Preliminary Characterization of the Urease and a 96 kDa Surface-expressed Polypeptide of *Ureaplasma urealyticum*

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(Received 29 December 1986; revised 24 April 1987)

Analysis by SDS-PAGE of *Ureaplasma urealyticum* (predominantly serotype 8), propagated in a growth medium containing 10% (v/v) foetal calf serum, revealed a complex series of polypeptides apparently free of medium contaminants. Serological analysis using an immobilized antibody reagent, and immunoblotting using a polyclonal serum, showed the presence of two major and several minor antigens. One major antigen, a putative surface component of apparent molecular mass 96 kDa was shown, with a monoclonal antibody, to be serotype-specific. Growth of the organism was partially suppressed in the presence of the antibody. The second major antigen had an apparent molecular mass of 76 kDa and was presumed to be an internal component since it failed to label with the Bolton and Hunter reagent, in contrast to the 96 kDa antigen. Another monoclonal antibody was characterized which detected the canonical urease enzyme of the organism serotype 8 and of the two other human serotypes tested. Purification of this urease antigen by affinity chromatography and electrophoretic analysis of polypeptides after denaturation revealed a single polypeptide of molecular mass 76 kDa, putatively related to the above major antigen. Enzymic activity could be recovered after purification and demonstrated by *in situ* techniques only when electrophoretic analysis was done under non-denaturing conditions suggesting that the functional enzyme is a multimeric complex.

INTRODUCTION

The mycoplasmas are the smallest and simplest of the self-replicating prokaryotes; they are bounded by a plasma membrane but lack a cell wall (for a review see Razin, 1978). Because of their simple structure and their lack of a number of conventional biosynthetic pathways, they require a relatively complex medium for growth *in vitro* (Rodwell & Mitchell, 1979).

Ureaplasmas are unique amongst the mycoplasmas in their ability to hydrolyse urea. In man, they have been associated with urogenital infections (e.g. see Shepard & Masover, 1979; Embree & Embil, 1980; Cassell & Cole, 1981). Very little information is available regarding their molecular properties. We have therefore initiated an investigation with the objective of exploring the molecular biology of this relatively simple free-living organism. In this study we have analysed the major polypeptides and antigens of *Ureaplasma urealyticum*, predominantly of the serotype 8, utilizing both polyclonal and monoclonal antibodies.

METHODS

Ureaplasma strains. Strains of *Ureaplasma urealyticum* serotype 1 (strain T7) and serotype 8 (strain T960) were a gift from Dr D. Taylor-Robinson (CRC, Harrow, UK); serotype 7 was obtained from the American Type Culture Collection (ATCC 27819). The animal ureaplasmas were a gift from Dr G. Jones (Moredun Institute, Edinburgh,

Abbreviations: c.c.u., colour change unit; PBS-N, phosphate-buffered saline containing Nonidet P40; RIA, radioimmune assay.

UK); they were *Ureaplasma diversum* (T44) and ovine and bovine field isolates (strains 7860/1 and E172/1 respectively). *Mycoplasma hominis* (WHO strain) was also a gift from Dr D. Taylor-Robinson.

Media and cell growth. Ureaplasmas were grown in medium containing 75% (v/v) PPLO broth (Difco), 20% (v/v) horse serum or 20% (v/v) calf serum or 10% (v/v) foetal calf serum and 2.5% (w/v) freshly prepared yeast extract, incorporating 0.1% (w/v) urea, 0.005% (w/v) phenol red and 10^3 IU penicillin G ml⁻¹. The media, unless otherwise stated, were buffered at pH 6.5 with 50 mM-HEPES/NaOH. Cultures were incubated until a pH of 7.5 was reached. Ureaplasmas were harvested using a Beckman J-21 rotor (25 000 g, 20 min), the pellets washed once in Dulbecco's phosphate-buffered saline 'A' (PBS) (Dulbecco & Vogt, 1954), resuspended and layered over 25% (w/v) sucrose and centrifuged at 45 000 g for 60 min, and the final pellet resuspended in PBS.

Determination of colour-change units (c.c.u.). Serial decimal dilutions of 0.2 ml of culture in vials containing 1.8 ml of growth medium were incubated at 37 °C until no further colour change of the phenol red indicator was apparent (normally 48 h). From the lowest dilution in which a colour change was seen the no. of c.c.u. per ml of original inoculum was determined.

Labelling of ureaplasmas with [³⁵S]methionine. Cells were labelled with [³⁵S]methionine [Amersham; 800 Ci mmol⁻¹ (29.6 TBq mmol⁻¹)] by growth in medium containing 30 µCi ml⁻¹ [³⁵S]methionine at inoculation. Ureaplasmas were harvested when the pH of the medium had risen to 7.5 by centrifugation in a Beckman SW40-1 rotor (70 000 g, 30 min, 4 °C) (Mouches *et al.*, 1981). Cell pellets were resuspended in PBS and stored at -20 °C until used.

