Int. J. Exp. Path. (1994) 75, 91–98

Intracellular location of *Mycoplasma genitalium* in cultured Vero cells as demonstrated by electron microscopy

J. SKOV JENSEN*, J. BLOM† AND K. LIND*

*Statens Seruminstitut, *Mycoplasma Laboratory, Neisseria Department, and †Laboratory of Molecular Cell Biology, Department of Clinical Biochemistry, Artillerivej 5, DK-2300 Copenhagen S, Denmark*

Received for publication 1 September 1993
Accepted for publication 26 November 1993

Summary. The original two strains of *Mycoplasma genitalium* were isolated from the human urogenital tract. No other strains have been isolated from this site since then. We have recently succeeded in propagating a third strain from a urogenital specimen from a patient with urethritis in Vero cell cultures.

By electron microscopy mycoplasmas were demonstrated intracellularly in about 10% of the examined Vero cells. Various stages of penetration into the cells could be observed. The flask-shaped organisms seemed to penetrate into the cells by the tip-end which included a rodlike structure. The intracellular location of normal mycoplasmas were in membrane-bound vacuoles very close to the nucleus, occasionally together with a few disintegrated organisms. In a few cells additional material was entangling the mycoplasmas in the cytoplasmic vacuoles. The potential for intracellular survival of *M. genitalium* may help the organism to evade the defence mechanisms of the human body. This trait may be considered a pathogenic property which supports the presumption that *M. genitalium* has clinical importance.

Keywords: intracellular mycoplasmas, *Mycoplasma genitalium*, Vero cells, ultrastructure

Mycoplasma (*M.*) *genitalium* was first isolated from the urethra of two men with non-gonococcal urethritis (NGU) (Tully *et al.* 1981; 1983), but since then there has been only putative evidence of its isolation from the urogenital tract (Taylor-Robinson *et al.* 1985). The significance of antibodies to *M. genitalium* in relation to pelvic inflammatory disease has been controversial (Møller *et al.* 1984; Lind & Kristensen, 1987). By means of the polymerase chain reaction (PCR) (Saiki *et al.* 1985), the presence of *M. genitalium* DNA in urogenital tract specimens has been demonstrated (Jensen *et al.* 1991; Palmer *et al.* 1991), and recently we have shown that *M. genitalium* was detected significantly more often in men with non-chlamydial NGU, than in men without symptoms of urethritis (Jensen *et al.* 1993).

Correspondence: Dr Jørgen Skov Jensen, Statens Seruminstitut, Mycoplasma Laboratory, Neisseria Department, Artillerivej 5, DK-2300 Copenhagen S, Denmark.

Some mycoplasma species have the ability to attach to cell surfaces. In *M. genitalium* a specific attachment protein (MgPa) has been described and found to be localized to the tip region (Hu *et al.* 1987). It has been difficult to resolve by light microscopy whether or not attached mycoplasmas are able to invade the cells. However, based on electron microscopy intracellular localization of both *M. fermentans* and *M. hominis* (Lo *et al.* 1989; Taylor-Robinson *et al.* 1991) and of a new species *M. penetrans* (Lo *et al.* 1992) has been described.

Recently we have succeeded in growing *M. genitalium* in Vero monkey kidney cells by inoculating a urethral specimen from a male patient with non-chlamydial NGU positive for *M. genitalium* DNA by PCR; results of studies on the growth conditions will be presented elsewhere.

In this paper we describe the ultrastructure of *M. genitalium* in Vero cells and present evidence of an intracellular location of this mycoplasma.

Materials and methods

Medium for Vero cells

Growth medium comprised Eagle's minimal essential medium containing 3% (of a 2.8% w/v solution) sodium bicarbonate, 2% Ultroser HY serum substitute (Gibco BRL, Life Technologies, Roskilde, Denmark), and 500 IU penicillin/ml. The cells were maintained in the same medium.

Vero cells

Vero African green monkey kidney cells were adapted to growth in the medium supplemented with Ultroser HY serum substitute by successive passages in medium with decreasing concentration of foetal calf serum and increasing amounts of Ultroser HY according to the procedure recommended by the manufacturer.

Confluent monolayers of cells grown in 10 ml of growth medium in 50-ml tissue culture (TC) flasks (Nunc, Roskilde, Denmark) (6×10^6 cells) were harvested by trypsinization and seeded into 100–150 ml of growth medium which was distributed in 10 or 50 ml portions in 50 or 260-ml tissue culture flasks, respectively, or in TC tubes with a flat side (Nunc) and incubated at 37°C with closed lids. Confluent growth was observed within 3–4 days.

