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Adherence of *Ureaplasma urealyticum* to Human Erythrocytes

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***Ureaplasma urealyticum* (four serotypes and two clinical isolates) were metabolically labeled with radioactive methionine to a high specific activity. Labeling allowed the study of the mechanism of adherence to human erythrocytes. The adherence mechanism was complex and partially mediated by proteinaceous surface components. The binding sites on the erythrocytes were partially sensitive to neuraminidase treatment, and adherence was inhibited by glycophorin and dextran sulfate, indicating recognition of sialyl residues and sulfated compounds.**

The ability to adhere is a prerequisite for pathogenesis by many microorganisms (2). The adherence mechanisms of a number of mycoplasmas, particularly that of *Mycoplasma pneumoniae*, have been extensively investigated (1, 5–9, 11–13, 15). It has been shown that the adherence of *M. pneumoniae* to both respiratory cells and erythrocytes utilizes similar mechanisms. For example, *M. pneumoniae* proteinaceous components mediate adherence to neuraminidase-sensitive regions on both respiratory epithelium and erythrocytes (6). Thus, the model system using erythrocytes has been used to study the adherence mechanisms of *M. pneumoniae*, *Mycoplasma gallisepticum*, and other mycoplasmas (1, 6, 12, 13).

Ureaplasma urealyticum is an inhabitant and possible pathogen of the human urogenital tract (18). The organisms have been observed adhering to various cells, including epithelial cells from urethra (18), spermatozoa (3), and animal cells in culture (8). However, the adherence mechanism of *U. urealyticum* is yet to be elucidated. The lack of quantitative studies has been mostly due to the difficulty of labeling the organisms to high enough specific activity by using the procedures that were successful with other mycoplasmas, e.g., incorporation of radioactive fatty acids (1) or L-[^{35}S]methionine (7, 14). We hereby present an adaptation of the method for metabolic labeling *Escherichia coli* with L-[^{35}S]methionine to label *U. urealyticum* cells after growth. By using this method, we obtained cells sufficiently labeled to allow evaluation and characterization of some features of their adherence mechanism.

U. urealyticum serotypes 2, 3, 7, and 8 and two clinical isolates were grown in 1 liter of medium containing PPLO broth, 10% horse serum, 5% fresh yeast extract, and 6.6 mM urea (17). The organisms were harvested and washed twice in 0.25 M NaCl, as previously described (17). The pellet was suspended in 0.8 ml of Hanks buffered salt solution from which sulfate-containing salts were omitted and replaced with NaCl (HBSS-M). Subsequently, 4 μl of L-amino acid mixture (alanine, 0.4 mM; arginine, 0.6 mM; asparagine, 0.32 mM; aspartate, 0.3 mM; glycine, 0.13 mM; isoleucine, 0.3 mM; leucine, 0.3 mM; lysine, 0.3 mM; phenylalanine, 0.3 mM; proline, 2.0 mM; serine, 4.0 mM; threonine, 0.3 mM; tryptophan, 0.1 mM, tyrosine, 0.1 mM; valine, 0.3 mM) (4) and L-[^{35}S]methionine (0.5 mCi; Amersham, Amersham, England) were added. The suspension was divided into four aliquots and incubated for 2 h at 37°C, and then 1 ml of HBSS-M containing 0.33 mM methionine (Sigma Chemicals) was added. After a 10-min chase, the suspension was centrifuged for 10 min at 15,000 rpm at 4°C in a microcentrifuge (Mikroliter; Hettich, Tuttingen, Federal Republic of Germany). The supernatant was discarded, and the pellet was washed twice in methionine-containing HBSS-M and suspended to a concentration of 50 μg of protein per ml in buffer A (50 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, pH 7.2) by forcing the pellet through a 26-gauge needle five times. The suspension was used in adherence experiments or solubilized and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 8% acrylamide gels (10).

The labeling conditions were selected after a series of experiments, including determination of optimal labeling time and washings. After 2 h of incubation, the cells were sufficiently labeled (about 16×10^6 cpm/mg of protein) to enable assessment of adherence and to receive a sharp fluorograph from X-ray films exposed (3 to 7 days at −70°C) to labeled proteins in dried gels after SDS-PAGE.

Urea is an essential growth factor of *U. urealyticum*, and hydrolysis of urea by its urease is involved in ATP generation (16, 18). Therefore, the influence of urea on the labeling procedure was investigated. The addition of 50 mM urea to the labeling mixture did not alter the amount of incorporation or the electrophoretic pattern of the labeled proteins.

