

MICROBIAL PATHOGENICITY

The effect of temperature on the interaction of *Haemophilus ducreyi* with human epithelial cells

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To investigate if temperature affects the interaction of *Haemophilus ducreyi* with human epithelial cells, nine strains were used to evaluate the adhesion kinetics of the organism at 33°C and 37°C. The effect of the free toxin on the epithelial cells at those temperatures was also assessed. The cyto-adherence kinetics of *H. ducreyi* to the epithelial cells was significantly greater at 33°C (10 times more) than at 37°C in all seven clinical isolates tested. There was a significant difference in cell-associated *H. ducreyi* at 33°C as compared with 37°C. Control strains showed similar adhesion properties at both temperatures. However, the virulent strain CIP542 adhered in larger amounts than the avirulent strain A77. Electron microscopy revealed that there was more tissue necrosis at the lower than the higher temperature. The effect of the free toxin was the same at each temperature. However, strain A77 had significantly lower toxicity than strain CIP542 and the clinical isolates. These results suggest that *H. ducreyi* displays a temperature-dependent interaction with human epithelial cells, and this feature may play a role in the virulence of the organism *in vivo*. While the overall toxic effect of viable bacteria depends on the metabolic activity of the bacteria and is, therefore, higher at 33°C than at 37°C with the same initial inoculum, the effect of the extracted toxin at molecular level with fixed concentrations is a temperature-independent event.

Introduction

Haemophilus ducreyi is the aetiological agent of chancroid, a sexually transmitted genital ulcer disease prevalent in developing countries [1, 2]. Chancroid has been associated with an increased risk for the acquisition and transmission of HIV in countries with a high prevalence of HIV infection [2–4].

H. ducreyi is strictly a human pathogen that primarily infects the skin. There is limited knowledge about the mechanisms by which this organism causes disease. Successful colonisation of a host by bacterial pathogens depends on the ability of the pathogen to interact with host cells. This is a complex event involving a pathogen-encoded ligand and a eukaryotic receptor [5]. A microbe has a choice of three types of host components with which it can interact: secreted cell products, host cell surfaces or extracellular matrices [6]. Adherence of *H. ducreyi* to host cell surfaces has been widely reported [6–9]. However, these studies

examined the interaction of the organism with these cells at temperatures of 35–37°C [4, 8, 10–12]. Interaction of a pathogen with host cells may depend on environmental factors such as temperature. *H. ducreyi* interaction with host cells *in vivo* is mainly with the skin, an organ of lower temperature (*c.* 33°C). This is also the optimal incubation temperature for the *H. ducreyi in vitro* [13, 14]. Therefore, temperature may play a role in the expression of virulence determinants of *H. ducreyi*. It was postulated that expression of attachment and virulence that affects keratinocytes may be upregulated at 33°C, because this is both the temperature of the host at the common site of infection and the optimal growth temperature for *H. ducreyi*. To test this hypothesis, this study compared the interaction of *H. ducreyi* with epithelial cells at the optimal *H. ducreyi* growth temperature of 33°C and the core body temperature of 37°C.

Materials and methods

Bacterial strains and inoculum preparation

Nine *H. ducreyi* strains were used; seven were clinical isolates from Durban, South Africa: SA26, SA42, SA60, SA63, SA68, SA71 and SA77. Reference strains

A77 and CIP542 served as avirulent and virulent controls, respectively (A. W. Sturm, unpublished data). The strains were cultivated on Modified Bieling (MB) plates containing Isovitalax 1% or yeast dialysate 2% [13]. The plates were incubated at 33°C in micro-aerobic conditions for 48 h. The purity of *H. ducreyi* was confirmed by colonial morphology and Gram's stain.

For preparation of the inocula, bacteria were harvested from plates and washed three times in phosphate-buffered saline (PBS). The suspension was vortex mixed and passed 10–15 times through 25-gauge needles to further break down large clumps. The suspension was allowed to stand for 1 h for larger clumps to settle by gravity. The supernate was aspirated and the OD was adjusted to 1.0 at 600 nm (Glock and Sturm, unpublished observations). Colony forming units (cfu) were estimated by plating out serial dilutions from the adjusted supernate.