Growth of a new strain (M 2300) of M. genitalium *in Vero cell culture*

Urethral swabs were collected in 1.8 ml of SP4 mycoplasma broth (Tully *et al.* 1977) as part of a study of men with urethritis with the purpose of determining the prevalence of *M. genitalium* by PCR (Jensen *et al.* 1993). One of the specimens was selected because it was culture negative both for ureaplasmas and for mycoplasmas capable of growth in modified Hayflick medium (Lind *et al.* 1984) and produced a strong positive signal in the PCR.

Three serial tenfold dilutions of the specimen were performed, and 0.2 ml of each dilution was mixed with 2 ml of Vero cell suspension immediately after the trypsinization (approximately 8×10^5 cells); the cultures were incubated in TC tubes with a flat side at 37°C for 3–4 days. The supernatant was aspirated and a 200-μl aliquot was used for PCR (Jensen *et al.* 1991) to monitor the growth. Adhering cells were trypsinized and resuspended in 4 ml of fresh medium; 2 ml were frozen in liquid nitrogen and 2 ml were incubated in TC tubes for another 3–4 days before repeated passage. At the third passage the culture was expanded to grow in 10-ml TC flasks by adding 1 ml of the *M. genitalium* infected cell culture to 10 ml of a suspension (ca 4×10^5 cells/ml) of uninfected Vero cells. The resulting 1 : 10 dilution was necessary to avoid the mycoplasmas overgrowing the culture and killing the cells. The mycoplasma present in the cell culture was identified as *M. genitalium* by its positive reaction in PCR with three different sets of primers (Jensen *et al.* 1991; 1993).

Preparation of infected cell cultures for transmission electron microscopy

Vero cells from three 10-ml TC flasks infected with *M. genitalium* strain M 2300 (uncloned) and grown for 3 days after the last passage were scraped off with a cell scraper (Costar 3010, Cambridge, USA). The pooled cell suspension was then pelleted by centrifugation for 5 min at 200 ***g***. The supernatant was aspirated and the pellet resuspended in 10 ml 3% glutaraldehyde in 0.01 M sodium cacodylate buffer, pH 7.2, containing 0.01 M $CaCl_2$. After fixation for 2 hours at room temperature, the cells were centrifuged again for 10 min (2800 ***g***) and the pellet was embedded in molten 1.5% (w/v) Noble Agar (Difco) at 45°C. After solidifying, the agar was cut into small cubes, and those with visible clusters of cells were transferred to 3% glutaraldehyde and fixed overnight at 4°C. The specimens were post-fixed for 1 hour at room temperature in 1% OsO_4 (w/v) in 0.1 M sodium cacodylate buffer, pH 7.2, containing 0.01 M $CaCl_2$ and then stained *en bloc* with 2% (w/v) uranyl acetate in 0.1 M barbiturate buffer, pH 7.3, for another hour. Procedures for dehydration and embedding in Vestopal-W (Martin Jaeger, Geneva, Switzerland) and further preparation of thin sections were carried out as described previously (Blom *et al.* 1976). The cells were examined in a Philips EM 201 C electron microscope at 60 kV.

Results

Propagation of M. genitalium *M 2300 in Vero cell cultures*

M. genitalium could be propagated in Vero cell cultures as shown by an increasing titre of the organisms determined by PCR after titration of the supernatant.

Amplified DNA from *M. genitalium* M 2300 showed the characteristic lack of the *Eco*RI restriction enzyme cleavage site present in the *M. genitalium* G 37 type strain at position 315 in the MgPa gene (Dallo *et al.* 1989) and in the six other strains isolated by culture (Jensen *et al.* 1991). At the time of the present study, we were not able to grow the mycoplasma on cell-free media, hence this investigation was performed without cloning the mycoplasma. We can not exclude the possibility that other micro-organisms may have been present in the culture.

Ultrastructure of M. genitalium

Most organisms were flask-shaped or round with a truncated body and a rather broad neck. The cells were bound by a single three-layer unit membrane (Figure 1). When the plane of sectioning was along the long axis, the internal structure disclosed a body part composed of loosely granular material (Figure 1) and occasionally a membrane-bound vacuole (Figures 1, 2, 3 and 6). However, some of these structures may represent deep cup-like invaginations of the outer limiting membrane. In the neck region rodlike terminal structures composed of homogeneous material ended in a specialized tip (Figures 1, 5 and 8) and the size of the whole rod-structure was about 60 × 200 nm. Most cells were 100–300 nm wide and 400–600 nm long.

