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Serotype 8- and Serocluster-Specific Surface-Expressed Antigens of *Ureaplasma urealyticum*

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The polypeptides of all 14 serotypes of *Ureaplasma urealyticum* were analyzed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. The electrophoretic patterns did not allow ready discrimination of individual serotypes or seroclusters. The analysis of the antigens of serotype 8 was reported previously (B. L. Precious, D. Thirkell, and W. C. Russell, J. Gen. Microbiol. 133:2659–2676, 1987). In this study, three of the surface-expressed membrane antigens of 16, 17, and 96 kilodaltons were further investigated, and monoclonal antibodies were raised against these three polypeptides. The major 96-kilodalton polypeptide was serotype 8 specific, and the 16-kilodalton polypeptide was present only in the larger serocluster. We describe monoclonal antibody probes that unequivocally differentiate serotype 8 from the other serotypes and that separate the two seroclusters of the organism.

Ureaplasma urealyticum is the mycoplasma most commonly isolated from the human urogenital tract, where it may be a pathogen or a commensal (12). It has been implicated in spontaneous abortion (14), nongonococcal urethritis, low birth weight, infertility, and chorioamnionitis (A. Naessens, W. Foulon, J. Breynaert, and S. Lauwers, Abstr. 7th Int. Congr. Mycoplasmol., abstr. no. 30, 1988). More recently, chronic infection by *U. urealyticum* of the central nervous system in preterm infants (23) and an association with infection of the lower respiratory tract with chronic lung disease and death in very-low-birth-weight infants (2) have been reported. The organism has been differentiated into 14 serotypes by using an expanded scheme involving a modified metabolic inhibition test and a colony indirect epifluorescence method (15), and the problems associated with serotyping the strains have been reviewed (20). Whereas some evidence is beginning to emerge that certain of these serotypes may predominate in disease processes (12, Naessens et al., 7th Int. Congr. Mycoplasmol.), definite probes for the differentiation of the two known seroclusters as determined by a variety of techniques (see Discussion) or of individual serotypes are awaited. Previously, we showed that an antigen of the canonical urease was conserved in all of the serotypes, and monoclonal antibody probes detecting four different epitopes were prepared that recognized this enzyme in all 14 serotypes (22). In this work we describe two other probes, one of which is serotype 8 specific and one of which differentiates the two seroclusters.

MATERIALS AND METHODS

***U. urealyticum* strains.** *U. urealyticum* serotypes 1 (T7), 4 (11860), 6 (1253), and 8 (T970) were gifts from D. Taylor-Robinson (Clinical Research Centre, Harrow, United Kingdom). *U. urealyticum* serotypes 2 (T23), 3 (DKF3), 5 (NIH5), 9 (9-Vancouver), 10 (10 Western), 11 (11-JsL2), 12 (JsL5), 13 (JsL6), and 14 (JsL11) were gifts from J. Robertson (University of Alberta, Edmonton, Canada). *U. urealyticum* serotype 7 (ATCC 27819) was obtained from the American Type Culture Collection.

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Medium and cell production. Except where stated, ureaplasmas were grown in medium containing 70% (vol/vol) PPLO broth (Difco Laboratories), 20% (vol/vol) horse serum (Northumbria Biologicals Ltd.), and 2.5% (wt/vol) freshly prepared yeast extract, incorporating 0.1% urea, 0.005% phenol red, and 10^3 IU of penicillin G ml^{-1} at a starting pH of 6.0. Cultures were incubated at 37°C until a pH of 7.6, corresponding to a cell density of 10^7 color change units (CCU) ml^{-1}, was reached. Determination of CCU was performed by making serial decimal dilutions of 0.2 ml of culture in vials containing 1.8 ml of growth medium; vials were incubated at 37°C until no further color change of the phenol red indicator was apparent (normally 48 h). From the lowest dilution in which color change was seen, the number of CCU per milliliter of the original inoculum was determined. The cells were harvested by centrifugation with a Beckman J-21 rotor (25,000 × *g*, 20 min), the pellets were washed in the phosphate-buffered saline A (PBS) of Dulbecco and Vogt (4), and the final pellet was suspended in PBS.

