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Variable Antigens of *Ureaplasma urealyticum* Containing Both Serovar-Specific and Serovar-Cross-Reactive Epitopes

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Currently there are 14 recognized serovars of *Ureaplasma urealyticum*, and it has been postulated that only certain ones may be associated with disease and that lack of serovar-specific antibody may be an important risk factor. Unfortunately, ureaplasma antigens important in the human immune response and disease pathogenesis are poorly defined. By using sera from ureaplasma-infected patients and antiureaplasma monoclonal antibodies, the present study has demonstrated, for serovars 3, 8, and 10, antigens which (i) are species specific, (ii) contain both serovar-specific and cross-reactive epitope(s), (iii) are produced not only in vitro but also in vivo, (iv) undergo a high rate of structural variation in vitro, (v) are present and structurally variable on invasive ureaplasma isolates (i.e., those from placenta, lung, and cerebrospinal fluid), and (vi) are among the predominant antigens recognized during infections in humans. Furthermore, we have shown that monoclonal antibodies to these antigens can inhibit the growth of the organisms in vitro, indicating the potential for these antigens to be important for host defense.

The role of *Ureaplasma urealyticum* in human disease, particularly as it relates to pregnancy outcome (3), is highly controversial. While the causative role of ureaplasmas in infertility, spontaneous abortion, and premature delivery remains controversial, recent studies indicate that *U. urealyticum* is a definite cause of chorioamnionitis (7) and is a significant cause of respiratory disease (4), meningitis (35), and death (6) in newborns. There is undeniable evidence that many of the respiratory infections are acquired in utero (4).

A major principle amply illustrated in these studies is that only in a subpopulation of individuals infected in the lower genitourinary tract do the organisms reach the upper tract and only in some of these individuals does disease ensue (5). Furthermore, it is apparent that only a subpopulation of infected infants, primarily those born before 30 weeks of gestation, are at risk for development of disease (4, 6, 35).

The initial difficulty in proving an etiologic role for ureaplasmas in adverse pregnancy outcome supplied a major impetus for the hypothesis that only certain subgroups of the species are truly disease associated. Distinct *U. urealyticum* serovars have been identified by a number of serological methods (14). At present there are 14 recognized serovars, which can to some extent be divided into two biotypes by DNA homology (11, 22, 23), manganese sensitivity (24), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (17, 19, 28, 32), and polymerase chain reaction analysis (2).

We have recently established a mouse model of ureaplasmal infection which closely mimics at least some aspects of human disease (26). Using this model, we have obtained preliminary evidence for a difference in virulence between serovars. While differences in the virulence of different serovars may explain why only a subpopulation of individuals infected in the lower genital tract develop upper tract disease, predisposing host factors are an equally likely explanation. With this animal model, we have also conclusively shown that disease susceptibility is age related (26). Furthermore, increased susceptibility of hypogammaglobulinemic patients (31) and serological study of these (34) and other (20, 21) patients suggest that lack of specific antibody may also be an important determinant in development of *U. urealyticum*-induced disease. Increased susceptibility to disease in infants of less than 30 weeks gestational age may also be related to their hypogammaglobulinemia (1, 10).

Studies attempting to prove an association of disease with only certain serovars by either culture or detection of specific antibody have yielded no conclusive results, and various studies dealing with analysis of antibody reactions (hyperimmune rabbit sera or sera from infected humans) to *U. urealyticum* antigens have failed to convincingly reveal either group- or serovar-specific polypeptides (8, 14). This can probably be attributed to the lack of well-characterized reagents and methods. There is, however, one recent study demonstrating, with the use of monoclonal antibodies (MAbs), a serovar-specific epitope in serovar 8 (32). This study described another MAb which defined an epitope common to all *U. urealyticum* serovars and, based on immunoblot analysis, allowed distinction between the two biotypes. With the exception of these potentially useful MAbs, there are no readily available or reliable methods to detect different serovars or serovar-specific antibodies, nor have the antigens relevant to the human immune response or disease pathogenesis been identified.

In the present studies, detailed analysis of the antigens in serovars 3, 8, and 10 has revealed an antigen recognized by infected humans that contains serovar-specific epitopes and that, based on immunoblotting, appears to be structurally similar to the previously described V-1 antigen of *Mycoplasma pulmonis* (38). This *U. urealyticum* antigen was also shown to vary in vitro, and if, like V-1, the ureaplasma antigen also varies in vivo, this may have important consequences for persistence of infection (35) and protection against disease.

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MATERIALS AND METHODS

Ureaplasmas and mycoplasmas. The reference strains of *U. urealyticum* serovars 1 through 8 used in this study were obtained from E. A. Freundt (Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark), and serovars 9 through 14 were obtained from J. A. Robertson (Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Alberta, Canada). An additional patient isolate of serovar 10 (26) was used for production of MAbs. Growth conditions for and harvesting of *U. urealyticum* have been described before (14). Subcloning of *U. urealyticum* was performed essentially as described for *M. pulmonis* (38). Briefly, 10-fold dilutions of actively growing cultures were spread onto agar plates and incubated at 37°C until growth was detected. Well-isolated colonies were picked, expanded into a 30-ml broth culture, harvested, and processed for electrophoretic analysis.

Mycoplasma pneumoniae (Eaton), *M. hominis* (PG21), *M. salivarium* (PG20), *M. fermentans* (PG18), *M. orale* (CH19299), *M. lipophilum* (MaBy), *M. faucium* (DC333), *M. genitalium* (G37), *M. buccale* (CH20247), *M. primatum* (B292), *M. arthritidis* (Campo), *Acholeplasma oculi* (27350), and *A. laidlawii* (PG8) were obtained from the National Institute of Allergy and Infectious Disease. Glucose utilizers were grown in SP-4 medium containing 10% agamma horse serum (GIBCO, Grand Island, N.Y.). Arginine utilizers were grown in Shephard 10B broth medium containing 0.2% arginine (Sigma Chemical Co., St. Louis, Mo.), 20% agamma horse serum, 2.5% dialyzed yeast extract, 0.5% CVA, 1% cysteine (Sigma), and 4% urea (ultrapure; Schwarz/Mann, Cambridge, Mass.). Cultures of each organism were harvested in late log phase and washed three times in phosphate-buffered saline (PBS, pH 7.3), and the final pellet was suspended in PBS. Protein content was estimated by the method of Lowry et al. (18) with bovine serum albumin as the standard.

