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The Immunodominant 90-Kilodalton Protein Is Localized on the Terminal Tip Structure of *Mycoplasma pneumoniae*

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Immunoblot analysis of convalescent-phase sera of experimentally infected chimpanzees or monoclonal antibodies (MAbs) specific to the 90- and 40-kDa proteins of *Mycoplasma pneumoniae* indicated that both proteins were present in cytadsorbing, pathogenic strains PI-1428, M129, and FH but absent in noncytadsorbing, nonpathogenic strain M129-B176. Adsorption of convalescent-phase chimpanzee sera with virulent strain PI-1428 removed reactivity, whereas adsorption with avirulent strain M129-B176 did not remove reactivity to these two proteins. By using proteolysis and specific MAbs, we demonstrated that the 90- and 40-kDa proteins were surface exposed. Immunoelectron microscopy employing specific MAbs showed that the 90-kDa protein is localized on the terminal tip attachment apparatus. However, the MAb specific for the 40-kDa protein failed to indicate a similar localization. Nevertheless, these data, taken together, indicate that the immunodominant 90- and 40-kDa proteins are surface exposed, are localized on the terminal tip apparatus, and might be involved in the attachment mechanism.

The ability of *Mycoplasma pneumoniae* to attach is a prerequisite for colonization of the respiratory mucosal epithelium and subsequent induction of disease in humans and animals (16, 17, 24, 42). Attachment has been correlated with virulence and pathogenicity (12, 35). *M. pneumoniae* possesses a characteristic terminal tip-like structure (11, 20, 42) that is oriented in close juxtaposition to the infected mucosal epithelium (14, 15, 24, 37). Previous studies have indicated that specific attachment of *M. pneumoniae* to target cells is mediated by the P1 adhesin protein (24) that is located at the surface of the terminal tip organelle (23). Treatment of virulent strain M-129-B16 with trypsin resulted in loss of P1, a smaller protein (P2), and the ability to attach. Regeneration of these proteins restored the ability of the organism to attach (24) and indicates that the P1 protein is the major surface component responsible for attachment (8, 23). Hu and colleagues (23, 27) have also shown that monoclonal antibodies (MAbs) directed against the P1 protein can inhibit attachment, and these findings were confirmed by Gestenecker and Jacob (21). However, subsequent studies have suggested that the molecular moiety of attachment involves multiple components (18). Several investigators have suggested that components in addition to the P1 adhesin are probably involved in adherence (9, 10, 22, 27, 30, 31) and that accessory proteins are required for the attachment process (8, 25, 29). Baseman et al. (8) showed that noncytadsorbing mutant M129-B176 produces the P1 protein but that P1 is not located at the terminal organelle. They also reported that the 30-kDa protein (P30) is an adhesin, associated with hemadsorption, that is localized at the terminal tip structure of *M. pneumoniae* on the basis of biochemical, immunological, and ultrastructural data (10). Stevens and Krause (40) reported that the high-molecular-weight membrane proteins associated with the cytoskeletonlike triton shell of *M. pneumoniae* are also localized primarily in the filamentous extension of the mycoplasma cell on the basis of biochemical and electron microscopy studies. They also discussed their function in cytadherence. The evidence suggested that the attachment element is an organized moiety composed of a number of organized protein components. This possibility is strongly supported by molecular cloning and sequence analysis studies of the P1 operon (27, 28, 41). These studies indicate that the P1 protein gene is part of an operon that consists of three open reading frames (ORF) in the order ORF4-P1-ORF6. Since analysis of the deduced amino acid sequence indicates that both the P1 gene and ORF6 possesses a membrane protein, a leader signal, and a stop-transfer sequence (28), it is likely that both are components of the attachment moiety. Recently, Sperker et al. (39) demonstrated that instead of an expected 130-kDa protein, 90- and 40-kDa proteins were identified as the gene products of ORF6. This indicates that the attachment moiety of *M. pneumoniae* has a complex organization.

The P1 protein has been established as an important disease-related immunogen (26). Antibodies specific for P1 were demonstrated in the respiratory secretions and sera of patients with culture-proven *M. pneumoniae* infections. Recently, Franzoso et al. (19) demonstrated that two additional proteins with approximate molecular masses of 86 and 35 kDa were highly immunogenic in experimentally infected chimpanzees (19a). In the present study, we showed that the 86- and 35-kDa surface proteins were identical to the 90- and 40-kDa proteins described earlier by Hu et al. (25). These proteins were present in each of three cytadsorbing, pathogenic strains examined but absent in noncytadsorbing strain M129-B176. We showed that the 90-kDa protein is localized at the terminal tip structure of the organism and that the 40-kDa protein may also be localized at the tip. These findings suggest that these two immunogenic proteins are also involved in the attachment process and play a role in the pathogenesis of disease.

