Haemophilus ducreyi Attaches to and Invades Human Epithelial Cells In Vitro

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Haemophilus ducreyi **is a sexually transmitted pathogen that causes genital ulcers and inguinal adenopathy. Because chancroidal ulcers are most commonly located on the foreskins of uncircumcised males, we utilized human foreskin epithelial cells (HFECs) to investigate the initial interaction of** *H. ducreyi* **with its host. The eight different strains of** *H. ducreyi* **that were studied varied in their abilities to attach to these epithelial cells, with six strains consistently attaching to ≥90% of HFECs and two strains attaching to <25% of HFECs. The strains with low levels of adherence also failed to exhibit chaining in broth culture and were avirulent in the rabbit model, suggesting that virulence in this model and attachment may be linked. The most adherent strain, LA228R, was further evaluated for its ability to invade HFECs and HEp-2 cells. Scanning electron microscopy and transmission electron microscopy of HFECs after interaction with LA228R produced images consistent with attachment, ingestion into vesicles, and escape from the vesicles into the cytoplasm. In addition, the gentamicin protection assay and inhibition of invasion by cytochalasin B and D indicated that LA228R was able to invade both HFECs and HEp-2 cells. Further examination of the mechanisms involved in the adherence and invasion of** *H. ducreyi* **into epithelial cells and their correlation with virulence will provide a better understanding of the pathogenesis of the disease caused by this important pathogen.**

Haemophilus ducreyi is the causative agent of chancroid, a genital ulcer disease highly prevalent in Africa and increasing in incidence in the United States (1, 9, 30). In addition, infection with *H. ducreyi* has been identified as a risk factor for the heterosexual transmission of the human immunodeficiency virus in Africa (26, 33). Conversely, infection with the human immunodeficiency virus reduces the effectiveness of antimicrobial therapy for chancroid and may increase transmission of *H. ducreyi* (15). Thus, these two diseases likely interact in such a way to mutually facilitate sexual transmission of one another. An improved understanding of the pathogenesis of *H. ducreyi* infection may enable us to devise strategies for prevention of chancroid and thus reduce human immunodeficiency virus transmission.

Chancroid is characterized by genital ulcers which histologically demonstrate a necrotic base consisting of epithelial cell debris, fibrin, and polymorphonuclear leukocytes with underlying proliferating epithelial cells, macrophages, and lymphocytes (22, 30). Fifty percent of chancroid cases have associated inguinal lymphadenopathy which may progress to suppurating buboes. Chancroidal ulcers are diagnosed much more frequently in men than in women and are most prevalent in uncircumcised men, in whom the ulcer is frequently located on the foreskin.

The mechanisms through which *H. ducreyi* produces genital ulceration and inguinal adenopathy are at present poorly understood. Lipooligosaccharides (LOS) appear to be important for the organism both for evading ingestion by polymorphonuclear leukocytes and for production of disease in the rabbit model (7, 23, 24). In addition, putative toxins specific for human epithelial cells, heat-shock proteins, and pilus-like structures have been described, but their roles in the production of disease remain unclear (8, 25, 28).

Attachment to mucosal surfaces is important for initiation of infection by other organisms and is often mediated by pili and other outer-membrane components. It is likely that *H. ducreyi* has also developed specific mechanisms to attach to and invade host epithelial cells. In addition, invasion by *H. ducreyi* into human cells might enable the organism to evade the immune response, contribute to tissue necrosis, and spread to surrounding tissue. *H. ducreyi* strains are able to attach to several cell types (3, 4, 6), but their ability to invade is controversial (4, 19).

In this study, we investigated the interaction of *H. ducreyi* with primary cultures of human foreskin epithelial cells (HFECs) on the premise that this is the actual cell type with which *H. ducreyi* initially interacts in nature. In addition, the interaction of *H. ducreyi* with HEp-2 cells was also studied because the HEp-2 cell line is more widely used and available and is easier to grow in vitro. We found that the organism attached to and invaded both cell types in vitro.

MATERIALS AND METHODS

Bacterial strains and growth media. The *H. ducreyi* strains used in this study were obtained from various sources around the world, and their identities were confirmed as described previously (32). Strain 35000 (12), a clinical isolate from Winnipeg, Manitoba, Canada, was obtained from William Albritton (University of Manitoba, Winnipeg). Peter Piot (Institute of Tropical Medicine) provided strains V149/91 and V-180, which were clinical isolates from Rwanda. Strains CIP A75 (12), CIP A77 (12), and CH2 (a clinical isolate from Thailand) were obtained from Leslie Slaney (University of Manitoba, Winnipeg). CF101 was isolated from a patient in Seattle, Wash. Strain LA228 was obtained from J. R. Greenwood (University of California at Los Angeles). Strain

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LA228R, a derivative of LA228, was isolated from a lesion in the temperature-dependent rabbit model.

Other bacteria were used as controls in the attachment and invasion assays. Rd, a strain of *Haemophilus influenzae* lacking pili (29), was obtained from M. Roberts (University of Washington, Seattle) and served as a negative control in the adherence assays. Strains SL1344 (13) and HB101(pMJK7) (17), which served as positive and negative controls, respectively, in the invasion assays, were obtained from Steve Moseley (University of Washington, Seattle). HB101(pMJK7) contains the CS31A adhesin gene and thus attaches well to epithelial cells but does not invade. Initial experiments indicated that the low level of invasion displayed by HB101(pMJK7) was comparable to that of strain DH5α, which was obtained from Gibco BRL (Gaithersburg, Md.).