Labelling of ureaplasmas with ¹²⁵I-labelled Bolton and Hunter reagent [N-succinimidyl 3-(4-hydroxy-5-[¹²⁵I]iodophenyl)propionate]. Fresh cultures grown in the medium containing 10% foetal calf serum were centrifuged (25 000 g, 20 min, 4 °C), the pellets resuspended in PBS and centrifuged as before. This procedure was repeated twice followed by resuspension of the pellets in 0.1 M-sodium borate buffer, pH 8.3, before centrifugation as above. The final pellets were resuspended in 0.1 M-borate buffer at approximately 10^9 c.c.u. ml⁻¹. Cells (0.1 ml) were then labelled for 15 min with 100 µCi ¹²⁵I-labelled Bolton and Hunter reagent (Amersham) at 4 °C according to the suppliers instructions. The reaction was stopped by the addition of 0.1 M-glycine in 0.1 M-borate buffer, pH 8.3. The cells were washed three times in PBS to remove any labelled internal components released by autolysis, resuspended in PBS and stored at -20 °C.

Precipitation of specific polypeptides by an immobilized antibody reagent. To prepare the immobilized reagent, antibody was first bound to Protein A-Sepharose (Sigma) at 20 °C, either directly or, in the case of monoclonal antibodies, by pretreatment of the Protein A-Sepharose with a sheep anti-mouse immunoglobulin (Scottish Antibody Production Laboratories). In each instance, excess antibody was removed by washing the Protein A-Sepharose three times in 20 mM-Tris/HCl, pH 7.6, containing 0.5 M-NaCl, 5 mM-EDTA and 1% (v/v) Nonidet P40 (Sigma). To prepare solubilized extracts, ³⁵S-labelled or ¹²⁵I-labelled cells were mixed with an equal volume of 20 mM-Tris/HCl, pH 7.6, containing 150 mM-NaCl, 5 mM-EDTA and 0.2% (v/v) Nonidet, P40, after which the suspensions were disrupted using a Kerry probe sonicator (3 × 10 s bursts). To these suspensions an equal volume of 20 mM-Tris/HCl, pH 7.6, containing 1.25 M-NaCl, 5 mM-EDTA and 0.2% (v/v) Nonidet P40 was added. After centrifugation (12 000 g, 15 min, 20 °C) (MSE Microfuge), the resulting supernatants were used as antigen. Immune absorption was then done on the radiolabelled extracts (Ross *et al.*, 1980). Antibody-bearing Protein A-Sepharose (0.1 ml) was mixed with 50 µl of the cell extract and incubated for 2 h at 20 °C before washing by centrifugation and resuspending in 20 mM-Tris/HCl, pH 7.6, containing 0.5 M-NaCl, 5 mM-EDTA and 1% (v/v) Nonidet P40. Final pellets were then denatured and submitted to analysis by electrophoresis as described below.

SDS-PAGE and fluorography. These were done as described by Russell & Blair (1977). Slab gels (15%, w/v, polyacrylamide) were used except where noted. Samples for electrophoresis in denaturing gels were always boiled for 2 min after the addition of an equal volume of a solution containing 5 M-urea, 2% (w/v) SDS, 3.5 M-β-mercaptoethanol and 0.1% (w/v) bromophenol blue. For detection of urease activity, samples were solubilized by adding SDS to 1% (w/v) without boiling. Silver staining was done using a Bio-Rad staining kit.

Urease activity in cell extracts. This was determined using a modification of the Berthelot reaction. Doubling dilutions of cell extracts were prepared in PBS and 50 µl of these was added to the wells of an ELISA plate. To these dilutions, 50 µl 30 mM-urea was added and the plate incubated at 20 °C for 30 min. Then to each well, 50 µl phenol nitroprusside and 50 µl alkaline hypochlorite were added (Sigma urea nitrogen colorimetric kit for the Berthelot reaction) and the plates left for 30 min for colour development, after which they were read at 600 nm on an ELISA plate reader. Values of urease activity were expressed as micromolar NH₄⁺ derived from a standard curve for NH₄Cl solutions processed under similar conditions. Urease enzyme activity was detected in samples after SDS-PAGE by the method of Fishbein (1969). After electrophoresis gels were washed three times in PBS containing 0.2% (v/v) Nonidet P40 (PBS-N) (3 × 30 min) followed by four washes in 0.5 M-sodium citrate buffer, pH 6.0, before staining.

