INFECTION AND IMMUNITY, May 1993, p. 1735–1742
0019-9567/93/051735-08$02.00/0

Vol. 61, No. 5

Haemophilus ducreyi Adheres to but Does Not Invade Cultured Human Foreskin Cells

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Received 21 September 1992/Accepted 5 February 1993

***Haemophilus ducreyi* is the etiologic agent of the localized genital ulcer disease known as chancroid. The pathogenesis of this organism is poorly understood. The role of attachment in the disease process has not been evaluated. In this study, ^{125}I-*H. ducreyi* was used to quantitatively evaluate the interaction of virulent and avirulent *H. ducreyi* strains with human foreskin cells. Using this in vitro model system, we demonstrated that, at 22 and 35°C, the attachment of virulent *H. ducreyi* 35000 to human foreskin cells was significantly more marked than that of avirulent *H. ducreyi* A77. Although *H. ducreyi* penetrated between human foreskin cells, internalization was not a major component. Our competition assay data suggest that the attachment mechanism of *H. ducreyi* may be similar to that of *Neisseria gonorrhoeae*. We speculate that the attachment and microcolony formation of virulent *H. ducreyi* may provide a mechanism for bacterial localization and evasion of host defenses.**

Haemophilus ducreyi was described as the etiologic agent of chancroid over 100 years ago, yet little is known regarding how this organism is able to cause genital ulcers. Early observations with humans revealed that *H. ducreyi* did not appear to be capable of penetrating keratinized epithelium (5). As discussed by Albritton (1) and Morse (14), the organism is thought to gain entry into the host through microbreaks in the epithelium. The potential role of *H. ducreyi* attachment to eukaryotic cells in the pathogenesis of this bacterium has not been extensively investigated. *H. ducreyi* remains localized to the superficial layers of the skin and does not invade subcutaneously or systemically via the blood (1, 7, 8, 13, 15). It does, however, appear to enter the lymphatic system and cause lymph node swelling, which ultimately produces the characteristic bubo. Whether the attachment of *H. ducreyi* to genital epithelial cells has any role in this localization of the disease process is not known.

Attempts to study the pathogenesis of *H. ducreyi* disease have focused on either animal models (4, 8, 14, 17, 22, 23) or in vitro tissue culture models (2, 3, 12, 18). Mouse and rabbit models have indicated that the lipopolysaccharide-mediated inflammatory response accounts for most of the effects seen when *H. ducreyi* is injected intradermally (4, 23). In the chilled rabbit model described by Purcell et al. (17), lipopolysaccharide alone was insufficient to elicit ulcer formation and only virulent *H. ducreyi* caused ulcers. Animal models do not readily accommodate studies of bacterial attachment. In vitro cell culture models (2, 3, 12, 18) have demonstrated that *H. ducreyi* has a cytopathic effect on certain cell lines. Although there is some evidence that *H. ducreyi* binds to some eukaryotic cell lines (2, 3, 12), little is known about the characteristics of the attachment process.

The goal of this paper was to characterize the attachment of virulent and avirulent strains of *H. ducreyi* to human foreskin cell line HFF by use of radiolabelling and viability assays as methods of quantitation.

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MATERIALS AND METHODS

Bacterial strains used. The *H. ducreyi* strains used in this evaluation included 35000 (isolated in Canada), A77, CIP542 (obtained from Institute Pasteur), and R018 (isolated in Kenya). (All *H. ducreyi* cultures were kindly provided by A. Ronald.) Other bacteria included *Haemophilus influenzae* type b (ATCC 10211), *Neisseria gonorrhoeae* (isolated in Canada), and *Escherichia coli* (ATCC 25922). The *H. ducreyi*, *H. influenzae*, and *N. gonorrhoeae* isolates were cultivated on chocolate agar plates consisting of Columbia agar base (Becton Dickinson Microbiology Systems), hemoglobin, and 0.01% (vol/vol) IsoVitaleX. The plates were incubated at 35°C in 5% CO_2 in a humidified incubator. The *E. coli* isolate was grown on blood agar plates incubated in a room atmosphere at 37°C. All bacterial strains were stored as frozen skim milk stock cultures at −70°C.

Radiolabelling. Bacteria were suspended in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (Sigma Chemical Co., St. Louis, Mo.) and briefly sonicated. Clumps were allowed to settle, and only the supernatant containing well-suspended bacteria was used for labelling. Carrier-free ^{125}I was obtained from Amersham. *H. ducreyi* was labelled with ^{125}I by the Iodogen method in accordance with the manufacturer's instructions (Sigma). Unbound ^{125}I was removed by washing the bacterial pellet five times with tissue culture medium. The preparations used were ≥90% precipitable by a final concentration of 10% trichloracetic acid (Fisher Scientific, Fairlawn, N.J.). In one representative experiment, 2×10^6 CFU of *H. ducreyi* 35000 was ^{125}I labelled, and the specific activity achieved was 1 cpm/CFU. The specific activity varied slightly from experiment to experiment and ranged from 0.1 to 1 cpm/CFU. The bacterial suspension used to inoculate the eukaryotic monolayers had no visible clumps when observed with an inverted-plate microscope.