All strains were maintained at −70°C in L broth (21) with 25% glycerol. To prevent changes in the bacterial phenotype which might occur after prolonged culture, all *H. ducreyi* strains were recovered from the frozen stocks weekly. For recovery from these stock cultures, a portion of the frozen medium was flaked off with a sterile applicator stick and used to inoculate a plate of chocolate agar with fetal bovine serum (CFS) which was then incubated for 2 days at 35°C in a candle jar. These CFS plates consisted of GC agar base (Difco Chemical Co., Detroit, Mich.) and 1% hemoglobin (BBL, Cockeysville, Md.), and 0.1% glucose, 0.01% glutamine, 0.026% cysteine, and 10% fetal bovine serum (FBS) (Gibco BRL). The glucose, glutamine, cysteine, and FBS were added after the medium was autoclaved. These plates were incubated for 2 days at 35°C in a candle jar, and then the bacterial growth was inoculated onto another CFS plate and incubated overnight before the bacteria were used in experiments. *H. influenzae* was cultured on chocolate agar which consisted of GC agar base, 1% hemoglobin, and 1% XV factor enrichment (PML Microbiologicals, Tualatin, Oreg.). *Escherichia coli* strain DH5α was cultured in L broth or agar (21).

The clear broth used for growth of *H. ducreyi* (Hd broth) consisted of GCP broth (GC agar base without the agar), 1% XV factor enrichment, 100 μg of catalase per ml (Sigma Chemical Co., St. Louis, Mo.), and 10% FBS (32). The optical densities at 540 nm ($OD_{540}s$) achieved after 24 h of growth in Hd broth differed slightly among strains but were approximately 0.9 to 2.0. *H. influenzae* Rd was grown under the same conditions except for being grown in Hd broth lacking the FBS.

Primary cell cultures of HFECs. Primary cultures of HFECs were derived from pooled discarded neonatal foreskins from local Seattle hospitals as previously described (5) and were cultured in keratinocyte growth medium (KGM) with added supplements (Gibco BRL) at 35°C in 5% CO_2. Briefly, two to four foreskins were washed three times in phosphate-buffered saline (PBS) and then incubated overnight at 4°C with 0.8% dispase (Boehringer Mannheim, Indianapolis, Ind.). The next day, the epithelial layer was removed with sterile tweezers, chopped with a scalpel, suspended in a 1/10 dilution of trypsin-EDTA (Life Technologies Gibco BRL) in PBS, and dispersed by repeated pipetting. The reaction was stopped by the addition of PBS with 10% FBS, and the cells were collected by centrifugation. After suspension in 10 ml of KGM, cells were transferred to tissue culture dishes (100 by 20 mm; Corning Glass Works, Corning, N.Y.) and incubated at 35°C in 5% CO_2. Only cells passed fewer than eight times in vitro (8 weeks of cultivation) were used in our analyses. After isolation, frozen stock cultures of HFECs in KGM with 10% dimethyl sulfoxide were maintained in liquid nitrogen.

HEp-2 cells. HEp-2 cells were obtained from the American Type Culture Collection, Rockville, Md., and were cultured on Earle's minimum essential medium (Sigma Chemical Co.) with 10% FBS.

Attachment assays. For attachment assays assessed by light microscopy, sterile 12-mm glass circles (Fisher Scientific, Pittsburgh, Pa.) were inserted in each well of a 24-well tissue microtiter plate (Cell Wells; Corning Glass Works). Plates were seeded with 5×10^5 HFECs contained in 2 ml of KGM and incubated for 24 h at 35°C in 5% CO_2 before the attachment assays. Following this procedure, the HFECs were not quite confluent in the wells, which allowed us to distinguish between bacteria attaching to the HFECs and those attaching to areas between the HFECs. Strains of *H. ducreyi* were grown to stationary phase by shaking overnight at 35°C in Hd broth. Bacteria were pelleted by centrifugation and suspended in KGM to an OD_{540} of 1.0, and 0.1 ml of the bacterial suspension was inoculated into each well of the microtiter plate containing the HFECs. After incubating for 2 h at 35°C to allow for bacterial attachment, the wells were washed extensively with PBS and the cells were fixed with methanol for >5 min prior to being stained with Giemsa with the Diff-Quik Stain Set (Baxter Scientific Products, Seattle, Wash.). The glass circles were removed from the wells and fixed to slides with Permount, and attachment was assessed by light microscopy. Because of the chaining and clustering of bacteria which made assessment of the number of bacteria per HFEC difficult, attachment was expressed as the percentage of HFECs with attached bacteria.

For assessment of the attachment of CF101 to microtiter plate wells with no cells, PBS, 1% bovine serum albumin (BSA), or 1% gelatin was added to the microtiter plate wells. The wells were allowed to incubate at 35°C for 2 h, washed with KGM, and then maintained in KGM for the attachment assay. Attachment of CF101 to KGM was assessed by the addition of KGM with no cells to the microtiter plate wells and then incubation of the wells overnight before the attachment assay.

For assessment of quantitative attachment, assays were performed as described above, except microtiter plates without glass circles were used. After the 2-h incubation at 35°C, the wells were washed with PBS and then HFECs with attached bacteria were harvested by treatment with trypsin-EDTA, as described below for the gentamicin protection assay.

Electron microscopy. For scanning electron microscopy (SEM) and transmission electron microscopy (TEM), HFECs were grown in 60-mm-diameter tissue culture dishes (Corning Glass Works). *H. ducreyi* was added as described above for the attachment assay and incubated for 3 h at 35°C before preparation for electron microscopy. Infected monolayers were fixed for 1 to 2 h at ambient temperature with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 4% sucrose and 4 mM $CaCl_2$. Following fixation, samples were washed in the same buffer, reacted with 1% osmium tetroxide for 1 h, washed in buffer again, then dehydrated through a graded series of ethanol, and embedded in Med Cast resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a JOEL 100 B or 1200 transmission electron microscope. For ruthenium red staining, ruthenium red in the following solutions at the percentages indicated was used: glutaraldehyde fixative, 0.2%; the intermediate wash step, 0.1%; and the osmium fixative, 0.05%.

