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Molecular Basis for Cytadsorption of *Mycoplasma pneumoniae*

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Hemadsorbing (HA^+) virulent *Mycoplasma pneumoniae* and spontaneously derived nonhemadsorbing (HA^-) avirulent mutants were compared by biochemical and ultrastructural techniques in an attempt to understand the molecular basis for cytadsorption. Lactoperoxidase-catalyzed iodination of intact mycoplasmas indicated that both virulent and avirulent mycoplasmas displayed similar surface protein patterns. A specific external protein, P1 (molecular weight, 165,000), previously implicated as a major ligand mediating attachment, was readily detected in HA^+ and HA^- mycoplasma strains. However, immunoferritin electron microscopy, with monospecific antibody against P1, revealed that differences in P1 topography existed among these strains. Only virulent mycoplasmas exhibited high concentrations of P1 at the terminal organelle. Avirulent mycoplasmas which possessed P1 showed no P1 clustering at the terminus. Both virulent *M. pneumoniae* and avirulent P1-containing mutants possessed numerous less dense P1 regions along the mycoplasma surface. Not surprisingly, an HA^- mutant lacking P1 exhibited only background immunoferritin labeling. Negative staining of intact mycoplasmas revealed a well-defined, naplike terminus (associated with P1 clusters) confined at the tip of virulent *M. pneumoniae*. Previous characterization of HA^+ virulent and HA^- avirulent strains of *M. pneumoniae* by one- and two-dimensional polyacrylamide gel electrophoresis suggests that identified groups of mycoplasma proteins, lacking in specific HA^- mycoplasmas, regulate the physical arrangement of P1 and the ultrastructure of the terminus, thus influencing adherence to the respiratory epithelium and virulence.

Surface parasitism of eucaryotic cells by virulent microorganisms is an initial and critical step in tissue colonization and subsequent disease pathogenesis. *Mycoplasma pneumoniae*, the smallest known free-living human pathogen (19), possesses a distinguishing tiplike terminal structure (2, 6, 22) which orients the mycoplasmas tip first, closely juxtaposed to the respiratory epithelium (5, 12). This host-parasite interaction is influenced, at least in part, by sialic acid-containing sites on the epithelium (5, 18). We previously implicated a trypsin-sensitive surface protein on *M. pneumoniae*, designated P1 (molecular weight, 165,000 daltons), as the prime mycoplasmal ligand (12). However, an avirulent *M. pneumoniae* strain which cannot parasitize the respiratory epithelium also possesses a surface-located P1 protein (8). Isolation of mutagen-derived (7) and spontaneous nonhemadsorbing (HA^-) mutants (16) of the hemadsorbing (HA^+) virulent *M. pneumoniae* strain permitted characterization of these mutants by protein gel profiles, attachment capabilities, and virulence (8–10, 16). These data linked additional mycoplasma proteins to the adherence event. By using an in vitro binding assay, we recently demonstrated the selective adherence of several of these mycoplasma proteins, including P1, to hamster trachea epithelial cells via a receptor-ligand mechanism of recognition (15).

In the study reported here, we employed immunoferritin electron microscopy with monospecific antiserum against P1 to define the topography of P1 in relation to the terminal organelle of *M. pneumoniae*. By so doing, we also distinguished between virulent and avirulent mycoplasmas, reinforcing the putative role of other mycoplasma proteins previously implicated in adherence.

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MATERIALS AND METHODS

Organisms and culture conditions. The cloned virulent *M. pneumoniae* strain M129-B25C and representative HA^- mutants described previously (7, 10, 16) were used in this study. The virulence of strain B25C and the avirulence of the HA^- mutants have been previously established (9, 10, 16). Monolayer cultures of *M. pneumoniae* were grown in 70 ml of Hayflick medium (11) at 37°C in 32-oz (ca. 960-ml) glass prescription bottles. Organisms were harvested in the late log phase 48 to 72 h after inoculation (when the phenol red indicator in the growth medium became orange in color). After three washes with phosphate-buffered saline (PBS; pH 7.2), mycoplasmas were collected by centrifugation at 9,500 × *g* for 15 min. For certain experiments, mycoplasma proteins were radiolabeled intrinsically with [^{35}S]methionine as described elsewhere (16) before being washed.

Preparation of *M. pneumoniae* protein P1 for immunization. Monospecific antiserum directed against P1 was produced as described by Tjian et al. (20) with modification. Approximately 10^{11} colony-forming units of *M. pneumoniae* were suspended in dissolving buffer (62.5 mM Tris [pH 6.8], 2% β-mercaptoethanol, 10% glycerol) and then solubilized with the addition of sodium dodecyl sulfate (SDS) to a final concentration of 2%. The preparation was boiled for 3 min and then centrifuged for 2 min in a Microfuge B (Beckman Instruments, Inc., Palo Alto, Calif.) to remove insoluble debris. Bromophenol blue was added to a concentration of 0.005%. A 1-ml amount of the resulting supernatant, containing approximately 4 mg of total protein, was layered on individual 1.5-mm-thick SDS-polyacrylamide slab gels (17). The gel system consisted of a 3% stacking gel and a 4.5% separating gel; no sample wells were constructed. After electrophoresis, the gels were stained overnight in 0.25% Coomassie brilliant blue dissolved in 50% methanol–10% glacial acetic acid. Gels were destained with gentle agitation in 25% methanol–7.5% glacial acetic acid until the protein bands were discernible. Protein band P1, identified by its mobility in gels, was sliced from the slab, macerated by forcing the gel slice through an 18-gauge needle, and lyophilized. The dried acrylamide containing P1 was ground to a fine powder with a mortar and pestle, divided into three equal samples, and stored in tightly sealed tubes at −20°C.

