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A *Mycoplasma genitalium* Protein Resembling the *Mycoplasma pneumoniae* Attachment Protein

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In previous studies with hyperimmune rabbit sera and monoclonal antibodies against the P1 protein of *Mycoplasma pneumoniae*, we obtained evidence of a shared antigenic determinant with a single protein of *Mycoplasma genitalium*. Because of biologic and morphologic similarities between these two human *Mycoplasma* species, attempts were made to characterize this cross-reacting protein of *M. genitalium* (designated MgPa). The protein was surface exposed and had an estimated molecular size of 140 kilodaltons. Electron microscopy with monoclonal antibodies produced against either MgPa or P1 demonstrated that MgPa is located over the surface of the terminal structure of *M. genitalium* which is covered by a nap layer. These immunologic and morphologic findings suggest that the MgPa protein of *M. genitalium* could be the counterpart of the P1 protein of *M. pneumoniae*.

Mycoplasma genitalium is a new mycoplasma isolated from the urethral specimens of human patients with nongonococcal urethritis (23, 30, 31). Although technical problems have impeded large-scale surveys for the prevalence of the organism, seroepidemiologic data and experimental infections suggest its pathogenicity. Antibodies specific to *M. genitalium*, but not to *Chlamydia trachomatis* and *Mycoplasma hominis*, have been demonstrated in 40% of 31 women with acute pelvic inflammatory disease (29). When inoculated intraurethrally, this organism induced urethritis in young male chimpanzees (28). The organism could be reisolated from the experimentally infected animals for at least 13 weeks, and the chimpanzees developed a marked antibody response. This evidence implicates *M. genitalium* in the etiology of human genitourinary tract disease.

Ultrastructural studies indicated that *M. genitalium* possesses a differentiated terminal structure covered by a peplomer-like nap (30). Furthermore, adherence of *M. genitalium* to Vero cell membranes appeared to be mediated by the nap area (31). This evidence suggests that *M. genitalium* may possess an attachment mechanism similar to that described earlier for *Mycoplasma pneumoniae* (2, 9, 12, 13). This possibility is supported by previous findings that *M. genitalium* shares extensive serologic cross-reactivity with *M. pneumoniae* (3, 11, 21) and that a monoclonal antibody specific to the P1 protein of *M. pneumoniae* was reactive with a single protein of *M. genitalium* by immunoblotting (7). In the present study, further characterization of this cross-reacting protein by immunological and ultrastructural means is described.

MATERIALS AND METHODS

Growth of mycoplasmas. *M. genitalium* G-37 (ATCC 33530) was purchased from the American Type Culture Collection (Rockville, Md.). *M. pneumoniae* M129-B12 (ATCC 29342) was from our stock. Monolayer cultures of mycoplasmas were grown in Edward-Hayflick medium in glass prescription bottles as previously described (13). Organisms were harvested by rinsing confluent monolayers with 0.01 M phosphate-buffered saline (PBS, pH 7.2) followed by scraping into ice-cold PBS and pelleting by centrifugation.

Antisera and monoclonal antibodies. Hyperimmune rabbit antisera to *M. genitalium* and *M. pneumoniae* were produced by subcutaneous injection of washed whole organisms (approximately 1 mg of protein) emulsified in complete Freund adjuvant followed by three alternate-week booster injections in incomplete Freund adjuvant. The procedures described by Shulman et al. (27) were used to produce monoclonal antibodies against *M. genitalium* proteins. Briefly, splenic cells from BALB/c mice immunized with whole *M. genitalium* organisms were hybridized with myeloma cells SP2/0 Ag14 (ATCC CRL-1581). Hybridomas producing antibodies against *M. genitalium* were screened with a solid-phase radioimmunoassay (17), and those specific to particular proteins were identified with Western immunoblotting (16). ^{125}I-labeled immunoglobulins were prepared by the chloramine T method (10). Monoclonal antibodies specific to the P1 protein of *M. pneumoniae* were available from our previous studies (7, 12, 15). Preimmune and immune sera from a young adult chimpanzee expermentally infected with *M. genitalium* were also available from a previous study (28).