Gentamicin protection assay. The gentamicin protection assay was performed as described by Finlay and Falkow (10) and modified by St. Geme and Falkow (31). Briefly, 10^6 HFECs contained in 2 ml of the appropriate tissue culture growth medium were seeded into 24-well tissue culture plates. The plates were incubated for 1 day, washed with tissue culture
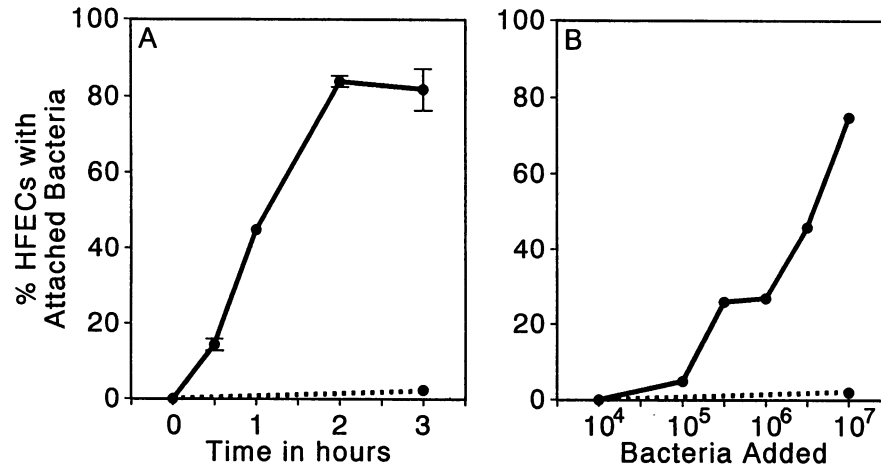
FIG. 1. Attachment of LA228 to HFECs is both time and concentration dependent. (A) Effect of time on adherence. The graph shows that attachment increased linearly with time; 2 h was required for optimal adherence. The bars indicate the standard errors calculated from two separate wells. (B) Effect of the multiplicity of infection. The graph shows that adherence increased linearly with bacterial concentration. Because a single well was used for each bacterial concentration, standard errors are not shown. In the panel A experiment, 10^7 bacteria per microtiter well were used; in the panel B experiment, 3 h was allowed for attachment. No attachment was seen with *H. influenzae* Rd. Solid line, LA228R; dotted line, *H. influenzae* Rd.

media to remove unattached cells, and then incubated for an additional day to achieve confluency for the invasion assay. Bacterial strains were grown to stationary phase by overnight incubation in the appropriate medium and conditions, which were L broth, stationary (SL1344); L broth with 100 μg of ampicillin per ml, stationary [HB101(pMJK7)]; and Hd broth, shaking (LA228R, CIP A75, and 35000). SL1344, a highly invasive strain of *Salmonella*, was used as positive control. HB101(pMJK7), a clone containing the CS31A adhesin gene, was used as a negative control because it attached well to the HFECs (data not shown). Thus, this strain was used to ensure that the gentamicin was able to kill noninternalized attached bacteria in the assay. The low levels of invasion of this bacteria were comparable to those of DH5α, a less-adherent bacterium (data not shown). After overnight growth, bacterial suspensions were pelleted by centrifugation and then suspended in the tissue culture growth medium (KGM for HFECs and

Earle's minimum essential medium for HEp-2 cells) to an OD_{540} of 1.0. Each microtiter plate well was inoculated with 100 μl of bacterial culture diluted to 10^7 to 10^8 CFU per ml. The plates were centrifuged (148 × g for 10 min) and then incubated for 2 h in 5% CO_2 at 35°C to allow attachment and invasion. Next, wells were washed three times with PBS to remove unattached bacteria, 100 μg of gentamicin per ml in tissue culture medium was added, and the incubation was continued for 2 h. Wells were next washed three times with PBS to remove the gentamicin, and then the cells were incubated with 0.2 ml of 1% trypsin–EDTA for 5 min at 35°C as described previously (31). L broth (0.8 ml) was subsequently added to each well, the contents were mixed by repeated pipetting, and 100 μl of the mixture was plated on culture plates containing the appropriate medium: L agar (SL1344), L agar with 100 μg of ampicillin per ml [HB101(pMJK7)], or CFS (LA228R and 35000). Each well was inoculated onto at

TABLE 1. Adherence to HFECs, rabbit virulence, and morphology in broth cultures of eight *H. ducreyi* strains

Strain	% HFECs (mean ± SE) with attached bacteria[a]	No. of bacteria/ HFEC[b]	No. of bacteria between HFECs[c]	% HEp-2 cells (mean ± SE) with attached bacteria[a]	Virulence in rabbits[d]	Chaining in broth
H. ducreyi						
LA228R	97 ± 2	4+	0	99 ± 1	+	+
V149/91	99 ± 2	3+	2+	94 ± 1	+	+
V-180	99 ± 2	3+	3+	71 ± 6	+	+
35000	97 ± 2	3+	2+	100 ± 1	+	+
CF101	100 ± 2	3+	4+	89 ± 2	+	+
CH2	98 ± 2	2+	4+	74 ± 5	+	+
CIP A77	18 ± 3	1+	0	21 ± 1	−	−
CIP A75	23 ± 5	1+	0	16 ± 3	−	−
H. influenzae						
Rd	<2	0	0	ND[e]	ND	ND