Tissue cultures. The eukaryotic cell lines used included HFF, a nontransformed cell line derived from pooled foreskins from children, and FS2-3, a nontransformed cell line derived from an adult foreskin (3). The eukaryotic cell cultures were cultivated in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.), 1

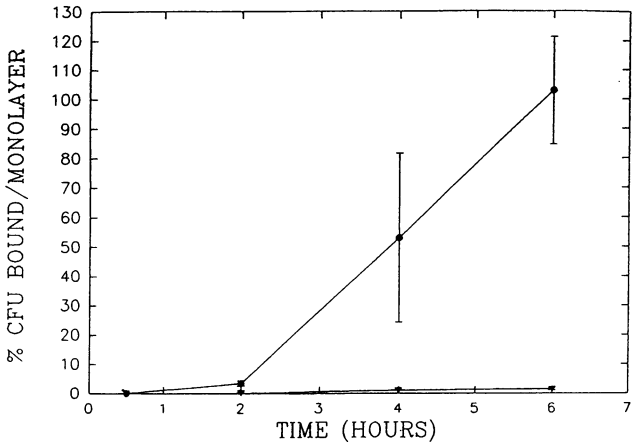


FIG. 1. Attachment of *H. ducreyi* to HFF cells at 35°C. Virulent *H. ducreyi* 35000 (●) and avirulent *H. ducreyi* A77 (▼) were inoculated onto HFF monolayers, and the numbers of bacteria bound were monitored at various times postinfection. The numbers of bacteria inoculated were as follows: *H. ducreyi* 35000, 1.3×10^7 CFU per well; *H. ducreyi* A77, 5.2×10^6 CFU per well. The results are expressed as the mean ± the standard deviation for three replicates.

mM Na pyruvate (Flow), and 2 mM L-glutamine (Flow). The tissue culture cells were incubated at 35°C in 5% (vol/vol) CO_2 in a water-saturated incubator. After 48 to 72 h of incubation, the cells were harvested by treatment with a 0.05% trypsin solution in 0.53 mM EDTA · 4Na (1×) (GIBCO). The cells were suspended in fresh tissue culture medium, and 10^5 cells were inoculated into each well of 24-well tissue culture trays (Corning Glass Works, Corning, N.Y.). The seeded trays were incubated for 48 h and then used in the attachment studies.

Attachment assays. In brief, for the attachment assays bacteria (either ^{125}I labelled or unlabelled) were added to the monolayer to achieve a multiplicity of infection of 5 to 10 bacteria per HFF cell. Because of variations in the labelling of the different bacteria, the input counts per minute per monolayer varied; therefore, the data from all radioactivity experiments are expressed as the percentage of input counts per minute bound per monolayer. The input counts per minute ranged from 5×10^5 to 1×10^6 per monolayer; this value represented 5×10^6 to 1×10^7 CFU per monolayer. The infected monolayer was incubated at the appropriate temperature, and at intervals of 0.5, 2, 4, and 6 h postinfection, the monolayer was washed four times with tissue culture medium. For viable-cell counts, the washed monolayer was resuspended (by use of a sterile scraper) in 0.4 ml of tissue culture medium. The suspension was briefly sonicated and then serially diluted 1/10. Portions consisting of 100 μl of each dilution were spread onto chocolate agar plates and incubated at 35°C in 5% CO_2 in a water-saturated incubator. After 48 h of incubation, the number of *H. ducreyi* colonies was counted. When ^{125}I-*H. ducreyi* was used for attachment experiments, the infected eukaryotic cell monolayer was washed four times with tissue culture medium and then the cells were collected onto a cotton swab. The amount of radioactivity on each swab was determined by use of a gamma counter (LKB) that had a counting efficiency of 86%.

HFF enrichment assay. Enrichment for adherent *H. ducreyi* 35000 and A77 and *H. influenzae* was done by use of