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TABLE 1. Characteristics of the chimpanzees used in this study

Identification no.	Size of inoculum (10^7 CCU[a]) used for experimental infection[b]	Vaccine used for immunization[c]
854	1	
881	1	
967	1	
810		OSU-1A
903		OSU-1A
1009		OSU-1A
957	3	
1028	3	
1157	3	
905		Acellular
918		Acellular
1008		Acellular
856	Unknown[d]	
1110	Unknown[d]	
854		None[e]
881		None[e]
902		None[f]
1161		None[f]

[a] CCU, color-changing unit.

[b] Chimpanzees were experimentally infected intratracheally with either 1×10^7 or 3×10^7 color-changing units of virulent strain PI-1428 of *M. pneumoniae*.

[c] Chimpanzees were immunized with either the OSU-1A formalin-inactivated vaccine or the acellular vaccine in 2 doses by the intraperitoneal priming and intratracheal booster immunization schedule or given sterile broth (positive control) twice with a 3-week interval and then challenged intratracheally 2 weeks later with 10^7 color-changing units of virulent strain PI-1428 of *M. pneumoniae*. The two chimpanzees with prior infections (see footnote *e*) were also rechallenged. The same immunization schedule protected hamsters against a challenge (4).

[d] The two negative-control chimpanzees inadvertently became infected during the course of the study.

[e] Previously infected.

[f] Sterile broth was administered (see footnotes).

MATERIALS AND METHODS

M. pneumoniae. The three cytadsorbing pathogenic strains used include low-passage, pathogenic, cytadsorbing strain PI-1428, obtained from R. M. Chanock; strain M129, from A. M. Collier; and strain FH, used in our previous study (3). Noncytadsorbing, nonpathogenic derivative strain M129-B176, passage 186 of strain M129 (36), was a gift from W. A. Clyde, Jr. The mycoplasmas were grown in 150-cm^2 Corning flasks containing Edward-Hayflick medium (2) at 36 ± 1°C for 5 to 7 days. The adherent colonies were scraped off into the medium, and the cells were harvested following centrifugation at 12,000 × *g* for 30 min at 4°C. The cell pellet was washed twice in phosphate-buffered saline (PBS) pH 7.2, aliquoted in 20 vials, and stored at −40°C until used.

Chimpanzees. The infected and immunized chimpanzees used in these studies were used in earlier studies (7, 19). The experimental protocol used is summarized in Table 1.

Chimpanzee sera. The sera used were also examined in earlier studies (5, 6, 19). In this study, the immunoblot patterns obtained with cytadsorbing, pathogenic strains PI-1428, M129, and FH were compared with the patterns obtained with noncytadsorbing, nonpathogenic, high-passage strain M129-B176. The paired preimmune and convalescent-phase sera examined were obtained from five groups, which included (i) six chimpanzees experimentally infected with virulent strain *M. pneumoniae* PI-1428; (ii) two negative control chimpanzee, 902 and 1061, that became inadvertently infected; (iii) six chimpanzees immunized with either a formalin-inactivated (chimpanzees 810, 903, and 1009) or an acellular (chimpanzees 905, 918, and 1008) vaccine and challenged; (vi) two previously infected chimpanzees (881 and 854) that were rechallenged; and (v) two nonimmunized chimpanzees that were challenged and served as positive controls. The convalescent-phase serum of chimpanzee 957 was used in surface proteolysis studies, and convalescent-phase sera from chimpanzees 902, 903, 1008, and 1009 were used in adsorption studies with avirulent strain M129-B176 and/or horse serum.

Human sera. Paired acute and convalescent-phase sera of a patient infected with *M. pneumoniae* were kindly supplied by George Kenny, Seattle, Wash. The diagnosis of infection was based on a fourfold rise in the titer of a specific antibody to *M. pneumoniae* and direct isolation of the pathogen.

MAbs. Production of MAb M-328, directed against an epitope(s) on the 169-kDa P1 adhesin protein, and MAbs M-337 and M-384, directed against the 90- and 40-kDa proteins, respectively, was described previously (23, 25). Ascites were produced in BALB/c mice by intraperitoneal injection of soft-agar-cloned hybridomas.