Antibody preparation. Polyclonal antiserum to *U. urealyticum* serotype 8 was produced as described previously (9). For the production of monoclonal antibodies (MAbs), BALB/c mice were immunized with ureaplasma serotype 8 organisms grown as described above. Washed cells (approximately 10^9 CCU ml^{-1}) on ice were sonicated (four 10-s bursts with intermittent cooling) by using a 0.5-cm microprobe on a Kerry ultrasonicator and then alum precipitated (7). Initial immunization (intraperitoneal) was with 0.2 ml of alum-precipitated cell extract followed by a second such injection 4 weeks later. A third injection (intravenous) of 0.1 ml of sonicated extract was given 4 weeks later, 3 days before fusion. Mouse antisera taken 2 days before fusion were screened by a radioimmune assay (10) for anti-ureaplasma antibodies with organisms grown in a medium with 10% (vol/vol) calf serum substituted for horse serum. Details of the production and screening of MAbs were as described previously (18). Screening for antibodies to ureaplasma-derived proteins was by radioimmune assay with ^{125}I-labeled protein A (Amersham Corp.) and by enzyme-linked immunosorbent assay with a goat peroxidase-linked anti-mouse immunoglobulin (Sigma Chemical Co.).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (17) with 15% (wt/vol) polyacrylamide slab gels. All samples for electrophoresis were boiled (2 min) after the addition of an equal volume of a solution containing 5 M urea, 2% (wt/vol) SDS, 3.5 M β-mercaptoethanol, and 0.1% (wt/vol) bromophenol blue (denaturing mix). Staining of polypeptides was achieved either with Coomassie stain or with the more sensitive silver stain (Bio-Rad Laboratories kit). Apparent molecular masses were determined, where appropriate, by comparison with protein standards of known molecular weight (Bethesda Research Laboratories, Inc.).

Immunoblotting. Transfer of polypeptides from polyacrylamide gels to nitrocellulose sheets was carried out on an Ancos model A semidry electroblotter according to the instructions of the supplier. The blots were blocked with 3% (wt/vol) bovine serum albumin (30 min, 20°C), washed three times for 10 min each with PBS containing 0.2% (vol/vol) Nonidet P-40 (Sigma) (PBS-N). Whole sheets or sheets cut into strips were incubated with MAbs (normally 1/500 in 50:50 [vol/vol] PBS-N–growth medium) for 1 h at 37°C. After preparations were washed three times with PBS-N, approximately 10^6 cpm of ^{125}I-labeled protein A (45 mCi mg^{-1}; Amersham) was added; after incubation (1 h, 20°C) and extensive washing with PBS-N, the nitrocellulose was subjected to autoradiography with X-ray film (Fuji-RX) and a Philips fast tungstate intensifying screen.

Labeling of serotype 8 organisms with ^{125}I-labeled Bolton-Hunter reagent. Fresh cultures grown in medium (with 10% [vol/vol] fetal calf serum substituted for horse serum) were centrifuged (25,000 × *g*, 20 min, 4°C), and the pellets were suspended in PBS and centrifuged as above. This procedure was repeated twice, followed by suspension of the pellets in 0.1 M sodium borate buffer (pH 8.3), before centrifugation as above. The final pellets were suspended in 0.1 M borate buffer at approximately 10^9 CCU ml^{-1}. Either whole cells (0.1 ml) or sonicated (as above) cell extracts were then labeled for 15 min with 100 μCi of *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate (Bolton-Hunter reagent; Amersham) at 4°C according to the instructions of the supplier. The reaction was stopped by the addition of 0.1 M glycine in 0.1 M borate buffer (pH 8.3). The ^{125}I-labeled whole cells were washed three times in PBS to remove any labeled internal components released by autolysis, suspended in PBS, and stored at −20°C. Sonicated ^{125}I-labeled cell extracts were centrifuged (300 × *g*, 4 min) through a 1-ml minicolumn of Sephadex G50 (coarse; Pharmacia) to remove unbound label and then stored at −20°C.