Preparation of sonicates

H. ducreyi was scraped off four plates and suspended in 10 ml of EMEM (*c.* 10⁴cfu/ml), then washed three times with PBS by centrifugation at 1200 rpm for 5 min. The pellet was resuspended in EMEM and sonicated with a Branson sonicator at 60% duty cycle for 5 min with 1-min cycles. The sonicate was centrifuged to remove the debris. The supernate was filtered through a 0.22- μ m pore size filter. The sonicates were stored at -20°C and diluted 1 in 4 with EMEM when required for experiments.

Tissue-culture systems

The HaCaT keratinocytes (provided by Professor Füssenig of the German Cancer Research Centre, Heidelberg) and HEC-1-A (ATCC HTB111) were used for attachment assay and cytotoxicity studies. HEC-1-A cells were cultivated in McCoy's 5a medium supplemented with fetal calf serum (FCS; Delta Bioproducts, Kempton Park, South Africa) 5%, non-essential amino acids (NEAA, BioWhittaker, MD, USA) 1%, penicillin 100 U/ml, streptomycin (BioWhittaker) 100 μ g/ml and amphotericin B 5 mg/ml. HaCaT cells were cultivated in EMEM supplemented with FCS 5 or 10% and other supplements similar to those used for HEC-1-A cells. The cell cultures were incubated at 37°C in humidified air with CO₂ 5%. Each well of 24-well cluster plates (Corning Glass Works, NY, USA) was seeded with 1 \times 10⁵ cells and incubated for 24 h before use in attachment studies. Cell viability was established by the trypan blue exclusion method. The number of cells/monolayer before infection was estimated at 3 \times 10⁵ (mean of three estimations) at 33°C and 37°C respectively. The cells were washed thoroughly with PBS and the medium was replaced with antibiotic-free medium before use in experiments.

Adhesion assay

This assay was performed by the method described by Lagergård *et al.* [15] with slight modifications. Briefly, 250 μ l of *H. ducreyi* suspension in EMEM-FCS were added to each well to yield a multiplicity of infection (MOI) of 10 bacteria/eukaryotic cell. The inoculated monolayers were incubated in air with CO₂ 5% at 33°C and 37°C. Tests were performed at 2, 5, 12, 16 and 24 h after inoculation. At the designated times, the infected monolayers were washed five times in PBS to remove unbound bacteria. Distilled water (1 ml) was added and the plates were placed on a platform shaker with gentle shaking for 5–10 min to release both internalised and adherent bacteria (total bacterial count). Two-fold serial dilutions were made from the suspension. Portions (20 μ l) of the dilutions were plated out in duplicate on MB plates and colony counts were done after incubation for 3–4 days. Internalised bacteria were counted by the amikacin protection/invasion assay. Briefly, after removal of non-adherent bacteria by washing in PBS, 1.5 ml of amikacin (30 μ g/ml in PBS) was added to each well. After 2 h, the antibiotic was removed by washing in PBS (five times) and the cells were lysed with distilled water to release intracellular bacteria. This was followed by plating out on MB agar and the numbers of cfu were counted after incubation as described above. The adherent bacteria were assessed from the equation:

$$\text{number of adherent bacteria} = \frac{\text{total number of bacteria} - \text{internalised bacteria}}{\text{total number of bacteria}}$$

Samples were fixed in glutaraldehyde 1% in EMEM-FCS for transmission electron microscopy (TEM). After removal of the fixative, the monolayers were washed and placed in EMEM-FCS and held at 4°C before TEM processing.