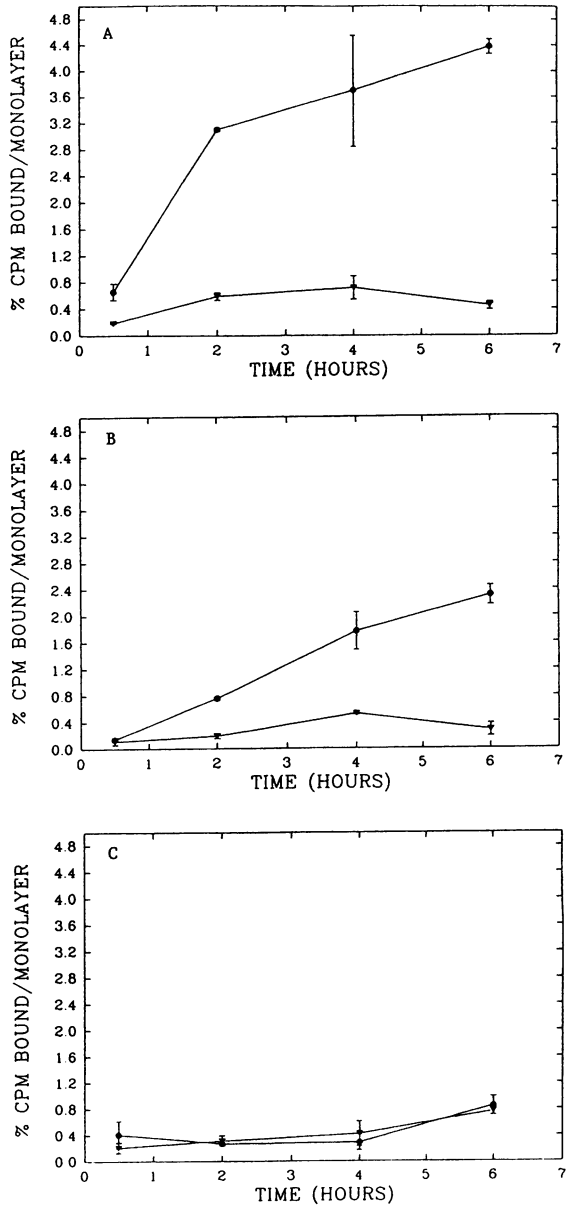


FIG. 2. Attachment of ^{125}I-*H. ducreyi* to HFF cells at 4 (C), 22 (B), and 35°C (A). HFF cells were infected with *H. ducreyi* 35000 (●) and *H. ducreyi* A77 (▼) at 5.5×10^5 and 1.7×10^6 cpm, respectively. The results are expressed as the mean ± the standard deviation for three replicates.

sequential passages in HFF cells. In brief, the bacteria were allowed to attach to an HFF monolayer for 4 h, unbound bacteria were washed off, and the monolayer with bound bacteria was scraped into 1 ml of tissue culture medium. A 50-μl aliquot of this suspension was then transferred to a fresh HFF monolayer and incubated overnight. This passage sequence was repeated on four sequential days. After the enrichment process in the tissue culture, the bacteria were isolated on chocolate agar and the protein profiles of the attached bacteria were compared with those of similar strains that had been passaged in tissue culture medium alone.

Invasion assay. The invasion assay used was that de-

TABLE 1. Survival of *H. ducreyi* in tissue culture medium

Temp (°C)	*H. ducreyi* strain tested	Viable bacteria (CFU/ml)[a] at the following h postinoculation:		
		0	2	6
22	A77	3.2×10^6	3.6×10^6	2.6×10^6
	35000	3.1×10^6	2.5×10^6	1.5×10^6
35	A77	1.2×10^7	1.8×10^7	6.9×10^7
	35000	8.1×10^6	1.4×10^7	1.2×10^7

[a] Averages for triplicate experiments. The SEM was <5% for all of the results presented.

scribed by St. Geme and Falkow (21). In brief, monolayers of HFF cells grown in antibiotic-free medium were infected with either *H. ducreyi* 35000 (1.3×10^6 CFU per monolayer) or *H. ducreyi* A77 (4.4×10^6 CFU per monolayer). At various intervals postinfection, unbound bacteria were washed off and either tissue culture medium containing 100 μg of gentamicin per ml or antibiotic-free medium was added. Monolayers with and without antibiotic were incubated at 35°C for 2 h to allow gentamicin to kill any external *H. ducreyi*. The monolayers were then washed four times with tissue culture medium without antibiotic, and viable counts of bound *H. ducreyi* were determined as described above for the attachment assay. Bacteria that are internalized are protected from the killing effect of gentamicin, which cannot effectively penetrate eukaryotic cells. Initial experiments demonstrated that *H. ducreyi* 35000 and A77 at a concentration of approximately 10^8 bacteria per ml were completely killed when exposed to 100 μg of gentamicin per ml for 2 h.

SEM. For scanning electron microscopy (SEM), HFF cells were grown on glass coverslips and infected with *H. ducreyi*. At specific times postinfection, the monolayers were washed three times with tissue culture medium to remove unbound bacteria. The monolayers and any adherent bacteria were fixed with 2% (wt/vol) glutaraldehyde for 5 min at 35°C. The monolayers were washed twice in SC buffer, which consisted of 0.1 M Na cacodylate and 0.01 M $CaCl_2$ at pH 7.4. Further fixation was achieved with 1% (wt/vol) osmium tetroxide in SC buffer and incubation at room temperature for 1 h. The coverslips were washed twice with SC buffer, dehydrated through a graded series of ethanol, and critical point dried in CO_2. The coverslips were then sputter coated with gold and viewed with a JEOL model 35C scanning electron microscope. The acceleration voltage was 15 kV.