[a] Attachment assays of HFECs were performed in triplicate in two separate experiments, and results are expressed as the mean ± the SE of all six values. Attachment assays with HEp-2 cells were performed in duplicate from a single experiment. One hundred cells were counted in each assay.
[b] The numbers of bacteria per HFEC were estimated relative to LA228 (designated 4+) and Rd (designated 0).
[c] The amounts of attachment between cells were estimated relative to CF101 (designated 4+) and LA228 (designated 0).
[d] Virulence in the rabbit model was defined as the ability to form eschars at an inoculum of <10^8 CFU. All the strains except CIP A75 and CIP A77 elicited lesions after injections of 1 × 10^5 to 5 × 10^5 CFU. CIP A75 and CIP A77 did not elicit ulcers even after injections of 10^8 CFU.
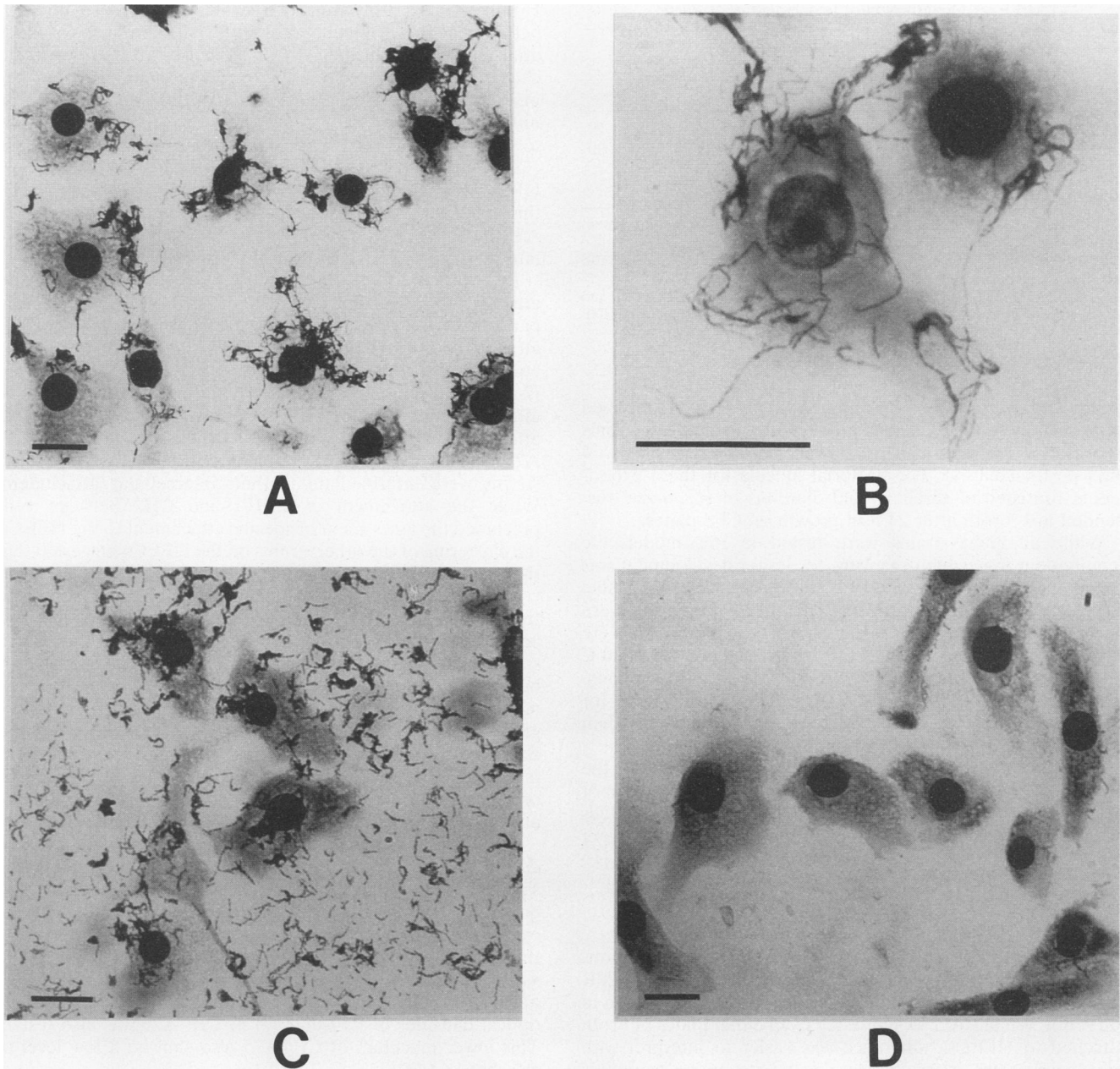[e] ND, not done.

FIG. 2. Attachment assays showing the interactions of three different *H. ducreyi* strains with HFECs. (A and B) Two different magnifications showing LA228R attached to HFECs. (C) CF101 attached between HFECs. (D) Limited attachment of CIP A75 to HFECs. Bar = 25 μm.

least two culture plates, and levels of invasion were calculated from at least three wells in each experiment. Levels of invasion were calculated as percentages of the initial inoculum determined from serial 10-fold dilutions plated in triplicate for each experiment. Gentamicin at a concentration of 100 μg/ml was sufficient to kill 100% of the inoculum after 2 h of incubation without HFECs or HEp-2 cells in all strains examined. In these experiments, 100% of the inoculum was present on the cells after centrifugation. The number of bacteria during the 2-h incubation period increased approximately fivefold, and this increase was similar to that of the *E. coli* control. In experiments analyzing the effects of cytochalasin B and D on invasion, doses of 5 μg of these drugs per ml were added to the HFECs 1 h before the addition of the bacteria and these levels

were maintained throughout the experiment. Cytochalasin B and D had no effect on the viability of strain LA228R.

Temperature-dependent rabbit model. Eight *H. ducreyi* strains (see Table 1) were tested for their ability to elicit dermal lesions in male New Zealand White rabbits housed at 18 to 20°C as described previously (27). This temperature-dependent rabbit model differs from the rabbit model of Hammond et al. (12) originally used to test strains of *H. ducreyi* for virulence. In the temperature-dependent model, only viable organisms elicit lesions and a much lower inoculum (10^5 versus 10^7 CFU) is required for lesion development. Briefly, the backs of male New Zealand White rabbits were shaved to remove the fur, 0.1 ml of the appropriate *H. ducreyi* suspension was injected in duplicate into each rabbit, and the animals were

TABLE 2. Quantitation of attachment of *H. ducreyi*

Strain	Inoculum viable count (CFU) in:		% Attachment (mean ± SE) in[a]:	
	Expt 1[b]	Expt 2[c]	Expt 1	Expt 2
LA228R	2×10^6	2×10^7	30 ± 3	57 ± 19
CF101	4×10^6	1×10^7	33 ± 1	60 ± 20
CIP A75	4×10^7	7×10^7	4 ± 1	$2 \pm <1$
		6×10^6		4 ± 1

[a] The percent attachment is expressed as CFU per well after the attachment assay divided by inoculum CFU times 100.

[b] In experiment 1, the bacterial inocula were adjusted to an OD_{540} of 0.1 and 100 μl was added to each of three wells.

