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Identification and Characterization of Serotype 4-Specific Antigens of *Ureaplasma urealyticum* by Use of Monoclonal Antibodies

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Monoclonal antibodies against *Ureaplasma urealyticum* serotype 4 were produced by immunizing BALB/c mice with whole-cell antigens of the *U. urealyticum* serotype 4 reference strain. Ten monoclonal antibodies differentiated into two groups were found: one group included five monoclonal antibodies recognizing a band in immunoblotting that had a molecular mass of 81 kDa, and a second group included another five monoclonal antibodies recognizing three bands in immunoblotting that had molecular masses of 81, 75, and 71 kDa. Fifteen clinical *U. urealyticum* isolates were selected for serotyping with serotype 4-specific monoclonal antibodies and polyclonal antisera 1 to 14. The results obtained with polyclonal and monoclonal antibodies suggest the existence of heterogeneity of the serotype antigens among clinical isolates of *U. urealyticum* serotype 4.

Ureaplasma urealyticum is an organism frequently isolated from the lower genital tract of men and women (1, 16). The role of this microorganism in diseases of the genital tract is difficult to establish because of its high isolation rate in asymptomatic patients. However, in some instances it has been shown that *U. urealyticum* can interfere with normal fetal and neonatal development (1–4, 6, 7, 16, 19). Probably, only a small number of all women colonized with *U. urealyticum* will ultimately develop a severe ureaplasma infection. Therefore, it seems interesting to look for strains of *U. urealyticum* that are more pathogenic than others, a characteristic which could explain why only certain patients develop an adverse effect from *U. urealyticum* colonization. Serotyping is one of the methods that can be used to differentiate the *U. urealyticum* strains. To date, 14 serotypes of *U. urealyticum* arranged in two serogroups are recognized (12, 15). In a previous serotyping study, using polyclonal antibodies, serotype 4 was found more often among patients with recurrent miscarriages, suggesting that serotype 4 might be more pathogenic (10). However, serotyping of *U. urealyticum* with polyclonal antisera remains to some extent a test which is difficult to interpret because of the cross-reactions between different serotypes. This makes it difficult to associate certain serotypes of *U. urealyticum* with diseases. A more objective and standardized serotyping method and a better understanding of the antigens of *U. urealyticum* are needed (5, 9, 13, 15). Recently, serotype 3-, 8-, and 10-specific antigens have been identified by means of monoclonal antibodies, and a monoclonal antibody distinguishing the two serogroups in immunoblotting has been described (17, 20). More interestingly, both serotype-specific and cross-reacting epitopes were found within one antigen, which explains, to some extent, the cross-reactions among serotypes. Monoclonal antibodies appear to be a good tool to analyze membrane antigens of *U. urealyticum*. In the present study, we produced monoclonal antibodies against *U. urealyticum* serotype 4 and used them to serotype clinical isolates of *U. urealyticum*.

Reference strains of *U. urealyticum* serotypes 1 to 10 were supplied by E. A. Freund (Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark), and those of serotypes 11 to 14 were supplied by J. A. Robertson (Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Canada).

Monoclonal antibodies were produced by injecting BALB/c mice (4 to 6 weeks old; Charles River Wiga, Sulzfeld, Germany) intraperitoneally every 2 weeks with 0.5 ml of antigen of the *U. urealyticum* serotype 4 reference strain. Complete Freund adjuvant was added to the first injection, and a total of six injections were given. The humoral immune response of the mice was monitored by the colony indirect immunofluorescence assay (colony-IFA). When the antibody titer was more than 1/80, a final booster dose of 0.2 ml of antigen was given through tail vein injection 3 days before fusion.

Spleen cells from immunized mice and nonsecreting P3-X63-Ag 8.653 mouse myeloma cells were mixed in a 10:1 ratio, and cell fusion was initiated by the addition of 1 ml of prewarmed (37°C) 50% polyethylene glycol (molecular weight, 1,300 to 1,600; Sigma). Fused cells were washed with serum-free RPMI 1640, resuspended in RPMI 1640 containing 15% fetal calf serum and supplemented with hypoxanthine, thymidine, and aminopterin (GIBCO), and added to 96-well plates. The hybridoma clones were screened for the production of antibodies by colony-IFA, by using the serotype 4 reference strain as the antigen. The colony-IFA was performed as described previously (11) by using sterile 24-well tissue culture plates filled with A7 ureaplasma differential agar medium (14).