The different strains were labeled to the same extent, and the majority of the ureaplasmal polypeptides were labeled, as judged from the comparison of silver stain and fluorograph (Fig. 1). The advantages in using a metabolic label with ureaplasmas are as follows. (i) The elimination of labeling contaminating medium components is a serious problem with these fastidious organisms requiring a complex growth medium containing serum (14, 18). (ii) Protein labeling is preferred over fatty acid labeling, since it allows tracking of proteinaceous components involved in adherence.

The availability of metabolically labeled *U. urealyticum* with a high specific activity enabled us to assess some features of the ureaplasmal mechanism of adherence to human erythrocytes.

Adherence was assayed in plastic tubes as previously described (1). Briefly, the assay contained 0.05 ml of buffer A, 0.05 ml of 2% fresh human erythrocytes, and 0.1 ml of a

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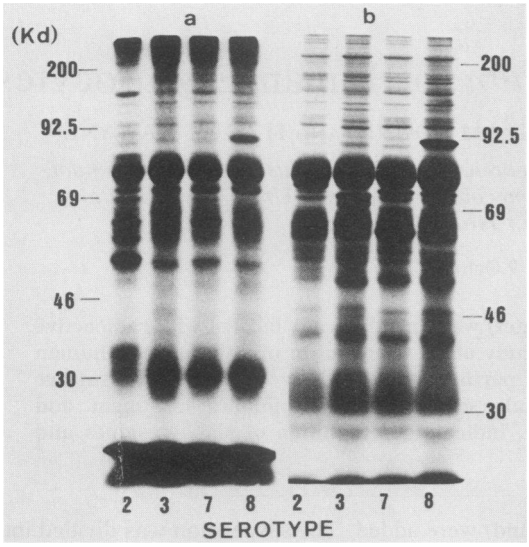

FIG. 1. Electrophoretic pattern of labeled polypeptides from different serotypes of *U. urealyticum*. Serotypes 2, 3, 7, and 8 were labeled with L-[^{35}S]methionine for 2 h. After denaturation and reduction, 10 μg of labeled proteins was loaded in each slot and subjected to SDS-PAGE. One part of the gel was soaked with Amplify solution (Amersham), dried, and exposed to X-ray film (a). The remaining part was stained with silver (b). Molecular mass markers were run in the flanking slots.

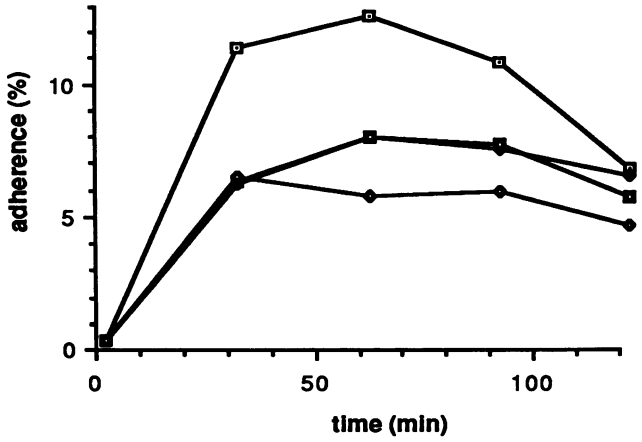

FIG. 2. Adherence of different serotypes of *U. urealyticum* to human erythrocytes. Serotypes 2 (□), 3 (◆), 7 (■), and 8 (◆) labeled with radioactive methionine were assayed for adherence to human erythrocytes. The remaining radioactivity on the washed erythrocytes was determined after various incubation times and was expressed as the percentage of total radioactivity added. The results represent a typical experiment out of 4.

suspension of labeled ureaplasmas. After a 30-min incubation at 37°C, 0.5 ml of cold buffer A was added and the unattached ureaplasmas were separated from the erythrocytes by centrifugation at 200 × *g* in a Sorvall GLC-2 centrifuge for 1 min (1). After two additional washings, the erythrocyte pellet was exposed to 1% SDS plus 1% H_2O_2 and the residual radioactivity was determined by liquid scintillation (1). The radioactivity measured from control tubes containing labeled ureaplasmas without erythrocytes was subtracted from the values obtained from the samples.

The adherence of the ureaplasmas tested in these experiments reached 4 to 12% after 30 to 60 min, depending on the serotype (Fig. 2). Prolonged incubation did not increase the attachment. Similar results (3.5 to 4% adherence of *U. urealyticum* serotype 8) were obtained when the experiments were performed on bovine Fallopian tube mucosa cells in culture (Saada et al., unpublished data). These are primary cultures of epithelial cells obtained from bovine Fallopian tubes, which served as a model of the female upper genital tract. These adherence values are comparable with those obtained in the same experimental system for several other mycoplasmas, e.g., *M. pulmonis* and *M. gallisepticum* (1, 13).