Antisera and MAbs. Methods for the production of anti-*U. urealyticum* rabbit sera and for the characterization of patient sera have been described previously (14). MAbs (ascites) directed to the *U. urealyticum* serovars (lysed organisms or affinity-purified antigen) were produced in conjunction with the Hybridoma Core Facility of the Multipurpose Arthritis Center at the University of Alabama at Birmingham by previously described procedures (38). MAbs reactive to antigens of specific molecular masses were screened by immunoblotting with nitrocellulose strips containing electrophoretically separated antigens of the serovar being examined. Enzyme-linked immunosorbent assay (ELISA) titers were determined as described previously (14).

Affinity chromatography. MAbs were partially purified from ascites by precipitation with ammonium sulfate (40%) and coupled to cyanogen bromide-activated Sepharose 4B (Sigma). Organisms were solubilized in PBS containing 1% Nonidet P-40 (NP-40) at 1 mg of organism protein per ml for 1 h at room temperature and 10 s of vortexing every 10 min. The preparation was clarified by centrifugation for 20 min at 20,000 × *g*. The clarified supernatant was diluted to 0.1 mg/ml in PBS with 1% NP-40 and passed slowly through the column. Unbound material was removed by washing the column with 30 bed volumes of PBS with NP-40, 30 bed volumes of PBS, and then 10 bed volumes of 0.2 M ammonium bicarbonate; no protein was detectable in the last 10 volumes of wash. Specifically bound antigen was eluted with 0.2% acetic acid and neutralized with ammonium bicarbonate. Antigen was detected in fractions (0.5 ml) by Western immunoblot (see below) with the same MAb as used for the affinity column. Positive fractions were pooled, concentrated, dialyzed against PBS, and used for MAb production or immunoblot analyses.

SDS-PAGE and immunoblots. SDS-PAGE was performed on 10% polyacrylamide gels as previously described (38). In some experiments, the organisms were harvested and prepared for electrophoresis in the presence of protease inhibitors (10 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin A per ml, 0.3 trypsin-inhibiting units of aprotinin per ml, 2 mM EDTA) as described previously for *M. pulmonis* (38). With standard gels, 20 μg of protein was loaded per well, and 10 μg was loaded for mini-gels (Bio-Rad). Separated proteins were transferred to nitrocellulose by the method of Towbin et al. (33), and immunological reactions were performed and identified by peroxidase-labeled conjugates as described previously (38). Rabbit antisera (1:1,000) and human sera (1:100) were diluted in culture medium (adjusted to pH 7.3 and containing 0.05% Tween 20 [Sigma]). MAb ascites (1:2,000) were diluted in blocking buffer (PBS containing 10% horse serum and 0.05% Tween 20). Biotinylated molecular weight standards (Bio-Rad) were used with immunoblot experiments to allow comparison of molecular masses between experiments.

MI test. The metabolism inhibition (MI) test was performed essentially as described by Taylor-Robinson (30). Medium 10B at pH 6.1 (25 μl) containing 1% guinea pig serum was added to each well of a 96-well microtiter plate (Costar, Cambridge, Mass.). MAbs (ascites) to be tested were diluted 1:20 in 10B medium and heat inactivated at 56°C for 30 min; 25 μl was added to the first well and then diluted in twofold serial steps. Antigen control wells received 25 μl of 10B only. Fifty microliters of a *U. urealyticum* culture containing 500 color-changing units was added to each well. Medium control wells received 50 μl of 10B without organisms. Finally, all wells received 125 μl of 10B, and the plates were incubated at 37°C in a humidified atmosphere. The MI titers were determined when the color change in the antigen control wells was easily detectable (approximately pH 6.7). The titers were recorded as the dilution of antibody which noticeably prevented a color change compared with the antigen control wells.

Competitive binding of anti-*U. urealyticum* antibodies. *U. urealyticum* antigen (300 μg of whole organism protein) was loaded across the entire length of an SDS-PAGE gel, and proteins were separated and transferred to nitrocellulose. Individual strips were cut and reacted with the appropriate antibodies. All antibody dilutions were made in *U. urealyticum* growth medium. To determine competition of binding, strips were reacted first with the appropriate MAb (1:100) for 2 h at room temperature and then washed and reacted for 2 h with the human serum (1:100). These strips were then washed and reacted with anti-human immunoglobulin conjugates. As a control, some strips were reacted with patient sera only (1:100) or with MAbs only (1:2,000), and the reactions were visualized after reaction with anti-human or anti-mouse immunoglobulin conjugates, respectively.

Competitive binding between individual MAbs of different isotypes (but directed to the same antigen) was similarly evaluated. Nitrocellulose strips containing separated *U. urealyticum* antigens were first incubated with a primary MAb (1:50) of a known isotype for 2 h at room temperature, and then the strips were incubated for 2 h with a secondary MAb of a different isotype. The strips were then washed and reacted for 1 h with rabbit antiserum specific for the isotype

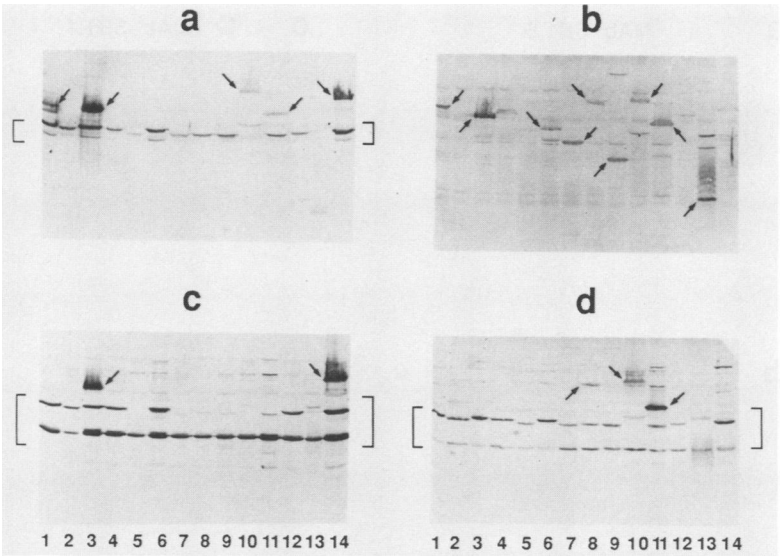

FIG. 1. Immunoblots of human serum reacted with the 14 *U. urealyticum* serovars. Proteins from the 14 serovars were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with the appropriate antibodies. Reactions were visualized by using peroxidase-labeled conjugates. Lanes 1 to 14 contained proteins from serovars 1 to 14, respectively. Patient sera used: (a) serum B2, (b) serum K1, (c) serum P2, (d) serum S2. The areas of strongly reactive bands which appeared to be common to multiple serovars are indicated by the brackets. The major bands, excluding these serovar-common bands, are identified by arrows. The molecular masses of these latter bands for the reacting serovars are: serovar 1 (panels a and b), 85 kDa; serovar 3 (panels a, b, and c), 71 kDa; serovar 6 (panel b), 61 kDa (and the next lower band of 55 kDa); serovar 7 (panel b), 54 kDa; serovar 8 (panels b and d), 88 kDa; serovar 9 (panel b), 45 kDa; serovar 10 (panels a, b, and d), 97 kDa; serovar 11 (panels a, b, and d), 70 kDa; serovar 13 (panel b), 30 kDa; and serovar 14 (panels a and c), 88 kDa.