Tissue culture studies

Mycoplasma genitalium infected cultures of Vero cells revealed attachment and invasion of mycoplasmas in about 10% of the examined cells. Both live (Figure 2) and dead cells (Figure 3) were found surrounded by attached mycoplasmas. In some of the healthy looking cells (Figure 2) various stages of penetration into the cells could be observed.

Most of the mycoplasmas were oriented with the tip-like structure invaginating the cell membrane (Figures 1, 2 and 5), but cell contact with the body of the organism was also seen (Figures 1, 2, 4 and 5). If this was the case an electron-lucent zone was seen in the mycoplasmas closely parallel to the contact zone (Figures 1, 4 and 5). To a lesser degree this could also be seen when mycoplasmas were found adhering to each other (Figures 1, 4 and 5). In several of these contact zones between cells and mycoplasmas as well as between mycoplasmas a striated or granulated material could be seen (Figures 1, 4 and 5); this material may be part of the previously described nap (Tully *et al.* 1983). Most of the intracellular mycoplasmas had a normal morphology and were found in membrane-bound vesicles often wrapped up in membrane debris (Figures 2 and 6). In the same vacuoles could also be seen mycoplasmas with degenerative changes (Figures 2 and 6). The vacuoles containing mycoplasmas were most often located near the nuclei (Figures 2 and 6). In a few cells mycoplasmas were found within membrane-bound irregular structures in which the mycoplasma organisms were entangled in a festoon-like network of homogeneous electron-dense material (Figures 7 and 8). The ultrastructure of the embedded mycoplasmas looked normal (Tully *et al.* 1983) and some appeared to be dividing (Figures 7 and 8). There was no loose membrane debris in these structures (Figure 8). The Vero cells in which the intracellular mycoplasmas were found looked healthy with normal mitochondria (Figures 2 and 7) and nuclei (Figures 2 and 7).

Discussion

We have previously described the ultrastructure of 7-day-old cultures of *M. genitalium* (Lind *et al.* 1984); in the present study of infected Vero cell cultures the characteristic rodlike structure in the neck region of *M. genitalium* was seen again. A similar structure has also been found in a few other mycoplasma species including *M. pneumoniae*, *M. gallisepticum*, *M. alvi* and *M. sualvi* (Biberfeld & Biberfeld 1970; Allen *et al.* 1970; Gourlay *et al.* 1977; 1978); it has some resemblance to the densely packed tip-like structure recently described in *M. penetrans* (Lo *et al.* 1992).

In *M. genitalium* a specific attachment protein designated MgPa (Hu *et al.* 1987) has been localized to the surface of the tip-region which is covered by a nap layer. *Mycoplasma pneumoniae* adhesion has been shown to be mediated at least partially through a sialoglycoconjugate receptor on the host cell (Gabridge *et al.* 1979; Loomes *et al.* 1984). The receptor for *M. genitalium* probably has a similar composition because neuraminidase treatment of human erythrocytes abolishes haemadsorption (Tully *et al.* 1983).

Penetration of mycoplasmas into eucaryotic cells may be achieved in several ways. The recently described *M. penetrans* (Lo *et al.* 1992) seems to penetrate the cells by the specialized tip structure and this is apparently also the most common route for *M. genitalium*, as evidenced by the present study. However, in *M. fermentans* and *M. hominis* such tip structures are not found so other mechanisms for penetration may be involved. In this study *M. genitalium* often adhered to the Vero cells also by the anterior (proximal) two-thirds of the body part. This conforms well to the findings that the attachment