Cytotoxicity assay

Epithelial monolayers were prepared by adding 10⁴ cells to each well of a 96-well flat-bottomed micro-titration plate (Greiner Labortechnik, Frickenhausen, Germany). Portions of the sonicate (50 μ l) were added to each well containing 200 μ l of antibiotic-free medium. The monolayers were exposed to the crude toxin or viable bacteria for 60 h, followed by aspiration of the medium and washing of the monolayers with PBS. The medium was replaced with 50 μ l of MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide) (Sigma, Pinetown, South Africa) at a concentration of 5 mg/ml in EMEM-FCS and incubated for a further 4 h at 37°C. This was followed by addition of 100 μ l of the lysin reagent, which consisted of SDS:N,N-dimethylformamide (DMF) 1:1. After overnight incubation at 37°C, the OD was measured at 570 nm with an ELISA reader, employing the lysin reagent as the blank.

TEM

Cells were grown on tissue-culture slides (Nunc, Illinois, USA). Briefly, 250–300 μl of 10^4 cells/ml were seeded in each well of the chamber 24–48 h before infection with *H. ducreyi*. Portions (50 μl) of the previously prepared inoculum were added to each of the wells and incubated for the same time intervals as above. The previously fixed samples were retrieved from the refrigerator and washed again before further processing. Samples were treated with osmium tetroxide 1% at 4°C for 1 h, washed four times with PBS, then dehydrated through a graded series of ethanol and embedded in Spurr resin (Sigma). Ultra-thin sections were cut and double-stained with uranyl acetate and lead citrate. Viewing was done with the JEOL 1010 transmission electron microscope with an accelerating voltage of 60–80 kV. Photographs were taken on Ilford fine grain plate film.

Statistical analysis

Student's *t* test was applied; *p* values were considered significant if they were < 0.05 . Duncan's multiple range test [16] was applied to determine the similarities between strains.

Results

Adherence studies

The adherence of the control strains to the cells is shown in Fig. 1. Both showed similar adhesion kinetics at 33°C and 37°C. Adherence was rapid, reaching a peak at 16 h for strain A77. Strains A77 and CIP542 showed different adhesion kinetics, with a significantly lower adherence of strain A77 at both temperatures. No peak was reached within 24 h at either temperature with strain CIP542. The adherence kinetics of the seven clinical isolates are summarised in Fig. 2. There

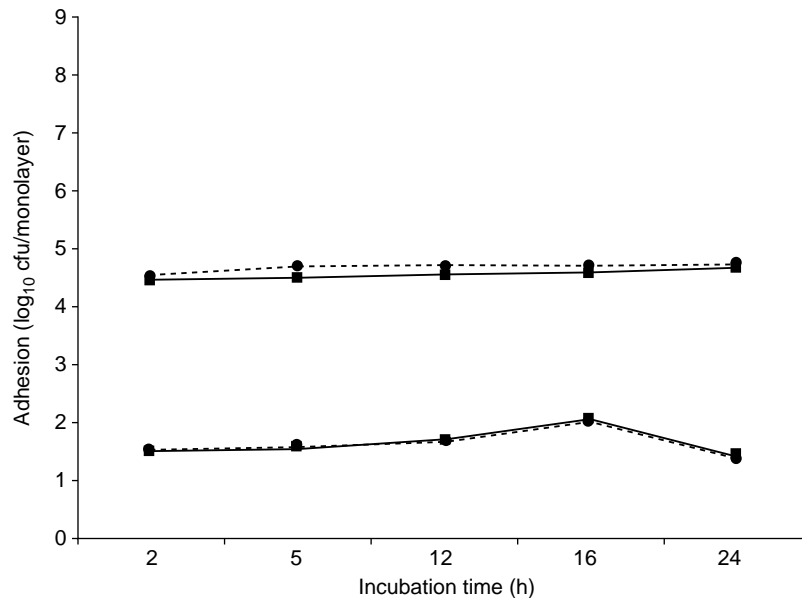


Fig. 1. Adhesion kinetics of *H. ducreyi* control strains CIP542 (top) and A77 (bottom) to epithelial cells at 33°C (---) and 37°C (—); $p > 0.05$.