Immunoprecipitations. Bolton-Hunter reagent-labeled whole cells were lysed in detergent-containing buffer A (10 mM Tris hydrochloride [pH 7.2], 5 mM EDTA, 0.5% Nonidet P-40, 0.65 M NaCl, 0.1% NaN_3, 1 mM phenylmethylsulfonyl fluoride) and sonicated as described previously. Soluble antigen extracts were obtained in the supernatant after centrifugation in a microfuge (12,000 × *g*, 10 min). Samples (50 μl) of soluble antigen were incubated (1 h, on ice) with 5 μl of appropriate ascitic fluid (used undiluted), and the immune complexes were isolated on an excess of a fixed suspension (20 μl of a 10% [wt/vol] suspension per μl of ascitic fluid) of Cowan strain A of *Staphylococcus aureus* by incubation for 1 h on ice (6). The immune complexes of *S. aureus* were pelleted (microfuge, 12,000 × *g*, 10 min), suspended in buffer A containing 10% (wt/vol) sucrose, and centrifuged as above. Suspension of the pellet in this buffer and centrifugation were repeated three times, after which the final pellet was suspended in 100 μl of denaturing mix and boiled for 3 min. After further centrifugation as above, the supernatant was subjected to SDS-PAGE. After electrophoresis, the gels were either silver stained and then dried or dried without prior staining, and labeled polypeptides were visualized by autoradiography as above.

Phase partitioning of extracts of serotypes 1 and 8 with Triton X-114. The phase partitioning method of Bordier (1) was modified to include centrifugation stages (16). Sonicated cell suspension (0.2 to 1.0 mg ml^{-1}) was prepared in 200 μl of buffer B (10 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, and 1% [vol/vol] Triton X-114 [Sigma]) and maintained on ice for 10 min. Residual particulate matter was removed by centrifugation (2,500 × *g*, 3 min), and the supernatant was overlaid on 200 μl of buffer B containing 6% (wt/vol) sucrose and 0.01% (vol/vol) Triton X-114 in an Eppendorf tube. After incubation (30°C, 3 min) and centrifugation (300 × *g*, 3 min), a small oily droplet pelleted; the upper aqueous supernatant was removed and made 0.5% (vol/vol) with respect to Triton X-114, and the phase partition was repeated. The final aqueous phase was made 2% (vol/vol) with respect to Triton X-114; after phase separation, the resultant detergent phase was discarded. With buffer and Triton X-114, both final detergent and aqueous phases were made to the same volume (200 μl) and to 1% (vol/vol) with respect to Triton X-114.

Portions of both phases were mixed with an equal volume of denaturing mix and boiled (2 min) before being subjected to SDS-PAGE followed by either silver staining or immunoblotting with appropriate MAbs and autoradiography as described above.

RESULTS

Electrophoretic analysis of the serotypes. All 14 serotypes grown in a medium with 10% (vol/vol) fetal calf serum substituted for horse serum were analyzed by SDS-PAGE and gave very complex polypeptide patterns, which were, however, remarkably similar to each other (data not shown) and to the pattern given by serotype 8 (Fig. 1, lane A). Some minor differences in polypeptides and in the quantitative amounts of individual polypeptides were noted between the serotypes, but these differences were not considered sufficiently striking to justify either serotyping or differentiation into seroclusters by this technique.

Antigenic analysis. The antigens of *U. urealyticum* serotype 8 which were recognized by homologous polyclonal serum were established either by immunoprecipitation of sonicated cell extracts labeled with ^{125}I-labeled Bolton-Hunter reagent, followed by SDS-PAGE and autoradiography, or by SDS-PAGE, immunoblotting, and autoradiography. Both techniques gave similar results; the results achieved by immunoprecipitation are shown in Fig. 1 (lane B). The antigens detected in the ^{125}I-labeled cell extracts by immunoprecipitation with homologous polyclonal serum (Fig. 1, lane B) differed in some instances from the polypeptides of serotype 8 visualized with the silver stain (Fig. 1, lane A). Such differences were not unexpected, since minor polypeptides not easily visible in lane A may be highly antigenic; moreover, since labeling with Bolton-Hunter reagent is dependent upon interaction of the reagent with free ε-amino groups of lysine in a polypeptide, only a subset of antigens would be labeled. In previously reported work (9, 22), nine MAbs were raised against serotype 8, four of which recognized a 96-kilodalton (kDa) antigen. In addition, 18 MAbs were raised against purified urease from serotype 8. Of the MAbs prepared against serotype 8 in the study

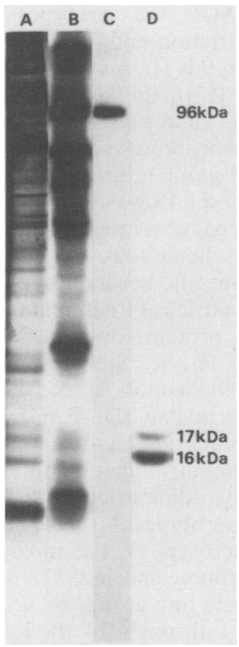

FIG. 1. Lane A: electropherogram of the polypeptides of *U. urealyticum* serotype 8 by SDS-PAGE visualized with silver stain. Lanes B through D: autoradiograms of immunoprecipitations of sonicated cell extracts labeled with ^{125}I-labeled Bolton-Hunter reagents with homologous polyclonal serum (B), MAb UU8/29 (C), and MAb UU8/39 (D). Note that lane A was derived from an electrophoresis parallel to that portrayed in lanes B through D.