Urease enzyme 'catch test'. Partially purified (see below) monoclonal antibody UU8/1 (shown to cross react with *U. urealyticum* ureases) was diluted to 10 µg protein ml⁻¹ in PBS. Of this, 100 µl was added to each well of a 96-well ELISA plate which was then incubated at room temperature for 2 h after which the excess antibody was removed; 200 µl 3% BSA in PBS was then added to each well, the plate was left at room temperature for 30 min, and then

washed five times in PBS-N. Appropriate dilutions of ureaplasma cell extracts in PBS-N or of culture fluids diluted 1:2 (v/v) in PBS-N (100 µl) were added to each well of the antibody-coated plate and incubated at room temperature for 2 h or at 37 °C for 1 h. Excess antigen was then removed and the plate washed five times with PBS-N followed by five washes with PBS. To each well 100 µl 15 mM-urea (freshly deionized) in PBS was added and the plate was then incubated at 20 °C for 30 min after which the Berthelot reagents were added (see 'Urease activity'). Plates were read on an ELISA plate reader at 600 nm. Control 'blanks' were incorporated using wells to which no antibody had been added. It should be noted that Nonidet P40 is inhibitory to the Berthelot reaction and wells must be thoroughly washed with PBS before the addition of urea.

Immunoblotting experiments. Electrophoretic transfer of proteins from slab polyacrylamide gels to nitrocellulose sheets was done as described by Russell & Precious (1982). For immunoblotting, the nitrocellulose blots were 'blocked' for 30 min with 3% (w/v) BSA in PBS, washed three times in PBS-N and incubated at 37 °C for 2 h with the appropriate antibody (normally at 1/500 dilution). After washing three times with PBS-N, approximately 10^6 c.p.m. ^{125}I -labelled Protein A was added; the blots were incubated for 2 h at 20 °C and then washed extensively with PBS-N. Monoclonal antibodies that had been shown not to bind Protein A were either iodinated directly using chloramine T (Hunter, 1978) and used as a probe or, alternatively, a sheep anti-mouse Ig second antibody was added to the blots at a dilution of 1/500 for 2 h at 37 °C before probing with ^{125}I -labelled Protein A. All nitrocellulose blots were then subjected to autoradiography using X-ray film (Fuji RX). Where appropriate a Phillips fast tungstate intensifying screen was used. Protein A was also labelled with ^{125}I using chloramine T. Protein staining of nitrocellulose strips was done, without prior blocking with BSA, with 0.2% Naphthalene Black in acetic acid/methanol/water (1.5:5, by vol.) for 5 min before destaining.

Antibody preparation. Polyclonal antisera to *U. urealyticum* serotype 8 and to *M. hominis* (WHO strain) were produced in rabbits by intramuscular injections of approximately 5×10^9 c.c.u. ml^{-1} (grown in medium containing horse serum) with Freund's complete adjuvant, followed by a series of further intramuscular injections in complete adjuvant at 3 week intervals for 3 months. Animals were test-bled periodically and antisera monitored by a radioimmune assay (RIA) (Randall *et al.*, 1984). Further booster injections were given intramuscularly at 3 month intervals.

For the production of monoclonal antibodies, Balb/C mice were immunized with ureaplasma serotype 8 organisms grown in medium containing horse serum. Washed cells (approximately 10^9 c.c.u. ml^{-1}) were sonicated extensively and then alum precipitated (Mautner & Wilcox, 1974). Initial immunization (intraperitoneal) was with 0.2 ml 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 d before fusion. Mouse antisera taken 2 d before fusion were screened by RIA for anti-ureaplasma antibody using organisms grown in medium containing calf serum. Details of the production and screening of monoclonal antibodies were described by Russell *et al.* (1981). Screening for antibodies to ureaplasma-derived proteins was by RIA using ^{125}I -labelled Protein A and by ELISA using a goat peroxidase-linked anti-mouse immunoglobulin.

Affinity column chromatography. Monoclonal antibody UU8/1 was partially purified by precipitation with 50% saturated ammonium sulphate and, after dialysis against PBS, by passing through a column of Sephadex G-200 (Fahey & Terry, 1978). Fractions from the column were analysed by SDS-PAGE and peak immunoglobulin-containing fractions pooled. Approximately 60% of the total protein in these fractions was ascribed to immunoglobulins. Of this immunoglobulin fraction, 5 mg was then coupled to 1 g cyanogen-bromide-activated Sepharose 4B (Sigma) by standard techniques (Axen *et al.*, 1967).

Metabolic inhibition test. The method used was that described by Taylor-Robinson (1983); the mycoplasma culture contained 10^2 c.c.u. ml^{-1} .