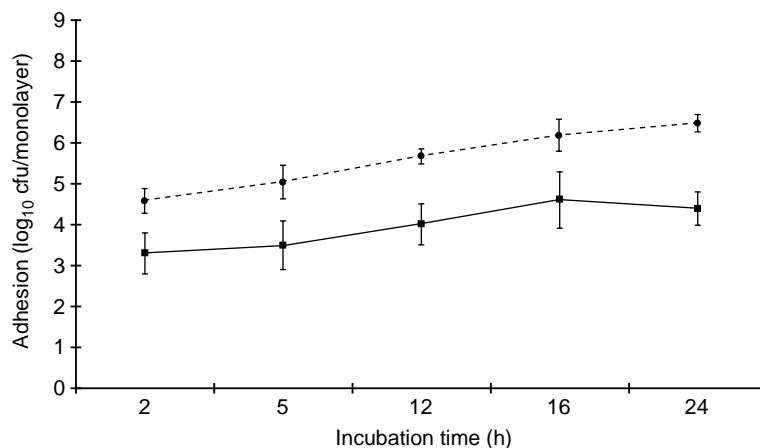


Fig. 2. Adhesion kinetics of seven clinical isolates (means) at 33°C (---) and 37°C (—); $p = 0.0001$.

was a significant difference between the adhesion at 33°C and 37°C, with *H. ducreyi* isolates adhering 10-fold more at 33°C ($p = 0.00016$). At 2 h after infection, an average of 60% of *H. ducreyi* had adhered at 33°C whereas *c.* 35% had adhered at 37°C. The intra-strain variation at 33°C was 47–69% and 16–45% at 37°C, with an SD of 0.43. Adherence at 33°C continued to rise throughout for the full 24 h of the experiment. However, at 37°C, a peak of adherence was reached at 16 h. Results of individual adhesion kinetics of the clinical isolates are illustrated in Fig. 3. When the strains were classified according to the magnitude in difference of adherence to epithelial cells at the two temperatures, it was found that strain SA68 was a unique isolate with the rest of the clinical isolates clustering in one group. The reference strains CIP542 and A77 formed a third group (Table 1). When classified according to adhesion at 33°C and 37°C, strain CIP542 grouped with the clinical isolates, whereas strain A77 was unique (Tables 2 and 3).

Cytotoxicity assay

The nine *H. ducreyi* sonicates were tested for their ability to cause death of the epithelial cells at the two

Table 1. Duncan grouping illustrating grouping of *H. ducreyi* strains in terms of their ability to attach to epithelial cells

Strain no.	Difference in mean* log ₁₀ cfu/monolayer	Duncan grouping
SA68	2.9300	A
SA42	2.5110	A B
SA60	2.2570	A B
SA71	2.0090	B C
SA63	1.4960	C D
SA26	0.9830	D
SA77	0.9660	D
CIP542	0.0050	E
A77	-0.0080	E

*Indicates the differences in means at 33°C and 37°C. Means with the same letter are not significantly different.

temperatures (33°C and 37°C). In all strains, cell death was the same at both temperatures (Fig. 4). The ability to cause cell death varied from isolate to isolate, with strains SA26 and SA71 causing the lowest cell death of *c.* 40%. The highest cell death of *c.* 80% was shown by strains SA42 and SA77. Although toxicity was the same at both temperatures for all clinical isolates, strain SA77 had significantly lower toxicity than strain CIP542 and the clinical isolates. Toxicity with live bacteria was greater at 33°C than at 37°C (Table 4).

