Association of *Haemophilus ducreyi* with cell-culture lines

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Summary. The association of *Haemophilus ducreyi* with epithelial cell cultures was studied by light microscopy, electronmicroscopy and viable counts. Associated organisms were engulfed by epithelial cells and sequestered from the cell-surface environment. Large numbers of organisms within epithelial cells appeared to induce cell lysis and release of *H. ducreyi*. Such a mechanism occurring *in vivo* may assist *H. ducreyi* to evade the bactericidal action of polymorphonuclear leucocytes and may explain some of the tissue damage seen in genital ulcers caused by *H. ducreyi*.

Introduction

Haemophilus ducreyi is a major cause of genital ulcers in the tropics. The organism causes localised cutaneous lesions that in some cases progress to involve inguinal lymph nodes and result in buboes. *H. ducreyi* has been isolated with increased frequency from clinical lesions^{1,2} now that suitably enriched media are available. Despite this increase in frequency of isolation, *c.* 30% of genital ulcers fail to yield *H. ducreyi*.³

In our *in-vivo* studies with a rabbit model of *H. ducreyi* infection organisms appeared to be susceptible to polymorphonuclear leucocytes (PMNLs) (unpublished data). It is not known how a lesion progresses to form an ulcer and cause tissue damage despite the lack of viable organisms, but it is possible that there is a mechanism by which *H. ducreyi* evades the bactericidal action of PMNLs and causes progressive infection. To investigate this possibility we have examined the association of *H. ducreyi* with cultured epithelial cell lines by light microscopy, electronmicroscopy and viable count studies.

Materials and methods

Bacterial strains

H. ducreyi isolates from cases of chancroid in the Gambia and from other geographical locations were

stored in heat-inactivated horse serum at -70°C . Strains were cultured on Columbia Agar Base (Oxoid) supplemented with defibrinated sheep blood (Tissue Culture Services) 10%, fetal calf serum 5% and Vitox (Oxoid) (HD medium).² Plates were incubated for 24 and 48 h at 33°C in the presence of CO_2 6% in a humidified atmosphere.

Preparation of inocula

Inocula were prepared by harvesting the 18-h growth of *H. ducreyi* from agar plates into phosphate-buffered saline (PBS), washing twice and resuspending in antibiotic-free tissue-culture growth medium (see below). After sonication for 5 s at 80 W to break up clumps and resuspend the organisms uniformly, the turbidity of suspensions was adjusted to *c.* 10^8 cfu/ml. In some experiments, inocula were heated for 30 min at 100°C to kill bacteria or sonicated with five 20-s bursts at 80 W to lyse bacterial cells.

Preparation and inoculation of cell culture monolayers

Tissue culture cells lines—Human cervical carcinoma cell line (HeLa, Flow Laboratories), Chinese Hamster Ovary (CHO), MRCC and C16⁴—were grown in Eagle's Minimum Essential Medium supplemented with fetal calf serum 10%, 2 mM glutamine, bicarbonate 0.04%, penicillin 100 IU/ml and streptomycin 100 µg/ml (TC medium) in 6-well cluster plates (Flow Laboratories). When cell monolayers were *c.* 90% confluent, TC medium was replaced with antibiotic-free TC medium after two washes with PBS. Monolayers were then inoculated with 20 µl of *H. ducreyi* suspension (10^8 cfu/ml).

For some experiments involving electronmicroscopy, HeLa cells were incubated for 18 h in antibiotic-free TC medium containing a 100-fold dilution of

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Thoria (PolySciences). Cells were washed three times in PBS to remove surface-associated Thoria, then incubated for 1 h at 37°C in PBSA followed by a further wash in PBS. Fresh antibiotic-free TC medium was added before inoculation with *H. ducreyi*. In experiments where HeLa cells were pre-treated with cytochalasin B, washed monolayers were incubated in antibiotic-free TC medium containing cytochalasin B (Sigma) 1.5 or 10 µg/ml, at 37°C for 30 min before inoculation with *H. ducreyi*.