Production of antiserum. One aliquot of the powdered acrylamide, containing approximately 150 μg of P1 (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, Calif.), was mixed with distilled water to a final volume of 0.8 ml and emulsified with 0.8 ml of Freund complete adjuvant. A male New Zealand white rabbit (from which preimmunization serum had been collected) was injected subcutaneously in the neck region and subcutaneously and intramuscularly in the hind legs. Booster injections, with Freund incomplete adjuvant substituted for Freund complete adjuvant, were administered 2 and 5 weeks after the initial injection. Blood was collected from the ear beginning 5 days after the final booster. Serum was aliquoted and stored at −70°C. Rabbit antiserum raised against whole *M. pneumoniae* organisms was obtained by subcutaneous and intramuscular injection of 10^{10} *M. pneumoniae* organisms in Freund complete adjuvant, followed 3 weeks later by a booster of 10^{10} mycoplasmas in Freund incomplete adjuvant. Bleedings were initiated 1 week after the booster injection.

Analysis of serum specificity. The reactivity of prebleed serum and immune anti-P1 antiserum was examined by radioimmunoprecipitation (1, 14). [^{35}S]methionine-labeled *M. pneumoniae* were solubilized in TDSET (10 mM Tris [pH 7.8], 0.2% sodium deoxycholate, 0.1% SDS, 10 mM tetrasodium EDTA, and 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride. After 5 min at room temperature, ovalbumin was added to a final concentration of 1 mg/ml. The preparation was incubated at 37°C for 15 min and then centrifuged at 100,000 × *g* for 45 min at 4°C on a cushion of TDSET containing 5% sucrose. The supernatant was removed, diluted to the desired volume (1 to 2 ml), and divided into 100-μl samples. A 20-μl amount of serum (concentrated or diluted in PBS) was added to each tube containing radiolabeled *M. pneumoniae* proteins; PBS alone was added to the serum-free control sample. The antigen-antibody mixtures were incubated for 15 min at 37°C and then overnight at 4°C. Precipitation of antigen-antibody complexes was achieved by the addition of 50 μl of a 10% suspension of formaldehyde-treated organisms of protein A-bearing *Staphylococcus aureus* Cowan 1. After 1.75 h at 4°C, the *S. aureus* organisms were washed four times with TDSET, and *M. pneumoniae* proteins reactive with the sera were eluted by boiling the *S. aureus* organisms for 4 min in 40 μl of dissolving buffer containing 2% SDS and 0.005% bromophenol blue. The bacteria were pelleted, and the supernatants were removed and applied to SDS-polyacrylamide gels (3% stacking gel and 7.5% separating gel). After electrophoresis, the gels were processed for fluorography by the method of Bonner and Laskey (3).

Lactoperoxidase-mediated radioiodination. Cultures of wild-type and HA^- *M. pneumoniae*, grown and harvested as described above, were washed extensively and suspended in PBS. Carrier-free Na^{125}I (Amersham Corp., Arlington Heights, Ill.) was added to each suspension at a final concentration of 300 μCi/ml. Lactoperoxidase (100 IU/mg; CalBiochem, La Jolla, Calif.) was introduced at a final concentration of 30 μg/ml, and labeling was initiated with the addition of 25 μl of 0.0012% H_2O_2. Ten minutes later, 25 μl of fresh 0.0012% H_2O_2 was added. The reaction was terminated after an additional 10 min with 5 ml of cold phosphate-buffered iodide (similar to PBS, with NaI substituted for NaCl). The labeled mycoplasmas were washed three times with phosphate-buffered iodide and then prepared for SDS-polyacrylamide gel electrophoresis (PAGE).

After electrophoresis, the gel was fixed overnight in 50% methanol–10% acetic acid, rehydrated in 7% acetic acid, and dried under a vacuum onto filter paper for autoradiography. Autoradiograms were scanned by using an Ortec model 4310 densitometer.