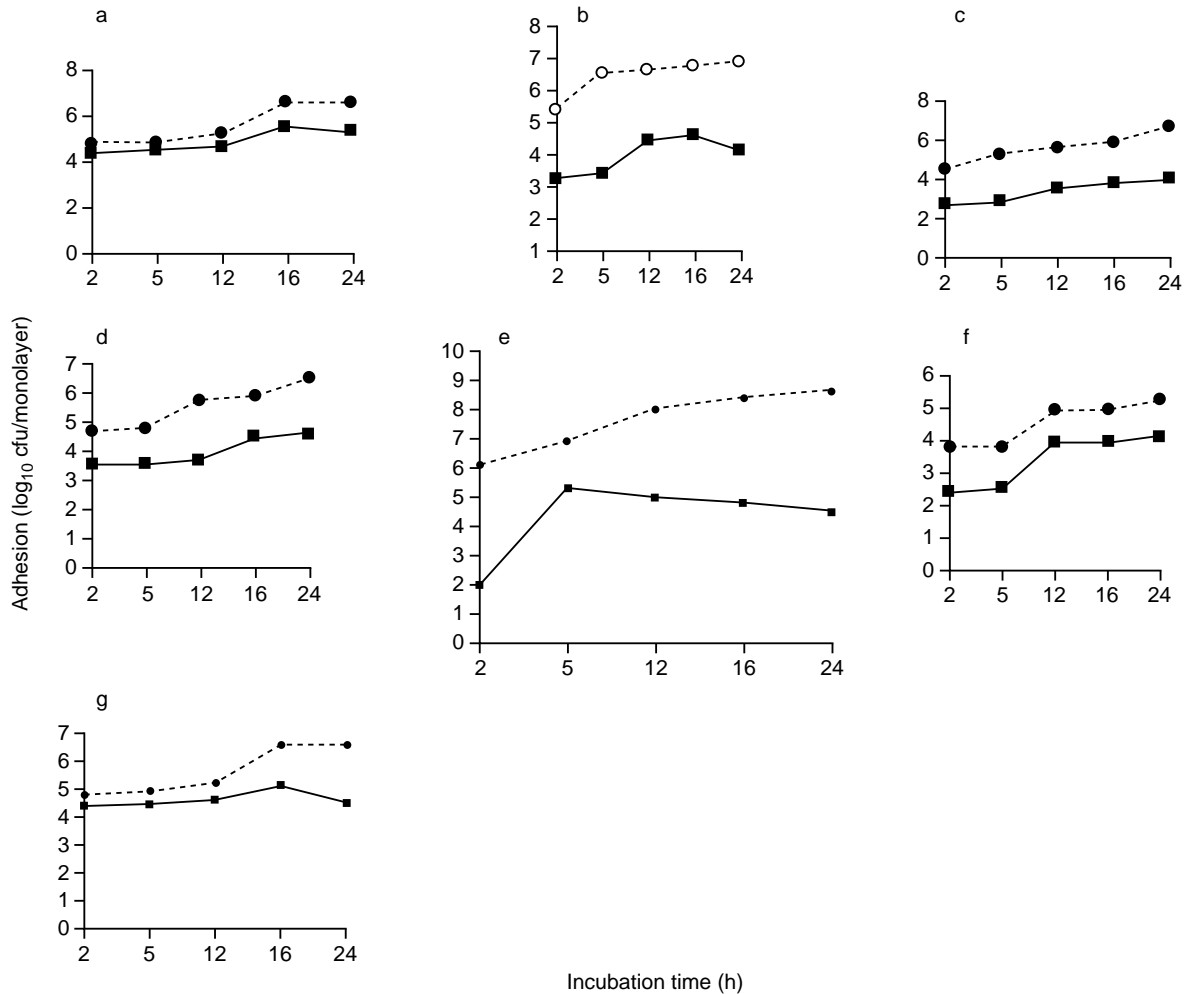


Fig. 3. Adhesion kinetics of individual clinical isolates at 33°C (---) and 37°C (—).

Table 2. Duncan grouping illustrating grouping of *H. ducreyi* strains in terms of their ability to attach to epithelial cells at 33°C

Strain no.	Difference in mean* log ₁₀ cfu/monolayer	Duncan grouping
SA68	7.567	A
SA26	5.607	B
SA60	5.562	B
SA63	5.467	B
SA42	5.443	B
SA71	5.325	B
SA77	4.995	B C
CIP542	4.575	C
A77	1.646	D

*Indicates the differences in means at 33°C and 37°C. Means with the same letter are not significantly different.