It was also reported that decreased adherence occurred following many passages of laboratory strains (12). As the *U. urealyticum* serotypes that we examined originated from numerous passages, we compared the adherence of serotype 8 with that of two clinical isolates (less than five passages). These strains adhered to the same extent as serotype 8 did.

The ability to adhere was not influenced by the addition of 50 mM urea or by the urease inhibitor Flurofamide (50 μM) (Norwich Eaton Pharmaceuticals). This indicates that the ureaplasmal urease, which is essential in *U. urealyticum* energy metabolism (16, 18), does not participate in the adherence process.

The optimal temperature for attachment was 37°C. The decrease of adherence by 63% ± 3% at a lower temperature (0°C) may be indicative of an alteration of the metabolic state of the organism or of the mainly nonhydrophobic nature of these interactions. Heat treatment (30 min at 55°C) of the labeled ureaplasma prior to the adherence assay resulted in the loss of 18% ± 2% binding capacity. These findings indicate the involvement of ureaplasmal proteins. The results were further confirmed by the finding that labeled ureaplasmas (50 μg of protein per ml) treated for 1 h with papain (0.5 μg/ml), chymotrypsin (0.2 μg/ml), or trypsin (2.5 to 5.0 μg/ml) adhered 33 to 39% less than cells treated with these proteases in the presence of their respective inhibitor (iodoacetic acid, 10 mM; phenylmethylsulfonyl fluoride, 0.5 mM; 1-chloro-3-tosylamide-7-amino-L-2 heptanone, 30 μg/ml). Proteolytic digestion, as judged by SDS-PAGE, resulted in the loss of some minor polypeptide bands of molecular mass above 95 kDa (data not shown).

Sialyl receptors on the surface of host cells have been reported to mediate adherence of many mycoplasmas (5, 6, 12, 15). We investigated this in ureaplasmas, using neuraminidase-treated erythrocytes. The findings that asialoerythrocytes (1) bound 29% ± 3% fewer ureaplasmas confirmed this to some extent. The ability of glycophorin, but not asialoglycophorin, to interfere with adherence further strengthened the aforementioned result (Table 1).

The sialoglycoproteins, α_1-acid glycoprotein and fetuin, interfered less than glycophorin with the adherence of *U. urealyticum* (Table 1). Bovine serum albumin was included in these experiments as a negative control (Table 1). These results and the finding that free *N*-acetylneuraminic acid (2 mM) did not interfere with adherence indicate the importance of the linkage in which the sialic acid residues are bound. This is common to other microbial adhesins and was previously reported for *M. pneumoniae* and *M. gallisepticum* (5, 15).

Krivan et al. (9) recently reported the importance of sulfatide receptors in *M. pneumoniae* binding. The similarity with *U. urealyticum* was shown by our findings that the addition of 10 μg of dextran sulfate (M_w, ~500,000) in the

TABLE 1. Effects of sialoglycoproteins and asialoglycophorin on adherence of *U. urealyticum* to human erythrocytes

Treatment[a]	% Inhibition[b]
Glycophorin	52 ± 20
Asialoglycophorin	0
α_1-Acid glycoprotein	29 ± 10
Fetuin	8 ± 6
Bovine serum albumin	4 ± 4
Buffer (control)	0

[a] α_1-Acid glycoprotein, asialoglycophorin, fetuin, or glycophorin (0.1 mg/ml) was added to the suspension of *U. urealyticum* (serotype 8) prior to the erythrocytes in adherence experiments. In control experiments, bovine serum albumin or buffer was added.

[b] Results are expressed as percentage inhibition compared with controls with buffer (5.5 to 7% adherence). Each value is the mean ± standard error of eight experiments.

binding assay inhibited adherence by 53% ± 10%, while dextran (M_w, ~500,000) inhibited adherence by 16% ± 6%.

The adherence of *U. urealyticum* seems to be of a complex nature, since it is mediated by proteinaceous and other surface components that recognize sialyl residues and/or sulfated compounds. These findings suggest that *U. urealyticum* possess adherence mechanisms resembling those found in other pathogenic mycoplasmas, such as *M. pneumoniae*, *M. gallisepticum*, and *M. pulmonis* (5, 6, 9, 12, 13, 15).

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