TABLE 2. Competition for ^{125}I-*H. ducreyi* 35000 binding to HFF cells[a]

Competing bacterium	% Inhibition
None	0[b]
H. ducreyi 35000	91.6
H. ducreyi A77	89.2
H. influenzae	34.8[c] ($P = 0.002$)
N. gonorrhoeae	89.1
E. coli	47.1[c] ($P = 0.0004$)

[a] Each monolayer was infected with a mixture of ^{125}I-*H. ducreyi* 35000 (10^4 cpm, equivalent to 10^4 CFU) and an unlabelled competing bacterium. The ratios of unlabelled CFU to ^{125}I-*H. ducreyi* 35000 CFU were as follows: *H. ducreyi* 35000, 1,000/1; *H. ducreyi* A77, 200/1; *H. influenzae*, 3,600/1; *N. gonorrhoeae*, 1,200/1; and *E. coli*, 2,200/1. All experiments were performed in triplicate at 35°C.

[b] For ^{125}I-*H. ducreyi* 35000 with no competing unlabelled bacterium, 3.5×10^3 cpm bound per monolayer. All other results are expressed as a percentage of this result.

[c] The ability to inhibit the binding of ^{125}I-*H. ducreyi* 35000 was significantly lower than that of the homologous control (as assessed by use of the unpaired *t* test).

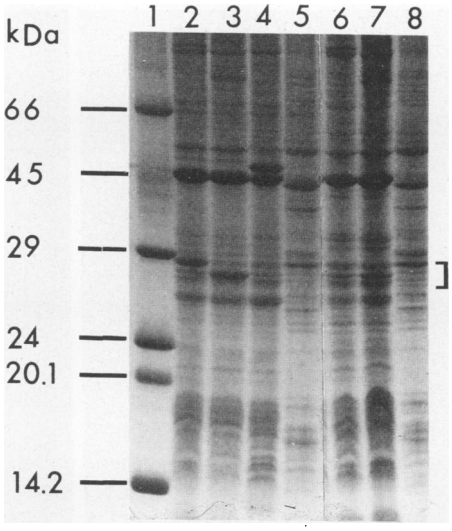
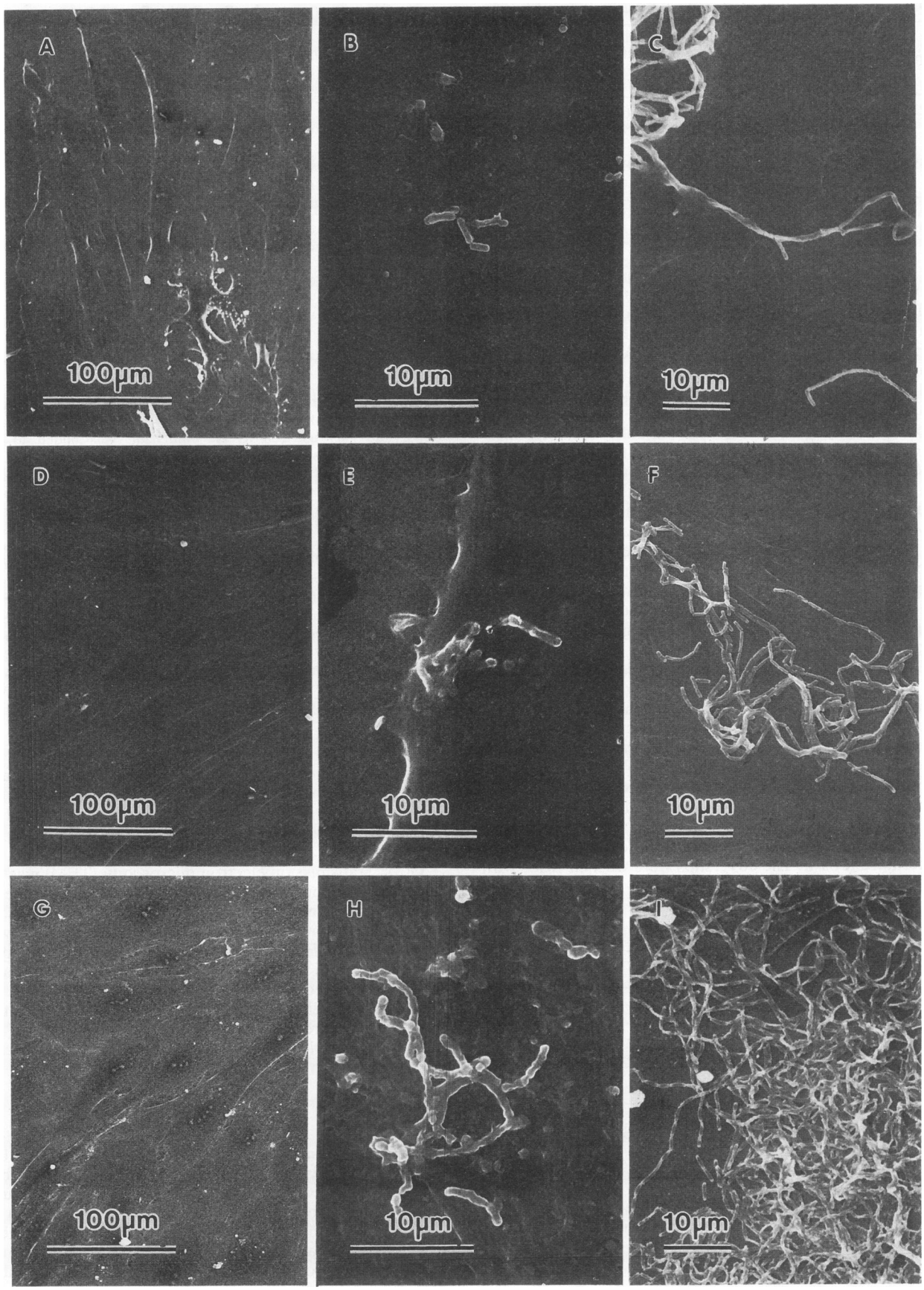