Table 3. Duncan grouping illustrating grouping of *H. ducreyi* strains in terms of their ability to attach to epithelial cells at 37°C

Strain	Difference in mean* log ₁₀ cfu/monolayer	Duncan grouping
SA68	4.637	A
SA26	4.624	A
SA60	3.305	C
SA63	3.971	B
SA42	2.932	C
SA71	3.316	C
SA77	4.029	B
CIP542	5.570	A
A77	1.650	D

*Indicates the differences in means at 33°C and 37°C. Means with the same letters are not significantly different.

Microscopy

Fig. 5 shows electron micrographs of HaCaT cells infected with *H. ducreyi*. Large numbers of bacteria were seen to be associated with epithelial cells. A

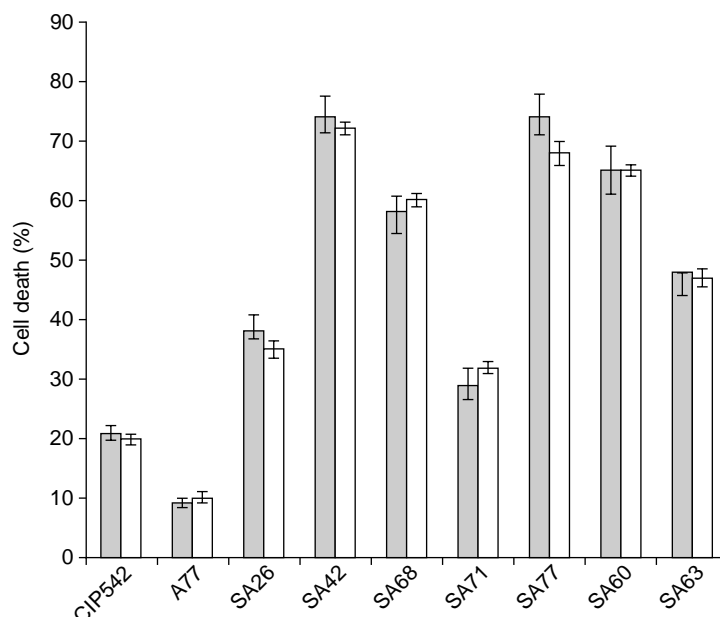
Table 4. Cytotoxicity of live bacteria for epithelial cells at 33°C and 37°C

Strain no.	Cell death (%) at	
	33°C	37°C
Control	0	0
CIP542	29	30
A77	8	8
SA42	40	28
SA60	42	34
SA68	71	37

prominent feature at both temperatures was an electron-dense area with no recognisable membrane where the bacteria were associated with the epithelial cells (Fig. 5a). Some bacteria were located within the epithelial cells; those were enclosed within vesicles. Epithelial cell necrosis was more apparent at 33°C (Fig. 5b) than at 37°C (Fig. 5c). The membrane-bound necrotic area may represent a phagolysosome. There were changes in nucleus morphology with nuclei being pushed towards the cell periphery at 33°C, whereas at 37°C nuclei had only slightly changed morphology.

Discussion

This study examined the interaction of *H. ducreyi* with cultured human epithelial cells and compared this interaction at 33°C and 37°C. Numerous studies have reported on in-vitro systems to investigate the interaction of *H. ducreyi* with epithelial cells [6, 8, 10, 17]. However, these reports used the standard approach in virulence studies for human pathogens, i.e., incubation temperatures of 35–37°C. Because the optimal growth temperature for *H. ducreyi* is 33°C [9, 13, 14], as

**Fig. 4.** Cytotoxicity assay results with crude sonicates showing the same toxicity at 33°C (■) and 37°C (□); $p > 0.95$.