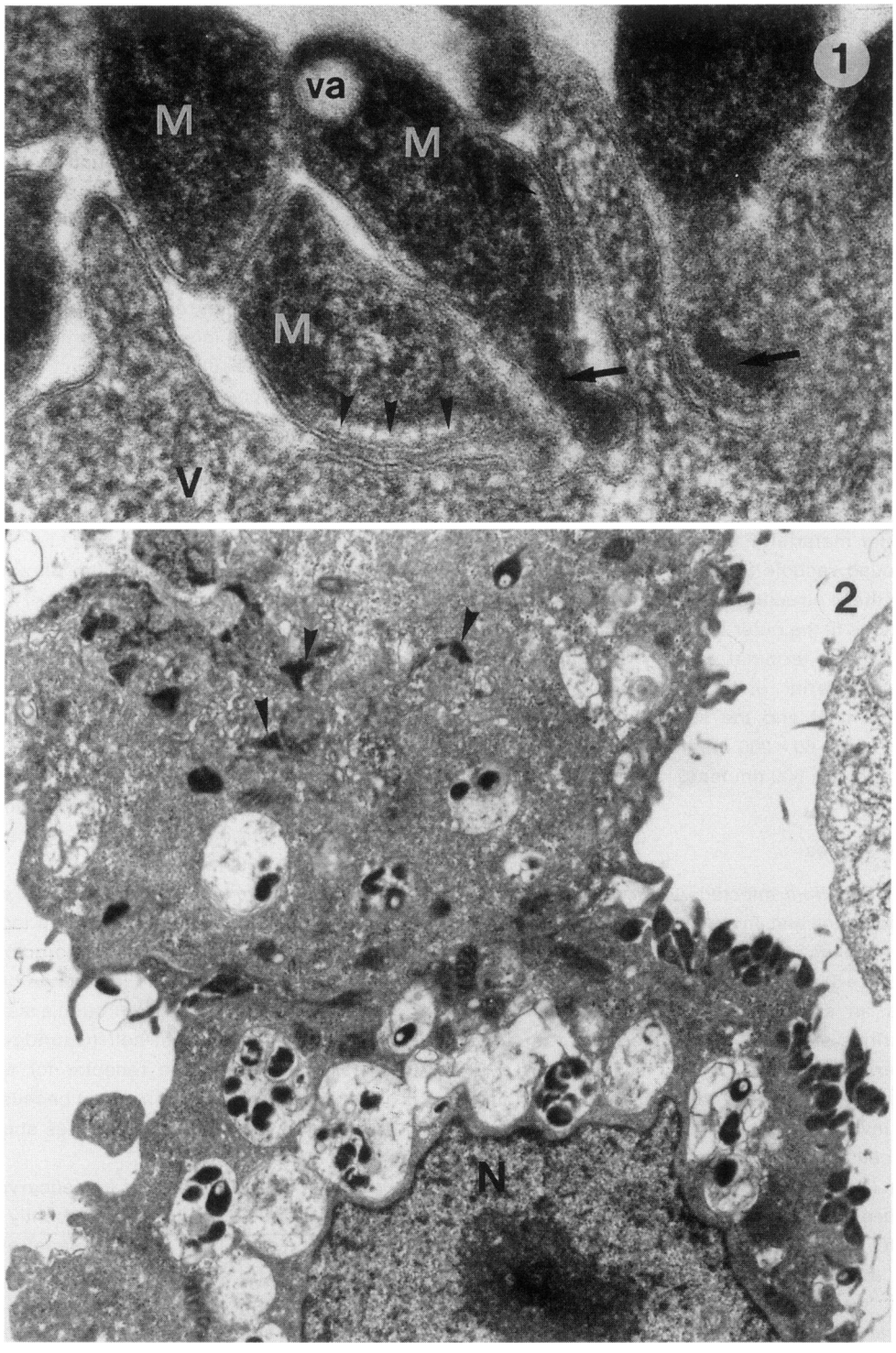

Figure 1. *M. genitalium* (M) adherent to a Vero cell (V) 72 h after infection. The characteristic rodlike structure (arrows) is seen in two of the mycoplasmas. Electron-lucent areas (arrow heads) are found in mycoplasmas closely parallel to the contact zone with the Vero cell. Va denotes vacuole. × 104 000.

Figure 2. Two Vero cells with adherent and intracellular *M. genitalium* 72 h after infection. The mycoplasmas are localized in vacuoles close to the nucleus (N) or in the cytoplasma wrapped in cellular material (arrow heads). × 13 400.

