Cytopathic effect of *Haemophilus ducreyi* for human foreskin cell culture

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Summary. An explant adult foreskin cell culture (FS2-3) was compared with human lung carcinoma cell culture (A549) with regard to the ability of *Haemophilus ducreyi* to produce a cytopathic effect. The survival of *H. ducreyi* for up to 26 days in FS2-3 cells was far greater than in any previously described in-vitro culture system. *H. ducreyi* survived for up to 7 days in A549 cells. The *H. ducreyi* cells grew and formed “fungal-like” microcolonies on the eukaryotic monolayer. Portions of the microcolonies remained attached despite extensive washing. Transmission electronmicroscopy indicated that, at 48 h after infection, the *H. ducreyi* cells did not penetrate the FS2-3 cells but they were closely associated with them; there was only a 2–5 nm gap between the *H. ducreyi* cell wall and the FS2-3 membrane. The virulent *H. ducreyi* strains RO18 and 35000 produced a cytopathic effect on FS2-3 cells that did not appear to be due to a soluble toxin. These strains did not produce any CPE on A549 cells. *H. influenzae* and the avirulent *H. ducreyi* strain CIP542, inoculated in the same concentration and incubated for the same length of time, did not produce CPE on FS2-3 cells. This study demonstrated that the use of FS2-3 foreskin cell culture provided an in-vitro approach for evaluating the cytopathic effect of virulent *H. ducreyi* whereby, unlike in other in-vitro systems, viability of the micro-organism could be readily maintained.

Introduction

Haemophilus ducreyi is the causal agent of chancroid. Although *H. ducreyi* was described over 100 years ago, little is known about its pathogenicity. Clinically, chancroid is characterised by a genital ulcer that, if untreated, can result in inguinal lymph node involvement, leading to bubo formation and supuration. As reviewed by Albritton,¹ it is assumed that *H. ducreyi* gains entry through breaks in the epithelial layer, but nothing is known about its attachment or localisation mechanisms or the cytopathic capacity that ultimately leads to ulcer formation. A rabbit model for chancroid was introduced by Reenstierna,² but in this system, a large inoculum of 10^8 organisms^{3,4} was required to produce a lesion and reproducibility was frequently a problem. Heat-killed *H. ducreyi* did not produce lesions in rabbits.³ With this model, avirulent strains CIP542 and A77 have been recognised.⁴ Recently, a CBA mouse model was described by Tuffrey *et al.*,⁵ in which ulcers similar to those observed in man were produced consistently, although, again, the inoculum required was large (10^7 organisms). Both heat-killed and viable organisms could produce ulcers. Histology of these lesions indicated that the characteristic histology of chancroid that was observed in man⁶ was also apparent in the

mouse lesions.⁷ *H. ducreyi* does not survive for long in either the rabbit or the mouse model and the lesions heal spontaneously.^{4,5,7} Neither of these animal models was designed for attachment or cytotoxic studies. There have been no published descriptions of cell-culture models for *H. ducreyi* infection.

The primary aim of this study was to determine whether *H. ducreyi* could grow in human lung carcinoma (A549) or adult foreskin explant (FS2-3) cell cultures and to determine whether any specific cytopathic effect was produced in either system.

Materials and methods

Bacterial strains

All *Haemophilus* isolates were maintained as frozen stocks at -70°C in skimmed milk and all subcultures were on chocolate agar incubated at 35°C in CO_2 5%:air 95% with high humidity. The test strains included *H. influenzae* type b ATCC 10211 and *H. ducreyi* clinical isolates 35000 (Winnipeg strain), RO18, RO27 (Kenyan strains) and CIP542 (Institut Pasteur).