Identification of *M. genitalium* surface proteins. Surface proteins of *M. genitalium* were identified as described recently by Engleberg et al. (8) by using antibodies absorbed onto freshly propagated organisms in combination with immunoblotting, instead of iodination (18). Briefly, monolayer culture grown to confluence in a 96-oz (ca. 2.839-liter) prescription bottle was washed three times with PBS. To this, 0.2 ml of hyperimmune rabbit antiserum to *M. genitalium* diluted in 20 ml of PBS was added, and the culture was stored at 4°C overnight. The supernatant was discarded, and the mycoplasma monolayer was thoroughly washed with PBS. After the final wash, 2.0 ml of elution

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buffer (0.2 M NaCl, 0.2 M glycine, pH 2.8) was added. The bottle was incubated at room temperature for 30 min with gentle rocking to release the absorbed antibodies. The elution buffer containing the released antibodies was recovered, the pH was titrated immediately to 7.5 with 2.0 M Tris, and the mixture was centrifuged at 27,000 × *g* for 30 min at 4°C. The recovered antibodies were used in immunoblotting for the identification of *M. genitalium* surface antigens.

Trypsin treatment of *M. genitalium*. As described previously, the age of the mycoplasma monolayer cultures was critical to the effectiveness of enzyme treatment (13); cultures incubated for 24 to 36 h were found to be optimal. The growth medium was decanted, and the monolayers were washed with PBS before receiving 20 ml of minimal essential medium. Crystalline trypsin (type XI, diphenyl carbamyl chloride treated, 7,500 BAEE units per mg of protein; Sigma Chemical Co., St. Louis, Mo.) was then introduced to final concentrations ranging from 25 to 500 μg/ml. Trypsin-treated cultures were incubated at 37°C for 0 to 30 min, and the reaction was terminated by the addition of 2 ml of fetal calf serum. Control cultures were treated similarly, except that trypsin was omitted. The monolayer cultures were then washed three times with PBS, scraped into 2.0 ml of PBS, and processed for sodium dodecyl sulfate-gel electrophoresis.

Sodium dodecyl sulfate-gel electrophoresis and Western blotting. The procedures for sodium dodecyl sulfate-gel electrophoresis of mycoplasma proteins and preparation of Western blots were those used previously (16).

Electron microscopy and immunostaining. The morphology of *M. genitalium* was examined by electron microscopy with both negative staining procedures and thin sectioning as described by Tully et al. (31). For negative staining, the young monolayer culture was scraped from the glass bottle into a small amount of fresh medium and pelleted in a Brinkmann microfuge. The pellet was suspended in 1% ammonium acetate. A small drop was applied to an electron microscope grid and stained with 2% ammonium molybdate as described by Cole and Popkin (5). For immunostaining, *M. genitalium* grown on cover slips in 24-well microtiter plates was fixed with 2.0 ml of 5% formaldehyde in PBS for 2 h, washed thoroughly with PBS, and incubated with 1.0 ml of monoclonal antibody Mg-209 (heat-inactivated ascites fluid diluted 1:20 in PBS containing 2% immunoglobulin G-free horse serum) overnight at 4°C. The cover slips were washed extensively again with PBS and incubated with 1.0 ml ferritin-conjugated goat antibody to mouse immunoglobulin G (Cappel-Worthington Biochemicals, Malvern, Pa.) diluted 1:20 or 1:100 in PBS containing 2% immunoglobulin G-free horse serum for 6 h at 4°C. After extensive washing with cold PBS, the treated monolayers of mycoplasmas were then fixed with a solution of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight, washed three times with phosphate buffer with 6.8% sucrose, and then subjected to secondary fixation with 1% osmium tetroxide in phosphate buffer for 1 h at room temperature. After dehydration the samples were embedded in Luft's Epon 812. Thin sections were cut with an LKB Huxley ultramicrotome and stained with 5% uranyl acetate followed by Reynolds lead citrate. Electron microscopy was carried out with a Zeiss model EM-10A electron microscope at an accelerating voltage of 60 kV.

RESULTS

Monoclonal antibodies specific to MgPa protein of M. genitalium. Five monoclonal antibodies, designated Mg-209, Mg-124, Mg-182, Mg-229, and Mg-240, were specifically reactive with MgPa by immunoblotting (Fig. 1, lanes 2 through 6). The variable intensity of these monoclonal antibody reactions may be related to their concentration, affinity of binding, or specificity for different epitopes of the MgPa molecule. Monoclonal antibodies Mg-199 (lane 7) and Mg-169 (lane 8), which react with two other *M. genitalium* protein bands with molecular masses estimated 66 and 57 kilodaltons (kDa), respectively, were included as controls. The absence of visible bands at the position of 140 kDa in lanes 7 and 8 indicates that the recognization of MgPa by monoclonal antibodies in lanes 2 through 6 is specific. Monoclonal antibody Mg-209 (lane 2), because of its strong reactivity, was chosen later for immunostaining to determine the location of the MgPa on the organism.