Molecular mass markers. In SDS-PAGE, molecular masses were determined, where appropriate, by comparison with those ascribed to adenovirus type 2 polypeptides (Philipson, 1983), using both purified virus and [^{35}S]methionine-labelled adenovirus-infected cell extracts (Russell *et al.*, 1981).

RESULTS

Polypeptide analysis of ureaplasmas by SDS-PAGE

Previous investigators (Sayed & Kenny, 1980; Mouches *et al.*, 1983) have noted contamination of ureaplasmas by medium components and this has seriously impeded their molecular characterization. In the present study very significant purification was evident, however, when 10% foetal calf serum was used in the growth medium instead of horse serum. We were able to obtain reproducible electrophoretic patterns even with relatively simple differential centrifugation procedures (see Methods).

Fig. 1 shows the electrophoretic patterns obtained with preparations *U. urealyticum* of serotypes 8, 7 and 1 grown in medium containing 10% foetal calf serum and for comparison, the

Fig. 1. Analysis of the polypeptides of whole cells of *U. urealyticum* by SDS-PAGE. Lane A, serotype 8 (strain T960) grown in medium containing 20% (v/v) horse serum and stained with Coomassie blue; lanes B, C and D, serotypes 8, 7 (strain ATCC 27819) and 1 (strain T7) respectively, grown in medium containing 10% (v/v) foetal calf serum visualized with silver stain; lane E, whole medium containing 10% foetal calf serum and visualized with silver stain. The molecular mass values indicated (right) are those of the polypeptides of purified adenovirus type 2 run on the same gel as B, C, D and E. Three ureaplasma polypeptides are identified by their molecular mass values (left).

medium itself, using the sensitive silver-staining technique. Fig. 1 also demonstrates the major contamination with medium components that occurred when the organisms were grown in medium containing 20% horse serum (lane A). In agreement with previous investigators (Sayed & Kenny, 1980), a major polypeptide of apparent molecular mass 96 kDa was noted in serotype 8 (lane B). The general patterns of polypeptide bands given by the different serotypes were also similar with polypeptides ranging in apparent molecular mass from about 200 kDa to 10 kDa. Serotypes 7 (lane C) and 1 (lane D) showed a considerable number of polypeptides that co-migrated with respect to those of serotype 8. Serotype 1 showed a greater variation in the electrophoretic pattern of its polypeptides, particularly with respect to the major polypeptides corresponding to the 76 kDa and 47 kDa polypeptides of serotype 8, which had a distinctly faster mobility. Since these serotypes were grown in the same medium, it is consistent with the apparent variations in mobilities of the polypeptides to conclude that no significant contamination with medium components occurred.

Fig. 2. Analysis of radiolabelled polypeptides of *U. urealyticum* using specific antibodies. (a) Fluorogram of $[^{35}\text{S}]$ methionine-labelled extracts of *U. urealyticum* serotype 8, grown in medium containing 20% (v/v) horse serum, after SDS-PAGE. Lane A, whole cell extract; lane B, whole cell extract after precipitation of polypeptides by immobilized polyclonal rabbit antibody raised against serotype 8; lane C, $[^{35}\text{S}]$ methionine-labelled polypeptides of extracts of adenovirus-infected HeLa cells as molecular mass markers. (b) Autoradiogram of ^{125}I -labelled cell extracts of *Ureaplasma urealyticum* grown in medium containing 10% (v/v) foetal calf serum and of Protein A-Sepharose-bound ^{125}I -labelled antigen-antibody complexes after SDS-PAGE analysis. Lanes A, D and F, whole cell extracts of serotypes 8, 7 and 1 respectively; lane H, ^{125}I -labelled growth medium containing 10% foetal calf serum; lanes B, E and G, whole cell extracts of serotypes 8, 7 and 1 respectively, after precipitation of specific ^{125}I -labelled polypeptides by an immobilized polyclonal rabbit antibody raised against serotype 8; lane I, as lanes B, E and G but with whole ^{125}I -labelled medium as antigen; lane C, as lane B but using monoclonal antibody UU8/5. The molecular masses of the ureaplasma antigens in this and the subsequent figures are shown (left).

$[^{35}\text{S}]$ Methionine- and ^{125}I -labelling of ureaplasmas

Radiolabelling of *U. urealyticum* serotype 8 polypeptides was achieved by growing the organisms in medium containing $[^{35}\text{S}]$ methionine. However, only relatively poor incorporation of this label was obtained. After SDS-PAGE and fluorography, exposure for long periods (6 weeks) was necessary to obtain reasonable patterns. Fig. 2(a) shows such a fluorogram with about 25 bands (lane A), some of which corresponded to major polypeptides seen in the stained polypeptide profiles (Fig. 1, lane B), those of molecular mass, viz. 76 kDa, 54 kDa, 47 kDa and 44 kDa. It is interesting that a band of apparent molecular mass 85 kDa was labelled although this was a relatively minor band in the silver-stained polypeptide pattern. It is also noteworthy that the prominent 96 kDa polypeptide seen in the stained patterns was not labelled in this

experiment. These variations between the stained and labelled polypeptide profiles probably reflect variations in the methionine content of the polypeptides.