FIG. 3. Protein profiles of *H. ducreyi* after sequential passages in HFF cells. Approximately 5×10^5 CFU each of *H. ducreyi* 35000, R018, and A77 as well as *H. influenzae* was added to HFF monolayers or tissue culture media. After sequential passages for 4 days (as described in Materials and Methods), the protein profiles of these bacteria were assessed by use of a 12.5% polyacrylamide running gel and a 2.5% polyacrylamide stacking gel. The protein bands were visualized with Coomassie blue stain. Bacteria passaged sequentially in tissue culture medium only are shown in the following lanes: 2, *H. ducreyi* R018; 3, *H. ducreyi* 35000; 4, *H. ducreyi* A77; and 5, *H. influenzae*. Bacteria that adhered to HFF cells and were enriched by four sequential passages are shown in the following lanes: 6, *H. ducreyi* R018; 7, *H. ducreyi* 35000; and 8, *H. influenzae*. Molecular mass markers (lane 1) consisted of alpha-lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), egg albumin (45 kDa), and bovine albumin (66 kDa). The bracket indicates areas in which polypeptide changes occurred.

TEM. Transmission electron microscopy (TEM) was performed by the method of Alfa (3). In brief, human foreskin cells were grown on Metricell GN-6 membrane filters and then infected with *H. ducreyi*. The Metricell filters were prepared for TEM by fixation with 2% glutaraldehyde, postfixation with 1% (wt/vol) osmium tetroxide, dehydration through a graded series of acetone-propylene oxide, and embedding in JEM Bed 812 medium. After polymerization, thin sections were cut and poststained with lead citrate. The sections were viewed with a Philips model 201 electron microscope at an acceleration voltage of 60,000 eV.

RESULTS

Attachment assays. *H. ducreyi* R018 and 35000 were both virulent clinical isolates, whereas *H. ducreyi* A77 had been passaged extensively in vitro and was shown to be avirulent in the rabbit model (8). These strains were used in the current study to determine whether there were differences in their abilities to adhere to eukaryotic cells. The attachment of *H. ducreyi* to HFF cells at 35°C was monitored by determining the number of viable bacteria bound to the


A
100μm
B
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D
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FIG. 4. SEM cells infected with *H. ducreyi*. Human foreskin cells (HFF) were grown on coverslips and infected with *H. ducreyi* A77 (B, E, and H) or *H. ducreyi* R018 (C, F, and I) at a multiplicity of infection of 10:1. Uninfected monolayers are also shown (A, D, and G). At 6 (A, B, and C), 24 (D, E, and F), and 48 (G, H, and I) h postinfection, nonadherent bacteria were removed by washing of the monolayers. The monolayers were then fixed with glutaraldehyde, dehydrated, and coated with gold as described in Materials and Methods.

monolayer. The data shown in Fig. 1 demonstrate that the number of avirulent *H. ducreyi* A77 cells bound per monolayer at 6 h was significantly ($P = 0.001$) smaller than the number of virulent *H. ducreyi* 35000 cells bound per monolayer. The use of viable count determination as a method for monitoring attached bacteria did not allow the differentiation of bound input bacteria versus newly replicated bacteria. Since the number of viable *H. ducreyi* 35000 cells bound at 6 h (Fig. 1) was larger than 100% of the input bacteria, it is apparent that some of the bacteria bound resulted from replication. When replicating, *H. ducreyi* form chains and clumps that are difficult to break up, a problem that makes viability assays somewhat variable. In an attempt to obtain more reliable quantitative data, radiolabelling of *H. ducreyi* with ^{125}I was used to study attachment. Figure 2 shows the attachment of ^{125}I-*H. ducreyi* 35000 and ^{125}I-*H. ducreyi* A77 at 4, 22, and 35°C. The attachment of *H. ducreyi* 35000 to HFF cells at 4°C showed the same pattern as that of *H. ducreyi* A77 (Fig. 2C). At 22 and 35°C, virulent *H. ducreyi* 35000 showed significantly higher levels of binding than avirulent *H. ducreyi* A77 (Fig. 2A and B). The differences in the attachment patterns observed in the ^{125}I attachment assays were not due to a lack of viability of *H. ducreyi* A77, since this organism was able to survive as well as *H. ducreyi* 35000 in tissue culture medium alone (Table 1). Maximal binding was achieved at 35°C. To ensure that the bound radioactivity was due to attached organisms, we carried out a competition assay with unlabelled bacteria. The attachment of ^{125}I-*H. ducreyi* was specific, since unlabelled *E. coli* did not inhibit the binding of ^{125}I-*H. ducreyi* to the same extent as unlabelled *H. ducreyi* A77 or 35000 (Table 2). These data suggest that avirulent *H. ducreyi* A77 attaches by a mechanism similar to that of virulent *H. ducreyi* 35000 but that it does so significantly less efficiently at 22 and 35°C (Fig. 2). This suggestion implies that active replication of virulent *H. ducreyi* 35000 enhances its adherence ability compared with that of avirulent *H. ducreyi* A77. Of the heterologous bacteria tested, *N. gonorrhoeae* could compete with ^{125}I-*H. ducreyi* 35000 and decrease its attachment to HFF cells more effectively than either *H. influenzae* or *E. coli* (Table 2).