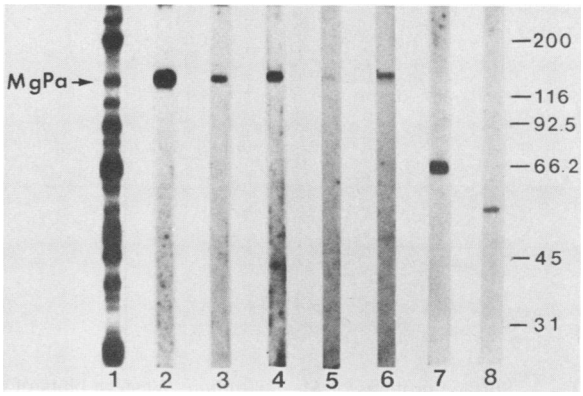

FIG. 1. Monoclonal antibodies specific to MgPa protein of *M. genitalium*. Western blots of *M. genitalium* proteins separated on a 7% sodium dodecyl sulfate gel were incubated with rabbit hyperimmune serum and imaged with ^{125}I-labeled goat anti-rabbit immunoglobulin G (lane 1) or incubated with monoclonal antibody Mg-209 (lane 2), Mg-124 (lane 3), Mg-182 (lane 4), Mg-229 (lane 5), or Mg-240 (lane 6) and then imaged with ^{125}I-labeled goat anti-mouse immunoglobulins. In lanes 7 and 8, two additional monoclonal antibodies, Mg-199 and Mg-167, which reacted with two *M. genitalium* proteins other than MgPa, were included as specificity controls. The absence of visible bands at the position of 140 kDa in lanes 7 and 8 indicates that the recognization of MgPa by monoclonal antibodies in lanes 2 through 6 is specific. Molecular masses (kDa) of protein standards are indicated by numbers.

Identification of surface-exposed proteins. One of the requirements to support our hypothesis that the MgPa protein of *M. genitalium* may serve the same function as P1 of *M. pneumoniae* is that MgPa must be a surface component. To examine this, rabbit antiserum was placed on monolayers of glass-attached *M. genitalium* organisms. After washing, surface-bond antibodies eluted from the monolayer culture were used to probe Western blots of *M. genitalium* proteins. Eleven major protein bands including MgPa were identified by the surface-bound antibodies (Fig, 2, lane 1), whereas the unabsorbed rabbit antiserum recognized many more bands (lane 2). None of these protein bands reacted with normal preimmune rabbit serum (lane 3). These results indicate that MgPa is one of the surface-exposed proteins of *M. genitalium*.

Sensitivity of MgPa to trypsin treatment. Evidence that MgPa is a surface protein was extended by determining whether it could be removed by trypsin treatment. Preliminary experiments indicated that MgPa is more resistant to trypsin treatment than the P1 protein of *M. pneumoniae* (removed by 25 μg of trypsin per ml for 15 min). However, when the concentration of trypsin was increased up to 500

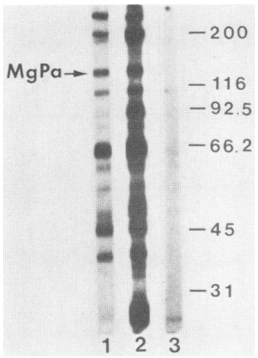

FIG. 2. Surface proteins of *M. genitalium*. Western blots of *M. genitalium* proteins were incubated with (lane 1) surface-bound rabbit antibodies eluted from a monolayer culture of *M. genitalium* as described in Materials and Methods, (lane 2) unabsorbed rabbit antiserum or (lane 3) normal preimmune rabbit serum. Blots were imaged with ^{125}I-labeled immunoglobulin G fraction of goat anti-rabbit immunoglobulin G, and radioautographs were produced by exposure to X-ray film at −70°C overnight.