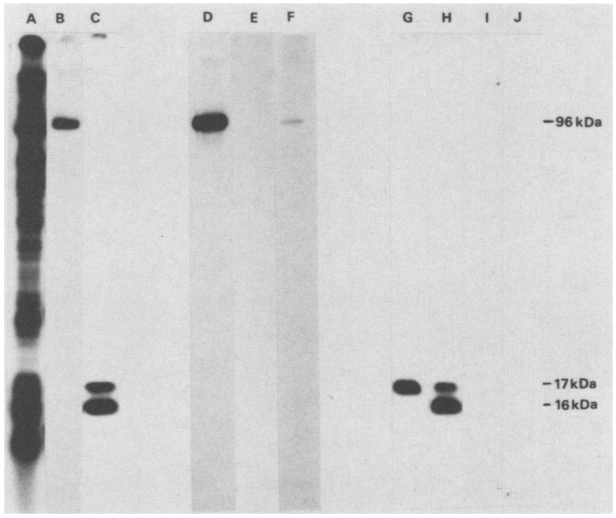

FIG. 2. Lane A: autoradiogram of whole cells of *U. urealyticum* serotype 8 labeled with ^{125}I-labeled Bolton-Hunter reagent. Lanes B and C: immunoprecipitations of soluble extract of ^{125}I-labeled whole cells of *U. urealyticum* serotype 8 with MAbs UU8/29 (B) and UU8/39 (C). Lanes D through F: immunoblots with MAb UU8/29 and ^{125}I-labeled protein A of the phases obtained after partitioning of *U. urealyticum* serotype 8 in the detergent phase (D) and in the aqueous phase (E) (both after overnight exposure of the X-ray film) and in the aqueous phase after 3 days of exposure to X-ray film (F). Lanes G through J: immunoblots with MAb UU8/39 and ^{125}I-labeled protein A after phase partitioning of *U. urealyticum* serotypes 1 and 8 in the detergent phase from serotype 1 (G), detergent phase from serotype 8 (H), aqueous phase from serotype 1 (I), and aqueous phase from serotype 8 (J).

reported here, 12 were recognized both by immunoprecipitation with sonicated ^{125}I-labeled cell extracts and by immunoblotting; of these, 10 were against the 96-kDa antigen and were designated UU8/29 through UU8/38. An autoradiogram of an immunoprecipitation with UU8/29 is seen in Fig. 1 (lane C). The other two MAbs, designated UU8/39 and UU8/40, recognized two polypeptides in serotype 8 with apparent molecular masses 16 and 17 kDa. This doublet pattern was consistent with the two different clones by immunoblotting, one that produced an immunoglobulin G (UU8/39) reacting directly with ^{125}I-labeled protein A and another (UU8/40) that did not react directly with ^{125}I-labeled protein A. Both MAbs gave similar results by immunoprecipitation of sonicated cell extracts labeled with ^{125}I-labeled Bolton-Hunter reagent, and an autoradiogram of an immunoprecipitation with UU8/39 is shown in Fig. 1 (lane D).

The 16-, 17-, and 96-kDa polypeptides are surface-expressed membrane antigens. Whole cells of serotype 8 were surface labeled with the Bolton-Hunter reagent (Fig. 2, lane A). The number of ^{125}I-labeled polypeptides detected after whole cells were labeled was considerably less than the number detected after sonicated cell extracts were labeled (data not shown). Nevertheless, the data suggest that the organism has a complex surface protein pattern, although it is possible that, despite extensive washing of labeled whole cells, some internal, more hydrophobic components may have become associated with the cells. Immunoprecipitations of the ^{125}I-labeled whole cells were carried out with a typical anti-96-kDa MAb (UU8/29) and with MAb UU8/39, which recognizes the 16- and 17-kDa polypeptides of this serotype (Fig. 1, lanes C and D). The results suggest that these three polypeptides are surface expressed, and the data here with respect to the 96-kDa polypeptide confirm results reported previously (9).