(Bio-Rad) of the secondary MAb. Reactions were visualized after washing and incubation with peroxidase-labeled goat anti-rabbit immunoglobulin antibody.

RESULTS

Analysis of antibody response in humans. Previous studies with one of the most commonly isolated serovars (*U. urealyticum* serovar 3) and immunoblotting with rabbit antisera (8, 14) identified a strongly reactive antigen (calculated molecular mass of 71 kDa) which appeared (compared with the other serovar 3 antigens) to have the fewest cross-reacting counterparts in the other serovars. To determine whether this potentially unique serovar 3 antigen was also an antigen recognized in human infections, sera from 30 *U. urealyticum*-infected patients were screened for immunoblot reactivity with this antigen. The 30 patients were randomly selected from patients attending an infertility clinic at the University of Alabama at Birmingham for whom cultures were positive for *U. urealyticum* (9). Twelve of the sera reacted with a 71-kDa serovar 3 band, corresponding to the molecular mass of the major antigen recognized by rabbit antiserum (8). Analysis of three of the sera which reacted strongly with the 71-kDa band (B2, K1, and P2) and one which showed no reaction with this band (S2) is shown in Fig. 1.

Each serum showed extensive cross-reactions among all serovars. However, in addition to the 71-kDa band of serovar 3, many of the most intense reactions indicated in the figure also suggested the presence of some bands which were tentatively unique to other individual serovars (arrows in each panel).

Specificity of anti-*U. urealyticum* MAbs. To more fully characterize the antigens recognized by the patient sera, MAbs were generated to selected *U. urealyticum* antigens. MAbs were screened by immunoblot for reactivity corresponding to the molecular mass of the antigens shown to be recognized by the human sera (indicated by arrows in Fig. 1). The first series of MAbs were produced by immunization with a lysate of whole serovar 3 cells. Several of these MAbs reacted strongly with a serovar 3 71-kDa band and reacted faintly with bands in some of the other serovars. In addition to the strong reaction with the 71-kDa band, there were also multiple, much less intensely staining bands forming a symmetrical ladder pattern beneath the 71-kDa band. This specificity is represented by MAb 3B1.5 in Fig. 2a and is similar to the pattern shown previously for a major antigen of *M. pulmonis* (15, 36–38). As was also shown for the *M. pulmonis* antigen (38), this multiple-banded (MB) antigen was unaffected by the inclusion of protease inhibitors. The faint reactions with serovars other than 3 may be nonspecific, since this blot was overdeveloped to make the multiple bands of the ladder pattern more easily seen. Another MAb (11A5.1) which reacted strongly with a doublet on all serovars (data not shown) was also identified and used as a control in some experiments.

To obtain MAbs to additional epitopes, the serovar 3 71-kDa MB antigen was affinity purified by using MAb 3B1.5 (yielding the complete ladder pattern by immunoblot) and then used for immunization. Most of these new MAbs had a specificity similar to that shown for MAb 3B1.5. One additional MAb (10C6.6) which showed strict serovar 3 specificity (Fig. 2b) was also used for further study. Another specificity obtained is represented by MAb 5B1.1 (Fig. 2c), which showed a cross-reaction between the 71-kDa band of serovar 3 and an 88-kDa band of serovar 14 similar to that reported previously with rabbit antiserum to the same antigens (14) and also similar to the human serum reactions shown in Fig. 1a and c. A third specificity with more extensive serovar cross-reactivity is illustrated by MAb 8B5.2 in Fig. 2d. This MAb recognized bands in all serovars