Ten clones secreting monoclonal antibodies against *U. urealyticum* were selected by colony-IFA by using the serotype 4 reference strain as the antigen. A strong fluorescence on more than 90% of the colonies of *U. urealyticum* was demonstrated by all 10 monoclonal antibodies. The reactivity of these 10 monoclonal antibodies against *U. urealyticum* serotypes 1 to 14 was first analyzed by colony-IFA. No cross-reaction with any of the other 13 *U. urealyticum* serotypes was found by this method, suggesting that the 10 monoclonal antibodies selected were serotype 4 specific.

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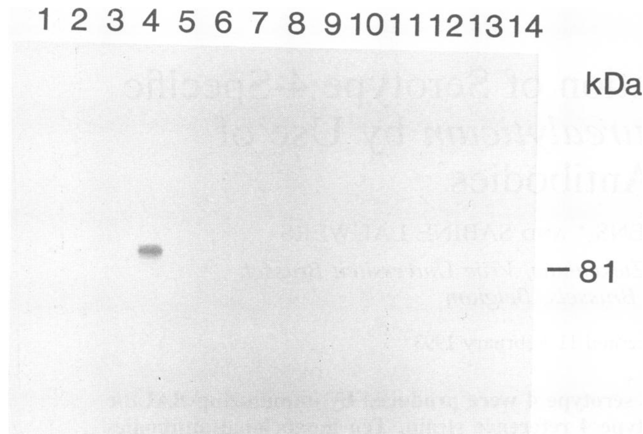

FIG. 1. Immoblot showing the reaction of monoclonal antibody 11A3 (immunoglobulin G2a; representative of group 1 monoclonal antibodies). Lanes 1 to 14 are antigens of serotypes 1 to 14 of *U. urealyticum*, respectively.

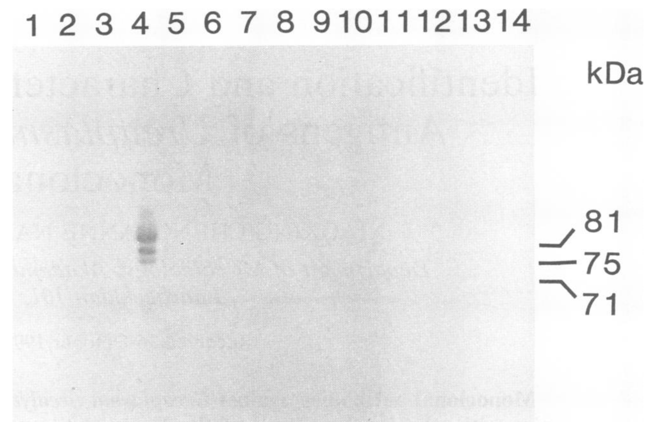

FIG. 2. Immunoblot showing the reaction of monoclonal antibody 17D11 (immunoglobulin M; representative of group 2 monoclonal antibodies). Lanes 1 to 14 are antigens of serotypes 1 to 14 of *U. urealyticum*, respectively.

The 10 monoclonal antibodies were further analyzed by immunoblotting. This was performed as follows. The samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared by heating the antigen at 95°C for 4 min in sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris [pH 6.8]). SDS-PAGE was performed by the method of Laemmli (8) with a 10% separating gel and a 4% stacking gel. Antigen transfer from gel to nitrocellulose sheets was carried out by the method of Towbin et al. (18). The nitrocellulose sheets were blocked by incubation with 3% bovine serum albumin (fraction V; Sigma) in phosphate-buffered saline (PBS-BSA) at room temperature for 2 h. Monoclonal antibodies (supernatant) were diluted 1/10 in PBS-BSA and added to the nitrocellulose sheets. The sheets were incubated overnight at room temperature. After being washed four times in PBS, peroxidase-labeled goat anti-mouse polyvalent antibodies (Sigma) diluted in PBS-BSA were added to the sheets and allowed to incubate for 2 h at room temperature. The sheets were developed with 4-chloro-1-naphthol substrate (horseradish peroxidase color development reagent; Bio-Rad). Biotinylated SDS-PAGE standards (low-molecular-weight range; Bio-Rad) were used for molecular mass determinations of antigens.

Immunoblot analyses of the 10 monoclonal antibodies showed two different patterns of reactivity. The first pattern was found with five monoclonal antibodies reacting with a single band that had a molecular mass of 81 kDa (group 1 monoclonal antibodies; Fig. 1). The second pattern was found by another five monoclonal antibodies reacting with three bands in the immunoblotting assay (group 2 monoclonal antibodies; Fig. 2). In addition to the 81-kDa band, another two bands that had molecular masses of 75 and 71 kDa were recognized. To check if these two groups of monoclonal antibodies recognized different epitopes, a competitive binding test was performed with these two groups of monoclonal antibodies and with type 4-specific polyclonal antibody. Both groups of monoclonal antibodies were unable to inhibit competitively the binding of the other group of monoclonal antibodies, whereas type 4 polyclonal antiserum inhibited the binding of both groups of monoclonal antibodies in the immunoblot. These results suggest that the two groups of monoclonal antibodies recognized different epitopes.