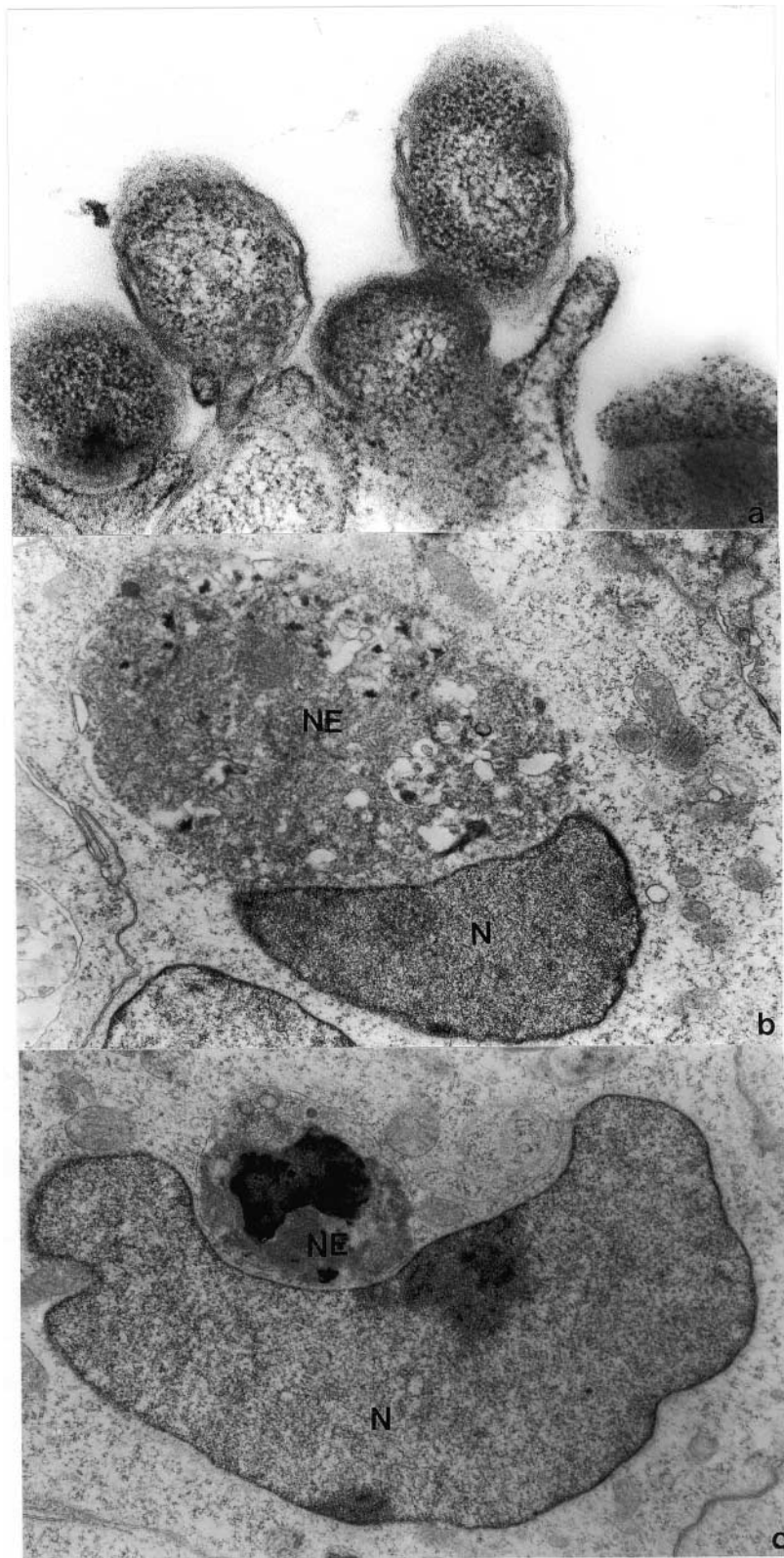


Fig. 5. Numerous bacteria associated with a HaCaT cell (a) as revealed by electron microscopy. Necrosis (NE) is more apparent at 33°C (b) as compared with 37°C (c). Note the nucleus (N) which is pushed to the cell periphery at 33°C. Magnifications: a, $\times 108\,000$; b, $\times 18\,400$; c, $\times 12\,000$.