In vitro labelling of freshly prepared and washed ureaplasmas was achieved by using the Bolton and Hunter reagent. Fig. 2(b) lane A shows the ^{125}I -labelled polypeptide pattern obtained from serotype 8 after SDS-PAGE and autoradiography. A number of polypeptides were well-labelled. Since the majority of these were lost after treatment of the labelled ureaplasmas with $50\text{ }\mu\text{g trypsin ml}^{-1}$ (Sigma) at 37°C immediately after labelling (data not shown), most of these were likely to be membrane components. Thus, the 96 kDa polypeptide was clearly labelled by this technique and was removed by trypsin treatment implying an external location in agreement with the suggestion by Sayed & Kenny (1980) that the 96 kDa polypeptide was membrane-associated.

Antigenic analysis of ureaplasmas

Initial experiments using a polyclonal rabbit hyperimmune serum prepared against serotype 8 and precipitation of specific polypeptides by antibody bound to Protein A–Sepharose from ^{35}S -labelled cell extracts showed a major antigen of molecular mass 76 kDa, another of 47 kDa and faint bands at 85, 65 and 54 kDa (Fig. 2a, lane B).

Monoclonal antibodies were prepared using *U. urealyticum* serotype 8 as antigen (see Methods). After screening by ELISA and RIA, nine monoclonal antibodies strongly positive by ELISA were selected for further study. Ascitic fluids were prepared and the monoclonal antibodies designated UU8/1–9. These antibodies were used to identify specific antigens associated with the organism. In preliminary studies it soon became evident that four of these monoclonal antibodies recognized the same antigen and one of these antibodies, designated UU8/5, was accordingly studied in more detail. Another four did not appear to react in any of the tests described below and were not further examined. The remaining monoclonal antibody, UU8/1, recognized the urease antigen and proved to be an extremely useful tool (see below).

Immunoaffinity gel bearing antibodies from a polyclonal serum was used to absorb ureaplasma-specific antigens from extracts of the more conveniently ^{125}I -labelled organisms, and the absorbed antigens were analysed using SDS-PAGE and autoradiography. In Fig. 2(b), the major antigen observed appeared to possess a molecular mass of 96 kDa (lane B) and was also recognized by monoclonal antibody UU8/5 (lane C).

Analysis of cross-reactions with serotypes 7 and 1 (lanes E and G) using the immunoaffinity gel bearing antibodies raised against serotype 8 indicated that a number of polypeptides shared antigenic epitopes with those of serotype 8. However, no cross-reactions were observed with either serotype 7 or serotype 1 in similar experiments using immobilized UU8/5 monoclonal antibody. No polypeptides were detected on the autoradiograms with either immobilized polyclonal or UU8/5 monoclonal antibodies when using ^{125}I -labelled growth medium as antigen (lane I) even after prolonged exposure of the X-ray film to the dried gel.

Immunoblotting procedures (see Methods) were done in conjunction with SDS-PAGE of whole cells of serotypes 8, 7 and 1 using the rabbit hyperimmune serum raised against serotype 8 as the specific probe. Fig. 3(a) shows the immunoblots thus obtained. In the case of the homologous reaction (lane A), the major antigen of molecular mass 96 kDa, formerly described, can be discerned. A number of other antigens were also labelled, but the 47 kDa polypeptide noted in the [^{35}S]methionine-labelling studies (Fig. 2a, lane B), was not detected. Immunoblotting of extracts from serotype 7 (lane B) and serotype 1 (lane C) confirmed that cross-reacting antigens occurred in both of these serotypes, although there was less reactivity with antigens of serotype 1. Lanes E–H show that monoclonal antibody UU8/5 recognized the 96 kDa antigen by this test and did not react with antigens of other serotypes. It was notable that monoclonal antibody UU8/1 did not react in any of these procedures.