To determine whether sequential passages in tissue cultures altered the characteristics of *H. ducreyi*, we compared the protein profiles of strains after serial passages in HFF cells or tissue culture medium alone by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Serial passages in HFF cells essentially enriched the adherent *H. ducreyi* population. No viable *H. ducreyi* A77 was detected after the second passage in HFF cells; therefore, no data are shown for this isolate. Association with eukaryotic cells appeared to cause changes in the protein profiles of *H. ducreyi* R018 and 35000 (Fig. 3).

Microscopy. For assessment of eukaryotic-prokaryotic interactions, SEM was done on infected and uninfected monolayers. Virulent *H. ducreyi* R018 not only bound to the eukaryotic cells but also, as observed by SEM, produced microcolonies which, by 48 h postinfection, had enlarged to completely coat the HFF cell surface (Fig. 4). Very few avirulent *H. ducreyi* A77 cells bound to the monolayer and, even after 48 h of incubation, only occasional bacteria were observed on the HFF cells (Fig. 4). The SEM characteristics of *H. ducreyi* A77 and R018 bound to human foreskin cell line FS2-3 were similar to those observed for the HFF cell line (data not shown). TEM revealed that, by 48 h postinfection, virulent *H. ducreyi* R018 cells were attached to and in some cases embedded in the foreskin cell membrane (Fig. 5). Although bacteria were observed in between the junctions of foreskin cells, internalized bacteria were not observed.

Invasion assay. To further clarify whether *H. ducreyi* cells were internalized, we performed experiments with gentamicin treatment to kill external bacteria (20, 21). As described by St. Geme and Falkow (20, 21), gentamicin is bactericidal and kills susceptible bacteria that are not internalized by eukaryotic cells. The protection of internalized bacteria is due to the inability of gentamicin to effectively penetrate eukaryotic cells. The data shown in Table 3 indicate that, although the number of bound bacteria increased over time, the number of *H. ducreyi* cells that were protected was minimal, even after 4 h of incubation with HFF cells at 35°C prior to exposure to gentamicin.

DISCUSSION

The current evaluation further extends our knowledge regarding the ability of *H. ducreyi* to attach to foreskin cells in the in vitro model initially described by Alfa (3). Attachment was time and temperature dependent (Fig. 1 and 2), with maximal binding occurring 4 to 6 h postinoculation. ^{125}I labelling of *H. ducreyi* was a reproducible method that effectively quantitated attachment but was independent of bacterial replication. Our data demonstrated that less than 1% of the input avirulent *H. ducreyi* A77 adhered to HFF cells. This low level of attachment was apparent regardless of the temperature evaluated (Fig. 2). However, for virulent *H. ducreyi* 35000, sixfold more bacteria attached at 35°C than at 4°C (Fig. 2). Although strain A77 survived in tissue culture medium as well as strain 35000 (Table 1), it appears that the attachment and replication of bound *H. ducreyi* and the formation of adherent microcolonies are unique to virulent strains of *H. ducreyi*. This conclusion was confirmed by SEM (Fig. 4). Previous histological and microscopy studies of chancroid ulcer biopsy material demonstrated that *H. ducreyi* forms extracellular clumps, or 'schools of fish' (7, 8, 10, 11, 13). These may represent smaller in vivo equivalents of the microcolonies observed in the foreskin cell culture model (Fig. 1).