Cell culture

The eukaryotic cell cultures evaluated were A549—a transformed cell line derived from a human lung

carcinoma (ATCC CCL21)—and FS2-3—a primary, non-transformed cell line derived from adult foreskin. The FS2-3 adult foreskin cell culture was prepared by scraping the mucosal layer of an adult foreskin (obtained from an elective circumcision) into phosphate-buffered saline (PBS). The mucosal cells were collected by centrifugation at 500 *g* and then trypsinised for approximately 10 min. The resulting cell suspension was washed with RPMI medium containing penicillin 2×10^6 IU/L and streptomycin 2000 mg/L and then seeded into 25-cm³ tissue-culture flasks containing 5 ml of RPMI culture medium. The resulting cell culture consisted mainly of fibroblasts. All experiments were performed on cultures between passage numbers 5 and 15.

The cell cultures were maintained as frozen stocks at -150°C in cell-culture media containing fetal bovine serum 20% v/v and dimethyl sulphoxide 10% v/v (DMSO). The cell lines were passaged in RPMI medium consisting of: RPMI (1640) base (Gibco) supplemented with 0.4 mM L-glutamine, 0.2 mM sodium pyruvate, fetal bovine serum (Gibco) 10% v/v, penicillin 1×10^6 IU/L and streptomycin 1000 mg/L. Cell cultures were incubated at 37°C in CO₂ 5%:air 95% in a water-saturated incubator. The 96-well trays used for experiments were seeded with 3×10^4 eukaryotic cells/well in antibiotic-free RPMI medium. For tests of the survival of bacteria in broth medium, Mueller-Hinton Broth supplemented with Isovitalex (MHI; Becton Dickinson and Co., Cockeysville, MD, USA) was used.

Quantitation of *H. ducreyi*

At appropriate intervals, the supernatant medium was aspirated by pipette and the cell monolayer was scraped to suspend it in the tissue culture medium. The resulting cell suspension was sonicated for *c.* 5 s, and then pipetted with an Eppendorf micropipette to disperse the cells. Examination by light microscopy revealed that the eukaryotic cells were adequately dispersed by this procedure. The resultant suspension was diluted serially in 10-fold steps in tissue culture medium and *H. ducreyi* cfus were counted by the spread-plate method on chocolate agar.

Transmission electronmicroscopy (TEM)

FS2-3 cells were seeded on to Metricell GN-6 membrane filters (Gelman Sciences Inc., Ann Arbor, MI, USA) in 24-well trays at a concentration of 1×10^5 cells/well. The cells were incubated for 2 days and then infected with *H. ducreyi* at a multiplicity of infection (MOI) of approximately 10:1. After incubation for 2 days at 37°C , the monolayers grown on the filters were pre-fixed by addition of glutaraldehyde (Eastman Kodak Co., Toronto, Ontario, Canada) to the tissue culture medium to a final concentration of 2% v/v. The monolayers were fixed overnight and then given three 5-min washes in 0.1 M sodium cacodylate, 0.01 M

calcium chloride (Fisher Scientific Co., Fairlawn, NJ, USA) pH 7.4 (rinse buffer). Freshly prepared osmium tetroxide (Polyscience Inc., Warrington, PA, USA) 1% w/v in rinse buffer was used to post-fix the monolayer by incubation at 4°C for 30 min. The monolayer was washed three times, each for 5 min with rinse buffer. To increase the rigidity of the membranes, the monolayer was overlaid with agarose 3% v/v in rinse buffer.

The agarose-coated monolayer was processed by standard TEM procedures through graded series of acetone, propylene oxide (Polysciences Inc.) and JEM Bed 812 embedding medium (J.B. EM Services, Dorval, Quebec, Canada). Strips of the membrane were placed in embedding medium and polymerisation was achieved by incubating at 37°C overnight, then at 45°C for 10 h, followed by 60°C for 18 h. Thin sections were prepared and stained for 10 min with saturated uranyl acetate (Polysciences Inc.) 50% v/v in ethanol and post-stained for 5 min with lead citrate (Polaron Equipment Inc., Watford) 0.25% in 0.1 N NaOH. The sections were viewed with a Philips model 201 electron-microscope, at an acceleration voltage of 60 000 eV.