Immunoferritin labeling. *M. pneumoniae* preparations used for electron microscopy were grown at 37°C for 48 h in SP-4 medium (21). Adherent organisms were scraped into the growth medium, and glutaraldehyde was added to a final concentration of 0.8%. After 1 h at room temperature, the mycoplasmas were collected by centrifugation with an Eppendorf Microfuge and washed three times with wash buffer (0.01 M Tris [pH 7.2], 0.14 M NaCl, 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$). The mycoplasma pellet was suspended in 1.0

ml of wash buffer to which was added 0.2 ml of a 1:10 dilution of rabbit serum (prebleed or monospecific). After 2 h at room temperature, mycoplasmas were washed five times with wash buffer. The mycoplasma pellet was suspended in 1 ml of wash buffer, and 0.2 ml of this suspension was combined with 0.2 ml of a 1:20 dilution of a ferritin-conjugated immunoglobulin G (IgG) fraction of goat anti-rabbit IgG (Cappel Laboratories, Cochranville, Pa.). Incubation was continued for 2 h at room temperature, followed by five washings in buffer and blending in a Vortex mixer. Mycoplasmas were suspended in 1% ammonium acetate, applied to a coated grid, and examined in an electron microscope (Hitachi model HU-11C; operated at 75 kV). The terminal surface nap of *M. pneumoniae* was identified by negative staining of intact mycoplasmas (11a).

RESULTS

Characterization of monospecific P1 antiserum. Purification of *M. pneumoniae* protein P1 from acrylamide slab gels resulted in an electrophoretically homogeneous preparation that produced a single protein band of 165,000 daltons when reexamined by SDS-PAGE.

The calculated molecular weight of P1 indicated here and in our earlier papers (15, 16) was determined by using 5 and 7.5% acrylamide concentrations (17) and appropriate molecular weight standards (range, 25,700 to 200,000). This value of P1 differs from that recently reported by others (11a). In the latter case, it is unclear how molecular weight determinations were derived.

The specificity of rabbit antiserum against P1 was examined by radioimmunoprecipitation (Fig. 1). No bands were visible in the serum-free control preparation (Fig. 1a, lane B), indicating little or no nonspecific binding of mycoplasma proteins to the fixed *S. aureus*. Several faint bands were precipitated by prebleed serum (Fig. 1a, lanes C and D). This may be attributed to minimal cross-reactivity in normal rabbit serum. In contrast, the anti-P1 antiserum reacted intensely with *M. pneumoniae* protein P1 (Fig. 1a, lanes E and F). The profile of total mycoplasma proteins precipitated by rabbit anti-*M. pneumoniae* antiserum is provided for comparative purposes (Fig. 1a, lane G). Molecular weights were determined by coelectrophoresis of ^{14}C-labeled molecular weight standards (Fig. 1a, lane H).

To further establish the purity of the monospecific antiserum reagent, we tested for the presence of rabbit immunoglobulin which reacted with *M. pneumoniae* proteins but did not directly bind to *S. aureus* protein A (Fig. 1b). After overnight incubation of solubilized, radiolabeled mycoplasma preparations with immune or normal rabbit serum, 10 μl of the IgG fraction of goat anti-rabbit immunoglobulin (IgG, IgM, and IgA; Cappell Laboratories) was added, and the preparation was incubated for 2 h at 4°C. All *M. pneumoniae* antigen-rabbit immunoglobulin complexes were then precipitated with *S. aureus* and processed as before. The profiles from the double-antibody precipitation (Fig. 1b) were identical to those obtained in the radioimmunoprecipitation described above (Fig. 1a).

Lactoperoxidase-catalyzed radioiodination of *M. pneumoniae* mutants. Previous examination of the protein profiles of spontaneous HA^- mutants by SDS-PAGE revealed that all but one mutant category (class IV) (16) possessed apparently normal levels of proteins P1, as determined by the intensity of Coomassie brilliant blue staining and fluorography (16). Lactoperoxidase-mediated radioiodination, combined with SDS-PAGE and autoradiography, was previously used to demonstrate the exposure of P1 on the outer surface of virulent HA^+ *M. pneumoniae* (12). Therefore, this approach was employed to determine the degree of surface exposure of P1 on the HA^- mutants relative to that seen with the wild-type strain. The results (Fig. 2) revealed

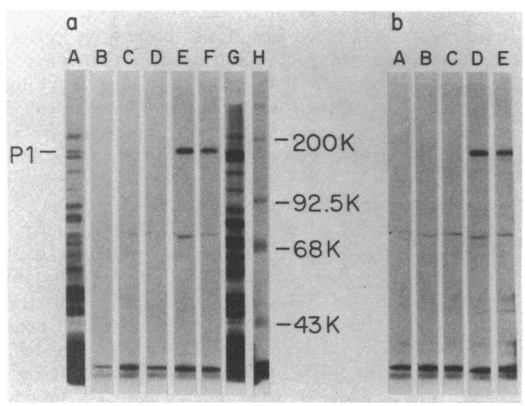

FIG. 1. Radioimmunoprecipitation analysis of anti-P1 antiserum. Single-antibody radioimmunoprecipitation (a) was done as described in the text. For double-antibody radioimmunoprecipitations (b), goat anti-rabbit immunoglobulin was added to test for the presence of antigen-antibody complexes which were not precipitated by protein A-bearing *S. aureus*. (a) Lanes: A, total [^{35}S]methionine-labeled *M. pneumoniae* protein profile; B, no serum control; C and D, undiluted and 1:10 dilution of prebleed serum, respectively; E and F, undiluted and 1:10 dilution of anti-P1 antiserum, respectively; G, anti-*M. pneumoniae* antiserum; H, ^{14}C-labeled molecular weight standards (myosin, 200,000; phosphorylase *b*, 92,500; bovine serum albumin, 68,000; and ovalbumin, 43,000), as indicated. (b) Lanes: A, goat anti-rabbit immunoglobulin control (no rabbit serum); B and C, undiluted and 1:10 dilution of prebleed serum, respectively; D and E, undiluted and 1:10 dilution of anti-P1 antiserum, respectively.