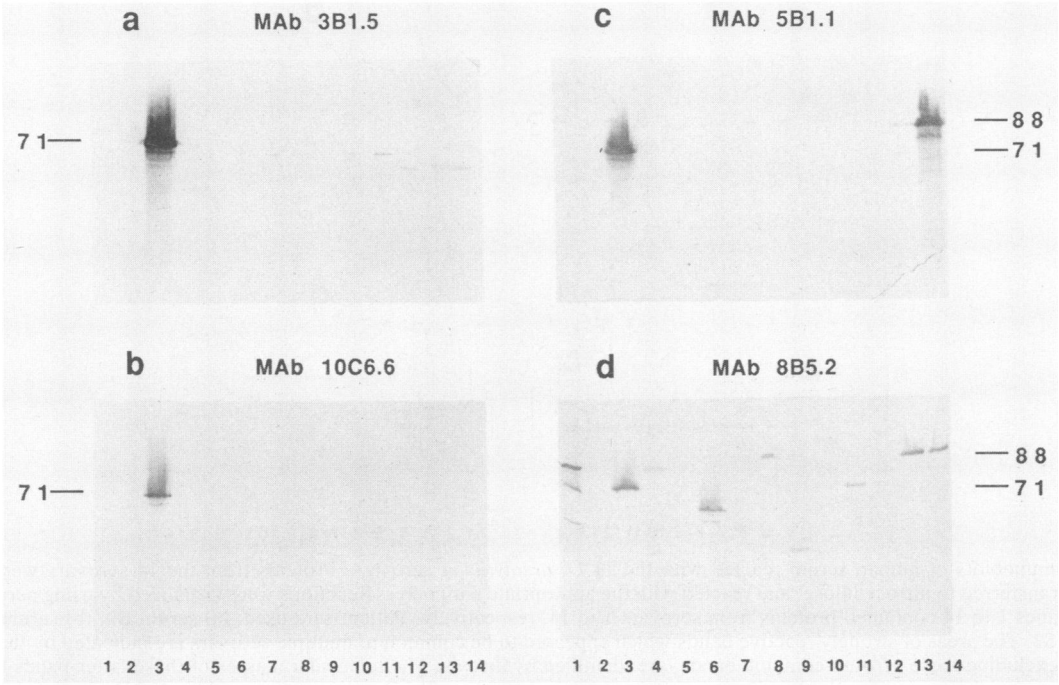

FIG. 2. Immunoblots showing the serovar specificities of anti-serovar 3 MAbs. Lanes in all panels were loaded left to right with serovars 1 to 14. MAbs used were (a) MAb 3B1.5 (IgM), (b) 10C6.6 (IgG2b), (c) MAb 5B1.1 (IgG1), and (d) MAb 8B5.2 (IgG2b). Molecular mass standard were run with each immunoblot to provide a more accurate comparison of different blots. Molecular masses (in kilodaltons) for the serovar 3 (71 kDa) and the serovar 14 (88 kDa) antigens are indicated. Molecular masses of bands in panel d for each lane (serovar) were: lane 1, 85 kDa, 74 kDa, and 58 kDa; lane 3, 71 kDa; lane 4, 81 kDa; lane 6, 61 kDa and 55 kDa; lane 7, 54 kDa; lane 8, 88 kDa; lane 9, 45 kDa; lane 10, 97 kDa (very faint); lane 11, 70 kDa; lane 12, 77 kDa (very faint); lane 13, 88 kDa; and lane 14, 88 kDa.

(weakly in some cases) except 2 and 5 and, based on calculated molecular masses, recognized the same serovar 3 and 14 cross-reactive antigens as MAb 5B1.1. It is also interesting that the strongest reactions of this MAb were with serovars 1, 3, 6, 13, and 14, which, with the exception of 13, correspond to the serovars of biotype 1 (32). The molecular masses of the antigens reacting with MAb 8B5.2, with the obvious exception of serovar 13, also corresponded to most of the antigens strongly recognized on the same serovars by the human sera shown in Fig. 1 (note particularly panel b). By immunoblotting, none of these MAbs showed reactivity with the other mycoplasmas known to occur in humans, including *M. pneumoniae*, *M. hominis*, *M. salivarium*, *M. fermentans*, *M. orale*, *M. lipophilum*, *M. faucium*, *M. genitalium*, *M. buccale*, *M. primatum*, *M. arthritidis*, *A. oculi*, and *A. laidlawii* (data not shown).

MAbs were also generated to two representatives of biotype 2 (32), serovars 8 and 10. The serovar 8 strain was the reference laboratory strain, and the serovar 10 strain was a patient isolate used in our laboratory for developing a mouse model of ureaplasmal disease (26). MAbs generated to serovar 8 were screened by immunoblot for reaction with an 88-kDa band which corresponded to the molecular mass of the serovar 8 band recognized by patient sera as identified in Fig. 1b and d. The serovar 10 MAbs were screened for reaction with a 97-kDa band which corresponded to the molecular mass of the serovar 10 band recognized by patient sera shown in Fig. 1a, b, and d.

One of the anti-*U. urealyticum* serovar 8 MAbs (146.5) to this 88-kDa antigen showed a pronounced multiple banding pattern (also unaffected by inclusion of protease inhibitors), with the most intense band at 88 kDa, but cross-reactive to some extent (note particularly serovar 11) with several other serovars (Fig. 3a). This multiple banding pattern of serovar 8 differed from that seen for serovar 3 (Fig. 2a) in that it lacked the symmetry of spacing between the different bands and the staining intensity of the multiple bands was greater (i.e., the multiple bands constituted only a small proportion of the total antigen). Another of the anti-*U. urealyticum* serovar 8 MAbs (182.10) reacted strongly with the 88-kDa antigen, but the multiple bands were only faintly visible, and it was also specific (Fig. 3b). The serovar 8, 88-kDa MB antigen was affinity purified with MAb 182.10, and it was found by immunoblotting (data not shown) that the anti-serovar 8 MAbs 146.5 (Fig. 3a) and 182.10 (Fig. 3b) and the cross-reactive anti-serovar 3 MAb 8B5.2 (Fig. 2d) reacted with this purified 88-kDa antigen. This verified that all three MAbs were recognizing the same antigen but different epitopes.

Four anti-serovar 10 MAbs were found which all appeared to react specifically with the serovar 10 97-kDa antigen. This specificity is represented by MAb 8A1.2 in Fig. 3c. Also, as for the serovar 8 antigen, it was found that the affinity-purified serovar 10 97-kDa antigen was recognized by the anti-serovar 3 MAb 8B5.2.

Competition of binding between human sera and MAbs. Blocking experiments were performed to verify that the antigens recognized by the serovar 3, 8, and 10 MAbs were identical to the major bands indicated in Fig. 1 which were recognized by patient sera in the same serovars. MAb 11A5.1, which reacted with all serovars, was used as a negative control in these experiments.

Sera from the four patients shown in Fig. 1 were evaluated for competition of binding with the serovar 3 antigen and the anti-serovar 3 MAbs. Similar results were obtained for MAbs 3B1.5, 5B1.1, 10C6.6, and 8A5.2. The results for 3B1.5 (which showed a more pronounced ladder pattern than

