Isolation and purification of viable *Ureaplasma urealyticum* cells free from medium components

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A procedure was devised to produce *Ureaplasma urealyticum* preparations free of adsorbed components of the growth medium, which contains high concentrations of serum. The ureaplasmas were cultivated in a medium containing PPLO-serum fraction as a replacement for horse serum. High titres of ureaplasmas ($> 10^7$ c.f.u. ml⁻¹) were obtained. Harvested cells were then purified by Urografin density gradient centrifugation. By use of ³H-labelled ureaplasma cells and ¹²⁵I-labelled medium components, a distinct band of viable cells devoid of serum constituents was demonstrated. The absence of medium components was verified by immunoblotting cells from this band with antiserum to medium components. Medium components that had been present before the purification procedure were undetectable in the purified cell fraction obtained. The viability of the purified ureaplasma cells represented an 85% recovery rate and their antigenicity, examined with anti-serotype specific antiserum, remained intact. This easy and reproducible procedure can be used to prepare purified ureaplasmas for investigation of ureaplasma antigens and their expression and/or role in disease.

Introduction

The human mycoplasma *Ureaplasma urealyticum* inhabits the urogenital system of 40–80% of the sexually active population and is associated with numerous diseases in men, women and infants (non-gonococcal urethritis, pelvic inflammatory disease, chorioamnionitis, recurrent and spontaneous abortions, neonatal morbidity and mortality, and infertility) (Cassell *et al.*, 1986, 1987; Taylor-Robinson, 1986; Hillier *et al.*, 1988).

It has been suggested that only some of the 14 known serotypes of *U. urealyticum* may be pathogenic and that the others are not involved in disease (Robertson *et al.*, 1986; Cassell *et al.*, 1987; Naessens *et al.*, 1988). To establish the pathogenic potential of individual serotypes requires identification of serotype-specific antigens. Such identification has been hindered by the high level of cross-reactivity noted among the serotypes.

In addition to true antigenic relatedness between serotypes (Robertson & Stemke, 1982; Stemke & Robertson, 1985; Horowitz *et al.*, 1986; Razin *et al.*, 1986; Thirkell *et al.*, 1989), another reason for cross-reactivity is the adsorption to ureaplasma cells of medium components and especially constituents of serum present in high concentrations in ureaplasma media. Such components adsorb to the surface of

cultured strains which may subsequently be used both for preparation of reference antisera and as antigens in the detection assays. Therefore, the availability of purified ureaplasma cells, free of medium contaminants, should enhance the definition of ureaplasma antigens, including those common to all serotypes and those that are serotype-specific, thus facilitating investigation of the role of these antigens in pathogenesis.

The aim of this study was to develop a procedure for the purification of ureaplasma cells suitable for antigenic research and for the development of specific detection assays.

Methods

Strains. *U. urealyticum* reference serotypes were kindly donated by G. H. Cassell, Birmingham, Alabama, USA. Serotypes 1 to 8 were originally obtained from E. A. Freundt (Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark). Serotypes 9 to 14 were originally obtained from J. A. Robertson (Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Alberta, Canada).

Growth and harvesting. *U. urealyticum* cells (serotype 4) were cultured in Shepard's 10B broth, but containing 5% (v/v) agamma horse serum (Gibco) or 1% (v/v) PPLO-serum fraction (Difco). Both types of 10B media were supplemented according to Shepard (1983) with CVA enrichment (5 ml l⁻¹) (Gibco) and glycyl-histidyl-lysine tripeptide

(20 $\mu\text{g l}^{-1}$) (Sigma). Cultures were incubated at 37 °C in an atmosphere of 5% (v/v) CO_2 to limit excessive pH increase. When the cultures reached pH 7.2–7.3 cells were harvested by centrifugation (Sorvall RSCB centrifuge, rotor SS-34) at 27000 g for 40 min at 4 °C. After two washes with phosphate-buffered saline (PBS; 0.13 M-NaCl, 0.014 M-sodium phosphate, pH 7.3), the final pellet was resuspended in a small volume of PBS (1 ml per litre of original culture). Non-inoculated broth controls were incubated identically and the pH adjusted to 7.2 before centrifugation.

Quantitative determination of organisms. The number of viable organisms in culture and during the isolation procedure was assessed by determining the number of colony-forming units (c.f.u.) ml^{-1} on A8 agar plates (Shepard, 1983). Growth curves of the ureaplasmas in broth were determined by monitoring both the increase in culture pH and the change in the number of c.f.u. during cultivation.