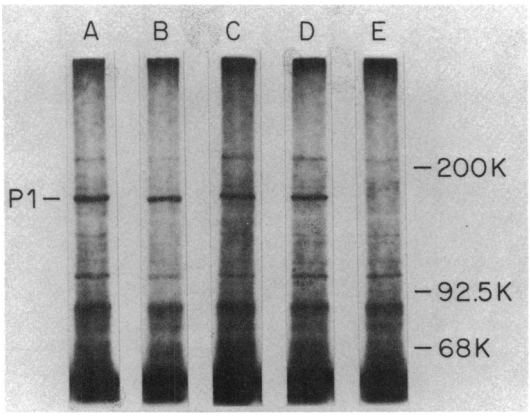

FIG. 2. Autoradiogram of lactoperoxidase-mediated radioiodination profiles of wild-type and representative HA$^-$ mutant classes of *M. pneumoniae*. A, Wild-type B25C; B to E, spontaneous HA$^-$ mutants from classes I, II, III, and IV, respectively (16). *M. pneumoniae* protein P1 is indicated on the left, and the positions of molecular weight markers are on the right. A scan of the autoradiogram and calculation of the area under the peak corresponding to P1 in each strain resulted in the following relative absorbances: B25C, 250 U; class I, 245 U; class II, 216 U; class III, 245 U; and class IV, 0 U.

that no substantial differences existed between the surface labeling of P1 in those mutants possessing P1 (Fig. 2B–D) and the parent strain B25C (Fig. 2A), confirming the surface location of P1. As expected, no labeled band was observed in the area of the gel corresponding to P1 in the HA$^-$ mutant lacking that protein (Fig. 2E).

***M. pneumoniae* characterization by electron microscopy.** To clarify the apparent functional contradiction between the role of P1 in cytadsorption of virulent *M. pneumoniae* and the markedly reduced ability of P1-containing HA$^-$ mutants to bind to the respiratory epithelium (7, 10, 16), high-resolution electron microscopy and immunoferritin labeling were employed. This combination of techniques permits assessment of the tip structures of virulent *M. pneumoniae* and its isogenic HA$^-$ mutants and also provides a basis for determining P1 topography.

The distinguishing terminus of negatively stained, intact virulent *M. pneumoniae*, with its characteristic naplike and truncated structure, is shown in Fig. 3a. Note the clear demarcation of the nap, which is restricted to the tip region. Figure 3b is a companion micrograph which defines the physical arrangement of P1 on the