The 10 monoclonal antibodies were shown to be serotype 4 specific in the immunoblotting assay as well as in the colony-IFA. The high yield of serotype-specific monoclonal antibodies seems surprising, especially since we used a crude antigen mixture for immunization. However, this can be explained by our screening method. In this study, the colony-IFA was used for screening monoclonal antibody production throughout the experiment. The clones were first screened with the serotype 4 reference strain and subsequently checked for cross-reactivity. This indicates that screening of the hybridomas with the colony-IFA would allow good detection of serotype-specific monoclonal antibodies.

To evaluate our type 4 monoclonal antibodies, 15 clinical isolates were selected to be tested in a serotyping assay. These clinical isolates were selected because of the serotyping results obtained with them in a previous assay when only polyclonal antibodies were used (10). Some strains were selected because they exhibited a strong reaction to serotype 4, and others were selected because of their strong reaction to another serotype. One strain was selected because it reacted with more than three polyclonal antisera and was designated a multireacting strain.

The 15 clinical strains were tested in the colony-IFA simultaneously with polyclonal antibodies 1 to 14 and with four type 4-specific monoclonal antibodies. From group 1, monoclonal antibodies 11A3 and 10D8 were selected; from group 2, monoclonal antibodies 17D11 and 12D7 were selected.

As shown in Table 1, monoclonal antibodies of group 1 reacted with five strains of the 15 clinical isolates, and the monoclonal antibodies of group 2 also recognized 5 strains. Only one strain (N5) reacted with both groups of monoclonal antibodies. We considered the weak reactivity of group 1 monoclonal antibodies (a fluorescence that was not as strong as the one observed with the reference strain) with two clinical isolates as a positive reaction. The seven strains reacting with polyclonal antiserum 4 were detected by at least one of the four monoclonal antibodies. In addition, one multireacting strain and two of seven strains which were not defined as serotype 4 by polyclonal antibodies did show a

TABLE 1. Analysis of 15 clinical isolates by serotype 4-specific monoclonal antibodies and polyclonal antibodies by colony-IFA

Antibody[a]	Reactions of clinical isolate[b]														
	B43	V110	G13	B64	N5	C30	P23[c]	P26	E16	Ve61	A15	P28	G10	G12	L29
Group 1 MAbs															
11A3	–	–	–	–	w	+	+	+	+	–	–	–	–	–	–
10D8	–	–	–	–	w	+	+	+	+	w	–	–	–	–	–
Group 2 MAbs															
17D11	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–
12D7	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–
PAbs															
Antiserum 4	+	+	+	+	+	+	w	w	w	+	–	w	w	w	w
Other antisera	–	–	–	–	10	8	w	10	10	10	10	3	3/6	6	3

[a] MAbs, monoclonal antibodies; PAbs, polyclonal antibodies.
[b] +, positive reaction; w, weak reaction; –, negative reaction. The numbers indicate the reaction of the isolates with the respective antiserum.
[c] Multireactive strain.

reaction with some of our monoclonal antibodies. Two strains showed a strong reaction with polyclonal antiserum 10 and only a weak reaction with polyclonal antiserum 4. The strain which was considered by polyclonal antibodies to be multireactive also showed strong fluorescence with group 1 monoclonal antibodies. It is interesting to note that not all ureaplasma colonies of the clinical isolates reacted with the monoclonal antibody. Whereas all of the colonies of the *U. urealyticum* clinical isolates showed a strong fluorescence in the colony-IFA when polyclonal antibodies were used, only some of the clinical strains reacted with 100% of the colonies when monoclonal antibodies were used.

Four clinical isolates (B43, V110, G13, and P23) were subcloned by the procedure of Robertson and Stemke (12), and their reactions in immunoblots with type 4-specific monoclonal antibodies were examined. Strains B43, V110, and G13 reacted only with group 2 monoclonal antibodies, while P23 reacted only with group 1 monoclonal antibodies (Fig. 3). The clinical isolates showed immunoblot patterns different from that of the reference strain, and none of the clinical isolates showed a pattern similar to those of the other clinical isolates. This indicates the high variation rate of antigens between clinical *U. urealyticum* strains as described by Watson et al. (20).