[c] In experiment 2, the bacterial inocula were adjusted to an OD_{540} of 1.0 and 100 μl was added to each of three wells. In addition, a 1:10 dilution of the CIP A75 inoculum was added to an additional three wells.

observed daily for lesion development for 1 week as described by Purcell et al. (27). Lesions produced by virulent organisms appeared as pustules which developed into eschars at 3 to 4 days postinoculation. The bacterial inocula for these experiments consisted of serial 10-fold dilutions of *H. ducreyi* suspended in L broth after 24 h of growth on CFS plates.

While all eight strains were tested in this model, the requirement for viable organisms for lesion development was tested only with LA228R. In these experiments, viable LA228R cells elicited lesions when 10^5 to 10^8 organisms were injected. L broth alone caused no lesions, and none were caused by heat-killed LA228R (10^8 organisms per ml at 100°C for 10 min) or UV-treated LA228R (10^8 organisms per ml 6 in. [ca. 15 cm] from an inverted UV transilluminator exposed for 10 min with shaking; 1,000 viable organisms remained) when injected in parallel with the virulent untreated strain.

Statistical analysis. Student's *t* test was used to calculate the significance of the differences between the percent invasion of *H. ducreyi* and that of the noninvasive *E. coli* control. *P* values were considered significant if they were <0.05%. Values were reported as means ± standard errors.

RESULTS

Attachment of *H. ducreyi* to HFECs. To identify conditions that would affect adherence, we compared *H. ducreyi* LA228R grown overnight on CFS plates with the same strain grown in Hd broth. While LA228R harvested from either plates or broth attached to HFECs, adherence was easier to interpret with organisms grown in broth because the organisms were less aggregative than organisms grown on CFS plates (data not shown). Attachment of LA228R to HFECs was time and dose dependent, requiring at least 2 h for optimal attachment (Fig. 1A) and increasing linearly with bacterial concentration (Fig. 1B). There was no attachment of *H. influenzae* Rd after 3 h of incubation, indicating that attachment of LA228R was specific and not due to inadequate washing of the microtiter plate wells. Attachment of LA228R to HFECs was heat labile; this organism attached to only 16% ± 0.5% of the HFECs after being heated for 0.5 h at 56°C. In subsequent assays, *H. ducreyi* strains were grown in Hd broth, 2 h was allowed for adherence, and 10^7 organisms per well were added to wells containing 5 × 10^5 HFECs.

We compared eight different *H. ducreyi* strains in this assay and found that they differed in their abilities to attach to HFECs (Table 1). Strains LA228R, V149/91, V-180, 35000, CF101, and CH2 attached to >90% of the HFECs, while strains CIP A77 and CIP A75 attached poorly (<30%).

However, even the latter two strains attached significantly better than Rd, a nonadherent strain of *H. influenzae*, which attached to <2% of the HFECs. Because the *H. ducreyi* cells chained and clustered on the HFECs, quantitation of the number of bacterial cells per HFEC was difficult. However, the number of bacterial cells per HFEC was estimated to be >50 to 100 per HFEC for LA228R and 1 to 10 per HFEC for CIP A75. Thus, for all eight strains, the number of bacteria per HFEC was semiquantitatively determined for each strain. By this analysis, LA228R attached in the greatest numbers, CIP A75 and CIP A77 attached the least, and the other strains were intermediate (Table 1 and Fig. 2).

In addition to attaching to HFECs, several strains also attached to areas between the cells. These two different patterns of attachment are shown in Fig. 2. Strain LA228R attached specifically to the HFECs (Fig. 2A and B), and CF101 attached between as well as to the HFECs (Fig. 2C). The eight *H. ducreyi* strains tested varied in the observed amounts of attachment between the HFECs, with strains CF101 and CH2 attaching the most between the HFECs and LA228R, CIP A75, and CIP A77 never attaching between cells. The other strains were intermediate in their intercellular attachment. While the attachment of CF101 and CH2 between cells precluded the assessment of specific attachment to the HFECs, the clustering of the other strains on the HFECs suggested that they were attached specifically to the HFECs (Fig. 1).

The attachment of CF101 between cells was examined further. No attachment of CF101 to glass coverslips preincubated with PBS, 1% gelatin, 1% BSA, or KGM without added supplements was seen. However, CF101 attached frequently but not always to wells coated with KGM with added supplements. The supplements added to KGM included epidermal growth factor and pituitary extract (Life Technologies Gibco BRL), two biologically important molecules. In all experiments, CF101 attached between the cells when either HFECs or HEp-2 cells were present. As expected, LA228R did not attach to the wells in any of these experiments.

The bacterial inocula of three representative strains of *H. ducreyi*, LA228R, CF101, and CIP A75, were adjusted to the same OD_{540} reading, and attachment of these strains was quantitatively measured by viable counts (Table 2). In these experiments, the percentages of the inocula that attached to the HFECs were 30 to 60% for LA228R and CF101 and 2 to 4% for CIP A75. Because the CFU count of CIP A75 was much higher than those of LA228R and CF101 at comparable optical densities, a 1:10 dilution of CIP A75 was also tested. This lower inoculum of CIP A75 also showed a low level of attachment (4%).

The appearance of *H. ducreyi* strains after growth in Hd broth varied significantly. Strains LA228R, V149/91, V-180, 35000, CF101, and CH2 all formed chains, while strains CIP A77 and CIP A75 appeared as individual cells (data not shown).