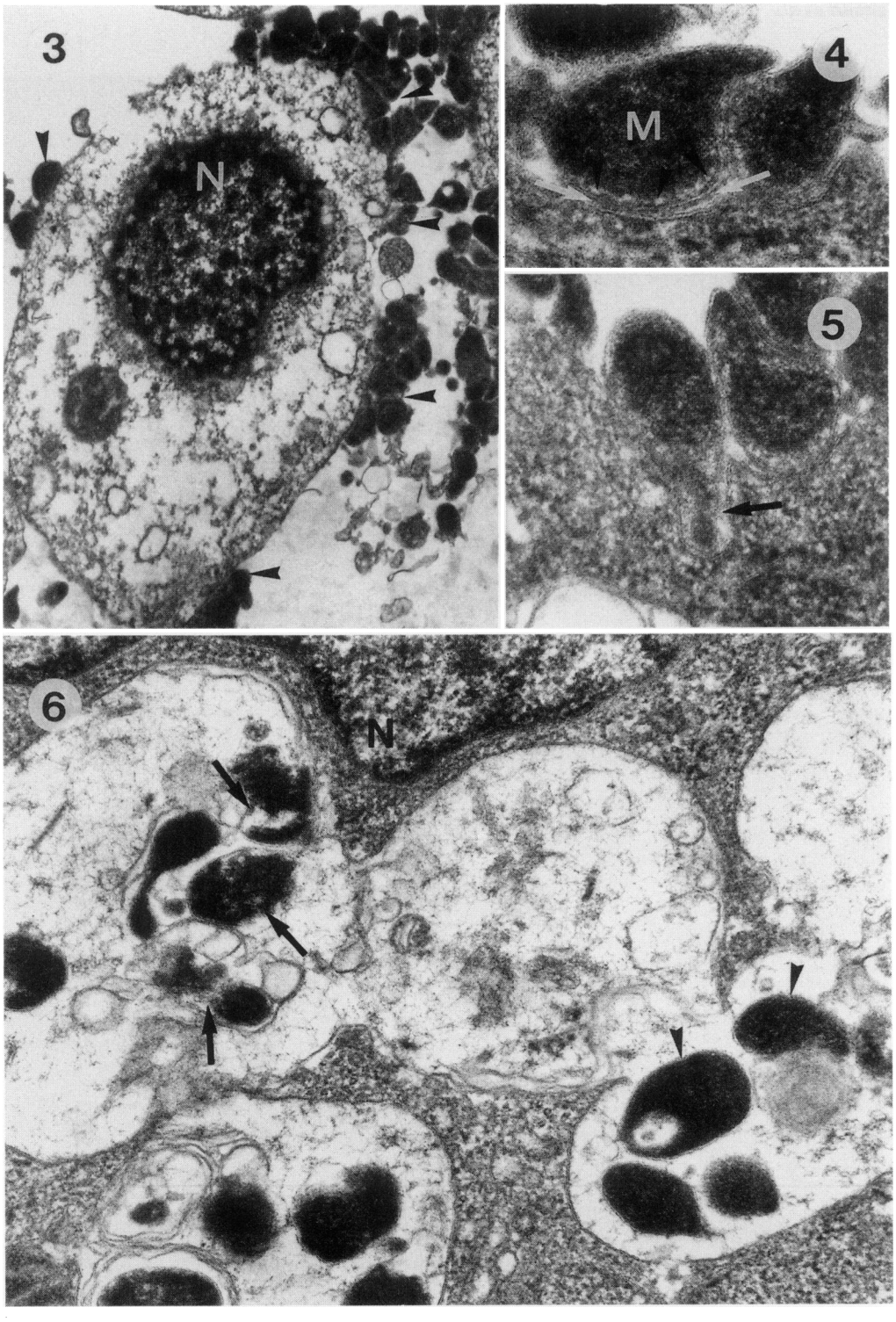

Figure 3. Dead Vero cell surrounded by adherent mycoplasmas (arrow heads). N denotes nucleus. × 18 300.

Figure 4. *M. genitalium* (M) adherent to the Vero cell by the proximal part of the body 72 hours after infection. Closely parallel to the contact zone is seen an electron-lucent area in the organism (arrow heads). Between the cells a striated or granulated material is seen (arrows). × 68 200.

Figure 5. *M. genitalium* penetrating into the Vero cell by the tip-end (arrow), while the neighbour organism is adherent with the proximal part of the body 72 hours after infection. × 68 200.

Figure 6. Higher magnification of some of the vacuoles containing both healthy looking (arrow heads) and degenerated (arrows) mycoplasmas together with membrane debris seen in Figure 2. N denotes nucleus. × 45 500.