The initial interaction of *H. ducreyi* with the human host occurs in genital tract epithelial or mucosal areas. Initial evaluations (10, 11) showed that *H. ducreyi* could not penetrate the keratinized epithelium of intact arm or thigh skin. However, when the intact skin was first scarified, *H. ducreyi* could successfully infect and cause ulcers on the arms or thighs of the patients tested. This procedure was evaluated by Heyman et al. (10) as a method of diagnosis of chancroid. These preliminary studies had led subsequent investigators to propose that the mechanism of pathogenesis necessitates microbreaks in the epithelium before infection

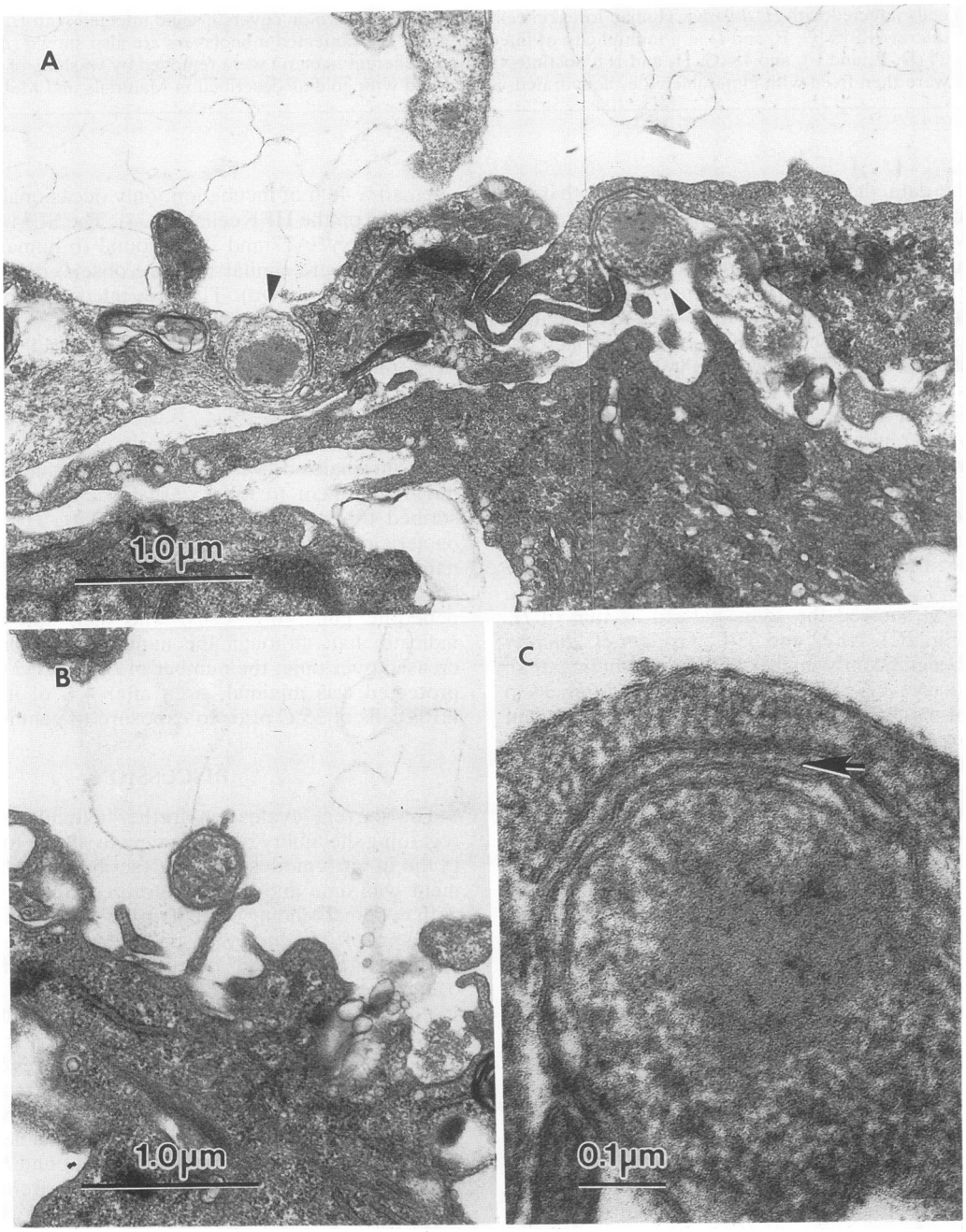

FIG. 5. TEM of human foreskin cells infected with *H. ducreyi*. Human foreskin cells (FS2-3) were grown on Metricell GN-6 filters and infected at a multiplicity of infection of 10:1 with virulent *H. ducreyi* R018. After 48 h of incubation at 35°C, the monolayers were fixed, sectioned, stained, and observed as described in Materials and Methods. (A) *H. ducreyi* cells embedded in the surface of FS2-3 cells or located between foreskin cells (arrowheads). (B) *H. ducreyi* bound to the ends of eukaryotic microvilli. (C) Fibrillar matrix between *H. ducreyi* and the human foreskin cell membrane (arrow).

can occur. The necessity of microbreaks for *H. ducreyi* infection of mucosal surfaces has not been studied. Our data demonstrate that virulent *H. ducreyi* readily attaches to foreskin cells in vitro and support the need for further studies to characterize the attachment mechanism.