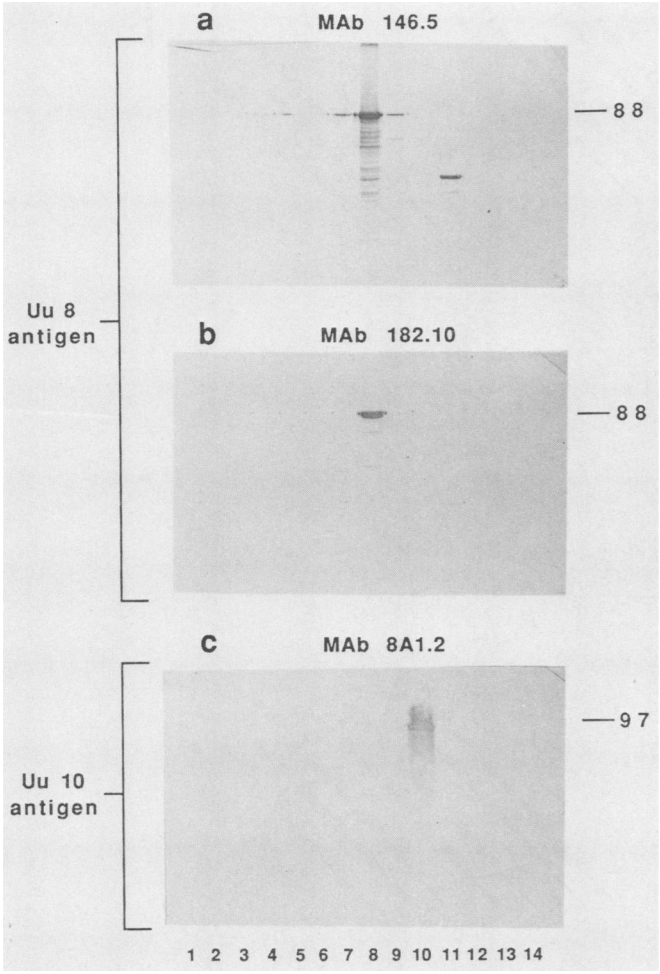

FIG. 3. Serovar-specificity of anti-serovar 8 (Uu 8) and anti-serovar 10 (Uu 10) MAbs. Immunoblots contained serovars 1 to 14 loaded in lanes 1 to 14, respectively. Panel a was reacted with the anti-serovar 8 MAb 146.5 (IgG2a) showing the multiple banding pattern with serovar 8 and some cross-reactions with other serovars. Panel b was reacted with the serovar 8-specific MAb 182.10 (IgG3). Panel c was reacted with the serovar 10-specific MAb 8A1.2 (IgM). The molecular masses (in kilodaltons) of the most intense bands are indicated.

the other MAbs) are shown in Fig. 4a. The blots in this figure were intentionally overdeveloped to make the multiple bands more obvious, but the loss in resolution of the more intense reactions makes it difficult to discern the upper band of the pattern. The 71-kDa bands of sera B2 (section 2; also faintly showed the multiple bands), K1 (section 3), and P2 (section 4) were specifically blocked by 3B1.5 and unaffected by 11A5.1, and no other bands were affected. Serum S2 (section 5), which showed no detectable reaction to the serovar 3 71-kDa antigen, was completely unaffected by the blocking.

Patient sera B2 and P2 (Fig. 1a and c) reacted appreciably with serovar 14, and a blocking experiment with serovar 14 antigen and MAb 5B1.1 (which cross-reacts with serovar 14, Fig. 2c) was performed. The results for serum B2 are shown in Fig. 4b. MAb 5B1.1 reacted strongly with an 88-kDa band of serovar 14 (lane 1, Fig. 4b), with less intensely staining multiple bands beneath. This MAb at least partially blocked the serum B2 reaction with serovar 14, indicating that the major reaction with serovar 14 found in serum B2 is also with an antigen containing multiple bands, similar to the serovar 3 antigen.

Although none of the patient sera tested reacted very strongly with serovar 8 or 10 antigens, serum S2 (Fig. 1d) reacted to some extent with a serovar 8 88-kDa band and a serovar 10 97-kDa band. Blocking experiments with serovar 8 (Fig. 4c) or serovar 10 (Fig. 4d) antigens and the respective MAbs showed that the 88-kDa band and the 97-kDa band recognized by this serum also corresponded to antigens containing multiple bands in these serovars. Lane 1 of Fig. 4d shows the faintly staining, tightly spaced multiple bands of the serovar 10 antigen.

The anti-serovar 3 MAbs 3B1.5 (Fig. 2a), 5B1.1 (Fig. 2c), and 8A5.2 (Fig. 2d) all recognized different epitopes on the serovar 3 71-kDa antigen; MAb 10C6.6 may recognize the same epitope as 3B1.5 (compare Fig. 1a and b). These MAbs were also evaluated by immunoblotting for competition of binding to the serovar 3 antigen. The results shown in Fig. 4e demonstrate that binding of MAb 3B1.5 prevented the subsequent binding of the remaining three MAbs. This gel was run with 7.5% acrylamide, conditions which were found to resolve the upper band of the serovar 3 antigen into three bands of 73, 71, and 69 kDa (71 kDa being the strongest-reacting band). Although very faintly staining, the 73- and 69-kDa bands were recognized by all MAbs. Under these conditions, the multiple bands beneath the 71-kDa band were very diffuse and difficult to discern, but except for a small amount of residual staining, 3B1.5 was shown to effectively block the reaction of the other 3 MAbs. Essentially identical results were obtained when MAb 5B1.1 was used for blocking (data not shown).

Analysis of clinical isolates with MAbs. Randomly selected, low-passage-number *U. urealyticum* isolates from cerebrospinal fluid (CSF), lung, or placenta of 21 infected patients (isolates not previously serotyped) were reacted with the MAbs to compare the antigen expression of the isolates with that shown for reference strains. Five of the isolates (two from placenta, two from lung, and one from CSF) reacted with the anti-serovar 3 MAbs, one (from lung) reacted with anti-serovar 10 MAb, and none reacted with anti-serovar 8 MAb. The reaction of the serovar 10 isolate with the anti-serovar 10 MAb was identical to that for the reaction with the serovar 10 reference strain (Fig. 3c). Two representative isolates (both from lung) reactive with the anti-serovar 3 MAb 3B1.5 are shown in Fig. 5a. The isolate in lane 6 showed a strong band at 71 kDa, and the isolate in lane 12 showed a strong band at a slightly lower molecular mass, 70 kDa. In addition, the lane 6 isolate showed a relatively strong variant band at 60 kDa; two (one from lung and one from placenta) of the five isolates showed this variant band. The ladder patterns were only faintly visible on the isolates compared with the serovar 3 reference strain in lane 13.