μg/ml (Fig. 3A) and the incubation time extended up to 30 min (Fig. 3B), complete removal of MgPa was evident with 250 μg of trypsin per ml for 20 to 30 min. In addition, another protein band with an estimated molecular mass of 108 kDa was also lost in the trypsin-treated preparation. When a Western blot prepared from an SDS-gel similar to that shown in Fig. 3A was incubated with monoclonal antibody Mg-209, the reactive epitope of MgPa was lost after treatment with 100 μg or more of trypsin per ml for 30 min (Fig. 3C). This was accompanied by the appearance of a low-molecular-weight band that was reactive with the antibody (most prominent in lane 4 of Fig. 3C), indicating removal of this epitope from MgPa by trypsin. The increased prominence of the new band (lane 4) may be due to exposure by trypsin of more accessable reactive sites than are present in the intact MgPa molecule. The reactive residue diminished with increased amounts of trypsin (Fig. 3C, lanes 5 and 6). Together, these data indicate that MgPa is sensitive to trypsin under the experimental conditions used. Technical differences may explain recently published information suggesting that the 140-kDa protein of *M. genitalium* is insensitive to the effects of trypsin (24). The differing sensitivities of MgPa of *M. genitalium* and P1 of *M. pneumoniae* to trypsin treatment implies that these proteins, although serologically related, could be significantly distinct in terms of their physicochemical properties. Nevertheless, this observation adds additional evidence that MgPa is surface exposed.

Electron microscopy and immunostaining. Electron microscopy of negatively stained organisms revealed that single *M. genitalium* cells were flask shaped (Fig. 4A), with a well-demarcated outer layer of nap covering approximately one-third of the cell at the differentiated terminus. This confirms features described previously by Tully et al. (30, 31). The nap structure is similar to that described in *M. pneumoniae* (12), but is much more extensive in area. When *M. genitalium* was incubated with one of the monoclonal antibodies (Mg-209) specific to MgPa followed by ferritin-conjugated rabbit antibody to mouse immunoglobulins, the monoclonal antibody reacted only with the region covered by the nap structure (Fig. 4B). A monoclonal antibody (M-525) specific to P1 protein of *M. pneumoniae* and previously shown to cross-react with protein MgPa of *M. genitalium* by immunoblotting (7) also was used for immunostaining; identical results were observed (Fig. 4C). When either of the monoclonal antibodies was omitted in the immunostaining procedure, binding of the ferritin-labeled second antibody was not evident (Fig. 4D). Thus, it appears that MgPa and P1 share both immunologic relatedness and association with the terminal organelle surface nap.

Immunogenicity of MgPa. Previous studies indicated that the P1 protein of *M. pneumoniae* is a major immunogen, since antibodies specific to P1 can be demonstrated in sera of both hamsters infected with *M. pneumoniae* by inhalation and human patients with natural infection (14, 16). Similar results supporting the role of P1 protein as an immunodominant antigen were reported by Leith et al. (20). Moreover, antibodies specific to P1 of both immunoglobulin G and secretory immunoglobulin A classes were demonstrated in respiratory secretions from those human subjects examined, suggesting its role in the stimulation of protective immunity (14). Comparable studies on MgPa of *M. genitalium* have been precluded due to the technical difficulty of isolating the organism from patients and, in turn, the lack of sera or secretions from culture-proven infections. Recently, Morrison-Plummer et al. reported evidence that experimentally infected chimpanzees developed antibodies to the 140-kDa protein of *M. genitalium* by using sera from two animals in a radioimmunoprecipitation technique (24). Sera from a third animal available to us from the same study (28) were tested from MgPa antibody by the Western blot method. Antibodies to several *M. genitalium* proteins developed by 8 weeks postinfection, including one at the gel location of MgPa (Fig. 5A). To determine whether this is the same protein recognized by monoclonal antibody Mg-209, comparable blots were treated first with the chimpanzee immune serum. After washing, Mg-209 was reacted with the same blot. The chimpanzee antibodies significantly blocked