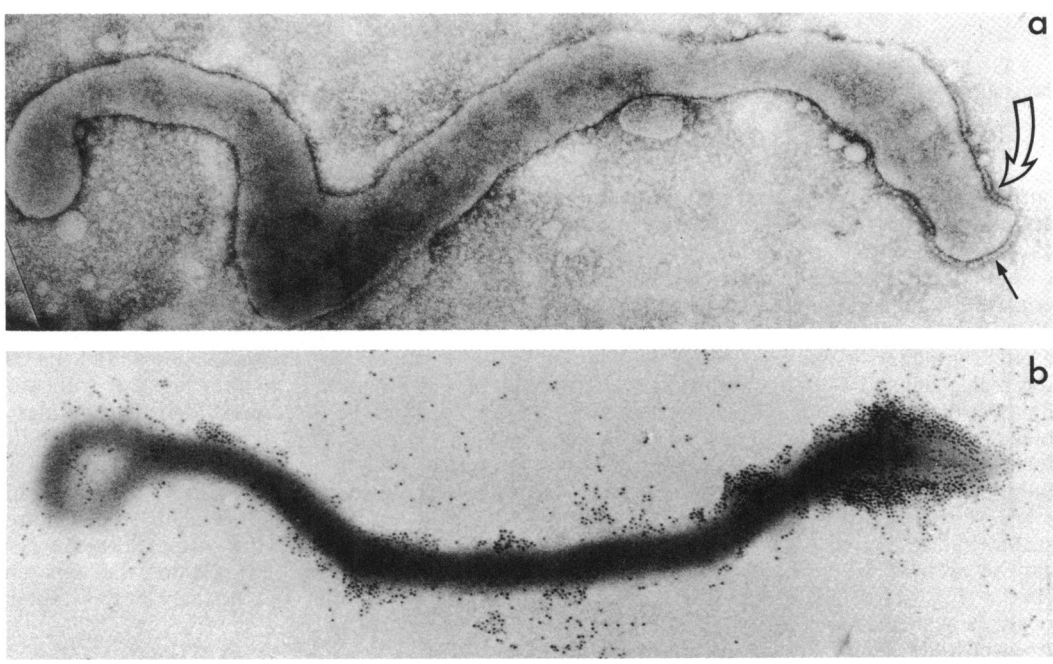

FIG. 3. (a) Negatively stained, intact virulent *M. pneumoniae* demonstrating its characteristic nap (solid arrow) and truncated terminus (open arrow). Magnification, ×125,000. (b) Immunoferritin-labeled virulent *M. pneumoniae* exhibiting the highly dense clustering of P1 sites at the terminus, with less dense but frequent sites along the membrane surface. Magnification, ×76,000.

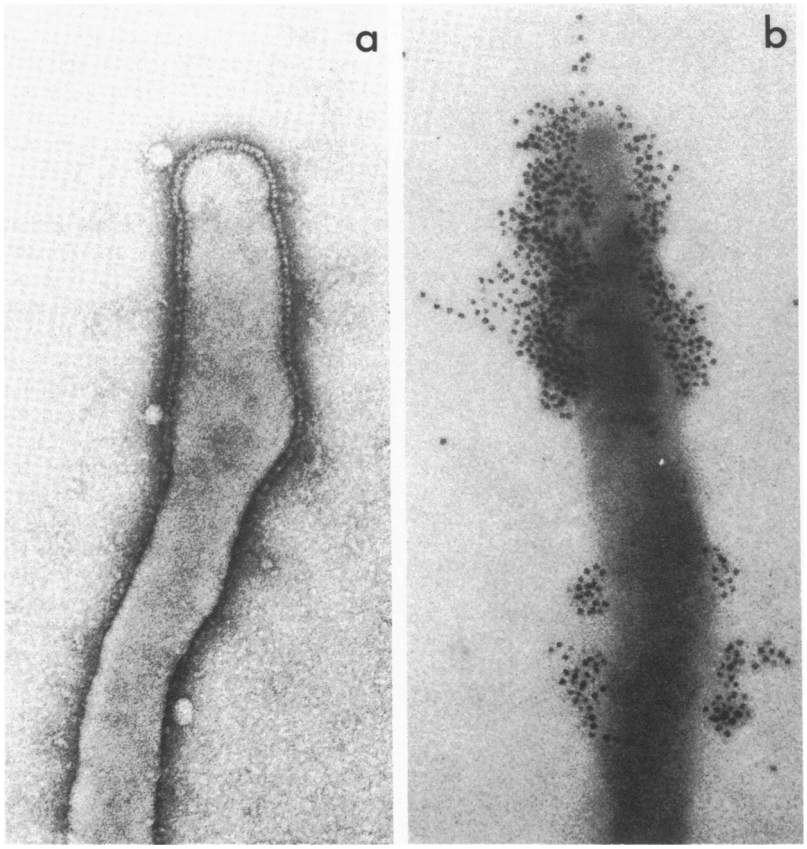

FIG. 4. High-resolution electron microscopy of (a) *M. pneumoniae* nap and (b) P1 clusters at the tip region. Magnification: (a) ×130,000; (b) ×150,000.

surface of *M. pneumoniae*; monospecific P1 antibody and immunoferritin labeling were used as a probe. There is an obvious clustering of P1-containing sites at the terminal organelle. Less dense P1 regions can be detected in many places along the outer mycoplasma membrane. Figure 4 offers a higher-resolution view of the nap and the highly dense P1 configurations at the terminus. Virulent *M. pneumoniae* exposed to prebleed serum showed little association of the ferritin marker (Fig. 5). Interestingly, P1-containing HA^- mutants displayed P1 sites at numerous surface locations, as did the virulent strain. In direct contrast, no concentration of P1 can be seen at the terminal regions of mutant class I (Fig. 6a) or among other mutant classes possessing surface-located P1 (data not shown). As expected, the mutant lacking P1 (16) exhibited only background immunoferritin labeling (Fig. 6b).

To further understand the possible relationship between the naplike terminus and protein P1, we compared negatively stained, intact virulent *M. pneumoniae* with representative P1-positive HA^- mutant classes. Figure 7a shows the wild-type HA^+ virulent mycoplasma with the truncated nap; Fig. 7b shows a class I mutant (16) which possesses P1 but has spontaneously lost a series of high-molecular-weight proteins, as determined by one-dimensional SDS-PAGE (16). In this mutant, neither nap nor truncated terminus can be discerned. Figure 8 provides a comparison at higher magnification between the terminal region of another mutant class (Fig. 8a), which possesses P1 but lacks three proteins detected only by two-dimensional gel electrophoresis (8, 16), and the terminal region of a class I mutant (Fig. 8b). Notice the apparent truncated terminus but no nap in this mutant class (Fig. 8a).