Light microscopy

Cell lines inoculated with *H. ducreyi* were examined by light microscopy after incubation at 37°C for 18, 48 and 72 h. Viability of cell monolayers was assessed by light microscopy after washing twice in PBS and adding trypan blue 1% in distilled water. In some experiments, after 18 h inoculated cell monolayers were washed twice with PBS and fresh antibiotic-free TC medium was added. Cell monolayers were then incubated for a further 48 h or 72 h and then examined by light microscopy.

Electronmicroscopy

For transmission electronmicroscopy (TEM), all the cell lines, except HeLa cells, were fixed as monolayers after incubation with *H. ducreyi* for 18 h. HeLa cells were fixed after 0, 1, 2, 4, 18, 48 and 72 h. Cell monolayers were washed three times in PBS and fixed in glutaraldehyde 3% in 0.1 M sodium cacodylate (pH 7.4) containing ruthenium red 0.1% (CR buffer) for 2 h at room temperature. After washing three times with CR buffer, cells were post-fixed in osmium tetroxide 1% in CR buffer for 3 h at room temperature. Cells were scraped off the dishes and embedded in low-temperature gelling agarose (Sigma) 2%. Blocks were then cut, dehydrated through a graded series of ethanol, and then embedded in Spurr resin. Sections were cut on an LKBIII ultramicrotome and stained with uranyl acetate and lead citrate. The sections were examined in a JEOL 1200EX electron-microscope.

Viable counts

The number of viable *H. ducreyi* in suspension over, and associated with, HeLa cells was assessed immediately following inoculation (0 h) and after incubation for 1, 4, 18, 48 and 72 h. The TC medium over HeLa cells was aspirated and sonicated for 5 s at 80 W, and a 0.1-ml sample was serially diluted from 1 in 10 to 1 in 10⁸. A 20-µl sample of each dilution was seeded on to HD agar plates in duplicate to determine the number of organisms in suspension. To count cell-associated *H. ducreyi*, cell monolayers were washed

three times with PBS immediately after aspirating the TC medium and dispersed with 0.1 ml of trypsin 0.25% for 5 min at room temperature. The action of trypsin was neutralised by adding 1 ml of antibiotic-free TC medium. Cells were then pelleted by centrifugation at 13 000 *g* for 5 min, resuspended in 0.2 ml of antibiotic-free TC medium and sonicated at 80 W for 5 s. The suspensions were serially diluted as above and seeded on HD agar. All viable counts were determined after incubation for 48 h at 33°C.

The number of *H. ducreyi* in suspension over plastic and adherent to plastic were similarly determined in control cultures in unseeded tissue-culture dishes.

Results

Light microscopy

All four epithelial cell lines (HeLa, CHO, C16 and MRCC) showed a stippled appearance after incubation for 18 h with *H. ducreyi* (fig. 1). Monolayers were not uniformly affected. At 48 h, this effect was more pronounced but remained patchy. Cell lines MRCC and C16 showed a more rapid deterioration of the monolayers after inoculation. However, the trypan blue dye test showed that dye was excluded by all the adherent cells in the monolayer.

All isolates produced similar changes in the cell monolayers. There were variations in intensity of the

Fig. 1. (a) Stippling effect of *H. ducreyi* on HeLa cells seen at 18 h by light microscopy $\times 160$. (b) Control uninoculated HeLa cells.

stippling effect depending on the size of the inoculum. Inocula $> 10^6$ cfu/ml produced changes in monolayers discernible by light microscopy after incubation for 18 h; inocula $\leq 10^5$ cfu/ml required incubation for 48 h before similar changes were discernible. The stippling effect in a cell monolayer was sustained even after several washes and, on further overnight incubation with fresh TC medium, washed cell monolayers did not recover but the morphological changes continued to progress as in unwashed controls. With heat-killed *H. ducreyi*, 10^6 cfu/ml were required to effect the morphological changes in the cell monolayers after 48 h and the stippling effect was less pronounced than that produced by viable *H. ducreyi*. Furthermore, the changes in the monolayer were not progressive when the cells were washed and incubated in fresh TC medium. Sonicated *H. ducreyi* failed to produce any morphological changes in the monolayers.