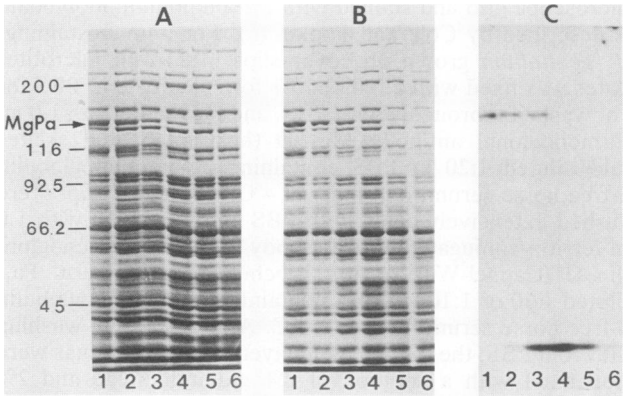

FIG. 3. Electrophoretic analysis and immunoradioautographs of *M. genitalium* proteins after trypsin treatment. (A) Monolayers of glass-grown *M. genitalium* were incubated at 37°C for 30 min with increasing trypsin concentrations of (lane 1) 0, (lane 2) 25, (lane 3) 50, (lane 4) 100, (lane 5) 250, and (lane 6) 500 μg/ml. Electrophoresis was carried out in a 7% sodium dodecyl sulfate gel, and the gel was stained with Coomassie blue. Complete removal of MgPa is seen in lanes 5 and 6. (B) *M. genitalium* monolayer cultures treated with 250 μg of trypsin per ml for (lane 2) 0, (lane 3) 5, (lane 4) 10, (lane 5) 20, and (lane 6) 30 min at 37°C; lane 1 is an untreated control. The absence of MgPa band is evident in lanes 5 and 6. (C) A Western blot prepared from a gel similar to that in A was incubated with monoclonal antibody Mg-209 and imaged with ^{125}I-labeled goat anti-mouse immunoglobulins. Loss of reactive sites at the position of the MgPa band is clearly seen in lanes 4 through 6, and residues recognized by the antibody appear near the bottom of the blot.