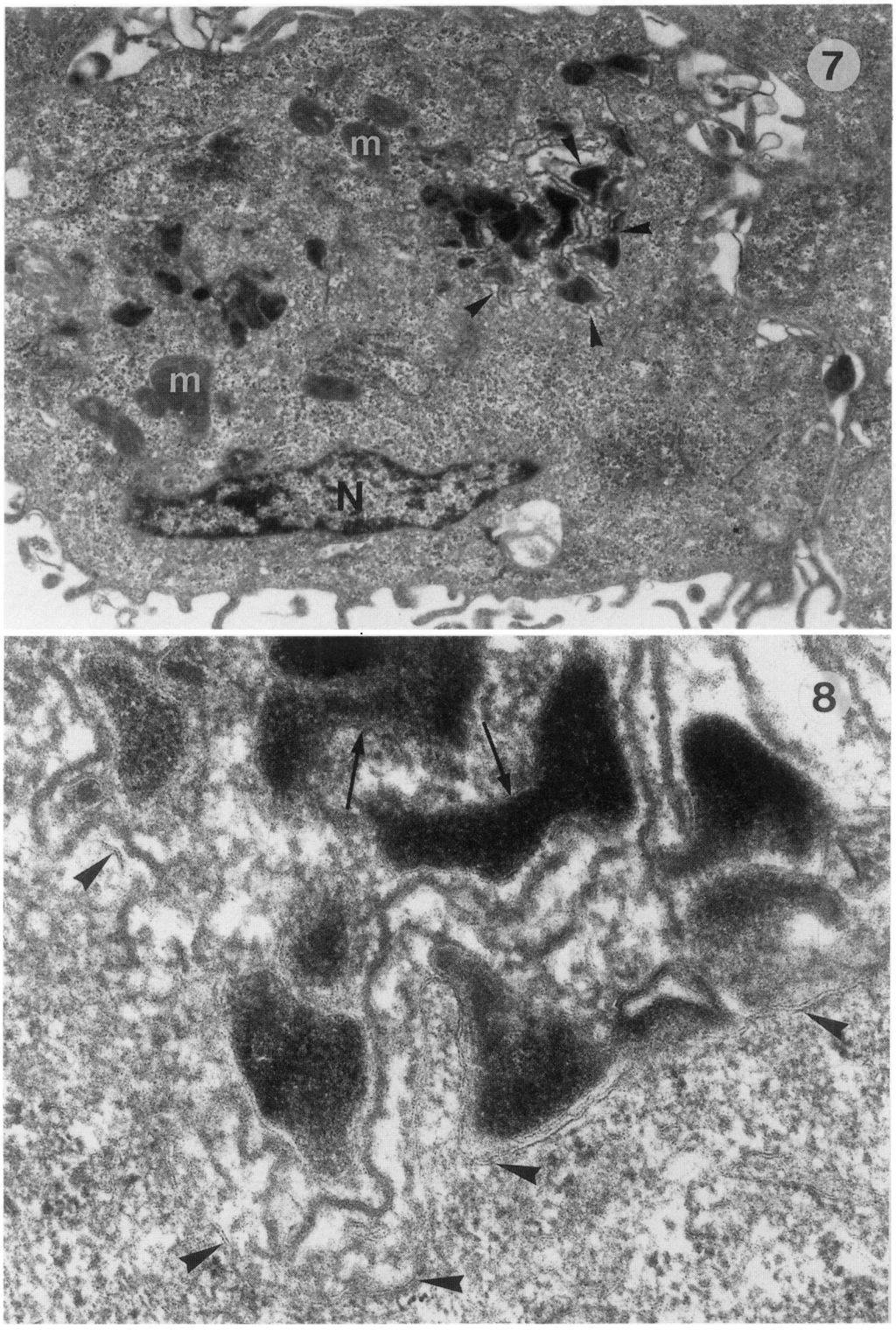

Figure 7. Vero cell with intracellular *M. genitalium* wrapped in festoons of electron-dense material (arrow heads) 72 hours after infection. N denotes nucleus and m mitochondria. × 16 800.

Figure 8. Higher magnification of area from Figure 7 shows that the mycoplasmas are separated from the cytoplasma by a membrane (arrow heads). Some of the mycoplasmas (arrows) may be in the process of division. × 68 200.

protein is found in the nap-layer (Hu *et al.* 1987). Closely parallel to the contact zones, electron-lucent zones were seen in the mycoplasmas; the significance of this observation is not clear. The capacity to adhere to cells with more than the tip-region has also been described for *M. pneumoniae* (Gabridge *et al.* 1979) and it may be another way to penetrate the cells; possibly it could add to the cytopathic effect described to be the result of an *M. genitalium* infection (Tully *et al.* 1983); indeed the present study showed several dead cells with *M. genitalium* attached to them. The striated or granulated material which could be seen in the contact zones between cells and mycoplasmas as well as between mycoplasmas may represent substances present in the nap. In *M. pneumoniae* the P1 adhesin has been shown to cluster mainly at the tip-region, but also in regions throughout the surface length (Baseman *et al.* 1982). The P1 clusters outside the tip region could explain the finding that *M. pneumoniae* is able to adsorb to guinea-pig erythrocytes and human lung fibroblasts by membrane sites other than the tip (Brunner *et al.* 1979; Gabridge *et al.* 1979). It is possible that clusters of MgPa at the neck and side of *M. genitalium* cells could mediate adhesion to Vero cells in a similar way.