Results

To determine how well *H. ducreyi* survived in the cell cultures, compared with survival in broth medium alone, organisms were seeded into 96-well trays containing either 10^4 eukaryotic cells/well or broth (RPMI or MHI) only. At various times after infection, the number of viable organisms (cfu/ml) was assessed by the spread-plate technique. Each value recorded represented the mean of three replicate counts. *H. ducreyi* died within 4 days when inoculated into MHI broth (fig. 1). Although survival was better (up to 12 days) in tissue culture medium containing FBS 10%, *H. ducreyi* survived for extended periods (26 days) when growing with FS2-3 cells (fig. 1). The cell lines were infected separately with *H. ducreyi* strains 35000, RO27, CIP542 and RO18, and *H. influenzae* type b and the infected monolayers were monitored by light microscopy to assess visible growth of the bacteria and any apparent cytopathic effect (CPE). On both FS2-3 and A549 cell culture, the *H. ducreyi* strains grew as branching fungal-like colonies, unlike *H. influenzae* type b that grew as a uniform cell suspension with no apparent microcolony formation. The large complex microcolonies of *H. ducreyi* (fig. 2) were observed only when eukaryotic cells were present. The microcolonies appeared to associate with or adhere preferentially to eukaryotic cells. When an MOI of 10:1 was used, a CPE was detected by day 3. This CPE was apparent with *H. ducreyi* strains RO18, RO27 and 35000. Fig. 3 (G,H) shows the typical CPE on FS2-3 cells detected at days 3 and 7 after infection. There were no apparent differences; therefore, only the RO27-infected cell culture has been shown. On day 1, all infected monolayers were unaltered and were similar to the

Fig. 1. Survival of *Haemophilus* spp. in cell culture and broth medium. Cell lines A549 (—△—) and FS2-3 (—○—) were seeded (3×10^4 cells/well) in 96-well trays and incubated at 37°C in CO₂ 5%:air 95% for 24 h. MHI (—●—) and tissue culture medium (—■—) was dispensed into wells with no eukaryotic cells. The monolayers and broths were inoculated with *H. ducreyi* CIP542 1×10^5 cfu/ml (A), *H. ducreyi* RO18 9×10^4 cfu/ml, (B) *H. ducreyi* 35000 3×10^5 cfu/ml (C) and *H. influenzae* 3×10^5 cfu/ml (D). Samples were removed at the indicated times after infection and the numbers of viable *Haemophilus* cells were evaluated. Each point represents the average from triplicate tests.

uninfected controls. The CPE of *H. ducreyi* for adult foreskin cells was species-specific; inoculation of *H. influenzae* at the same MOI produced no detectable CPE on FS2-3 cells within the same time period (fig. 4C, D). The avirulent *H. ducreyi* CIP542 produced no detectable CPE on FS2-3 cells at days 3 or 7 after infection (fig. 3; E, F). If a higher inoculum was used to achieve an MOI of 500:1, *H. ducreyi* CIP542 could

produce a weak CPE. Tissue specificity was also apparent, because the CPE produced by *H. ducreyi* isolates on A549 cells (fig. 4) was not as dramatic as that observed with FS2-3 cells (fig. 3), whereas *H. influenzae* produced no CPE on FS2-3 cells, but had a significant CPE on A549 cells.

Transmission electronmicroscopy (TEM) was used to determine whether *H. ducreyi* invaded the foreskin

Fig. 2. Morphology of *H. ducreyi* microcolonies grown in foreskin cell culture. Flasks of FS2-3 cells were seeded with *H. ducreyi* RO18 3×10^5 cfu/ml. The flasks were incubated at 37°C in CO₂ 5%:air 95% for 24 h. The monolayers were viewed by light microscopy. Bar, 50 µm.