DISCUSSION

We have attempted to utilize a multifaceted approach to understand the molecular basis of cytadsorption by virulent *M. pneumoniae*. What initially was explained by a single mycoplasma

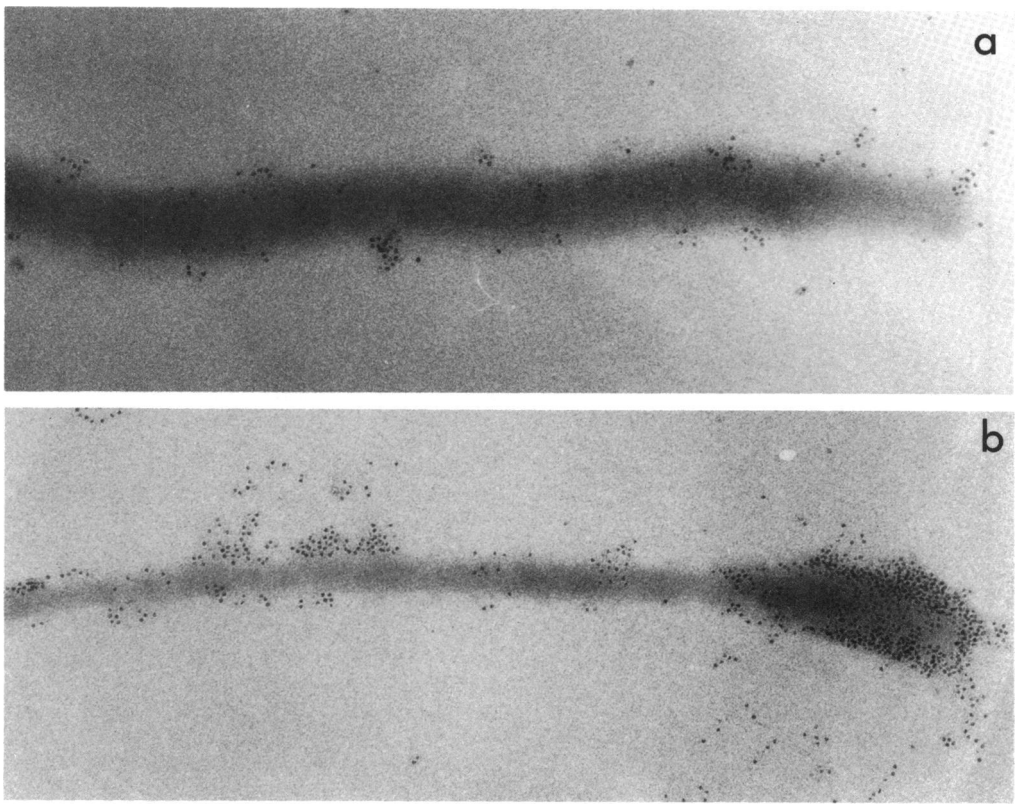

FIG. 5. Ferritin labeling of virulent *M. pneumoniae* with (a) prebleed antiserum and (b) immune monospecific antiserum against P1. Note the low background level of ferritin marker in *M. pneumoniae* exposed to preimmunized serum. Magnification: (a) ×100,000; (b) ×86,000.

macromolecule, protein P1, which appeared to mediate attachment of intact mycoplasmas to neuraminidase-sensitive regions on the respiratory epithelium (12), was later complicated by the detection of this protein on avirulent, weakly adhering mutants (7, 8, 16). The isolation and characterization of HA^- mutants with greatly diminished ability to parasitize respiratory cells indicated that groups of proteins, in addition to P1, appeared essential for adherence. Several diverse approaches were employed to account for the apparent complexity of the observations.

One technique made possible the identification of *M. pneumoniae* proteinaceous ligands via an in vitro binding assay (15). We observed the highly selective and avid association with tracheal cells of individual mycoplasma proteins, including P1 and several other proteins previously implicated by HA^- mutant analysis. Competitive binding studies reinforced the specific nature of the receptor-ligand interactions. Another series of experiments permitted the isolation of HA^+ revertants from HA^- mutants by a selective enrichment procedure (9; unpublished data). A direct functional or structural involvement (or both) of specific proteins in adherence was shown. In all instances, HA^+ revertants simultaneously regained the ability to synthesize these proteins and to adhere to the respiratory epithelium. Thus, the biological event(s) which enabled resynthesis of particular groups of proteins normally found in the virulent HA^+ parent strain also fully restored attachment capabilities.

In this report, we more clearly defined the role of protein P1 in cytadsorption. We showed that P1 localizes in high concentration at the tiplike terminus of virulent *M. pneumoniae*, providing a dense clustering of these molecules in association with the naplike structure detailed in negatively stained, intact wild-type *M. pneumoniae*. A similar finding has recently been described (11a). The reduced attachment capabilities of mutants possessing P1 is explained by the absence of both nap and P1 clusters at the tip structure. Among the mutants examined, the truncated terminus may or may not be present (Fig. 7 and 8).