As discussed by Finlay and Falkow (6), adhesins are common and important aspect of most pathogenic bacteria. Bacterial adhesins fall into two broad categories: pili (fimbriae) or other surface proteins. Spinola et al. (19) recently described and characterized pili in almost all of the *H. ducreyi* strains examined. We did not observe distinct pili in our TEM preparations; however, TEM revealed a fibrillar matrix in the 10- to 20-nm gap between *H. ducreyi* and foreskin cells (Fig. 5). Whether this matrix represents the fine tangled mesh of 3-nm-diameter pili of *H. ducreyi* 35000 described by Spinola et al. (19) requires further evaluation. Our competition studies suggest that *H. ducreyi* adhesins may have foreskin binding sites that are similar to those of *N. gonorrhoeae*. Although the data are only preliminary, they demonstrated that *N. gonorrhoeae* was the only heter-

TABLE 3. *H. ducreyi* attachment to versus internalization into HFF cells[a]

Time (h)	CFU of *H. ducreyi* bound/monolayer			
	35000		A77	
	Adherence	Invasion	Adherence	Invasion
0.5	6.5×10^3	6.0×10^1	4.7×10^3	0[b]
2	9.2×10^4	2.8×10^1	3.5×10^4	0
4	1.2×10^6	1.1×10^2	5.2×10^3	0

[a] *H. ducreyi* 35000 and A77 were inoculated at concentrations of 1.3×10^6 and 4.4×10^6 CFU per monolayer, respectively. The gentamicin invasion assay was described in Materials and Methods. Viable counts were used to determine how many bacteria attached to versus how many invaded HFF cells and were protected from gentamicin. The results are averages for triplicate experiments performed at 35°C.

[b] 0, below the limit of detection (10 CFU/ml).

ologous strain that could inhibit the binding of ^{125}I-*H. ducreyi* as effectively as the homologous control (Table 2). Whether this result reflects similarities in pili, outer membrane proteins, or surface carbohydrates requires further analysis. Similarities between the oligosaccharides of *N. gonorrhoeae* and *H. ducreyi* have been described by Campagnari et al. (4). However, the role of oligosaccharides in attachment is not known. Our preliminary studies demonstrated that the association of virulent *H. ducreyi* with foreskin cells resulted in alterations in the protein profile in the molecular weight range of 30,000 to 45,000. This range corresponds to that of the outer membrane proteins of *H. ducreyi* characterized by Odumeru et al. (16). These preliminary results suggest that certain *H. ducreyi* proteins may be altered by association with eukaryotic cells. The role of these altered proteins as virulence factors or adhesins warrants further evaluation.

Studies have shown that, even for bacteria, such as *H. influenzae*, that are not intracellular parasites, the invasion of eukaryotic cells is an important virulence factor (20, 21). Our data (Fig. 5 and Table 3) differ from those of Lammel et al. (12) in that we did not observe any *H. ducreyi* cells that appeared to have been internalized by foreskin cells (Fig. 5). We did observe a close attachment with extensive invagination, and *H. ducreyi* cells were frequently observed in between the opposing surfaces of foreskin cells (Fig. 5). The microcolonies of virulent *H. ducreyi* strains seen on SEM (Fig. 4) suggest that it would be extremely difficult for these bacterial cells to be internalized by eukaryotic cells because of the complex interaction and chaining of progeny bacterial cells. This hypothesis is supported by the results of our evaluation of *H. ducreyi* in the gentamicin invasion assay. St. Geme and Falkow (20, 21) used the gentamicin invasion assay to demonstrate that at 4 h postinfection, the adherence/invasion ratio for *H. influenzae* that invaded Chang epithelial cells was approximately 650/1; that for noninvasive *H. influenzae* mutants was 4,000/1. Virulent *H. ducreyi* 35000 had an adherence/invasion ratio of 11,000/1 in our study (Table 3), a result supporting the conclusion that the invasion of foreskin cells is not a significant feature.

In summary, we have provided preliminary data related to the attachment and invasion characteristics of *H. ducreyi* for a tissue culture cell line derived from foreskin tissues. Viability measurements and radiolabelling were the quantitative approaches used to monitor attachment. Using this model, we determined that virulent strains of *H. ducreyi* are significantly more able than avirulent strains to attach to and form microcolonies on foreskin cells at 35°C. Our evaluation also demonstrated that the interaction of virulent *H. ducreyi* with foreskin cells provided some degree of protection from the killing effect of gentamicin but that internalization was not a major feature. *H. ducreyi* appeared to have attachment characteristics that were similar to those of *N. gonorrhoeae* in that it could penetrate between foreskin cells and the latter organism could significantly inhibit the attachment of *H. ducreyi* in competitive binding assays. This study provides evidence that the attachment of *H. ducreyi* to eukaryotic cells is a significant virulence factor that warrants further studies.

ACKNOWLEDGMENTS

This work was supported by grants from the St. Boniface Research Foundation and the Manitoba Health Research Council.

We acknowledge the skilled technical assistance of P. Hazelton, Department of Medical Microbiology, University of Manitoba, with the transmission electron micrographs presented in this paper. The skilled manuscript preparation of Joan Boughton is acknowledged.

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