Fig. 3. Effect of *H. influenzae* and *H. ducreyi* on FS2-3 cells. Monolayers in 96-well tissue culture trays (3×10^4 cells/well) were uninfected (A, B) or infected with *H. influenzae* type b (C, D), *H. ducreyi* CIP542 (E, F) or *H. ducreyi* RO27 (G, H) at 3×10^6 bacterial/well. The monolayers were incubated at 37°C in CO_2 5% : air 95% for 3 days (A, C, E, G) or 7 days (B, D, F, H) and then stained with Coomassie Blue in methanol 20% v/v, acetic acid 10% v/v for 15 min. Excess stain was washed off and the monolayers were viewed by light microscopy. Bar, 100 μm .

cells *in vitro*. Two days after infection, *H. ducreyi* cells had not become intracellular, although there was pronounced intracellular vacuolisation (fig. 5). The microcolony formation noted by light microscopy proved to be closely associated with the cell membrane of FS2-3 cells (fig. 5). To ensure optimal structural integrity, the infected monolayers used for TEM were not washed before fixation. However, subsequent experiments with light microscopy confirmed that portions of the microcolonies remained attached to the

eukaryotic cells despite extensive washing (results not shown). Inter cellular adhesion of *H. ducreyi* as well as electron-dense areas in the cytoplasm were apparent (fig. 6). Close interaction of the *H. ducreyi* cell wall and the FS2-3 membrane indicate that attachment does occur (fig. 6). Indeed, the gap of 2–5 nm between the prokaryote and eukaryote membranes is similar to the gap between prokaryotic cells bound to one another (fig. 6).

To determine whether a soluble toxin was respon-

Fig. 4. Effect of *H. influenzae* and *H. ducreyi* on A549 cells. Monolayers in 96-well tissue culture trays (3×10^4 cells/well) were uninfected (A, B) or inoculated with *H. influenzae* type b (C, D), *H. ducreyi* CIP542 (E, F) or *H. ducreyi* RO27 (G, H) at 3×10^6 bacteria/well. The monolayers were incubated at 37°C in CO₂ 5%:air 95% for 3 days (A, C, E, G) or 7 days (B, D, F, H) and then stained with Coomassie Blue in methanol 20% v/v, acetic acid 10% v/v for 15 min. Excess stain was washed off and the monolayers were viewed by light microscopy. Bar, 50 µm.

sible for the CPE observed, a suspension of *H. ducreyi* RO27 was placed in a tissue-culture insert (Anocell, England) that allowed free diffusion of media, but had a pore size of 0.2 µm, which prevented the bacteria from passing through to the cell-culture monolayer growing on the well beneath the insert. Even when a large inoculum of *H. ducreyi* RO27 (10^8 /well) was used, no CPE was detected for FS2-3 cells when the membrane insert, separating the bacteria from the monolayer, was present.

Discussion

The results of this study demonstrate that FS2-3 is a potentially valuable in-vitro cell-culture model for evaluating the interaction of *H. ducreyi* with eukaryotic cells. *H. ducreyi* strains survived for extended lengths of time only if eukaryotic cells were also present. Indeed, survival for at least 26 days after infection (fig. 1) (when the experiment was stopped) is far longer than the survival of 6 days noted in mice^{5,8}

Fig. 5. Transmission electronmicroscopy of FS2-3 cells infected with *H. ducreyi*. Monolayers of FS2-3 foreskin cells were grown on Metricell membranes. The monolayers were infected with *H. ducreyi* RO18 5×10^6 /membrane (an MOI of 5:1). After incubation for 48 h, the uninfected (A) and infected (B, C) monolayers were fixed and then processed for transmission electronmicroscopy. The *H. ducreyi* cells have intracellular electron-dense areas (small arrow).