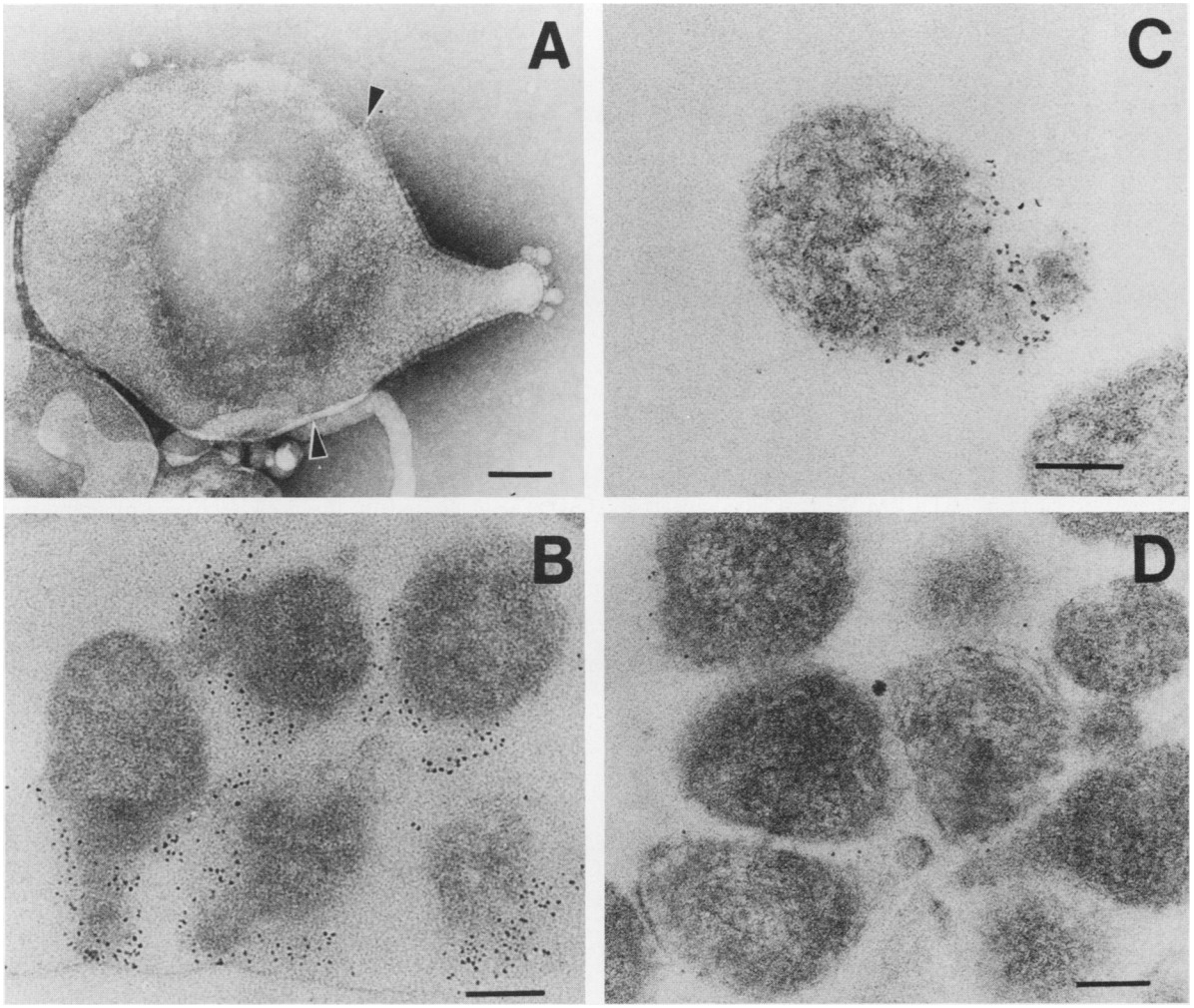

FIG. 4. Electron micrographs of *M. genitalium*. (A) Negative staining of an intact mycoplasma cell with ammonium molybdate. Note the terminus is covered with a nap extending peripherally to the area marked by arrowheads. (B) Thin section of *M. genitalium* organisms after incubation with monoclonal antibody (Mg-209) and indirect staining with ferritin-conjugated rabbit antibody to mouse immmunoglobulin G. The lower three organisms were attached to a cover slip surface represented by the thin horizontal line. Ferritin grains are clustered around the tip terminal corresponding to the area covered with the nap as shown in A. (C) Thin section of *M. genitalium* prepared in the same manner as (B), but incubated with a monoclonal antibody (M-525) specific to the P1 protein of *M. pneumoniae*. (D) Thin section of *M. genitalium* prepared in the same manner as B, but monoclonal antibody was omitted to serve as a negative control. Bars, 0.1 μm.

the immunoreactive sites recognized by Mg-209 (Fig. 5B). These data, other chimpanzee studies cited (24), recognition by immunized rabbits, and ease of monoclonal antibody production in mice suggest that MgPa is a significant immunogen of *M. genitalium*.

DISCUSSION

Several pathogenic *Mycoplasma* species including *M. pneumoniae* and *M. genitalium* have biologic features in common, of which the terminal tip structure (12, 19, 22, 28), adhesive properties (1, 6, 30, 31), and gliding motility (4, 25) appear the most striking. It is possible that these two *Mycoplasma* species may share proteins with biologic and immunologic similarities and parasitize host cells in like fashion. However, discrepancies exist in published studies seeking evidence of serologic cross-reactivity. Taylor-Robinson et al. (29) reported that there was little or no serologic cross-reaction between the human pathogens *M. genitalium* and *M. pneumoniae* by the metabolic inhibition test. In contrast, prominent cross-reactions were demonstrated between these two species by Lind (21) with a variety of serologic assays including the metabolic inhibition test. Using hyperimmune rabbit sera against *M. pneumoniae*, *M. genitalium*, and *Mycoplasma gallisepticum* in combination with immunoblotting, we have shown extensive serological cross-reactivities among these three species (11). However, this evidence does not necessarily imply that the cross-reactive proteins are identical or that they possess similar biologic functions, because the protein profiles of these two mycoplasma species have been shown to be markedly different from each other (3, 7).

An important virulence component of *M. penumoniae* is the surface protein P1, which mediates initial attachment of organisms to respiratory epithelium (3, 9, 12, 13). This specific attachment has been recognized as the prerequisite for colonization and subsequent development of *M. pneumoniae* disease in the lung (26). In view of the common morphologic and biologic features shared by *M. pneumoniae*, *M. genitalium*, and *M. gallisepticum* and the important