SDS-PAGE and immunoblot analyses. The polypeptide patterns were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 10 or 12% nongradient or 10 to 20% gradient gels, as described by Laemmli (34). Each lane was loaded with approximately 30 μg of total protein. The proteins were transferred to nitrocellulose membranes (BA85; Schleicher & Schuell, Inc., Keene, N.H.) at 140 mA for 4 h at 4°C. The nitrocellulose blots were incubated with agitation for 3 to 4 h in a blocking solution (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.25% Bloom gelatin, 0.05% Nonidet P-40, 2% horse serum, 0.01% sodium azide). They were then incubated overnight with a 1:100 dilution of serum or MAb in the blocking solution. Blots were rinsed three times, for 20 min each time, with the blocking solution and incubated for 4 h with either the ^{125}I-labeled or alkaline phosphatase-conjugated immunoglobulin G (IgG) fraction of anti-human or anti-mouse immunoglobulins. Blots exposed to ^{125}I-labeled IgG were rinsed once with blocking solution and three times with PBS. They were then air dried and exposed to film (Kodak X-Omat AR diagnostic film). Blots in which the alkaline phosphatase-conjugated secondary antibodies were used were developed with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) substrate from Protoblot Immunoscreening Systems in accordance with the manufacturer's (Promega, Madison, Wis.) recommendations. Goat anti-human IgG (Organon Teknika-Cappel Corporation) was labeled with ^{125}I as described previously (26) and served as the second antibody. Anti-mouse IgG ^{125}I-labeled secondary antibody was obtained from Amersham, Arlington Heights, Ill.

Adsorption with the avirulent strain. Strain M129-B176 was grown in 500 ml of broth medium (2), harvested following centrifugation, and processed as described earlier (19). The cell pellet was washed twice in PBS, and the cells suspended in three ml of PBS were dispersed by three passages through a 25-gauge needle. A 500-μl aliquot of a 1:10 dilution of convalescent-phase chimpanzee serum was mixed with 50 μl of the mycoplasma cells (approximately 10^7 CFU) and agitated gently at room temperature for 1 h. The cells were sedimented by centrifugation in an Eppendorf Microfuge (15,000 × *g*) for 20 min at 4°C, and the supernatant (adsorbed serum) was used in immunoblot assays against strain PI-1428.

Adsorption with horse serum. The horse serum used in the broth medium was heated at 70°C for 5 to 10 min in an Eppendorf tube. After cooling to room temperature, 50 μl of the heated horse serum was mixed with 500 μl of a 1:10 dilution of convalescent-phase chimpanzee serum and the mixture was incubated at room temperature for 1 h. Particulate material was sedimented in an Eppendorf Microfuge at 15,000 × *g* for 10 min at 4°C, and the supernatant (adsorbed serum) was used in immunoblot assays. These studies were done to eliminate antibodies, if any, directed against the horse serum component of the medium.

Surface proteolysis. A modification of the method reported by Barbour and coworkers (1) was used. A broth culture of pathogenic strain PI-1428 was sedimented by centrifugation, and the pellet was washed twice in 0.25 M NaCl. The cells were suspended by three passages through a 25-gauge needle, and the final protein concentration was adjusted to 100 or 500 μg/ml. Trypsin or proteinase K (Boehringer Mannheim, Indianapolis, Ind.) was added to 1.0 ml of the cell suspension to a final concentration of 100 or 50 μg/ml, respectively, and the mixture was incubated, with occasional shaking, for 30 to 60 min at either room temperature or 37°C. Trypsin proteolysis was terminated by adding 5 μl of a saturated solution (20 mg/ml) of phenylmethylsulfonyl fluoride in isopropanol to a final concentration of 100 μg/ml. Proteinase K proteolysis was terminated by addition of 20 μl of a 500 mM solution of EDTA to a final concentration of 10 mM. The cells were harvested following sedimentation at 15,000 × *g* at 4°C and washed four times with 0.25 mM NaCl containing 50 μg of PMSF per ml, and the pellet was stored at −40°C until used in the immunoblot assays.

Electron microscopy. To determine whether the 40- and 90-kDa proteins were localized on the surface of *M. pneumoniae*, organisms immunostained with MAbs were examined by electron microscopy as described previously (24, 25). Briefly, *M. pneumoniae* grown on glass coverslips in a 24-well microtiter plate was fixed with 2.0 ml of 0.5% glutaraldehyde in PBS for 10 min. The specimen was washed three times with PBS and incubated overnight at 4°C with PBS containing 5% IgG-free horse serum (PBS-AHS). The coverslips were incubated with 1.0 ml of MAb M-337 or M-384 (heat-inactivated ascites diluted 1:20 in PBS-AHS) for 2 h at 37°C. After being washed three times with PBS-AHS, the coverslips were incubated overnight at 4°C with 1.0 ml of gold-conjugated goat anti-mouse IgG (Amersham International) diluted 1:10 in PBS-AHS. The coverslips were washed three times with PBS-AHS, and the antibodies adsorbed onto the organisms were fixed overnight with a solution of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) containing 6.8% sucrose. The specimens were subjected to secondary fixation with 1% osmium tetroxide in phosphate buffer for 1 h at room temperature. Following dehydration, the samples were embedded in Luff's Epon 812 and thin sections were cut with an LKB Huxley ultramicrotome. The preparations were stained with 5% uranyl acetate, followed by Reynolds lead citrate, and sections were examined with a Zeiss EM-900 electron microscope at an accelerating voltage of 50 kV.