Repeated testing of the clinical isolates in the colony-IFA, by using monoclonal antibodies, over a period of several weeks always gave identical results. This is very important since we found previously that repeated testing of some strains by polyclonal antibodies was sometimes associated with the disappearance of a positive reaction when more than one specificity was found or by a shift from a negative to a positive reaction (10, 15). A comparison of polyclonal and monoclonal antibody results shows that serotyping with monoclonal antibodies is reproducible and that monoclonal antibodies might be promising tools for future serotyping studies.

However, serotype 4 clinical isolates do not appear to react with the two groups of monoclonal antibodies produced in our laboratory. While the serotype 4 reference strain reacted with both groups of monoclonal antibodies, clinical isolates most often reacted with only one group of monoclonal antibodies, and the reason for this is not yet clear. Apparently, it is not due to cross-reactivity of the monoclonal antibodies. Cross-reactivity was evaluated with two different methods (immunoblotting and colony-IFA) and could not be established. It is also not due to the method of

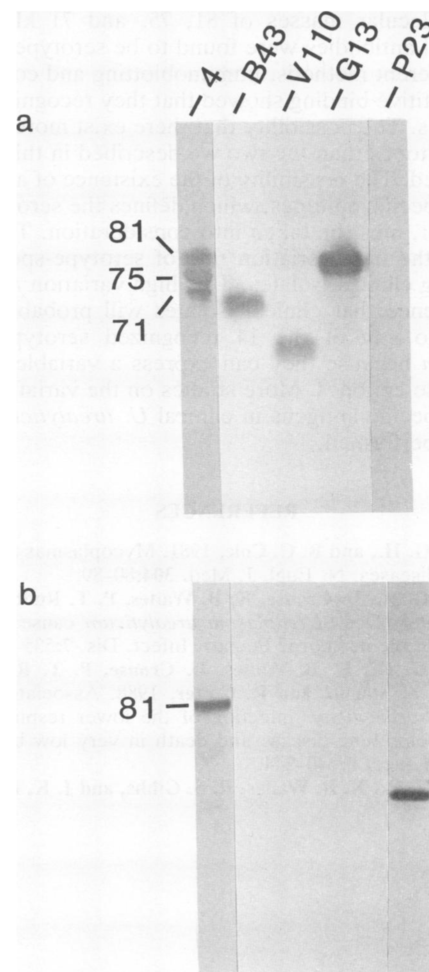

FIG. 3. (a) Immunoblots showing the reactions of selected clinical isolates with monoclonal antibody 17D11 (group 2 monoclonal antibody). (b) Immunoblots showing the reactions of selected clinical isolates with monoclonal antibody 10D8 (group 1 monoclonal antibody). Antigens from left to right are type 4 reference strain and clinical isolates B43, V110, G13, and P23. The approximate molecular masses of the antigens are as follows: B43, 68 kDa; V110, 59 kDa; G13, 78 kDa; P23, 55 kDa.

serotyping. Indeed, when the clinical isolates were serotyped by the immunoblotting technique, results similar to those of the colony-IFA were found: isolates reacting with only group 1 monoclonal antibodies in the colony-IFA also reacted with only group 1 monoclonal antibodies in immunoblotting, and vice versa. A different body site or patient population also was not responsible for this reaction pattern: strains reacting with either of the two groups of monoclonal antibodies were recovered from subjects with normal pregnancies as well as from subjects with pathological pregnancies and were isolated from the lower genital tract as well as from the higher genital tract. The serotype 4-specific antigens, which are found in the reference strain, are probably only partially expressed on the surface of the clinical isolates.

In conclusion, serotype 4-specific monoclonal antibodies were produced and used to characterize the antigens responsible for serotype specificity. Two different types of monoclonal antibodies were found: monoclonal antibodies recognizing a band having a molecular mass of 81 kDa or monoclonal antibodies recognizing three different bands having molecular masses of 81, 75, and 71 kDa. These monoclonal antibodies were found to be serotype 4 specific by two different methods, immunoblotting and colony-IFA, and competitive binding showed that they recognized different epitopes. The possibility that there exist more serotype-specific epitopes than the two we described in this report is not excluded. The possibility of the existence of a mosaic of serotype-specific epitopes, which defines the serotype of *U. urealyticum*, must be taken into consideration. This is suggested by the high variation rate of serotype-specific antigens among clinical isolates. This high variation rate has as a consequence that clinical isolates will probably not fall cleanly into any of the 14 recognized serotypes of *U. urealyticum* because they can express a variable combination of these epitopes. More studies on the variation rate of serotype-specific antigens in clinical *U. urealyticum* strains should be performed.