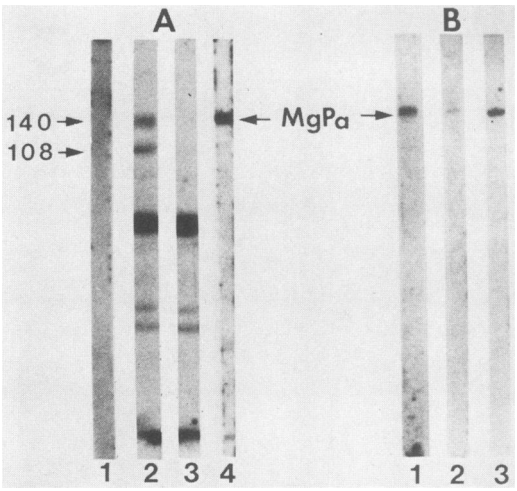

FIG. 5. Immunogenicity of MgPa. (A) Western blots prepared from *M. genitalium* separated on a 7% sodium dodecyl sulfate gel were probed with chimpanzee preimmune serum (lane 1) or immune serum (lane 2) and imaged with ^{125}I-labeled goat anti-human immunoglobulin G. Lane 4 is a similar blot incubated with monoclonal antibody (Mg-209) specific to MgPa (Fig. 1) and imaged with ^{125}I-labeled goat anti-mouse immunoglobulins. Lane 3 is a Western blot prepared from trypsin-treated *M. genitalium* monolayer cultures probed with chimpanzee immune serum. Loss of reactivity with MgPa at the 140-kDa position and another immunoreactive band at approximately the 108-kDa position is evident, indicating both are surface-exposed proteins. (B) Western blots similar to those in A were preincubated with chimpanzee preimmune serum (lane 1), immune serum (lane 2), or incubation buffer only (lane 3), washed, and then probed with monoclonal Mg-209 and imaged with goat anti-mouse immunoglobulins. Significantly reduced intensity of the 140-kDa band in lane 2 indicates the blocking of antigenic sites for monoclonal Mg-209 by the chimpanzee immune serum.

role of P1 protein in the pathogenesis of *M. pneumoniae* disease, it is pertinent to examine the possibility that *M. genitalium* and *M. gallisepticum* may possess a surface protein with function similar to the P1 protein of *M. pneumoniae*. Using a large collection of monoclonal antibodies, we were able to demonstrate that a monoclonal antibody specifically raised against P1 protein of *M. pneumoniae* was capable of reacting with a smaller protein of *M. genitalium*, and that monospecific antibodies raised by immunization of a rabbit with P1 protein reacted with a similar-sized protein of *M. gallisepticum* (7). These findings are compatible with our hypothesis that these flask-shaped pathogenic *Mycoplasma* species may parasitize their target host cells by similar mechanisms.

Previous efforts of others to demonstrate epitopes of *M. pneumoniae* P1 protein shared by *M. genitalium* and *M. gallisepticum* proteins were unsuccessful, probably due to the limited number of monoclonal antibodies used (3). However, a more recent study from the same laboratory reports similarities between the P1 protein and a 140-kDa protein of *M. genitalium*. The present report confirms this finding and provides evidence that the 140-kDa protein, which is designated MgPa, is surface exposed and located over the terminal structure of *M. genitalium*. The physical and immunological data imply that the MgPa protein of *M. genitalium* could be a counterpart of P1 in *M. pneumoniae* and might play a similar role in the pathogenesis of *M. genitalium* infections. It has been shown that attachment of *M. genitalium* to monkey kidney cells appeared to be mediated by the nap (31). However, the establishment of a more appropriate target cell model derived from human urogenital tract tissue will be essential to confirm the role of MgPa in attachment to natural host cells.

Another important aspect concerning the role of MgPa in disease pathogenesis is its immunogenicity for the host. We have shown that P1 protein of *M. pneumoniae* is recognized by all patients examined during recovery from natural infections, through the development of specific antibodies to it in sera and respiratory secretions (14). This immunologic property may have importance in protective immunity. Lack of comparable specimens from patients infected with *M. genitalium* have precluded similar observations, although preliminary studies reported here and elsewhere (24) with sera from experimentally infected chimpanzees suggest that MgPa is probably among the major immunogens of the organism; whether MgPa is as immunodominant as P1 of *M. pneumoniae* remains to be established.

Epidemiologic investigations of *M. genitalium* infections have been limited by the poor recovery of organisms with conventional culture systems and the possible nonspecificity of serologic reactions. The availability of monoclonal antibodies specific to MgPa and other *M. genitalium* proteins may aid in the development of improved diagnostic methods for the detection of *M. genitalium* in clinical samples. This effort should allow us to determine whether *M. genitalium* has a significant role in nongonococcal urethritis and pelvic inflammatory disease and the relationship of *M. genitalium* to other microorganisms isolated from patients with nongonococcal urethritis.

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