RESULTS

MAbs. MAbs M-337 (anti-90 kDa) and M-384 (anti-40 kDa) recognized the 86- and 35-kDa polypeptides, respectively, of pathogenic strain PI-1428 that were also recognized by the convalescent-phase sera of infected chimpanzee 902 at 8 weeks postchallenge and immunized and challenged chimpanzee 903 at 16 weeks postchallenge (Fig. 1). The 86- and 35-kDa proteins identified in the present study produced an intense cross-reaction with MAbs specific to the 90- and 40-kDa proteins reported by Hu et al. (25) and Sperker et al. (39), indicating that the 86- and 35-kDa proteins in our studies (5, 6) are probably the same as the 90- and 40-kDa proteins. Hu et al. (25) also reported that the 90- and 40-kDa proteins were present in pathogenic strain M129 but absent in its nonpathogenic, high-passage derivative M129-B176. Our studies have confirmed and extended these findings. The data presented in this report indicate that the 90-kDa protein (shown as 86 kDa in Fig. 2A) and the 40-kDa protein (shown as 35 kDa in Fig. 2B) were also present in two additional pathogenic strains, PI-1428 and FH. The findings also indicate that the 90- and 40-kDa polypeptides are conserved in pathogenic strains but not in the avirulent strain. The nonpathogenic strain does make the P1 protein, but it is not localized at the terminal attachment tip of the organism (8), indicating that the 90- and 40-kDa proteins are probably required for anchorage and tip localization of the attachment moiety.

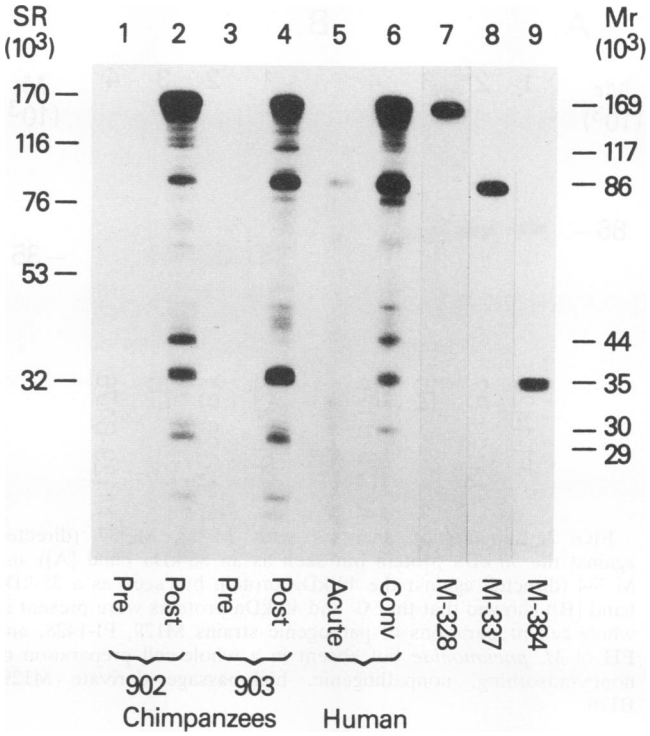

FIG. 1. When MAbs M-337 (anti-90-kDa protein) and M-384 (anti-40-kDa protein) were used for immunoblot analyses, strong reactions with the 86- and 35-kDa polypeptides of pathogenic strain PI-1428 were observed, indicating that the 90- and 40-kDa proteins of Hu et al. (25) are very similar or identical to our 86- and 35-kDa polypeptides. The pre- and postchallenge sera of chimpanzees 902 (at 8 weeks postchallenge) and 903 (at 16 weeks postchallenge) recognized the 169-, 90-, and 40-kDa proteins following challenge. The human acute and convalescent-phase sera used are described in Materials and Methods. Conv, convalescent phase; SR, standard reagent.

Convalescent-phase chimpanzee sera. Convalescent-phase serum of representative chimpanzee 903 (at 10 weeks postchallenge) recognized the 90-kDa protein band (shown as 86 kDa) and the 40-kDa protein band (shown as 35 kDa) in

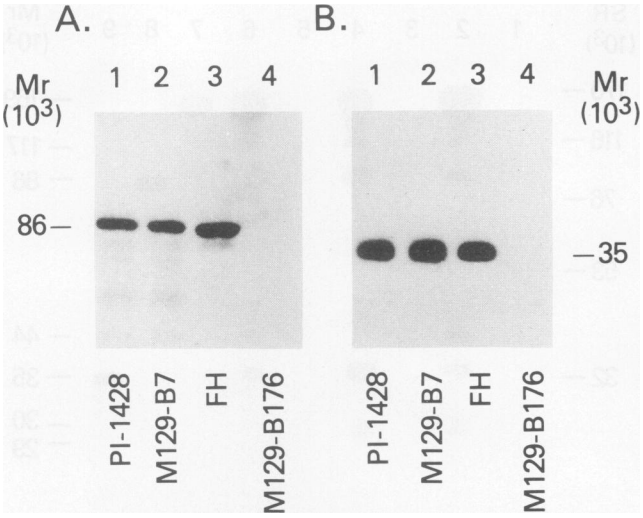

FIG. 2. Immunoblot analyses with MAbs M-337 (directed against the 90-kDa protein but seen as an 86-kDa band [A]) and M-384 (directed against the 40-kDa protein but seen as a 35-kDa band [B]) showed that the 90- and 40-kDa proteins were present in whole-cell preparations of pathogenic strains M129, PI-1428, and FH of *M. pneumoniae* but absent in a whole-cell preparation of noncytadsorbing, nonpathogenic, high-passage derivate M129-B176.