Detection of bands in the isolates which varied from the 71-kDa upper band of the serovar 3 reference strain was not unexpected since previous analysis of serovar 3 first-generation subclones had shown a structural variation in this antigen (8). An additional first-series subcloning of the serovar 3 reference strain was analyzed by immunoblotting with the anti-serovar 3 MAbs. One of these 28 first-series clones showed a drop in the upper band of the ladder pattern from 71 kDa in the parental strain to 60 kDa. This first-series 60-kDa variant was further subcloned, yielding the second-series clones shown in Fig. 5b and c. Most of these second-series clones had a 60-kDa upper band identical to the 60-kDa band from the original first-series clone from which they were derived. The molecular mass of this band (60 kDa)

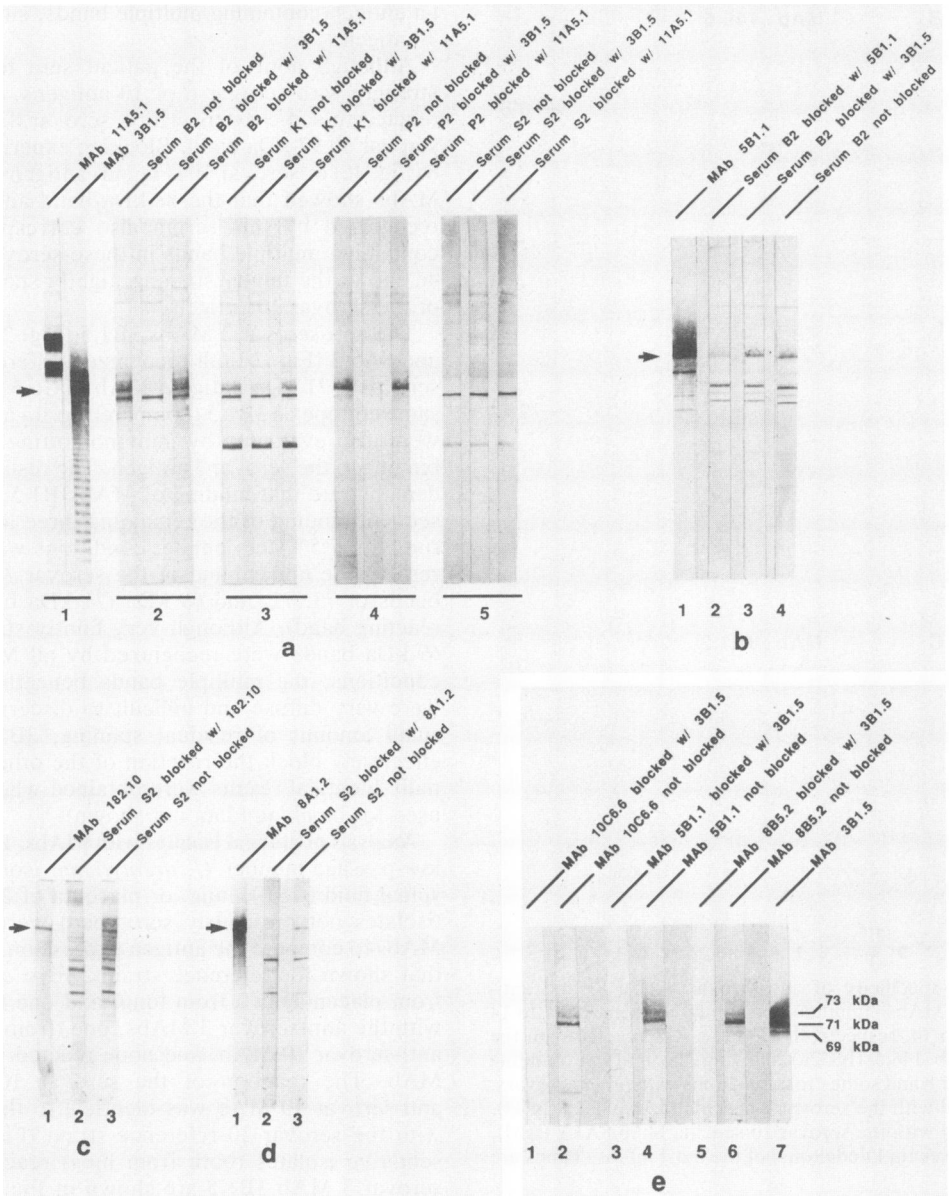

FIG. 4. Immunoblot results of patient sera evaluated for competition of binding with anti-*U. urealyticum* MAbs. Organism proteins were separated by SDS-PAGE and transferred to nitrocellulose, and individual strips were immunoblotted with the appropriate antibodies. Patient sera reactions were blocked by preincubation of the antigen strip with the MAbs. Section 1 in panel a and lane 1 in panels b, c, and d show reactions with the MAbs used for blocking. Panel a (serovar 3 antigen), sections 2 to 4, shows specific blocking by MAb 3B1.5 of the patient sera reaction with the 71-kDa (arrow) serovar 3 band. Serum S2 (section 5) showed no detectable response to the MAb 3B1-5 71-kDa antigen, and no other bands were affected by the blocking. Panel b was a similar experiment with serovar 14 antigen, serum B2, and MAb 5B1.1 and showed at least partial blocking of the patient serum reaction with the 88-kDa (arrow) band of serovar 14. Panels c (serovar 8 antigen) and d (serovar 10 antigen) were similar experiments showing blocking of the serum S2 reaction with the serovar 8 88-kDa band (arrow) and the serovar 10 97-kDa band (arrow) MAbs 182.10 and 8A1.2, respectively. Panel e contains serovar 3 antigen and demonstrates the blocking of three of the MAbs (of different epitope specificity) by a fourth MAb. MAb 11A5.1 (a serovar-common, non-ladder-pattern reactive antibody) showed no blocking in any experiments.

also corresponded to the molecular mass of the variant band of the isolate in lane 6 of Fig. 5a. Lane 7 of Fig. 5b shows a new variant with a molecular mass of 55 kDa for the most intense upper band of the ladder pattern. The rate of structural variation of this antigen has been calculated to be approximately 10^{-3} per cell per generation, which is similar to the rate of 2×10^{-3} shown for the *M. pulmonis* V-1 antigen (38).

It can be seen that all subclones in Fig. 5b and c still expressed a small amount of the original 71-kDa band, and lane 20 of Fig. 5c shows a slightly increased amount of this band compared with the other clones. It should also be noted that additional lower-molecular-mass bands of increased intensity were being expressed in several of the subclones (Fig. 5b, lane 11, and Fig. 5c, lanes 16, 18, 19, and 25). It has been found with both the *M. pulmonis* V-1 antigen (38) and *U. urealyticum* (data not shown) that further subcloning of the variants showing these bands of increased intensity