Virulence in rabbits. We tested all *H. ducreyi* strains for their abilities to elicit dermal lesions in the temperature-dependent rabbit model of Purcell et al. (27). Strains CIP A75 and CIP A77 were unable to elicit lesions, even after infecting doses of 10^8 organisms. All other strains of *H. ducreyi* were able to elicit lesions with inocula of <10^6 CFU and thus were considered virulent in this model (Table 1). The abilities of *H. ducreyi* strains to cause lesions in the rabbit model correlated with their abilities to attach to HFECs (Table 1). Both strains that were avirulent in the rabbit model, CIP A75 and CIP A77, attached poorly to HFECs, while the virulent strains were able to attach either directly to cells or to areas between cells.
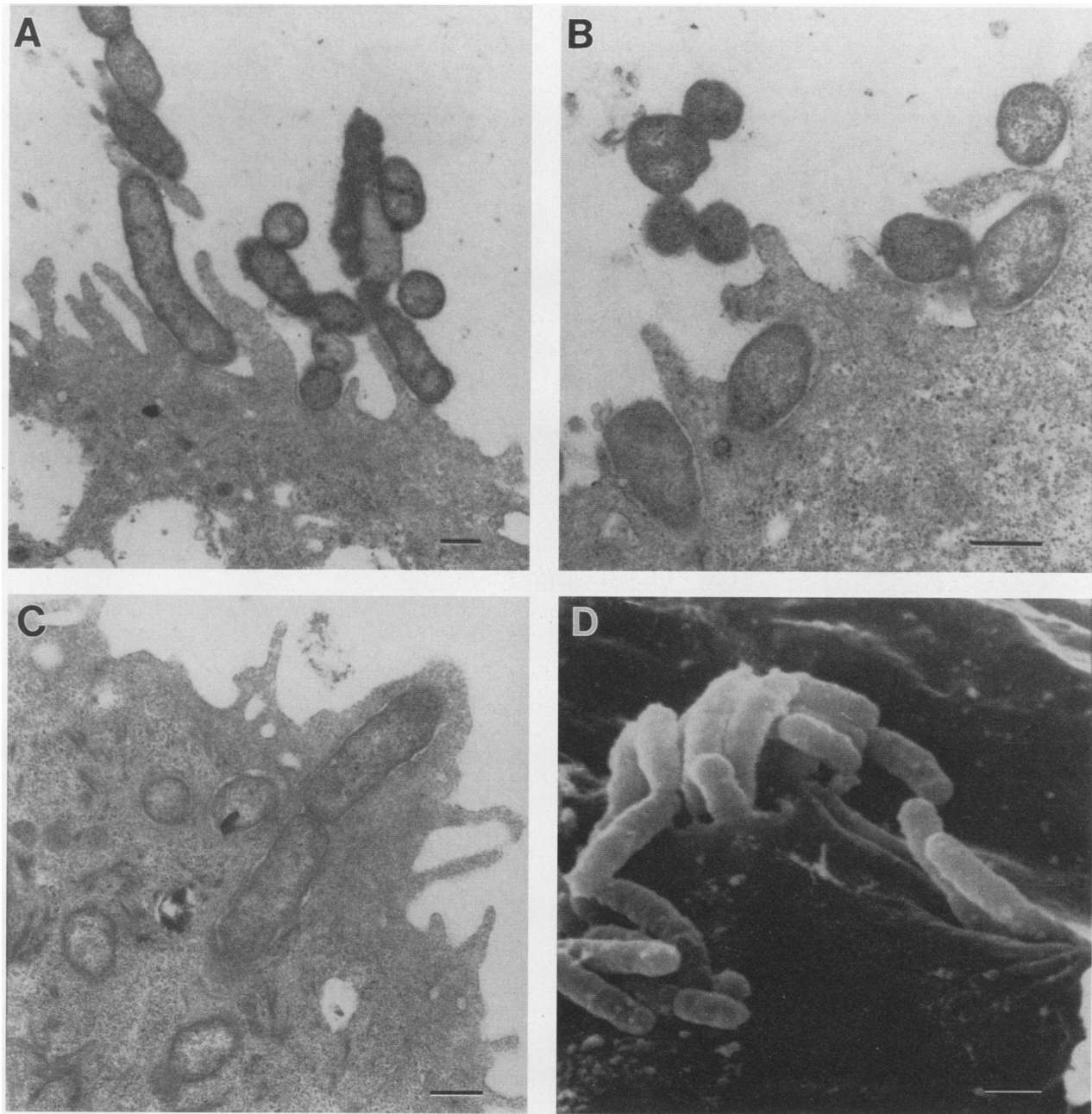
FIG. 3. The interaction of strain LA228R and the HFECs was examined by electron microscopy. Panels A, B, and C are transmission electron micrographs. (A) Bacteria attached to protrusions from the HFECs. (B) Bacteria engulfed by the cells. (C) Bacteria contained within vacuoles inside the HFECs. (D) Scanning electron micrograph showing bacteria which appear to be entering the HFEC. Bar = 0.5 μm (panels A, B, and C); bar = 1 μm (panel D).

Electron microscopy. The nature of the interaction of *H. ducreyi* LA228R and HFECs was examined by electron microscopy. By SEM, bacteria could be seen adhering to the HFECs and in certain areas appeared to be entering these cells (Fig. 3D). TEM was used to further characterize the interaction of LA228R with HFECs (Fig. 3). In these studies, LA228R appeared to be adherent to the HFECs (Fig. 3A), engulfed by the HFECs (Fig. 3B), and within vesicles inside the HFECs (Fig. 3C).