pathogenic strains PI-1428, M129, and FH but not in nonpathogenic strain M129-B176 (Fig. 3). The difference in the immunoblot patterns of chimpanzee 903 in Fig. 1 and 3 is attributable to the use of different serum bleeds. Identical results were obtained with the convalescent-phase sera of the other seven experimentally infected animals (data not shown), and the findings indicate that these two proteins are highly immunogenic. Nonetheless, no detectable differences were observed in the protein profiles of the pathogenic and nonpathogenic strains when SDS-PAGE with Coomassie blue or silver staining was used (data not shown). This is presumably due to the limited resolution of the one-dimensional SDS-PAGE gels. Similar findings were described in the earlier report of Hu et al. (25).

Immunoblot analyses revealed a weak 16-kDa band in the pathogenic strains and not in the nonpathogenic strain, but the identity or function of this protein is unknown.

Adsorption with the avirulent strain. The convalescent-phase sera of chimpanzees 902 (which became infected), 903 (which was immunized with the OSU-1A vaccine and challenged), and 1008 (which was immunized with acellular vaccine and challenged) were each adsorbed with whole-cell pellets of avirulent strain M129-B176. Following adsorption, the sera were examined by immunoblot analyses against pathogenic strains PI-1428 and M129 and avirulent strain M129-B176. The findings were similar for the three sera, and typical results for representative chimpanzee 902 are shown in Fig. 4. Adsorption completely removed the antibodies reactive with the P1 adhesin band, and it decreased reactivity with most, but not all, of the other bands. The notable exceptions were the 90- and 40-kDa protein bands. These proteins were not affected by adsorption with the avirulent strain, providing further evidence that these two proteins are not expressed in the avirulent strain. Conversely, adsorption with virulent strain PI-1428 completely inhibited reactivity with the 90- and 40-kDa proteins (data not shown).

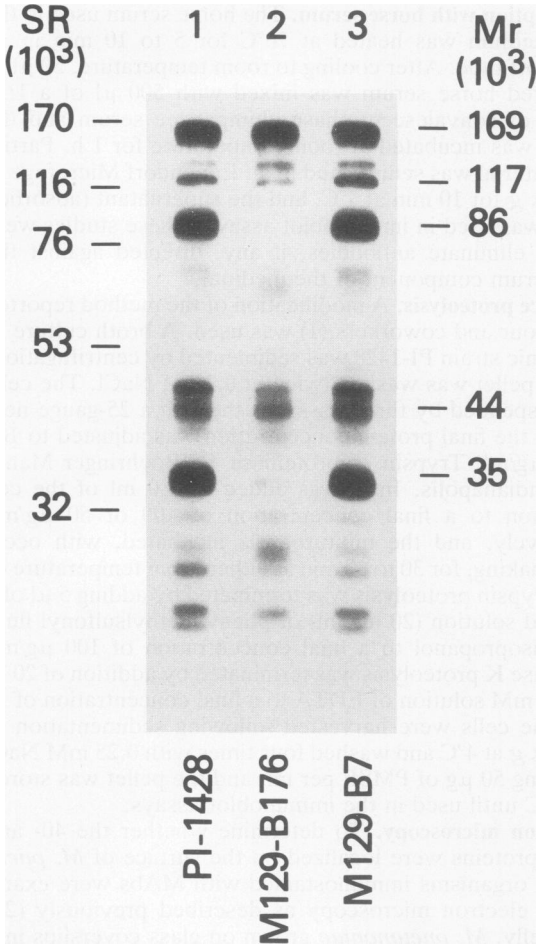

FIG. 3. In immunoblot analyses, convalescent-phase serum of representative chimpanzee 903, at 10 weeks postchallenge, recognized the 90- and 40-kDa bands in whole-cell preparations of cytadsorbing, pathogenic strains PI-1428 and M129 but not in a whole-cell preparation of noncytadsorbing, nonpathogenic strain M129-B176. SR, standard reagent.

Adsorption of convalescent-phase chimpanzee sera with horse serum did not affect the intensity of immunoblot reactions (data not shown) but did reduce the amount of background activity.