Identification of the urease

In preliminary experiments to examine different screening procedures for ureaplasmas, it was demonstrated that urease activity could be absorbed from extracts of ureaplasmas using the immobilized antibody reagent bearing polyclonal antibodies. These extracts were produced by

Fig. 3. Analysis of antigens of *U. urealyticum* using immunoblotting techniques with specific antibodies. (a) Autoradiogram of an immunoblot of whole cells of *U. urealyticum* grown in medium containing 10% (v/v) foetal calf serum. Lanes A, B and C, serotypes 8, 7 and 1 respectively, after probing with a hyperimmune polyclonal rabbit serum raised against serotype 8 followed by ¹²⁵I-labelled Protein A; lane D, whole medium containing 10% foetal calf serum treated as above; lanes E-H, as lanes A-D but monoclonal antibody UU8/5 was used as the probe followed by sheep anti-mouse Ig and ¹²⁵I-labelled Protein A. (b) SDS-PAGE (10%, w/v, polyacrylamide) of *U. urealyticum* serotype 8 grown in medium containing 10% foetal calf serum and solubilized in 1% SDS without boiling before application to the gel. Lane A, urease activity stained for by the method of Fishbein (1969); lane B, autoradiogram after immunoblotting and probing with ¹²⁵I-labelled monoclonal antibody UU8/1; lane C, as lane B but using a homologous hyperimmune rabbit serum as a probe followed by ¹²⁵I-labelled Protein A; lane D, as lane C but using a hyperimmune rabbit serum to *M. hominis* (WHO strain) as the probe.

first preparing a suspension of ureaplasma cells in PBS-N which was then sonicated (3×10 s bursts) and finally centrifuged at 12000 *g* for 15 min at 20 °C, the supernatant being used as antigen. After immunoabsorption and washing of the Protein A-Sepharose bearing the immune complexes with PBS-N followed by PBS, the enzyme could be detected in the Protein A-Sepharose pellet. This was achieved by addition of 30 mM-urea, incubation for 30 min at 20 °C and subsequent reaction with the Berthelot reagents. All the nine monoclonal antibodies were then screened. Only monoclonal antibody UU8/1 precipitated enzyme activity by this procedure. Monoclonal antibody UU8/1 also precipitated enzyme activity from extracts of

serotypes 7 and 1. However, as noted above, this monoclonal antibody did not recognize any polypeptides by the immunoabsorption procedure with ^{125}I -labelled antigens or by the immunoblotting procedures described above, suggesting that the epitope recognized by monoclonal antibody UU8/1 might be relatively labile and easily denatured.

This supposition was confirmed by the results of an experiment in which extracts of ureaplasmas were solubilized in 1% SDS (without boiling) followed by SDS-PAGE and blotting on to nitrocellulose; the blots were then probed with ^{125}I -labelled monoclonal antibody UU8/1 (see Fig. 3*b*). It is apparent that this monoclonal antibody recognized the same polypeptides as those demonstrated by staining for urease activity using the method of Fishbein (1969) (cf. lanes A and B). Here, as noted previously (Delisle, 1977), more than one band of enzyme activity was seen.

Partial purification of U. urealyticum urease

The urease antigen was partially purified to aid further characterization. As a first step, the UU8/1 immunoglobulin (non-Protein A reacting) was partially purified by precipitation with 50% saturation ammonium sulphate followed by gel filtration on a column of Sephadex G-200 and then coupled to cyanogen-bromide-activated Sepharose 4B (see Methods).

Extracts of cells in PBS from a 101 culture of *U. urealyticum* serotype 8 were prepared by sonication followed by centrifugation. The urease-containing supernatant was applied to the affinity column and the specifically bound antigen eluted with 0.1 M-borate buffer, pH 10.0. Potent urease activity was detected in the eluate, the recovery being about 60% of the original activity in the crude extract.

Analysis by SDS-PAGE under fully denaturing conditions yielded a single polypeptide of apparent molecular mass 76 kDa (data not shown). The purified enzyme so obtained was relatively stable provided it was not frozen. Accordingly the enzyme preparations were stored in 50% (v/v) glycerol in PBS at 4 °C or -20 °C.

An estimate of the activity of the purified enzyme (using the Berthelot reaction – see Methods) was made utilizing ammonium chloride standards to calibrate the intensity of colour produced. By this method the activity of the *U. urealyticum* urease was calculated to be approximately 65 000 units mg^{-1} (one unit of enzyme activity released 1 $\mu\text{mol NH}_3 \text{ min}^{-1}$ at 25 °C). By comparison, a preparation of crystalline Jack Bean urease (Sigma) titrated in parallel had an activity of 830 units mg^{-1} . It was notable that enzyme activity was irreversibly lost on lowering the pH to 3.0, and thus acid elution of the immunoaffinity column (see above) was not successful in retaining enzyme activity.

Urease enzyme 'catch tests'

Since the monoclonal antibody UU8/1 appeared to react with an epitope that was common to a number of ureaplasma serotypes (see above), and was presumably distinct from the active enzyme site, it was evident that monoclonal antibody UU8/1 might provide the means for a rapid, sensitive and specific assay for the detection of ureaplasmas. To study the sensitivity of this technique relative to standard culture techniques, experiments were done in parallel comparing the growth of ureaplasmas as monitored by development of c.c.u. and by enzyme assays. Samples of cultures were analysed for urease activity by disrupting the sedimented organisms and catching the released enzyme on plastic wells coated with the UU8/1 antibody ('enzyme catch test' – see Methods). By analysing dilutions of these extracts, it was possible to quantify the enzyme yields and to relate these to the growth of the organism as detected by pH changes.