Of special interest is the observation that less

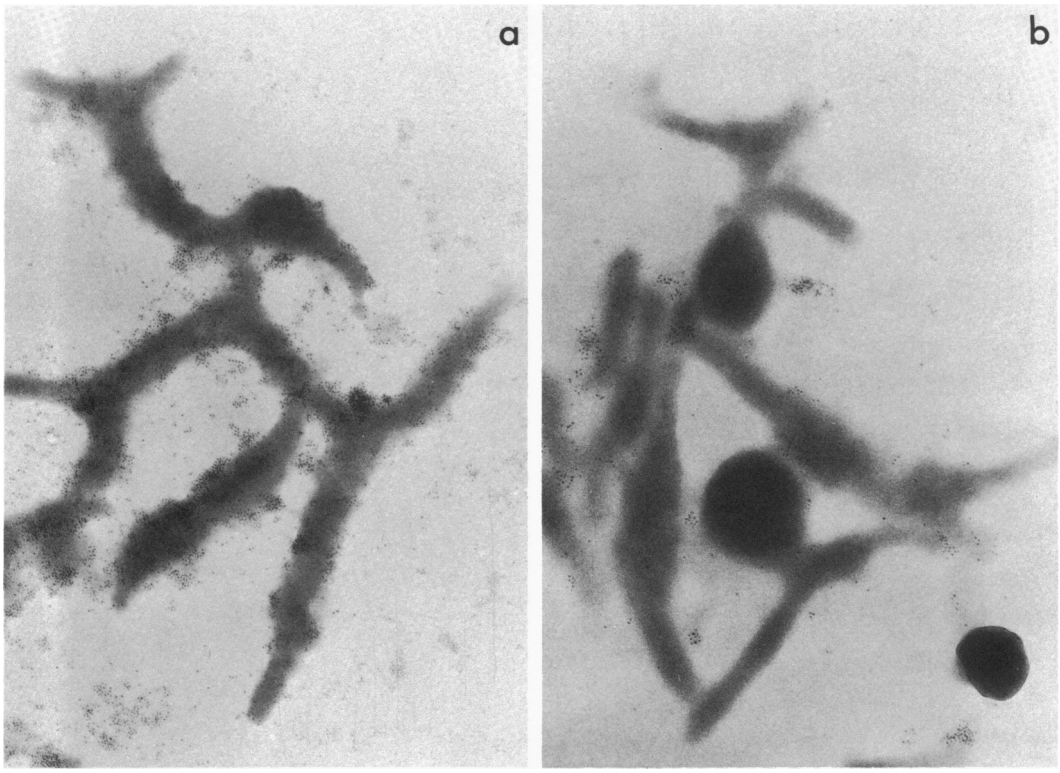

FIG. 6. Immunoferritin electron microscopy of HA^- mutants of *M. pneumoniae*. (a) *M. pneumoniae* mutant class I possessing P1. P1 sites can be observed along the mycoplasma surface, with no P1 concentration at the termini. (b) *M. pneumoniae* mutant class IV lacking P1 and showing little binding of ferritin marker. Magnification: (a) ×63,000; (b) ×55,000.

dense but clearly detectable P1-containing regions occur throughout the surface length of both virulent wild-type organisms and HA^- mutants possessing P1. At these P1 sites, no nap is observed. Thus, P1 need not be exclusively localized at either the tapered tip region or the naplike structure. This observation in part explains reports that *M. pneumoniae* may adhere to host cells by regions other than the terminus (4). It also clarifies our previous observations that mutagen-derived (10) or spontaneous HA^- mutants (16) still display residual binding to neuraminidase-sensitive and -insensitive sites on respiratory epithelial cells. In the case of neuraminidase-sensitive attachment, we have linked several of the previously referenced proteins (based on mutant analysis and two-dimensional SDS-PAGE [10, 16; J. B. Baseman, M. Banai, and I. Kahane, Infect. Immun., in press]) with sialic acid-dependent recognition. Furthermore, preliminary data indicate that monospecific antibody directed against P1 inhibits attachment of virulent *M. pneumoniae* to respiratory epithelium by approximately 75%, thus underscoring the importance of P1 in cytadsorption.

Taken together, the structure-function relationships among specific mycoplasma proteins, the naplike truncated terminus, the polarity of attachment of *M. pneumoniae* to sialic acid-containing regions on the respiratory epithelium, and virulence can be directly correlated to a series of identifiable mycoplasma proteins. The spontaneous loss of groups of these molecules results in morphological alteration in the terminal organelle as well as absence of a nap, corresponding to loss of hemadsorption capabilities (9, 13, 16), markedly decreased adherence to respiratory cells (16), and avirulence (10, 16). By employing a variety of approaches and techniques, the complexity of the cytadsorption event required for successful surface parasitism by *M. pneumoniae* is detailed. Several mycoplasma proteins (16) appear to be involved in direct attachment of *M. pneumoniae* to respiratory epithelium, as accessory proteins in the anchoring or functioning or both of protein P1, or as critical components in the unique structure and topography of the tip region. Cooperation between these proteins may also permit essential lateral mobility or activation of the mem-