or 72 hours in rabbits.³ This was to be expected, because there were no host-defence mechanisms present to eradicate the bacteria. In this respect, use of the foreskin-cell model would allow an assessment of cytotoxic effect independent of host factors. Although the viable counts of all three strains of *H. ducreyi* tested in the foreskin cell model (fig. 1) indicated only minimal growth of organisms (approximately a 10-fold increase), in comparison with that of *H. influenzae* (approximately a $\log_{10} 3$ increase), this may represent an underestimation, because *H. ducreyi* grew as microcolonies (fig. 2) that were difficult to disperse into single-cell suspensions. Examination by light microscopy showed that the number and size of the microcolonies did increase with time. The microcolony formation is probably a result of cell-to-cell adhesion (figs. 5, 6), intercellular adherence of *H. ducreyi* cells having been described.⁹ It is possible that this type of growth, which has also been observed *in vivo*, represents a virulence factor that prevents phagocytosis by macrophages or polymorphonuclear cells, thereby allowing *H. ducreyi* to proliferate.

Preliminary experiments demonstrated that, despite extensive washing, portions of the *H. ducreyi* microcolonies remained attached to the monolayer (as assessed by light microscopy). Adherence of *H. ducreyi* to erythrocytes has been shown to correlate to hydrophobicity.¹⁰ The role of hydrophobic interactions was not assessed in our evaluation. The monolayers used for TEM were not extensively washed before fixation.

This was done to ensure structural integrity of the microcolonies. *H. ducreyi* cells that were closely associated with the FS2-3 membrane were apparent. However, since special stains such as ruthenium red were not used, it is not possible to determine whether the matrix observed between the FS2-3 membrane and the *H. ducreyi* cell wall (fig. 6) contains material that may be involved in binding. However, it is apparent that *H. ducreyi* was not internalised (fig. 5). This is consistent with histological analysis of chancroid ulcers, in which the organism has been observed only as extracellular clumps.⁶ This tissue interaction of *H. ducreyi* is distinct from that described by Roberts *et al.*¹¹ for *H. influenzae*. Evaluations with monkey respiratory tissue demonstrated that *H. influenzae* was rapidly (180 min after infection) internalised.¹¹ Our TEM studies evaluated *H. ducreyi* internalisation at 48 h after infection; therefore, other intervals need to be evaluated to determine conclusively that internalisation does not occur.

The mechanism of ulcer formation in the mouse model has not been totally defined, but Tuffrey *et al.*⁷ demonstrated that viable organisms were not required and that lipopolysaccharide may be the crucial factor responsible for ulcer formation. *H. ducreyi* survives for long periods of time in man, but not in the current animal models. It is possible that there are factors that contribute to ulcer formation in man that cannot be detected in the mouse model because the organisms do not survive sufficiently long after intradermal injection

Fig. 6. Transmission electronmicroscopy of *H. ducreyi* RO18 cells grown in FS2-3 foreskin explant culture. Monolayers of FS2-3 foreskin cells were grown on Metricell membranes. *H. ducreyi* RO18 was inoculated at 5×10^8 bacteria/membrane (an MOI of 5:1). After incubation for 48 h, the monolayers were fixed and then processed for transmission electronmicroscopy. The *H. ducreyi* cells have tight intercellular adhesion (→) as well as close association with the FS2-3 membrane (→).

in mice. The human foreskin cell-culture model provides a method for assessing cytotoxicity in a more direct manner than do animal models. The results of this study show that the CPE produced by virulent *H. ducreyi* strains on FS2-3 cells required contact of the bacteria with eukaryotic cells and did not appear to be due to a diffusible toxin. How *H. ducreyi* caused the vacuolisation of FS2-3 remains to be elucidated. The factors responsible for the relative avirulence of *H. ducreyi* CIP542 are not known.

In summary, the FS2-3 foreskin cell culture model described in this paper provides an alternative to the animal models described previously.^{4,7} The greatest advantage of the FS2-3 in-vitro model was the distinct

CPE produced by virulent strains of *H. ducreyi* that was not apparent with either avirulent strains of *H. ducreyi* or other species such as *H. influenzae* or *H. parainfluenzae*. The foreskin cell culture model thus provides a means for studying the cytotoxicity of *H. ducreyi*, which in turn may help define the pathogenesis of chancroid.

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