REFERENCES

1. **Cassell, G. H., and B. C. Cole.** 1981. Mycoplasmas as agents of human diseases. N. Engl. J. Med. **304:**80–89.
2. **Cassell, G. H., D. Crouse, K. B. Waites, P. T. Rudd, and J. K. Davis.** 1988. Does *Ureaplasma urealyticum* cause respiratory disease in the newborn? Pediatr. Infect. Dis. **7:**535–541.
3. **Cassell, G. H., K. B. Waites, D. Crouse, P. T. Rudd, K. C. Canupp, S. Stagno, and R. Cutter.** 1988. Association of *Ureaplasma urealyticum* infection of the lower respiratory tract with chronic lung disease and death in very low birth weight infants. Lancet **ii:**240–244.
4. **Cassell, G. H., K. B. Waites, R. S. Gibbs, and J. K. Davis.** 1986. Role of *Ureaplasma urealyticum* in amnionitis. Pediatr. Infect. Dis. **5:**S247–S252.
5. **Cassell, G. H., H. L. Watson, D. K. Blalock, S. A. Horowitz, and B. L. Duffy.** 1988. Protein antigens of the genital mycoplasmas. Rev. Infect. Dis. **10:**S391–S398.
6. **Foulon, W., A. Naessens, M. Dewaele, S. Lauwers, and J. J. Amy.** 1986. Chronic *Ureaplasma urealyticum* amnionitis associated with abruptio placentae. Obstet. Gynecol. **68:**280–283.
7. **Foulon, W., A. Naessens, S. Lauwers, and J. J. Amy.** 1985. *Ureaplasma urealyticum* infections in infertility and pregnancy, p. 133–142. *In* B. L. C. Keith, G. S. Berger, and D. A. Edelman (ed.), Infections in reproductive health, vol. 2. Uncommon infections and special topics. M.T.P. Press, Lancaster, England.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
9. **Lin, J. L.** 1985. Human mycoplasmal infections: serologic observations. Rev. Infect. Dis. **7:**216–231.
10. **Naessens, A., W. Foulon, J. Breynaert, and S. Lauwers.** 1988. Serotypes of *Ureaplasma urealyticum* isolated from normal pregnant women and patients with pregnancy complications. J. Clin. Microbiol. **26:**319–322.
11. **Naessens, A., and S. Lauwers.** 1987. Modified indirect immunofluorescence test for serotyping large numbers of *Ureaplasma urealyticum* clinical isolates. J. Clin. Microbiol. **25:**191–192.
12. **Robertson, J. A., and G. W. Stemke.** 1982. Expanded serotyping scheme for *Ureaplasma urealyticum* strains isolated from humans. J. Clin. Microbiol. **15:**873–878.
13. **Romano, N., R. La Licata, and G. Scarlata.** 1977. Immunological analysis of plasma membranes of a T-strain of mycoplasma (*Ureaplasma urealyticum*). Infect. Immun. **16:**734–737.
14. **Shepard, M. C., and C. D. Lunceford.** 1976. Differential agar medium (A7) for identification of *Ureaplasma urealyticum* (human T mycoplasmas) in primary cultures of clinical material. J. Clin. Microbiol. **3:**613–625.
15. **Stemke, G. W., and J. A. Robertson.** 1985. Problems associated with serotyping strains of *Ureaplasma urealyticum*. Diagn. Microbiol. Infect. Dis. **3:**311–320.
16. **Taylor-Robinson, D., and M. W. McCormack.** 1980. The genital mycoplasmas. N. Engl. J. Med. **302:**1003–1010, 1063–1067.
17. **Thirkell, D., A. D. Myles, and W. C. Russell.** 1989. Serotype 8- and serocluster-specific surface-expressed antigens of *Ureaplasma urealyticum*. Infect. Immun. **57:**1697–1701.
18. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
19. **Waites, K. B., P. T. Rudd, D. T. Crouse, K. C. Canupp, K. G. Nelson, C. Ramsey, and G. H. Cassell.** 1988. Chronic *Ureaplasma urealyticum* and *Mycoplasma hominis* infections of the central nervous system in preterm infants. Lancet **ii:**17–22.
20. **Watson, H. L., D. K. Blalock, and G. H. Cassell.** 1990. Variable antigens of *Ureaplasma urealyticum* containing both serovar-specific and serovar-cross-reactive epitopes. Infect. Immun. **58:**3679–3688.