **Upchurch, S., and M. Gabridge.** 1981. Role of host cell metabolism in the pathogenesis of *Mycoplasma pneumoniae* infection. Infect. Immun. **31**:174–181.