Fig. 4 shows the result of such an experiment using serotypes 8, 7 and 1 and *U. diversum* (strain T44). The human serotypes were readily detected in five successive experiments using the enzyme assay, with a sensitivity equivalent to 10^3 – 10^4 c.c.u. ml^{-1} . On the other hand, no enzyme activity could be recovered by this method from extracts of *U. diversum* (strain T44) even using cell samples equivalent to 10^8 c.c.u. ml^{-1} . Similar experiments (data not shown) indicated that monoclonal antibody UU8/1 did not react with ureases derived from ovine or bovine field isolates or ureaplasmas, nor indeed with enzymes from Jack Bean or from seven species of

Fig. 4. Comparison of the growth of ureaplasma strains expressed as c.c.u. ml⁻¹ with detection of the urease by the 'enzyme catch' test. Each strain was diluted to give a starting titre of approximately 10² c.c.u. ml⁻¹. At each time point each strain was titrated in log₁₀ steps to determine the no. of c.c.u. ml⁻¹, samples (0.1 ml) of the cultures were assayed for urease enzyme activity. The histograms represent the titre of ureaplasma (c.c.u. ml⁻¹); the points (●) represent urease activity (expressed as the micromolar concentration of NH₄⁺ released). (a) *U. urealyticum* strain T960 (serotype 8); (b) *U. urealyticum* ATCC 27819 (serotype 7); (c) *U. urealyticum* strain T7 (serotype 1); (d) *U. diversum* strain T44 (no. of c.c.u. were measured at only four time points).

Klebsiella and four species of *Proteus*, all previously shown to be urease-positive. Similar results were obtained using a radioimmune assay (data not shown).

Metabolic inhibition using monoclonal and polyclonal antibodies

The ability of the polyclonal serum and monoclonal antibodies UU8/1 and UU8/5 to inhibit the growth of *U. urealyticum* serotype 8 was measured using various dilutions of the antibodies (see Methods). Inhibition by both the polyclonal serum and monoclonal antibody UU8/5 was clearly seen even at high dilutions (> 1/500). A more detailed investigation of this inhibition was undertaken at a single dilution of antibodies (Fig. 5). In this study, inhibition was measured both by analysing pH changes and by assaying urease activities by the 'enzyme catch' technique described above. Both assays showed that the polyclonal serum appeared to completely inhibit the growth of the ureaplasmas, whereas monoclonal antibody UU8/5 gave suppression of growth which could be overcome after a substantial lag. Such experiments were repeated three times with similar results but some slight variations in the time scale of growth. No such inhibition could be detected using rabbit polyclonal or monoclonal antibodies against a variety of prokaryotic and virus antigens at similar immunoglobulin concentrations. Similar experiments (data not shown) were done using serotypes 7 and 1, and although the polyclonal serum against serotype 8 showed some inhibitory effect on serotype 7 but not on serotype 1, the UU8/5 monoclonal antibody had no evident inhibitory effect on either.

DISCUSSION

This study sought to lay the foundation for further molecular investigations of *U. urealyticum* and has analysed some of the major features of the organism. One of the problems in analysing ureaplasmas related to the difficulty of removing tenaciously bound serum components. By

Fig. 5. Inhibition of metabolic activity by antibodies against *U. urealyticum* serotype 8 strain T960. The organism was diluted to give a starting titre of approximately 10^2 c.c.u. ml⁻¹ and incubated in the presence or absence of antibody. At each time point samples were taken for pH measurement and 0.1 ml samples assayed for urease enzyme activity. ●, pH in the absence of antibody; ○, urease activity in the absence of antibody; ■, pH in the presence of 1/100 monoclonal antibody UU8/5; □, urease activity in the presence of 1/100 monoclonal antibody UU8/5; ▲, pH in the presence of 1/100 homologous polyclonal rabbit antiserum; △, urease activity in the presence of 1/100 homologous polyclonal rabbit antiserum.

using foetal calf serum in the growth medium, we have been able, in the case of serotype 8, to obtain with relative ease purified preparations and to demonstrate at least 50 different polypeptides by one dimensional SDS-PAGE. Clearly a two-dimensional analysis (cf. Mouches *et al.*, 1981; Swenson *et al.*, 1983) would provide a greater separation of these polypeptides and reveal a much greater number.