Radiolabelling: ^3H -Labelled ureaplasmas were obtained by growing them in 10B broth containing [^3H]thymidine [0.1 $\mu\text{Ci ml}^{-1}$ (3.7 kBq ml^{-1}); Nuclear Research Center, Negev, Israel]. ^{125}I -Labelled components of either horse serum or PPLO-serum fraction were prepared by the Iodogen method (Markwell & Fox, 1978) (Amersham) and added to the culture medium to yield $2\text{--}3 \times 10^5$ c.p.m. per ml of 10B broth. The radioactivity of ^3H -labelled cells was measured by a beta counter (Beckman), and that of ^{125}I -labelled components by a gamma counter (Packard).

Isolation of ureaplasmas by density gradient. (1) *Continuous density gradient.* Labelled or unlabelled *U. urealyticum* cells ($10^8\text{--}10^9$ c.f.u.) were resuspended in PBS containing 10 mM- MgCl_2 and treated with DNAase I (2500 Kunitz Units mg^{-1} ; Sigma) to remove extracellular, labelled DNA fragments originating from dead cells. This was done at a concentration of 5 $\mu\text{g DNAase ml}^{-1}$ for 30 min at 37 °C. The cells were then washed once and layered onto a 20–60% (v/v) continuous Urografin gradient (a mixture of meglumin diatrizoate and sodium diatrizoate, 76% v/v, for injection, Schering, Berlin, FRG). Following centrifugation at 85000 g (Beckman ultracentrifuge, rotor SW 28) for 2 h at 4 °C, fractions of 1.0 ml were collected from the top of the gradient downwards. All fractions were measured both for the presence of viable organisms (c.f.u.) and for the presence of labelled material (c.p.m.). The density of the fractions was estimated based on the distance of migration into the gradient.

(2) *Discontinuous density gradient.* This consisted of 20, 32 and 40% (v/v) (8.5 ml each) and 60% (6 ml) Urografin layers. It was used as above.

Preparation of antigens for immunoblotting. Purified ureaplasmas collected from the density gradient were washed with PBS and centrifuged (85000 g for 1 h, Beckman SW 28 Rotor) to remove the Urografin. Washed pellets of cells obtained before and after purification by density gradient centrifugation were sonicated at 0 °C for 1 min at 80% power (50% duty cycle; Heat-Systems Ultrasonic Inc) in the presence of the protease inhibitor PMSF (0.01 mM). The sonicates were then treated with DNAase for 10 min at room temperature, followed by one wash, and resuspended in Laemmli sample buffer (Laemmli, 1970).

Antisera. Rabbit antiserum to serotype 4 was prepared and used as described previously (Horowitz *et al.*, 1986). Rabbit antiserum to antigenic medium components was prepared by immunizing rabbits with 1 mg of 10B broth pellet per each injection, given intradermally. This antigen was emulsified in Freund's complete adjuvant for primary immunization and in incomplete Freund's adjuvant for the booster injections that were given at 2, 4 and 6 weeks, intradermally. Antibody levels were measured by enzyme-linked immunosorbent assay (ELISA), as previously described (Horowitz *et al.*, 1986), but with slight modifications.

Immunoblotting. SDS-PAGE of ureaplasma antigens was done according to the method of Laemmli (1970) using a 3% (w/v, acrylamide) stacking gel and 10% resolving gel (1 mm thick). Transfer to nitrocellulose and immunoblotting were done according to the procedure of Towbin *et al.* (1979). Rabbit antisera were diluted 1:3200, and horseradish-peroxidase-conjugated swine anti-rabbit IgG (DAKO-immunoglobulin, Denmark) was used at a dilution of 1:1000. Antigens were visually detected by colour reaction with 3,3'-diaminobenzidine tetrahydrochloride dihydrate (Aldrich).

Results

Cultivation of ureaplasma in serum-free medium

Growth curves (pH change and viability titres) of *U. urealyticum* serotype 4 cultured in 10B broth/horse serum and in 10B broth/PPLO-serum fraction were compared. As shown in Fig. 1, identical growth curves were obtained in both these culture media, with the same final yield of 10^7 c.f.u. ml^{-1} . Serotypes 3, 6, 7, 9 and 14 were also cultured in 10B containing 1% PPLO-serum fraction and similar yields, in the range $8 \times 10^6\text{--}5 \times 10^7$ c.f.u. ml^{-1} , were obtained for each of these serotypes. The somewhat lower titres (3×10^7 c.f.u. ml^{-1}) obtained in the type of experiment depicted in Fig. 1 are probably due to interrupted incubation conditions resulting from the sampling procedure.

Isolation of Ureaplasma cells on a density gradient

U. urealyticum cells (serotype 4) were efficiently labelled upon cultivation in the presence of [^3H]thymidine. A resuspended pellet of ^3H -labelled cells (3×10^6 c.p.m. per 10^9 c.f.u.) was subjected to isopycnic centrifugation as described in Methods. A separate band of labelled

Fig. 1. Growth curves of *U. urealyticum* (serotype 4) in different media monitored by increase in culture pH (—) and by increase in c.f.u. ml^{-1} (---). Ureaplasmas were grown in 10B broth/5% horse serum (●) and in 10B broth/1% PPLO-serum fraction (○).