Vero monkey kidney cells have been used to study the internalization of obligate intracellular bacteria in non-phagocytic eucaryotic cells (Silverman *et al.* 1993), but whether the finding of *M. genitalium* in membrane-bound vacuoles reflects penetration of the mycoplasma or active endocytosis of the Vero cells is not clear.

We tried to stain the membranes of the Vero cells and the mycoplasmas with ruthenium red (Taylor-Robinson *et al.* 1991) in order to exclude the possibility that the presence of *M. genitalium* in vacuoles was an artifact caused by mycoplasmas being present in invaginations of the Vero cell membrane. The staining with ruthenium red, however, was very weak and did not allow us to draw any further conclusions. Regardless of this we feel that the location of *M. genitalium* close to the nuclei could be taken as strong evidence for truly intracellular localization. Most of the intracellularly located mycoplasmas looked healthy, but the loose membrane debris found in some of the vacuoles together with degenerated mycoplasmas could indicate that intracellular digestion was taking place in spite of the fact that no lysosomal structures were seen. Mycoplasmas were also found intracellularly entangled in a network of dense material arranged as festoons separated from the cytoplasma by a membrane. The mycoplasmas in these areas looked normal, and some might appear to be in the process of division.

Although the present study is purely morphological we suggest that *M. genitalium* may be able to survive or even multiply in eucaryotic cells and thereby escape the defence mechanisms of the human body. Whether the invasive properties of the examined strain are shared with the laboratory strains of *M. genitalium* remains to be clarified. We can not rule out the possibility that the invasive potential of *M. genitalium* M 2300 is caused by some other micro-organism being co-propagated in the cell culture because we did not have the possibility of cloning the strain in the usual fashion before introduction into the Vero cell culture. The simple passage of the Vero cells containing *M. genitalium* onto mycoplasma-free Vero cells also hampers exact determinations of the time elapsed between inoculation and fixation of the cells.

The potential for intracellular survival of *M. genitalium* cells may be considered a pathogenic property which supports the presumption that *M. genitalium* has clinical importance.

Note added in proof

After submission of this manuscript, Horner *et al.* (1993) published a study showing a significantly higher prevalence of *M. genitalium* in men with NGU as compared to men without urethritis. Their findings essentially confirm our results from the study mentioned in the Introduction (Jensen *et al.* 1993).

We have recently isolated and cloned strain M2300 and an additional strain 2341 of *M. genitalium* by subculturing the cell grown strains on Friis modified media.

References

Allen T.C., Stevens J.O., Florance E.R. & Hampton R.O. (1970) Ultrastructure of *Mycoplasma gallisepticum* isolate 1056. *J. Ultrastruct. Res* **33,** 318–331.

Baseman J.B., Cole R.M., Krause D.C. & Leith D.K. (1982) Molecular basis for cytadsorption of *Mycoplasma pneumoniae*. *J. Bacteriol.* **151,** 1514–1522.

Biberfeld G. & Biberfeld P. (1970) Ultrastructural features of *Mycoplasma pneumoniae*. *J. Bacteriol.* **102,** 855–861.

Blom J., Mansa B. & Wiik A. (1976) A study of Russell bodies in human monoclonal plasma cells by means of immunofluorescence and electron microscopy. *Acta Pathol. Microbiol. Scand. Sect. A.* **84,** 335–349.

Brunner H., Krauss H., Schaar H. & Schiefer H.G. (1979) Electron microscopic studies on the attachment of *Mycoplasma pneumoniae* to guinea-pig erythrocytes. *Infect. Immun.* **24,** 906–911.

Dallo S.F., Chavoya A., Su C.J. & Baseman J.B. (1989) DNA and protein sequence homologies between the adhesins of *Mycoplasma genitalium* and *Mycoplasma pneumoniae*. *Infect. Immun.* **57,** 1059–1065.

Gabridge M.G., Taylor-Robinson D., Davies H.A. & Dourmashkin R.R. (1979) Interaction of *Mycoplasma pneumoniae* with human lung fibroblasts: Characterization of the in vitro model. *Infect. Immun.* **25,** 446–454.

GOURLAY R.N., WYLD S.G. & LEACH R.H. (1977) *Mycoplasma alvi*, a new species from bovine intestinal and urogenital tracts. *Int. J. Syst. Bacteriol.* **27,** 86–96.

GOURLAY R.N., WYLD S.G. & LEACH R.H. (1978) *Mycoplasma sualvi*, a new species from the intestinal and urogenital tracts of pigs. *Int. J. Syst. Bacteriol.* **28,** 289–292.

HORNER *et al.* (1993) Association of *Mycoplasma genitalium* with acute non-gonococcal urethritis. *Lancet* **342,** 582–585.