related to its preference for colder parts of the body, it makes sense to investigate virulence attributes at that temperature as well. The primary organ infected by *H. ducreyi* *in vivo* is the skin, which has a physiological temperature of *c.* 33°C. The present study demonstrated

that clinical isolates of *H. ducreyi* had greater adhesion kinetics at 33°C than at 37°C ($p = 0.0016$).

The adhesion kinetics of reference strain A77 were low, and were similar at 33°C and 37°C. The finding of low

adhesion kinetics of this strain at 37°C is in keeping with those reported by other investigators [7, 18]. Hollyer and Alfa [7] reported low numbers of bacteria associated with epithelial cells, as well as low cytopathic effect. This may be attributed to the fact that this is an avirulent strain, and may be a result of multiple deficiencies in virulence properties. More work needs to be done to determine the difference between this strain and virulent strains. With this strain, the number of bacteria associated with the epithelial cells dropped after incubation for 16 h at both temperatures. This was also found with the clinical isolates, but only at 37°C. There are several explanations for this phenomenon. Bacteria could be dying due to environmental changes; in a tissue-culture system such changes result from cell metabolism. No differences in epithelial cell density were observed at the two temperatures studied and the bacterial replication rate was higher at 33°C; therefore, this explanation is unlikely. The phenomenon could also be due to bacteria being effectively internalised. This possibility was ruled out, as electron microscopy revealed no internal bacteria and also the amikacin protection/invasion assay showed no difference in invasion at either temperature with clinical isolates, while negligible invasion occurred with strain A77. Another explanation is that strain A77 loses the capacity to adhere to the cells over time at both temperatures while the clinical isolates do so at 37°C only. This supports the hypothesis that better adhesion at 33°C is related to virulence.

Reference strain CIP542 also showed similar adhesion kinetics to strain A77 at the two temperatures tested. Although higher numbers of bacteria were associated with the epithelial cells as compared with strain A77, the strains grouped together in Duncan's multiple range test. However, because CIP542 is a virulent strain, it is expected to display similar adhesion kinetics to the clinical isolates. With respect to adherence over time, strain CIP542 resembled clinical isolates. There was no decrease up to 24 h at both temperatures tested. An explanation for the difference in temperature attachment behaviour of these strains as compared with the clinical isolates could be that the two reference strains are relatively old strains with high passage numbers compared with the clinical isolates used; therefore, they have adapted to in-vitro conditions, i.e., growth at 35–37°C. Inter-strain variation was noted for adherence at both temperatures. This may be attributed to different levels of adhesin expression, thus allowing diversity in attachment [19]. Cytotoxicity studies showed a difference in the interaction of the free toxin at molecular as compared with the biological level. Whereas toxin preparations had a similar effect at both temperatures, with strain A77 having lower toxicity than strain CIP542 and the clinical isolates, the toxic effect of metabolically active bacteria was higher at 33°C. The clinical isolates also displayed a diverse toxic effect on the epithelial cells at a molecular level.

This report describes, for the first time, the interaction of *H. ducreyi* with human epithelial cells at 33°C. It has demonstrated that *H. ducreyi* clinical isolates display a temperature-dependent interaction with human epithelial cells. Furthermore, the overall toxic effect of whole bacteria depends on the metabolic activity of the bacteria and, therefore, was greater at 33°C than at 37°C. However, the effect of the free toxin at molecular level with fixed toxin concentrations is a temperature-independent event. These findings suggest that expression of adhesion to keratinocytes is upregulated at the lower temperature and that this is directly related to virulence.

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