Fig. 2

Fig. 3

Fig. 2. Isolation of ^3H -labelled cells of *U. urealyticum* (serotype 4) on a 20–60% Urografin density gradient. The distribution of ^3H -labelled material obtained from a culture of ureaplasmas (●), is compared with that of an uninoculated medium control (○). △, Density gradient.

Fig. 3. Viability of *U. urealyticum* (serotype 4) cells separated on a Urografin density gradient. Viable cells were determined as the number of (c.f.u. ml^{-1}) (○); and ^3H -labelled cells were determined by counting radioactivity (●). Note that the c.f.u. count is shown on a logarithmic scale while the radioactivity is on a linear scale.

Fig. 4

Fig. 5

Fig. 4. Isolation by density gradient centrifugation of purified cells of *U. urealyticum* (serotype 4) from a culture containing ^{125}I -labelled PPLO-serum fraction. The distribution of purified viable cells (○) is compared with that of ^{125}I -labelled medium components (●).

Fig. 5. Isolation by density gradient centrifugation of purified cells of *U. urealyticum* (serotype 4) from a culture containing ^{125}I -labelled horse serum. The distribution of viable cells (shown as c.f.u. ml^{-1} ; ○) is compared to that of radioactivity in cells containing ^{125}I -labelled components (●) or an uninoculated labelled medium control (△).

cells was visible at 35–37% Urografin (fractions 13–16) and it differed from the band obtained from the non-inoculated labelled-medium control (fractions 3–6) (Fig. 2). The distinct fraction of labelled material at a density of $1.23\text{--}1.25\text{ g cm}^{-3}$ (35–37% Urografin) coincided with the peak of viable cells and contained 80% of the total c.f.u. applied to the gradient (Fig. 3). In over 20 repeated experiments, the location of the cells in the gradient remained constant, and the yield of cells was 82–87%.

Removal of medium components from ureaplasma cells

The separation of medium components from viable ureaplasmas by the density gradients was verified by analysing cells that were grown in 10B medium containing either ^{125}I -labelled PPLO-serum fraction or ^{125}I -labelled horse serum. As shown in Fig. 4, the distribution of the ^{125}I -labelled PPLO-serum fraction components in the density gradient was distinct from

Fig. 6. Efficiency of the purification procedure. The percentage of viable *U. urealyticum* cells in the fractions obtained from a discontinuous Urografin density gradient is shown. The Urografin concentration in the gradient layers is indicated at the top of the Figure.

that of the ureaplasmas, and was located at 20–25% Urografin. Thus, the fractions containing viable ureaplasmas were devoid of ^{125}I -labelled components, as shown by their location in a separate area on this gradient. Somewhat different results were obtained with cells grown in 10B containing ^{125}I -labelled horse serum (Fig. 5). The ^{125}I -labelled components exhibited two distinct peaks, the first at 20–25% Urografin (as in Fig. 4), while the second (at 38–43% Urografin), partially overlapped the viability peak. Thus, 85% of the viable cells separated in this band (= peak) (fractions 9–15) were not contaminated with ^{125}I -labelled material. A sharp band of viable cells was also obtained by using a discontinuous gradient, which is very simple to prepare (Fig. 6). This band was obtained at the 32/40% Urografin interface, and the cells could be collected easily with high efficiency.

Antigenicity of purified *Ureaplasma* cells

The degree of purification of the ureaplasmas was further analysed by immunoblotting of cells before and after density gradient centrifugation. Equal amounts of ureaplasmas (5×10^7 c.f.u.) from the suspension applied to the gradient and from the resuspended pellet obtained from the gradient, were compared. This was done by immunoblotting with antiserum to the medium components and with anti-ureaplasma antiserum. As demonstrated in Fig. 7, ureaplasmas before gradient centrifuga-

Fig. 7. Immunoblot of purified *U. urealyticum* (serotype 4) cells. Ureaplasmas before purification (lanes 1 and 3) and after purification (lanes 2 and 4) were reacted with rabbit antiserum to medium components (lanes 1 and 2) and with rabbit anti-serotype 4 antiserum (lanes 3 and 4).

tion contained multiple medium components, as detected by their reactivity with antiserum to the medium components (lane 1). In contrast, the gradient-purified cells did not contain these proteins (lane 2). In comparison, genuine *U. urealyticum* antigens remained unaffected (lanes 3 and 4), as seen by their reactivity with rabbit anti-*U. ureaplasma* (serotype 4) antibodies.