Transmission electronmicroscopy (TEM)

Association of *H. ducreyi* with the cell cytoplasmic membrane and internalisation of bacterial cells was seen in all four epithelial cell lines examined. HeLa cells showed the maximum number of internalised organisms; therefore, the sequence of events leading to internalisation and the fate of the cells and organisms were studied in detail with HeLa cells. Single organisms or clumps of two or three organisms were seen associated with HeLa cells within 1 h. The outer membrane (OM) of the bacteria was closely associated with the plasma membrane of the HeLa cells (fig. 2a). After incubation for 2 h, invaginations of the plasma membrane close to bacteria were observed, and by 4 h the process of engulfment was usually complete (fig. 2b). Between one and three organisms were seen in membrane-bound vesicles in *c.* 10% of the cells examined.

Internalisation of bacteria was judged by the exclusion of ruthenium red stain from both the OM of the internalised bacteria and the innermost membrane of the phagocytic vacuole, compared with the stained cell plasma membrane and the stained OM of extracellular bacteria (fig. 2c). An increase in both the number of internalised organisms and the number of cells containing organisms was seen after incubation for 18 h and 48 h. In cells containing a large number of internalised *H. ducreyi*, a discernible vacuolar membrane was evident only in short tracts because of the plane of the ultrathin section. By 72 h, most of the cells were full of organisms, with many cells showing lysis and consequent release of bacteria (fig. 2d).

Electronmicroscopy of HeLa cells incubated with thoria-containing TC medium showed thoria-labelled primary and secondary lysosomes; at 48 h, *c.* 50% of internalised *H. ducreyi* had associated thoria.

Electronmicroscopy of HeLa cells treated with cytochalasin B 2 μ g/ml showed uptake of *H. ducreyi* similar to untreated controls; with cytochalasin B

10 μ g/ml the number of internalised *H. ducreyi* was significantly reduced but many of the cells examined by TEM still had internalised organisms (2–3 organisms/cell at 18 h).

Viable counts

Viable counts of *H. ducreyi* in suspensions over HeLa cells or plastic, and associated with cells or adherent to plastic, are shown in fig. 3. The counts are an average of two experiments which showed good reproducibility. *H. ducreyi* remained viable up to 18 h in TC medium but no viable organisms were retrieved after 48 h in this non-cell system. Approximately 33% of the inoculum adhered to plastic within 1 h and the number of viable *H. ducreyi* adherent to plastic remained constant up to 18 h. Approximately 10^5 cfu of *H. ducreyi*/ml were present in TC medium over HeLa cells for up to 48 h; counts were slightly reduced at 72 h. The number of *H. ducreyi* associated with HeLa cells was similar to the number adherent to plastic at 1 h but at 18 h the viable count of cell-associated *H. ducreyi* increased sharply to *c.* 10^9 cfu/ml; these counts fell slightly at 48 h but were still around 10^5 cfu/ml at 72 h.

Discussion

This study showed that *H. ducreyi* can associate with the epithelial cell culture lines HeLa, CHO, C16 and MRCC. The association of a large number of *H. ducreyi* with the cell surface produced a stippling effect on the cell monolayers evident by light microscopy after incubation for 18 h with *H. ducreyi*. The cell-associated organisms were able to withstand several washes, indicating that there was a strong interaction with epithelial cells. The TEM findings confirmed the close association of the OM of *H. ducreyi* and the plasma membrane of the epithelial cells. TEM also showed that organisms became internalised in membrane-bound vacuoles. The vacuolar membranes of these phagosomes were most evident in preparations incubated for 4 h when only a few *H. ducreyi* cells were internalised. Vacuolar membranes were not easily discernible in cells which were packed with large numbers of *H. ducreyi* as seen at 18 and 48 h. The organisms were sequestered into the phagosomes away from the cell-surface environment as indicated by exclusion of ruthenium red. The labelling of primary and secondary lysosomes of HeLa cells with thoria revealed that not all internalised *H. ducreyi* were associated with cell lysosomal contents. A similar observation has been made with internalised toxoplasma.⁵ It seems likely, therefore, that some phagosomes fuse with cellular lysosomes to form phagolysosomes and expose *H. ducreyi* to the bactericidal action of lysosomal enzymes, whilst *H. ducreyi* cells in unfused phagosomes or embedded