Ruthenium red dye exclusion. Ruthenium red staining was used to test whether the bacteria seen by TEM were truly inside the cell and therefore enclosed by membranes inaccessible to ruthenium red, which stains only surface-exposed membranes (20). In these experiments, dye was added with the fixative after *H. ducreyi* had been allowed to interact with the HFECs and the cellular membranes surrounding the organism were examined by TEM for staining. *H. ducreyi* LA228R could frequently be seen surrounded by membranes that were not stained by dye (Fig. 4B), consistent with organisms contained in vesicles. In addition, the organism was also seen free of host membranes (Fig. 4A), suggesting that it was able to enter the cytoplasm of the host cell.
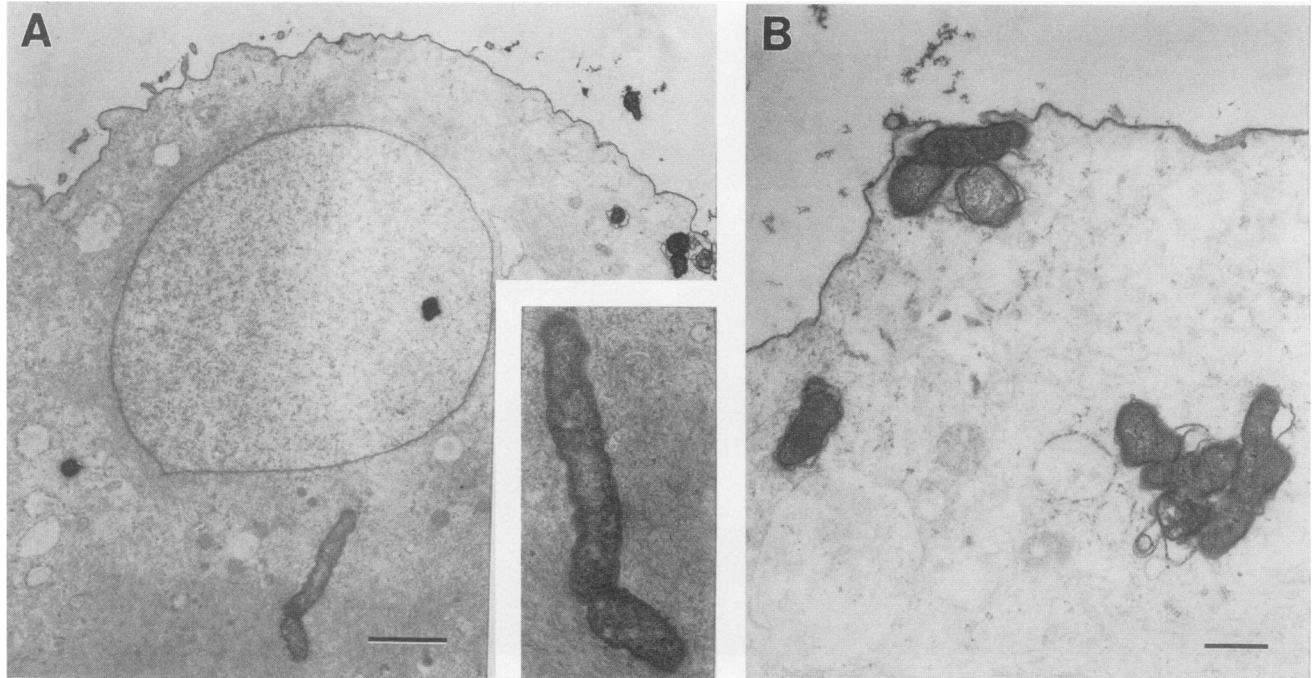
FIG. 4. Dye exclusion experiments suggesting intracellular location of strain LA228R. (A) *H. ducreyi* cells inside the HFEC, not surrounded by a vesicle. The inset is an enlargement of the bacteria seen in panel A. The enlargement shows the lack of a host membrane surrounding the bacteria. (B) *H. ducreyi* cells inside vesicles surrounded by membranes poorly stained with ruthenium red (compare the vesicle membranes with the cytoplasmic membrane, which is stained). Bar = 0.5 μm (panel A); bar = 2.0 μm (panel B).

Gentamicin protection assay. The gentamicin protection assay was used as an independent measure of the ability of *H. ducreyi* LA228R to invade both HFECs and HEp-2 cells. In the representative experiment detailed in Table 3, 1.4% of the LA228R inoculum was able to invade HFECs. This level of invasion was significantly greater than that of the adherent, noninvasive negative control, *E. coli* HB101(pMJK7). Results similar to this result were obtained in three separate experiments, and they indicated that LA228R was 33-fold ± 3.4-fold more invasive than HB101(pMJK7) ($P < 0.05$). In these and subsequent experiments, the levels of LA228R invasion into HFECs ranged from 0.7 to 1.4%, but LA228R was always at least 20-fold more invasive than HB101(pMJK7).

H. ducreyi LA228R was also able to invade HEp-2 cells, a transformed cell line of human respiratory epithelial cells (Table 3). Approximately 1.2% of the inoculum was able to invade HEp-2 cells, which is an amount significantly greater than that found for the noninvasive, adherent control

HB101(pMJK7). Again, results similar to this result were found in three separate experiments, in which the levels of invasion of LA228R were 0.7 to 1.7. In these experiments, LA228R was always at least 20-fold more invasive than HB101(pMJK7).

Other strains of *H. ducreyi* tested in the gentamicin protection assays were less invasive than strain LA228R. In a representative experiment, the percentages of invasion were LA228R, 0.7% ± 0.1%; 35000, 0.12% ± 0.05%; CIP A75, 0.21% ± 0.05%; and HB101(pMJK7), <0.03%.

Effects of cytochalasin B and D on invasion. Invasion experiments in which cytochalasin B and D were used indicated that these two microfilament inhibitors decreased the ability of LA228R to invade HFECs and HEp-2 cells (Table 4). Thus, in experiments in which cytochalasin B and D were present, invasion percentages of LA228R were 27 and 6%, respectively, of those with the control HFECs with no additives ($P < 0.05$ for both inhibitors). Similarly, with HEp-2 cells, LA228R invasion percentages with cytochalasin B and D were 19 and <1% of those with the control, respectively ($P = 0.05$ for cytochalasin B and $P < 0.05$ for cytochalasin D). These degrees of inhibition were comparable to those of the control, *Salmonella* strain SL1344 (Table 4).

DISCUSSION

In these studies, we have shown that *H. ducreyi* is able to attach to and invade primary cultures of HFECs and HEp-2 cells. The eight strains of *H. ducreyi* tested showed two different patterns of attachment: adherence specifically to the HFECs and adherence between cells. In addition, the strains that attached specifically to the HFECs varied in their abilities

TABLE 3. Invasion of HFECs and HEp-2 cells

Organism	Inoculum (CFU) for:		% Invasion (mean ± SE) of[a]:	
	HFECs	HEp-2 cells	HFECs	HEp-2 cells
S. typhimurium SL1344	2 × 10⁷	1 × 10⁶	14.7 ± 0.4	9.4 ± 0.3
H. ducreyi LA228R	1 × 10⁷	5 × 10⁵	1.43 ± 0.01	1.2 ± 0.3
E. coli HB101(pMJK7)	1 × 10⁷	5 × 10⁵	0.061 ± 0.001	0.01 ± 0.005

[a] The percent invasion was calculated as the percentage of the initial inoculum surviving treatment with gentamicin. Values were obtained in triplicate from each experiment. Similar results were obtained in three separate experiments.