HU P.C., SCHAPER U., COLLIER A.M., CLYDE W.A.J., HORIKAWA M., HUANG Y.S. & BARILE M.F. (1987) A *Mycoplasma genitalium* protein resembling the *Mycoplasma pneumoniae* attachment protein. *Infect. Immun.* **55,** 1126–1131.

JENSEN J.S., ØRSUM R., DOHN B., ULDUM S., WORM A.-M. & LIND K. (1993) *Mycoplasma genitalium*: a cause of male urethritis? *Genitourin. Med.* **69,** 265–269.

JENSEN J.S., ULDUM S.A., SØNDERGÅRD-ANDERSEN J., VUUST J. & LIND K. (1991) Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *J. Clin. Microbiol.* **29,** 46–50.

LIND K. & KRISTENSEN G.B. (1987) Significance of antibodies to *Mycoplasma genitalium* in salpingitis. *Eur. J. Clin. Microbiol.* **6,** 205–207.

LIND K., LINDHARDT B.Ø., SCHÜTTEN H.J., BLOM J. & CHRISTIANSEN C. (1984) Serological cross-reactions between *Mycoplasma genitalium* and *Mycoplasma pneumoniae*. *J. Clin. Microbiol.* **20,** 1036–1043.

LO S.C., DAWSON M.S., WONG D.M., NEWTON P.B., SONODA M.A., ENGLER W.F., WANG R.Y., SHIH J.W., ALTER H.J. & WEAR D.J. (1989) Identification of *Mycoplasma incognitus* infection in patients with AIDS: an immunohistochemical, in situ hybridization and ultrastructural study. *Am. J. Trop. Med. Hyg.* **41,** 601–616.

LO S.C., HAYES M.M., TULLY J.G., WANG R.Y., KOTANI H., PIERCE P.F., ROSE D.L. & SHIH J.W. (1992) *Mycoplasma penetrans* sp. nov., from the urogenital tract of patients with AIDS. *Int. J. Syst. Bacteriol.* **42,** 357–364.

LOOMES L.M., UEMURA K., CHILDS R.A., PAULSON J.C., ROGERS G.N., SCUDDER P.R., MICHALSKI J.C., HOUNSELL E.F., TAYLOR-ROBINSON D. & FEIZI T. (1984) Erythrocyte receptors for *Mycoplasma pneumoniae* are sialylated oligosaccharides of Ii antigen type. *Nature* **307,** 560–563.

MØLLER B.R., TAYLOR-ROBINSON D. & FURR P.M. (1984) Serological evidence implicating *Mycoplasma genitalium* in pelvic inflammatory disease. *Lancet* **1,** 1102–1103.

PALMER H.M., GILROY C.B., CLAYDON E.J. & TAYLOR-ROBINSON D. (1991) Detection of *Mycoplasma genitalium* in the genitourinary tract of women by the polymerase chain reaction. *Int. J. STD. AIDS* **2,** 261–263.

SAIKI R.K., SCHARF S., FALOONA F., MULLIS K.B., HORN G.T., ERLICH H.A. & ARNHEIM N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230,** 1350–1354.

SILVERMAN D.J., SANTUCCI L.A., MEYERS N. & SEKEYOVA Z. (1993) Penetration of host cells by *Rickettsia rickettsii* appears to be mediated by a phospholipase of rickettsial origin. *Infect. Immun.* **60,** 2733–2740.

TAYLOR-ROBINSON D., DAVIES H.A., SARATHCHANDRA P. & FURR P.M. (1991) Intracellular location of mycoplasmas in cultured cells demonstrated by immunocytochemistry and electron microscopy. *Int. J. Exp. Pathol.* **72,** 705–714.

TAYLOR-ROBINSON D., FURR P.M. & HANNA N.F. (1985) Microbiological and serological study of non-gonococcal urethritis with special reference to *Mycoplasma genitalium*. *Genitourin. Med.* **61,** 319–324.

TULLY J.G., TAYLOR-ROBINSON D., COLE R.M. & ROSE D.L. (1981) A newly discovered mycoplasma in the human urogenital tract. *Lancet* **I,** 1288–1291.

TULLY J.G., TAYLOR-ROBINSON D., ROSE D.L., COLE R.M. & BOVE J.M. (1983) *Mycoplasma genitalium*, a new species from the human urogenital tract. *Int. J. Syst. Bacteriol.* **33,** 387–396.

TULLY J.G., WHITCOMB R.F., CLARK H.F. & WILLIAMSON D.L. (1977) Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. *Science* **195,** 892–894.