Fig. 2. Association of *H. ducreyi* with HeLa cells shown by electronmicroscopy. (a) After incubation for 1 h: the ultrathin section was tilted 22° to eliminate superimposition and reveal the OM of the bacteria and the plasma membrane of the HeLa cell in close association; the plasma membrane beneath the arrowed bacterium shows slight invagination (bar, 0.5 µm). (b) After incubation for 4 h: a large invagination is seen containing many organisms (bar, 1 µm). (c) After incubation for 18 h: a phagosome containing *H. ducreyi*; the vacuolar membrane is clearly visible (▶) and the organisms are shown to be internalised as ruthenium red has been excluded; two organisms (↔) have ruthenium red on the outer membrane indicating incomplete internalisation (bar, 0.5 µm). (d) After incubation for 72 h: the HeLa cell is ruptured and organisms are released from large intracellular vesicles; most of the organisms appear to be healthy (bar, 5 µm).

Fig. 3. Viable counts of *H. ducreyi* associated with HeLa cells (●—●), in suspension over HeLa cells (■—■), in suspension over plastic (○—○) and adherent to plastic (□—□). Results shown represent combined data from two separate experiments.

in the folds of the HeLa cell membrane may multiply freely.

In viable count studies, increased numbers of viable

H. ducreyi were found only in association with tissue-culture cells. TC medium alone did not support the growth of *H. ducreyi*. At 48 h there was a 10² reduction in numbers of viable bacteria which may indicate some intracellular killing. This is supported by the presence of phagolysosomes in infected cells in the TEM studies. Despite the cell lysis seen by TEM at 72 h, cell-associated viable counts were maintained, perhaps because many "released" *H. ducreyi* cells remained cell-associated (fig. 3).

Heat-killed organisms were also internalised into HeLa cells, but the number of organisms seen to be internalised were few and the sequence of events did not progress as with viable *H. ducreyi*. HeLa cells treated with cytochalasin B still contained *H. ducreyi* cells, suggesting that the process of internalisation of *H. ducreyi* is not entirely a function of HeLa cells. The possibility that a heat-stable factor produced by *H. ducreyi* may be involved, as has been found with shigellae,^{6,7} requires further investigation. We have shown that internalisation of *H. ducreyi* into tissue-culture cells appears to be a combined function of both the HeLa cells and *H. ducreyi*. Similar mechanisms of internalisation *in vivo* would protect *H. ducreyi* from the rapid bactericidal action of PMNL and may also explain some of the tissue damage seen *in vivo*.

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References

1. Nsanze H, Fast MV, D'Costa LJ, Tukei P, Curran J, Ronald A. Genital ulcers in Kenya; clinical and laboratory study. *Br J Vener Dis* 1981; **57**: 378-81.
 2. Wall RA, Mabey DCW. *Haemophilus ducreyi* and genital ulcers. *Lancet* 1983; **2**: 1302-1303.
 3. Plummer PA, D'Costa LJ, Nsanze H *et al.* Antimicrobial therapy of chancroid: effectiveness of erythromycin. *J Infect Dis* 1983; **148**: 726-731.
 4. Phillips RJ. Clones of MRC-C cells may be superior to the parent line for the culture of 229E-like strains of human respiratory coronavirus. *J Virol Methods* 1983; **6**: 267-269.
 5. Jones T, Hirsch JG. The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. *J Exp Med* 1972; **136**: 1173-1194.
 6. Hale TL, Bonventre PF. Shigella infection of Henle intestinal epithelial cells: role of the bacterium. *Infect Immun* 1979; **24**: 879-886.
 7. Hale TL, Morris RE, Bonventre PF. Shigella infection of Henle intestinal epithelial cells: role of the host cell. *Infect Immun* 1979; **24**: 887-894.
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