Phase partitioning with Triton X-114 was carried out on serotypes 1 and 8, representatives of the two designated seroclusters of the organism. From each serotype, both the detergent and aqueous phases were subjected to SDS-PAGE and immunoblotting with MAb UU8/29 and with MAb UU8/39 (Fig. 2, lanes D through J). After overnight exposure of the X-ray film, all three polypeptides were exclusively within the detergent phase, which is consistent with their being predominantly hydrophobic membrane proteins. On prolonged exposure of the film (3 days), a small proportion of the 96-kDa polypeptide partitioned into the aqueous phase (lane F). It was also noteworthy that the 16-kDa polypeptide from serotype 8 was not recognized in either phase from serotype 1. Under similar conditions and with an anti-urease MAb, the cytosolic urease of the organism partitioned entirely into the aqueous phase (data not shown).

The 96-kDa polypeptide has a serotype 8-specific antigen. SDS-PAGE and immunoblotting with MAb UU8/29 was carried out on all 14 serotypes (Fig. 3). All of the anti-96-kDa MAbs, which recognize four distinct epitopes (D. Thirkell, A. M. Myles, and B. L. Precious, Zentralbl. Bakteriol. Mikrobiol., in press), gave similar results (data not shown). Thus, all of the anti-96-kDa MAbs recognize the 96-kDa polypeptide in serotype 8 only, do not cross-react with any other polypeptide in any of the other serotypes, and are therefore useful probes for the identification of serotype 8.

The 16- and 17-kDa polypeptides have antigens that are serocluster specific. SDS-PAGE and immunoblotting with MAb UU8/39 was carried out on all 14 serotypes (Fig. 4). UU8/39 recognized both a 16-kDa polypeptide and a 17-kDa polypeptide in serotypes belonging to the larger serocluster (group A) (see Discussion). However, only the 17-kDa

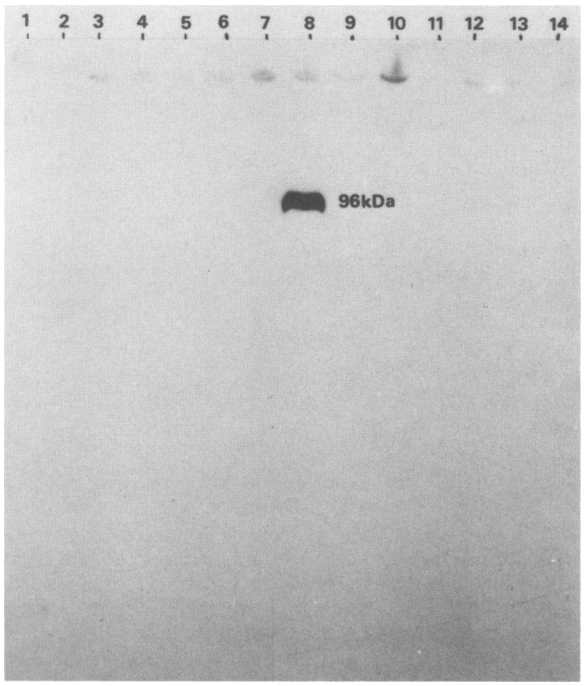

FIG. 3. Immunoblot of serotypes 1 through 14 of *U. urealyticum* probed with MAb UU8/29 and ^{125}I-labeled protein A.

polypeptide was recognized in the serotypes of the smaller serocluster (group B), implying that the 16-kDa polypeptide antigen is specific to those serotypes of the larger serocluster. It was noteworthy that serotype 13, whose serocluster designation is uncertain, falls into the larger serocluster in this system. Thus, this MAb is a useful probe for the differentiation of the two seroclusters of this organism.

DISCUSSION

It is important to attempt to recognize differences between the isolates of *U. urealyticum* that might correlate with pathogenicity (20). By a variety of techniques, e.g., DNA hybridization (with serotypes 1 through 8 [3]), one- and two-dimensional PAGE (12 serotypes [8]); (serotypes 1 through 9 [21]), restriction endonuclease digestion patterns (serotypes 1 through 9 [11]), and sensitivity to Mn^{2+} (serotypes 1 through 14 [13]), the serotypes of *U. urealyticum* have been shown to yield two seroclusters (20). One such cluster (group A) incorporates serotypes 2, 4, 5, 7, 8, 9, 10, 11, and 12; the other group (group B) incorporates serotypes 1, 3, 6, and 14. Serotype 13 was examined only with respect to Mn^{2+} sensitivity, gave an intermediate response, and was not included in the scheme (20).