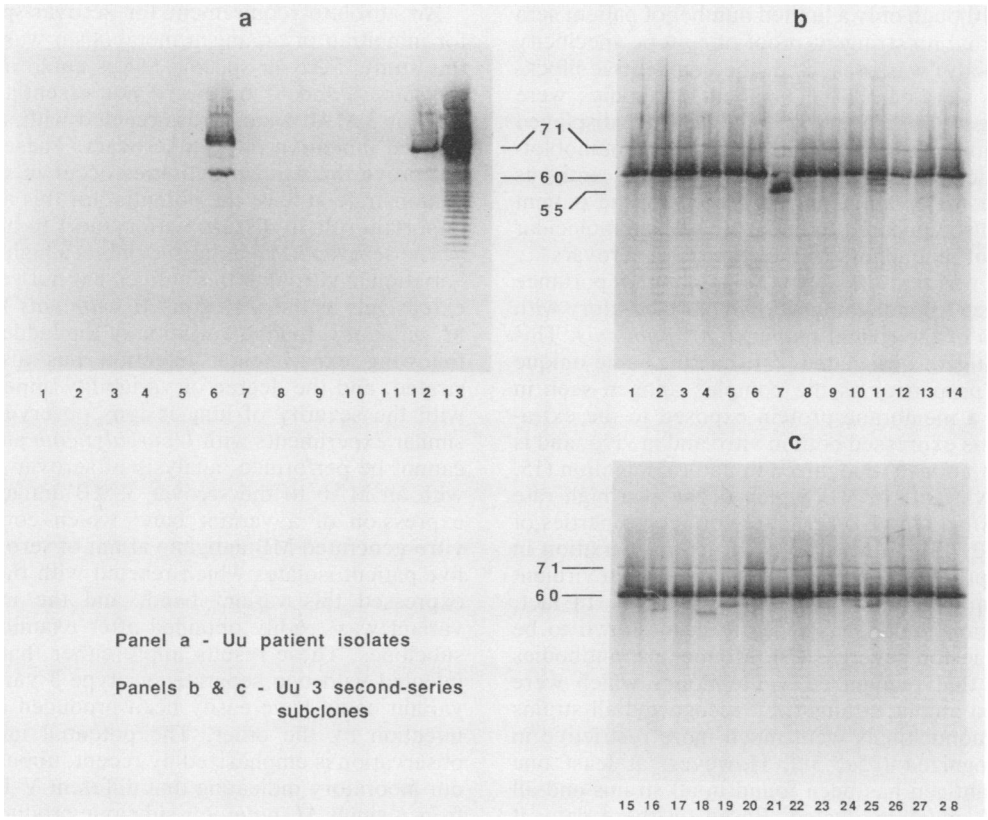

FIG. 5. Anti-serovar 3 MAb immunoblot reactions with *U. urealyticum* (Uu) patient isolates and the serovar 3 reference strain and subclones. All panels were reacted with MAb 3B1.5. Panel a contains 12 patient isolates (lane 1 to 12) and the serovar reference strain (lane 13, loaded with twofold more protein to show the ladder pattern). Panels b and c show the analysis of 28 second-series subclones derived from the serovar 3 reference strain. The upper bands of the ladder patterns are indicated; 71 kDa for the original serovar 3, 60-kDa variants, and a 55-kDa variant (lane 7, panel b). Molecular masses are indicated (in kilodaltons).

invariably results in at least one new variant with an upper band of the ladder pattern corresponding to the band of increased intensity of the parental clone. With the *M. pulmonis* V-1 antigen, it has been shown that subcloning of a low-molecular-mass V-1 variant frequently results in the generation of a new higher-molecular-mass variant (38). However, subcloning of *U. urealyticum* has thus far resulted only in the generation of variants (of the antigens recognized by the current MAbs) with molecular masses lower than those of the parental clone. This is suggestive of a deletion mutation event in the antigen, but the data are not yet extensive enough to allow this to be more than simple speculation.

MI test. The results of the in vitro inhibition of ureaplasma metabolism by the anti-*U. urealyticum* MAbs are shown in Table 1. The MAbs reactive with the MB antigens of serovars 3 and 8 showed MI for their homologous serovars only. The MAb which cross-reacted with the MB antigens of serovars 3 and 14 inhibited metabolism of both serovars, and the MAb specific for the serovar 10 MB antigen showed no MI for the homologous or heterologous serovars. The control MAb which recognized an antigen common to all serovars (not the MB antigen) also showed no inhibition for any serovar. The highly cross-reactive anti-serovar 3 MAb 8B5.2 (Fig. 2d) showed no inhibition of any serovar. However, the ELISA titer for this MAb (two separate ascites preparations) was >10-fold lower than for the other MAbs. Therefore, unless a high-titered preparation of this MAb can be obtained, the MI results will not be comparable to those for the other MAbs.

DISCUSSION

Antibody cross-reactions among the different *U. urealyticum* serovars have precluded attempts to use a patient's antibody response to make a reliable serovar-specific diagnosis (14). This problem derives, at least in part, from the paucity of available information on the *U. urealyticum* antigens relevant to the human immune response. In the

TABLE 1. MI with anti-*U. urealyticum* MAbs

MAb	Isotype	Serovar specificity[a]	ELISA titer[b]	MI[c]
3B1.5	IgM	3[d]	>10,000	3,200
10C6.6	IgG2b	3	>10,000	3,200
5B1.1	IgG1	3	>10,000	800
		14	>10,000	800
182.10	IgG3	8	>10,000	800
8A1.2	IgM	10	>10,000	0
11A5.1	IgG2b	1–14	>10,000	0

[a] Based on immunoblot reactions.
[b] Reciprocal of dilutions showing reaction with the serovar indicated; no detectable reaction with the other serovars.
[c] Reciprocal of dilution showing inhibition; there was no inhibition with other serovars.
[d] Immunoblot of 3B1.5 also showed slight reactivity with other serovars.

present study, although only a limited number of patient sera were evaluated and no stringent proof of epitope specificity (of patient antibody) was presented, the competitive blocking experiments verified that the patient antibodies were recognizing antigens of serovars 3, 8, and 10 which displayed a multiple banding pattern when visualized by immunoblotting with specific MAbs. Importantly, these MB antigens were among the major antigens recognized by the patient antibodies and also appeared to impart (based on molecular mass) a degree of uniqueness to the respective serovars.

Our attention was first drawn to the potential importance of this MB antigen from ongoing work in our laboratory with a similar antigen of the rodent pathogen *M. pulmonis*. This *M. pulmonis* antigen, designated V-1, has the same unique electrophoretic properties as the complex antigen seen in ureaplasmas, is a membrane protein exposed to the extracellular surface, is expressed both in vitro and in vivo, and is the predominant antigen recognized in natural infection (15, 36). Structural variants of V-1 which occur at a high rate both in vitro (38) and in vivo (29) alter surface properties of the organism (12, 13). Furthermore, structural variation in V-1 is the only protein difference detected between virulent and avirulent strains of *M. pulmonis* V-1 (35a). In fact, structural variation in V-1 has recently been shown to be associated with lesion severity (29). Monoclonal antibodies were produced that recognized V-1 epitopes which were highly conserved among strains (i.e., recognized all strains tested). Other monoclonals were much more restrictive in the strains recognized (35a, 38). However, at least one epitope of this antigen has been found in all strains and all subclones of *M. pulmonis* tested. Based on these data, it appeared that the MB antigen of *U. urealyticum* not only had the potential to play an important role in pathogenesis but was also likely to contain serovar-specific epitopes which would be of use in developing serovar-specific diagnostic strategies.