TABLE 4. Effect of microfilament inhibitors on bacterial invasion of HFECs and HEp-2 cells[a]

Inhibitor	% SL1344 cells (mean ± SE) invading:		% LA228R cells (mean ± SE) invading:	
	HFECs	HEp-2 cells	HFECs	HEp-2 cells
None	100	100	100	100
Cytochalasin B	36 ± 2	19 ± 9	27 ± 2	19 ± 11
Cytochalasin D	11 ± 5	<1	6 ± 1	<1

[a] Values were calculated as the percentage of the initial inoculum surviving gentamicin treatment with an inhibitor divided by the percentage of the inoculum surviving gentamicin treatment without an inhibitor.

to attach, with avirulent strains showing the least attachment. We selected the most adherent strain, LA228R, and found by the use of three different methods that it was able to invade epithelial cells. The methods included examination by SEM and TEM, ruthenium red dye staining (vesicles containing ingested bacteria did not stain), and the gentamicin protection assay. LA228R was also able to attach to and invade HEp-2 cells, a more accessible epithelial cell line suitable for the study of this important pathogen.

The different *H. ducreyi* attachment patterns (to the HFECs and between the HFECs) may reflect differences in adhesins contained by these different strains. Many pathogens contain multiple adhesins which provide diversity in attachment opportunities and whose functioning depends on the environment in which the organism finds itself. For example, *E. coli* contains an assortment of adhesins which provide multiple attachment capabilities (14). Alternatively, because *H. ducreyi* strains in culture possess different colony types (2, 22), it is possible that attachment is a function of the amount of each colony type present in the strain. Such differences in attachment capability associated with colony types are found with *Neisseria gonorrhoeae*, in which two different adhesins, pili and protein II, are subject to phase variation (18).

The two strains that demonstrated low levels of attachment to HFECs were also avirulent in the rabbit model. This may indicate that the ability to adhere is an important virulence factor in the rabbit model, either for initial attachment or for delivery of possible contact-dependent toxins to the appropriate cells. In studies reported by Alfa et al. (4), *H. ducreyi* strain CIP A77 attached poorly to human fibroblasts (3), indicating that avirulence and poor attachment may also be correlated in other cell systems. However, in our studies, the organisms were injected intradermally at high concentrations and thus potentially bypassed the need for normal attachment mechanisms. Alternatively, attachment may be mediated in part by LOS, which are also associated with virulence in the rabbit model (7, 24). The inabilities of the poorly adherent strains CIP A75 and CIP A77 to induce lesions in the rabbit model of Hammond et al. (12) have been previously associated with the unique LOS of the strains (24). Our results support this association in the temperature-dependent rabbit model of Purcell et al. (27). It is possible that the unique LOS in the avirulent strains also render the strains inefficient in attachment. Finally, the avirulent *H. ducreyi* strains may also be defective in other virulence factors besides LOS, including factors that mediate attachment to HFECs. Further studies are required to determine the nature of the adhesins, their specificity of attachment, and their role in virulence.

The results of our SEM and TEM studies were consistent with entry and internalization of LA228R into HFECs. Further studies confirmed that the organisms were inside the HFECs

because they were surrounded by membranes inaccessible to the surface-staining dye ruthenium red. The patterns of internalization of *H. ducreyi* revealed by TEM implied that the organism is ingested into vesicles in a manner similar to those of *Salmonella*, *Shigella*, and *Listeria* spp. However, unlike *Salmonella* spp., *H. ducreyi* was also seen free in the cytoplasm, suggesting a pattern of invasion similar to that of *Shigella* spp. in which the organism escapes from the vesicles into the cytoplasm (11).

Results of the gentamicin protection assay also supported the hypothesis that *H. ducreyi* is able to invade epithelial cells. While the level of invasion by *H. ducreyi* was significantly greater than that by the noninvasive *E. coli* control, it was lower than that by a highly invasive strain of *Salmonella*. However, the level of invasiveness displayed by *H. ducreyi* is not inconsistent with the levels of invasion displayed by other known invasive bacteria, such as enteroinvasive *E. coli* and *H. influenzae* (16, 31). Future studies on the regulation of genes required for invasion by *H. ducreyi* may reveal conditions under which invasion is enhanced. While the mechanism of invasion for LA228R is a subject for future study, the inhibition of invasion in the presence of cytochalasin B and D indicates that microfilaments are required for internalization, as they are for other invasive bacteria (10). Two other strains of *H. ducreyi*, 35000 and CIP A75, showed lower levels of invasion of HFECs than other strains, perhaps reflecting their lower attachment properties for this cell line.

The ability of *H. ducreyi* to invade host epithelial cells is consistent with the ulcerative lesions caused by this organism in infected patients. Invasion into epithelial cells would enable the organism to penetrate host tissue and cause the tissue destruction present in genital ulcers. While invasion of other cell types would enable the organism to penetrate more deeply and possibly be transported to the regional lymph nodes, the ability of *H. ducreyi* to invade other cell types is either controversial or unstudied. Lammel et al. (19) have shown by SEM and TEM that the pattern of interaction of *H. ducreyi* with human foreskin fibroblasts is consistent with invasion. However, using the gentamicin protection assay, Alfa et al. (4) were unable to demonstrate invasion of fibroblasts. Differences in the ability of *H. ducreyi* to invade HFECs and fibroblasts may reflect a specificity of invasion for epithelial cells or may reflect differences in the *H. ducreyi* strains tested or the experimental conditions used. The ability of *H. ducreyi* to invade other specific cell types and the correlation of this invasive ability with disease are clearly interesting avenues for further study.

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