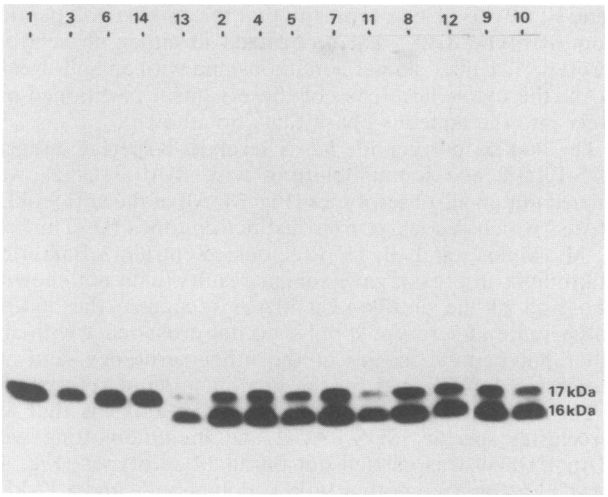

FIG. 4. Immunoblot of serotypes 1 through 14 of *U. urealyticum* probed with MAb UU8/39 and ^{125}I-labeled protein A.

With respect to specific serotypes, preliminary evidence was presented from protease treatment of cell lysates, which suggested that one protein (or peptide) carries serotype-specific antigenicity (G. W. Stemke and J. A. Robertson, Proc. Int. Org. Mycoplasmol., p. 55, 1982). With [^{35}S]methionine-labeled cells and an SDS-PAGE system with a 15 to 20% acrylamide gradient, it has been claimed that the two seroclusters of the organism can be differentiated (8). However, the use of two-dimensional gel electrophoresis to analyze unlabeled serotypes 1 through 9 revealed only a single protein (in serotype 9, the molecular mass was 41 kDa), which was serotype specific (21); others (5) have been unable to demonstrate any group- or serotype-specific proteins in serotypes 1 through 14 by immunoblotting with human sera from patients infected with a single serotype. Similarly, our own one-dimensional polypeptide analysis of serotypes 1 through 14 failed to demonstrate serocluster- or serotype-specific polypeptides on SDS-PAGE. On the other hand, the results suggest a high degree of homogeneity among the serotypes.

Probes that will differentiate the seroclusters or the serotypes, although useful in their own right, would, in addition, obviously assist investigation to determine whether specific seroclusters or specific serotypes predominate in disease processes.

Evidence has been presented here for three surface-expressed integral membrane protein antigens in serotype 8 with apparent molecular masses of 16, 17, and 96 kDa. All three polypeptides are recognized by homologous polyclonal serum and by specific MAbs by immunoblotting and by immunoprecipitation with surface ^{125}I-labeled cells. The predominance of MAbs against the 96-kDa polypeptide is consistent with it being a major polypeptide antigen in serotype 8 and, in that respect, confirms earlier reports by ourselves and others (9, 19). Prolonged exposure of an autoradiogram of an immunoblot of the aqueous phase with an anti-96-kDa MAb after phase partitioning of serotype 8 confirms that the polypeptide has major hydrophobic characteristics, and previous work (Thirkell et al., in press) has shown that the 96-kDa polypeptide has at least four distinct epitopes. However, it has been shown that this 96-kDa antigen, as detected by immunoblotting, is serotype 8 specific, and this is the first definite finding of a serotype-specific antigen in any of the 14 serotypes of *U. urealyticum*. These anti-96-kDa MAbs thus afford an unequivocable differentiation of serotype 8 from the other serotypes.

Furthermore, MAb UU8/39, raised against serotype 8, recognized the 16- and 17-kDa surface-expressed polypeptides in this serotype. The MAb cross-reacted with the other 13 serotypes in an interesting manner. The 16- and 17-kDa polypeptides were also recognized in all of the serotypes of the larger serocluster A, but the 17-kDa polypeptide only was recognized in the serotypes of the smaller serocluster B. On the basis of cross-reactions with serotype 13, the latter would appear to be placed in the larger serocluster A. This MAb is thus the first reported probe that will unequivocally

differentiate the two seroclusters of the organism. Whether the 16- and 17-kDa polypeptides in the larger serocluster A are in any way related by modification or whether two fairly similar-sized polypeptides exist which share a common epitope remains to be elucidated.

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