When the immunoblot reactions with the 14 serovars were examined, a striking similarity was found between patient sera cross-reactions and the cross-reactions of the anti-serovar 3 MAbs 5B1.1 and 8A5.2. The anti-serovar 3 MAb 8B5.2, generated to the affinity-purified MB antigen of serovar 3, cross-reacted with antigens in 12 of the 14 serovars. It also was found that the affinity-purified MB antigens from serovars 8 and 10 were recognized by MAb 8B5.2. Thus, this single cross-reactive MAb does, in fact, react with the most prominent band of this complex antigen of serovars 3, 8, and 10, and the data demonstrate that the MB antigens of these three serovars are not only structurally related but have at least one common epitope. The implication of these studies is that this antigen, which does contain serovar-specific epitopes, may also be a major contributor of epitopes responsible for the cross-reactions found in sera from *U. urealyticum*-infected patients.

Surface exposure of at least some epitopes of the MB antigen of serovars 3, 8, and 14 is indicated by the in vitro inhibition of organism metabolism by the MB antigen-specific MAbs. However, the MAb which specifically recognized the serovar 10 MB antigen showed no MI activity. This lack of MI activity with the anti-serovar 10 MAb may indicate (i) recognition of an epitope which was not accessible to the MAb (e.g., not surface exposed), (ii) recognition of an epitope yielding an antibody interaction which is not sterically favorable for MI activity, or (iii) that antibodies to the serovar 10 MB antigen are not metabolically inhibiting. MAbs to additional epitopes of the serovar 10 MB antigen will be required to resolve this question.

An absolute requirement for serovar-specific antibodies for inhibition of organism metabolism was not indicated in this study. Serovar-specific MAbs could inhibit (shown for serovars 3 and 8) but were not essential, since an anti-serovar 3 MAb which cross-reacted with serovars 3 and 14 showed inhibition of both serovars. These in vitro data do not prove that similar activities occur in vivo, but they do demonstrate at least the potential for this antigen to play an important role in disease with respect to host protection.

The serovar 3 MB antigen exhibits a high rate of structural variation in vitro, but this antigen has not yet been studied as extensively as the analogous *M. pulmonis* V-1 antigen. With *M. pulmonis*, in vivo variation of the ladder pattern antigen following experimental infection has also been demonstrated, and the degree of variability appeared to correlate with the severity of lung lesions observed (29). Although similar experiments with *U. urealyticum* and its natural host cannot be performed, analysis of serovar 3 patient isolates with an MAb to the serovar 3 MB antigen did reveal the expression of a variant band which correlated to an in vitro-generated MB antigen variant of serovar 3. Two of the five patient isolates which reacted with the serovar 3 MAb expressed this variant band, and the in vitro-generated variant was readily obtained after examination of only 28 subclones. These results imply either that the patient was infected with two separate serotype 3 variants or that one variant could have easily been produced after an initiating infection by the other. The potential importance of this observation is emphasized by recent, unpublished data from our laboratory indicating that different V-1 variants derived from a single *M. pulmonis* subclone produce diseases which differ in lesion severity and incidence of death. In light of the recent development of a mouse model for *U. urealyticum* disease (26), the prospects for exploiting similar properties of the *U. urealyticum* antigen are promising.

The serovar-specific MAbs generated to the *U. urealyticum* MB antigens have thus far proven useful for the identification of clinical isolates by immunoblotting, but these data must be interpreted with caution until a more extensive evaluation of these MAbs has been performed. None of the subclones (including variants) examined to date have lost expression of the serovar-specific epitopes recognized by the MAbs, and the same is true for the *M. pulmonis* V-1 antigen. However, this is a variable antigen which contains both serovar-specific and cross-reactive epitopes. Thus, the possibility does exist for a serovar to lose expression of a serovar-specific epitope or to begin expressing a previously nonexpressed cross-reactive epitope.

The competitive blocking experiments demonstrated that patient antibodies recognized the *U. urealyticum* MB antigens, but there was no evidence of preferential reactivity of the sera with a specific epitope. In fact, based on the results in Fig. 4a, each of the MAbs tested could individually effectively block the reaction of a patient's presumably polyclonal response to the same antigen. One implication of these data is that the epitopes recognized by the MAbs and patient antibodies are located close to one another or are overlapping. This hypothesis was supported by demonstrating that binding of a single MAb to this antigen prevented subsequent binding of MAbs to different epitopes on the same antigen. A second explanation for the observed competition of binding exists if the antigen, after SDS-PAGE and blotting, retains a sufficient degree of conformation; i.e., the binding of one MAb to its respective epitope could transmit a conformational change to a second, distal epitope and prevent interaction of an MAb specific for this second

epitope. The MB V-1 antigen of *M. pulmonis* has been shown previously to retain conformation after immunoblotting; some epitopes appear to be buried within the aggregate of subunits forming the upper band of the ladder pattern (the multiple bands being the result of partial disaggregation of this complex by SDS treatment). If the MB antigen of *U. urealyticum* has an analogous structure, then the concept of competition of antibody binding via conformational change is plausible. However, the present data do not allow distinction between these two possibilities.

Highly variable surface antigens may be quite common among the mycoplasmas, based on recent studies with *M. pneumoniae* (16), *M. hyopneumoniae* (39), *M. hominis* (8, 27), and *M. pulmonis* (38). Also, a recent study with *M. hyorhinis* (25) describes another mycoplasma ladder pattern antigen which is highly variable and appears to be analogous to the *M. pulmonis* V-1 antigen (37, 38) and to the *U. urealyticum* antigen described in the current study. Although the *M. hyorhinis* antigen has been shown to be lipid modified, neither the complex nature of the likely repetitive structure nor the mechanism of variation of these MB antigens is fully understood (25, 37). However, the potential for the variation of these or other structures to influence mycoplasma-host cell interactions provides an impetus for studies to elucidate their role in pathogenesis.

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