Discussion

We have shown that *U. urealyticum* can be cultured in medium in which PPLO-serum fraction is used to replace the horse serum commonly utilized for the cultivation of ureaplasmas. PPLO-serum fraction has been used previously to replace the serum components in culture media for other *Mycoplasma* and *Acholeplasma* species (Razin & Rottem 1976; Rottem *et al.*, 1977). The growth factors present in serum have been investigated (Rottem *et al.*, 1971; Washburn *et al.*, 1978; Johnson & Somerson, 1980), but not fully defined. A modified medium,

consisting of dialysate broth supplemented with whole serum, was previously used for antigenic analysis of *Ureaplasma* serotypes (Sayed & Kenny, 1980), but it still contained horse serum components. PPLO-serum fraction is a much less complex supplement than whole horse serum and contains mainly lipoproteins and albumin (Smith *et al.*, 1954; Washburn *et al.*, 1978). Therefore, whereas ureaplasma cells grown in media containing horse serum adsorb many serum constituents, as do other mycoplasma cells (Bradbury & Jordan 1972; Nicolet *et al.*, 1980), cells grown in media containing PPLO-serum fraction would be expected to be devoid of such constituents. We have also found that all 14 serotypes of *U. urealyticum* can be successfully cultured in the 10B broth/PPLO-serum fraction, resulting in high yields of cells (10^7 c.f.u. ml⁻¹). These titres are comparable and in some cases higher than the yields obtained in 10B broth/horse serum, as used in many laboratories. The reason for this better growth is unknown, but might be due to inhibitory factors which are known to affect certain batches of commercial sera used as supplements (Tully & Rose, 1983).

Further purification of ureaplasma cells grown in the PPLO-serum fraction medium was achieved by density gradient centrifugation. Sodium metrizoate has been used successfully for density gradient separation in various systems for the purification of white blood cells (Lymphoprep), macromolecules, subcellular organelles and viruses (Kirshner *et al.*, 1970; Grdina *et al.*, 1973; Serwer, 1975). A mixture of Metrizamide and sucrose was used for the separation of *Mycoplasma hyorhinis* from cultures (Wise *et al.*, 1982) and Urografin, a mixture of meglumin diatrizoate and sodium metrizoate was used for the purification of *Chlamydia trachomatis* (Caldwell *et al.*, 1981). However, we believe this to be the first report on the purification of viable *Ureaplasma* spp. by density gradient centrifugation. Purification of ureaplasmas on sucrose density gradients has been described previously (Rottem *et al.*, 1971) but the organisms obtained were non-viable. Isolation of viable cells by sucrose density gradient centrifugation has been reported for *Mycoplasma fermentans* (Williams, 1968), but not for *Ureaplasma*. Percoll gradient separation has been used for preparation of ureaplasma antigens (Lee & Kenny 1987), but details concerning the degree of purification, viability of cells and efficiency of the procedure were not reported. We have found that Urografin density gradients preserve viability more successfully than sucrose density gradients.

Final proof of the effective purification of ureaplasma cells was obtained by immunoblotting. Almost all of the medium components detected in a pellet of cells harvested from the medium were absent from the pellet of purified cells collected from the peak in the gradient.

In contrast, the reactivity of each ureaplasma antigen with antiserum specific to that serotype was maintained throughout the purification procedures. The few ureaplasma antigens that were undetectable after purification, when probed with rabbit anti-serotype 4 antiserum, could have represented antigenic medium components adsorbed to the cells used previously for immunization (Horowitz *et al.*, 1986). The very weak protein bands seen in the immunoblots of purified cells reacted with anti-medium antibodies could be due to cross-reactivity between antigenic epitopes present both in medium components and in ureaplasma cells. Most of the ureaplasma antigens remained unaffected by removal of the medium antigens.

Our results thus show that viable cells of *U. urealyticum* almost devoid of serum components can be isolated by means of Urografin density gradient centrifugation. This purification procedure is also applicable to ureaplasmas grown in traditional medium containing horse serum, albeit with a somewhat low efficiency.

The two major problems in isolating *U. urealyticum* and in identifying antigens and/or functions are, firstly, to obtain considerable numbers of cells, and secondly, to eliminate the medium components adsorbed to these cells. The procedure described in this paper makes it possible to obtain relatively large amounts of purified viable ureaplasma cells. These can be used to study functional aspects of intact cells (e.g. specific binding of purified ureaplasmas to host cell membrane, which otherwise may suffer from hindrance by serum components), and to determine their role in pathogenesis. This procedure will now enable us to investigate cross-reactivity among the serotypes, without interference from cross-reactive non-relevant medium components. We intend to use these cells for the identification of surface antigens which might have been masked and/or altered by the contaminating constituents. These antigens will be further analysed for expression in disease and possible value in developing a specific detection assay.

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