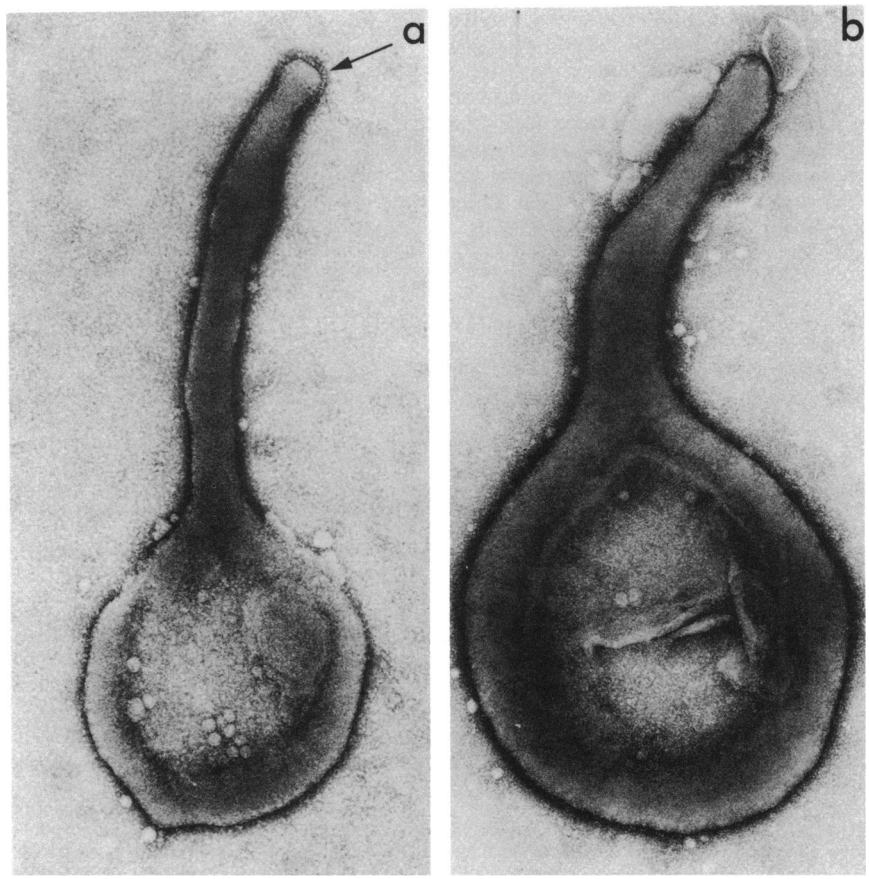

FIG. 7. Comparison of negatively stained wild-type virulent *M. pneumoniae* and HA^- mycoplasma mutant. (a) Virulent *M. pneumoniae* with its distinguishing nap (arrow) and unique flasklike appearance. (b) Avirulent HA^- mutant class I possessing P1 but lacking the nap. Magnification, ×83,000.

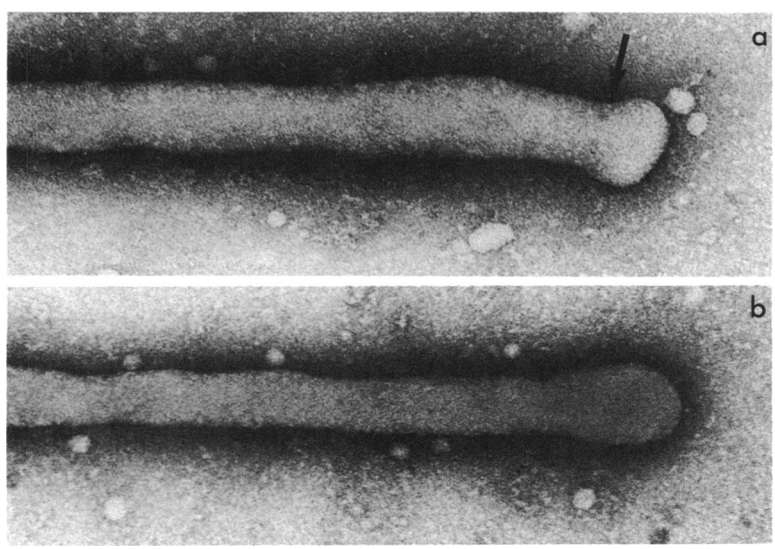

FIG. 8. Negative staining of different HA^- mutant mycoplasma classes. (a) Mutant class III with truncated tip (arrow) but no nap. (b) Mutant class I with neither truncation nor nap. Magnification, ×225,000.

brane for formation of the truncated naplike terminus. The combination of mutant analysis, biochemical characterization, and ultrastructural studies permits examination of protein-membrane associations at the molecular level and reinforces the usefulness of this model for elucidating membrane attachment proteins and mechanisms by which membrane macromolecules interact for proper biological function.

ACKNOWLEDGMENTS

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