Identification of the 90- and 40-kDa proteins as surface components in *M. pneumoniae*. When whole-cell pellets of pathogenic strain PI-1428 were treated with either trypsin or proteinase K at room temperature and then used in for immunoblotting, the 90-kDa protein (shown as 86 kDa) was digested by both of the enzymes within 30 min. The reaction was accelerated when the digestion was carried out at 37°C. Following treatment with either enzyme, MAb M-337 (anti-90-kDa protein) reacted with an 80-kDa degradation product, indicating that the epitope remains with the fragment following proteolysis (Fig. 5A).

Whereas trypsin may have induced only minimal digestion of the 40-kDa protein (shown as 35 kDa), proteinase K completely digested the protein within 30 min at room temperature. Following digestion with proteinase K, anti-40-kDa protein MAb M-384 did not react with any band, indicating that the specific MAb epitope(s) was destroyed (Fig. 5B).

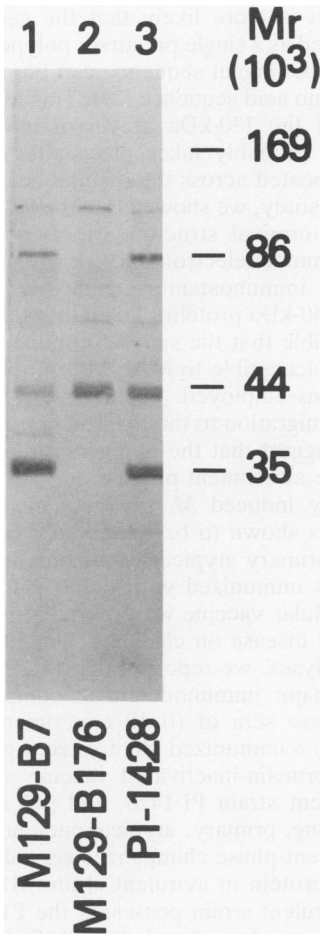

FIG. 4. Adsorption of convalescent-phase serum of chimpanzees 902 (at 10 weeks postchallenge) with whole-cell pellets of avirulent strain M129-B176, followed by immunoblotting, indicated that antibodies corresponding to several proteins, including the P1 adhesin band, were removed and the intensity of most, but not all, of the bands was decreased. The notable exceptions were the 90- and 40-kDa protein bands, indicating that the avirulent strain does not express these two proteins.

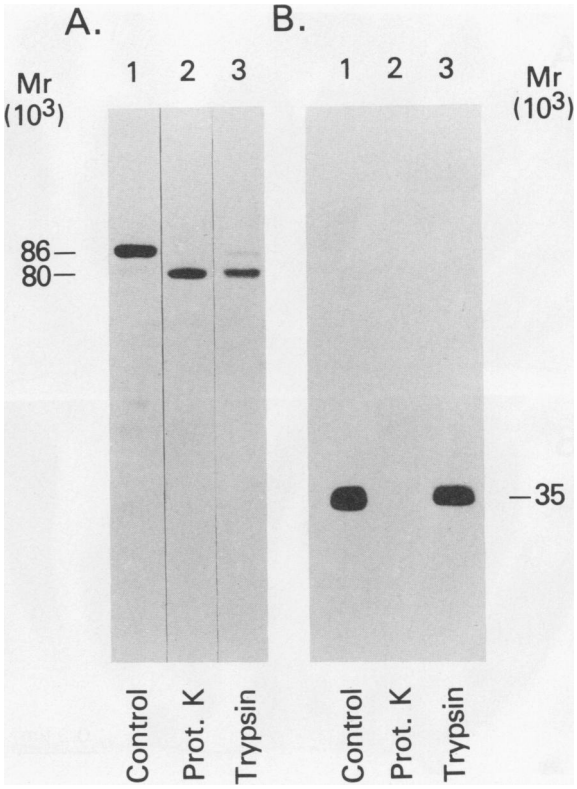

FIG. 5. Following surface proteolysis with proteinase K (Prot. K) and trypsin, MAb M-337 (anti-90-kDa protein [seen as an 86-kDa band]) reacted with an 80-kDa band (A), indicating that the major part of the 90-kDa protein of pathogenic strain PI-1428 was not exposed and thus was either integrated or located in the inner leaflet of the membrane. Following surface proteolysis with proteinase K, but not with trypsin, MAb M-384 (anti-40-kDa protein [seen as a 35-kDa band]) detected no bands, affirming that the binding epitope was cleaved.

Proteolysis with proteinase K, followed by immunoblotting with convalescent-phase chimpanzee sera, indicated that the patterns of the 169-, 90-, and 40-kDa proteins were altered. Trypsin altered the patterns of the 169- and 90-kDa proteins. These findings indicate that each of these proteins contains surface-exposed domains that were digested (data not shown). The 169-kDa P1 adhesin was shown to be a surface-exposed protein earlier by researchers at several laboratories (23, 24, 40). The data presented herein indicate that the 90- and 40-kDa proteins are also surface exposed. The other bands affected by proteolysis were the 148-, 117-, 30-, and 29-kDa proteins (data not shown).