U. urealyticum has been classified as a single species and 14 serotypes have been established on the basis of serological techniques (Robertson & Stemke, 1982). In this study, we have concentrated on serotype 8 but where appropriate, we used serotypes 7 and 1 for comparative purposes. These are appropriate serotypes since serotype 7 is closely related to serotype 8 whereas serotype 1 is more distantly related as determined by both immunological and DNA hybridization techniques (Christiansen *et al.*, 1981). Because of the inability to label antigens efficiently by *in vivo* labelling techniques, we used surface labelling by iodination and immunoblotting techniques to demonstrate the major antigens. Other workers have also shown antigenic determinants by immunoblotting and it has been suggested that all avian strains of *Ureaplasma* are closely related (Harasawa *et al.*, 1985).

One of the major antigens, detected by immunoprecipitation of radioiodinated antigens of ureaplasmas of serotype 8 with an immobilized polyclonal serum, had an apparent molecular mass of 96 kDa, appeared to be serotype-specific and was also presumably a surface antigen. It is of significance that this appeared to be the antigen most commonly recognized by the set of monoclonal antibodies which we tested. Furthermore, monoclonal antibody UU8/5, which also reacted with the 96 kDa polypeptide in both the immunoabsorption and the immunoblotting assays in a type-specific fashion, also specifically suppressed but did not completely inhibit the growth of serotype 8 as measured by the urease enzyme 'catch test' and also by the rate of increase of pH in the growth medium.

These observations suggest that this antigen may play a key role in the growth control of serotype 8 and as such may be categorized as a metabolic inhibitory antigen associated with the surface of the organism. The mechanism of this control and the process of inhibition by the appropriate antibody presumably is related to some signal from the cell membrane, perhaps via a specific receptor as noted in many other biological systems. The 96 kDa antigen was not readily detected using ³⁵S-labelled organisms (Fig. 2a), which may be a reflection of the paucity of methionine residues in the protein. It seems unlikely that the 96 kDa antigen originated from

the medium since it was not observed in other serotypes grown under identical conditions. However, it can be presumed that other type-specific antigens with similar properties occur in the different serotypes possibly with different molecular masses. Sayed & Kenny (1980) also suggested that a major polypeptide of molecular mass 96 kDa in serotype 8 was associated with membrane fractions. It is also interesting to note that, using the limited immunological tests described here, the rabbit polyclonal serum against serotype 8 appeared to distinguish the serotypes via the different patterns of reactivity, only a small number of polypeptides appearing to be antigenically and electrophoretically similar in the three serotypes examined (see Fig. 2*b*).

The other key component of the organism, urease, was also studied because we were able to select a monoclonal antibody, UU8/1, which recognized the ureases of all the three human serotypes examined by the enzyme 'catch test' or by radioimmune assay. The same monoclonal antibody did not recognize ureases derived from bovine or ovine ureaplasmas, other bacterial sources or from Jack Bean. Thus, it would appear likely that the *U. urealyticum* urease displays an epitope common to the human serotypes. Moreover, the UU8/1 monoclonal antibody seemed to react to a similar extent with extracts of serotypes 8, 7 and 1, the minimum level of antigen detection being equivalent to that in 10^3 – 10^4 c.c.u. ml⁻¹. It will be evident that this technique offers considerable advantages over standard culture methods, being rapid, specific and able to analyse many samples simultaneously. Furthermore, it offers the possibility of detection of ureaplasmas via this enzyme without recourse to growth in a complex growth medium.

The results presented for serotype cross-reactions with monoclonal antibody UU8/1 contrast with the findings of Eng *et al.* (1987), where a monospecific polyclonal antiserum surprisingly appeared to show inhibition of the heterologous enzyme, but did not significantly inhibit homologous activity. It is noteworthy that monoclonal antibody UU8/1 did not recognize antigen after complete denaturation, but nevertheless bound after solubilization of the urease with 1% SDS and removal of detergent after blotting. In this respect, monoclonal antibody UU8/1 behaved similarly to the polyclonal antiserum used by Eng *et al.* (1987) (G. E. Stemke, personal communication). It therefore seems likely that the urease requires secondary structure both for enzymic activity and for recognition of the UU8/1-specific epitope. Although interpretation of molecular masses from incompletely denatured gel electrophoretic patterns is problematic, it would appear from Fig. 3(*b*) that the minimum molecular mass for both functional enzymic activity and recognition by the monoclonal antibody UU8/1 would be of the order of 130 and 180 kDa. This suggests that the minimum functional unit of the urease is probably a dimer or trimer of the 76 kDa polypeptide.

This work is supported by a grant from the Scottish Home and Health Department. We are indebted to Mrs P. Braddock for the production of monoclonal antibodies and to Mr I. Armit for technical assistance.

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