Electron microscopy of immunostained organisms. When *M. pneumoniae* was incubated with MAb M-337 (specific for the 90-kDa protein), followed by gold-conjugated goat antibody to mouse IgG, electron microscopy indicated that MAb M-337 reacted with the tip structure of *M. pneumoniae* (Fig. 6A). This experimental approach and the results presented are similar to those of our previous study, in which MAbs specific for P1 were used to demonstrate that this adhesin was localized on the terminal tip (23). However, when MAb M-384, specific for the 40-kDa protein, was used, we failed to detect a positive signal. The inability of MAb M-384 to stain the tip structure may be due to the inaccessibility of the specific epitope to this MAb under the experimental conditions employed. Although immunostaining was unable to demonstrate an association, it seems reasonable to assume that the 40-kDa protein may also be associated with the tip structure of *M. pneumoniae*.

DISCUSSION

Two chemically distinct classes of molecules were shown to be receptors for attachment of *M. pneumoniae* to target tissues. They are the sialic acid-conjugated glycoproteins (38) and sulfated glycolipids (33). Together, these receptors account for 90% of the attachment to cell cultures. In addition to the 169-kDa P1 adhesin, several other proteins were identified with attachment of the organism to erythrocytes, including a 30-kDa protein (22, 30–32, 40). Therefore, adherence of *M. pneumoniae* to target tissues involves more than one receptor-adhesin complex.

Avirulent strain M129-B176, derived from virulent strain M129 (35) by continuous broth passages, lost the ability to colonize respiratory tissues in animals (35); the ability to produce histologic lesions, including ciliotoxicity and leukocyte recruitment in hamsters (3, 4); and the ability to attach

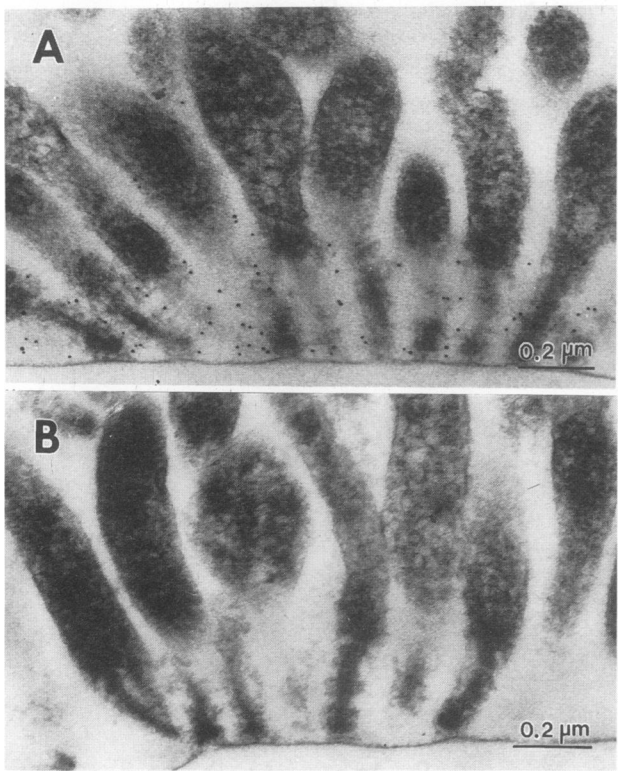

FIG. 6. Electron micrographs of thin sections of *M. pneumoniae* incubated with MAb M-337 (A) or M-384 (B) and immunostained with gold-conjugated rabbit anti-mouse IgG. In panel A, gold grains are clustered only around the terminal tip of *M. pneumoniae* organisms.

to cell cultures (13). The avirulent strain does contain the P1 adhesin protein (25), but immunoferritin staining showed that unlike in the pathogenic strains, the P1 protein is not localized on the terminal tip apparatus of the avirulent strain (8). These data supported the hypothesis that accessory proteins are missing in the avirulent strain (29, 31) and are required for anchorage and localization of the P1 adhesin.

The most convincing evidence that additional proteins are involved in the attachment process is based on cloning and nucleotide sequence analysis of the P1 operon (27, 28). These studies have shown that the P1-encoding gene is flanked by two ORFs, designated ORF4 and ORF6. ORF4 and ORF6 have respective coding capacities for proteins of 28 and 130 kDa. Since genes that interact functionally are often organized in operons, efforts were made by Sperker et al. (39) to identify the gene products of ORF4 and ORF6. They attempted to determine whether these products play a role in the attachment process. Mouse antisera directed against fusion proteins derived from the ORF6 sequence reacted with two proteins with respective molecular masses of 40 and 90 kDa rather than a single 130-kDa protein (39). Although the molecular mechanism(s) by which the precursor 130-kDa protein is cleaved to produce the 40- and 90-kDa proteins remains unknown, these data demonstrate that the 40- and 90-kDa proteins are the gene products of ORF6. The cleavage site has been recently identified at amino acid position 455 (R) on the basis of sequencing of the N terminus of the isolated 90-kDa protein (35). Sperker et al. (39) have suggested that the 90- and 40-kDa proteins represent two cotranslational cleavage fragments of the ORF6 gene product. However, it is more likely that the gene product of ORF6 is translated as a single precursor polypeptide because only a single leader signal sequence can be predicted from the deduced amino acid sequence (27). This feature suggests that cleavage of the 130-kDa precursor into the 40- and 90-kDa proteins probably takes place after the precursor protein is translocated across the membrane.

In the present study, we showed that the 90-kDa protein is located on the terminal structure of *M. pneumoniae*, as determined by immunoelectron microscopy using a specific MAb. Although immunostaining using one MAb, M-384 (specific for the 40-kDa protein), failed to produce a positive signal, it is possible that the specific epitope of the 40-kDa protein was not accessible to MAb M-384 under the experimental conditions employed. Further study is required to demonstrate its migration to the tip structure. Nevertheless, these findings suggest that the two gene products of ORF6 participate in the attachment process.

Experimentally induced *M. pneumoniae* pneumonia in chimpanzees was shown to be remarkably similar to naturally occurring primary atypical pneumonia in patients (4b, 5). Chimpanzees immunized with either a formalin-inactivated or an acellular vaccine were partially protected from colonization and disease on challenge (4a, 19). After using immunoblot analyses, we reported that the 90- and 40-kDa proteins were major immunodominant components in the convalescent-phase sera of (i) 10 experimentally infected chimpanzees, (ii) 8 immunized chimpanzees given either an acellular or a formalin-inactivated vaccine and then challenge with virulent strain PI-1428, and (iii) a patient with naturally occurring, primary, atypical pneumonia (19, 19a).

The convalescent-phase chimpanzee sera also recognized the P1 adhesin protein in avirulent strain M129-B176 (19). Because the avirulent strain possesses the P1 adhesin protein but has poor attachment activity (13), factors other than the 169-kDa adhesin protein are probably involved in the attachment process.

The present study has shown that the 90- and 40-kDa proteins identified previously by Hu et al. (25) were probably identical to the 86- and 35-kDa proteins reported by us (19, 19a). These two proteins were present in each of the three cytadsorbing, pathogenic strains examined but absent in noncytadsorbing, nonpathogenic strain M129-B176. These findings indicate that the 90- and 40-kDa proteins are highly conserved among cytadsorbing, pathogenic strains. The absence of these two proteins in noncytadsorbing, nonpathogenic strain M129-B176 was supported by the adsorption studies with convalescent-phase chimpanzee sera. In addition, antibodies specific to the 90- and 40-kDa proteins were removed by the whole organisms of pathogenic strains in the absorption studies, suggesting that these proteins are located on the cell surface. Recently, Yayoshi et al. (43) reported that sera of patients produced intense reactions with 168- and 85-kDa proteins of virulent strains of *M. pneumoniae* but not with those of avirulent strains.

Surface proteolysis studies provided additional evidence that the 90- and 40-kDa proteins were surface-exposed membrane components. Lack of complete proteolytic digestion suggests that these components are probably integral membrane proteins, because following treatment with proteinase K, MAb M-384 (specific for the 40-kDa protein) detected no protein bands. Either this protein was completely digested or the antibody-binding site was destroyed by digestion. On the other hand, the 40-kDa protein was digested by proteinase K but was resistant to trypsin. This

indicates that the arginine or lysine groups of the protein were not accessible to trypsin.

In summary, we have shown that the immunodominant 86- and 35-kDa proteins of *M. pneumoniae* described by Franzoso et al. (19) are identical to the 90- and 40-kDa proteins of *M. pneumoniae* described previously by Hu et al. (25) and Sperker et al. (39). They are present in pathogenic, cytadsorbing strains PI-1428, M129, and FH but absent in nonpathogenic, noncytadsorbing strain M129-B176. We have also reported that these two proteins are immunodominant components that were recognized by 18 convalescent-phase sera of either experimentally infected or immunized chimpanzees challenged with *M. pneumoniae*. The demonstration of these two components as surface-exposed membrane proteins was supported by adsorption studies, surface proteolysis, and electron microscopy in combination with immunostaining. The localization of the 90-kDa protein at the terminal tip apparatus suggests that it is an essential component involved in anchorage of P1 to the tip structure. These findings also suggest that the 90- and 40-kDa proteins encoded by ORF6 of the P1 operon are among the accessory proteins required, along with P1, for *M. pneumoniae* to adhere to the respiratory epithelium.

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