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Haemophilus ducreyi Infection Causes Basal Keratinocyte Cytotoxicity and Elicits a Unique Cytokine Induction Pattern in an In Vitro Human Skin Model

MARCIA M. HOBBS,* TERRY R. PAUL, PRISCILLA B. WYRICK, AND THOMAS H. KAWULA

Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

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***Haemophilus ducreyi* is the etiologic agent of the sexually transmitted genital ulcer disease chancroid. Predominantly a cutaneous pathogen, *H. ducreyi* is present in chancroid ulcers that are characterized by extensive neutrophil accumulation in intraepidermal lesions accompanied by a mononuclear infiltrate in the dermis. We used an in vitro human skin model composed of foreskin fibroblasts and keratinocytes to examine host skin cell interactions with *H. ducreyi* 35000. Bacteria replicated and persisted in artificial skin for at least 14 days. We observed *H. ducreyi* inside suprabasal keratinocytes using transmission electron microscopy. Although no bacteria were seen in the basal keratinocyte region, these cells were disrupted in infected cocultures. *H. ducreyi* infection stimulated increased secretion of interleukin-6 (IL-6) and IL-8 by skin cells. Conversely, tumor necrosis factor alpha and IL-1α levels were not elevated. IL-8 produced in response to *H. ducreyi* infection may be involved in recruiting polymorphonuclear leukocytes and other inflammatory cells, thereby contributing to the tissue necrosis and ulcer formation characteristic of chancroid.**

Chancroid, an ulcerating cutaneous infection caused by *Haemophilus ducreyi*, is one of the most prevalent sexually transmitted diseases in developing countries (61) and is endemic in some areas of the United States (6, 8, 16, 19, 36). Chancroid is a recognized cofactor for human immunodeficiency virus transmission (33, 40), making *H. ducreyi* the focus of considerable recent research aimed at understanding the molecular mechanisms of pathogenesis. Chancroid ulcers contain a superficial zone of polymorphonuclear leukocytes (PMNs) and extracellular debris accompanied by a dermal infiltrate of T cells and macrophages (20, 38, 61). The mechanisms by which *H. ducreyi* causes or induces epithelial destruction during infection are not known.

Several potential *H. ducreyi* virulence factors have been identified, including lipooligosaccharide (LOS) (11), pili (9), and the production of extracellular cytotoxin(s) (30, 31, 44) and hemolysin (41, 60). Understanding the relative contributions of these factors and identifying other bacterial components essential for *H. ducreyi* pathogenesis require appropriate models of infection. Several useful animal models have been described, including rabbits (25, 43), primates (59), and pigs (27), and experimental human challenge studies employ the natural host for *H. ducreyi* infection (52, 53). Absent from the repertoire of experimental chancroid systems has been a relevant in vitro human skin cell model.

Human tissue culture monolayers have been used as in vitro substrates for adherence and invasion studies with a number of sexually transmitted pathogens, including *Neisseria gonorrhoeae*, *Treponema pallidum*, and *Chlamydia trachomatis* (28, 48, 57). Similar studies with *H. ducreyi* and cultured human foreskin fibroblasts have yielded confusing and often contradictory results. Several independent studies have shown that *H. ducreyi* adheres to fibroblasts; some indicate that the bacteria are invasive (32), and others suggest that the bacilli are not found within these host cells (2, 3). While fibroblasts are present in the dermis, the relevance of *H. ducreyi* attachment to and invasion of these cells has not been demonstrated in vivo. Keratinocytes are the predominant epithelial cell in the epidermis and are likely the first host cells with which *H. ducreyi* interacts during infection. Recent studies with purified human foreskin keratinocytes or primary human foreskin epithelial cells grown in monolayers suggest that *H. ducreyi* attaches to (10) and invades (58) these cells.

Cultured cells offer host tissue relevance and ease of growth and manipulation; however, cellular morphology and the distribution of cell surface components in monolayers may not accurately reproduce the environment encountered by an organism infecting the human host. By using alternative tissue culture substrates and techniques, it is possible to maintain polarized epithelial cell layers in vitro with differentiated apical and basolateral surfaces and well-developed tight junctions. Artificial tissue systems have been constructed by layering relevant cell types. An artificial skin model has been developed using neonatal foreskin fibroblasts and keratinocytes in a coculture system composed of dermal, epidermal, and stratum corneum layers anchored to a nylon mesh support (17, 55). Important features of the model include the development of a basal lamina and anchoring zone (13) supporting the stratified epithelium composed of keratinocytes expressing the K1 keratin differentiation marker (50). K1 is expressed by epithelial cells in human foreskin and adult skin, but not by keratinocytes cultured in monolayers (50).

In the present study, we examined the interactions of virulent *H. ducreyi* 35000 with human foreskin keratinocytes and fibroblasts in infected artificial skin. Bacteria were observed inside suprabasal keratinocytes, and structural changes in the epidermis were consistent with the pathology of chancroid. We characterized the cellular interleukin-1α (IL-1α), tumor necrosis factor alpha (TNF-α), IL-6, and IL-8 responses to *H. ducreyi* infection and herein describe an unusual pattern of proinflammatory cytokine induction.

* Corresponding author. Mailing address: Department of Medicine, University of North Carolina School of Medicine, CB 7030, Chapel Hill, NC 27599. Phone: (919) 966-5050. Fax: (919) 966-6714. E-mail: mmhobbs@med.unc.edu.

MATERIALS AND METHODS

Human foreskin keratinocyte-fibroblast cocultures (KFCs). Artificial skin samples (Skin2 model ZK 1300) were obtained from Advanced Tissue Sciences (ATS; LaJolla, Calif.). Culture conditions have been previously described in detail (39). Briefly, human neonatal foreskin fibroblasts and keratinocytes were isolated and expanded into monolayer cultures (13). Fibroblasts seeded onto an inert nylon mesh grew submerged for 26 days, during which time they secreted an extracellular matrix (ECM) composed of mature collagen fibrils, fibronectin, and both sulfated and nonsulfated glycosaminoglycans (51). Keratinocytes were seeded onto the dermal substrate, grown submerged for 1 week, and then raised to the air-liquid interface. Cocultures were maintained for 13 days at ATS before squares (9 by 9 mm) were laser cut and placed on agarose containing growth medium with antibiotics for shipment and overnight delivery.

Upon receipt in our laboratory, KFCs were placed with the dermal side resting on the permeable membrane of 25-mm MilliCell culture inserts (Millipore, Bedford, Mass.) inside 35-mm chambers of six-well tissue culture plates (Corning Costar, New York, N.Y.). Cells were maintained at 35°C in a humidified atmosphere with 5% (vol/vol) CO_2 with 1 ml of tissue culture medium (Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 100 μg of ascorbate and 0.5 μg of hydrocortisone per ml) supplied beneath the insert and replaced daily. In this system, liquid medium contacts the lower surface of the culture inserts, but the artificial skin squares and bacterial inocula are maintained at the air interface and are not immersed in fluid. KFCs were equilibrated with antibiotic-free medium for 3 days prior to inoculation to ensure that residual antibiotics were removed from the tissue.

Microscopic analysis of uninfected KFCs confirmed the presence of a well-developed epidermis overlaying a dermal equivalent of fibroblasts embedded in secreted ECM material (see Fig. 2). Basal keratinocytes formed a characteristic columnar layer with several suprabasal squamous cell layers covered by a thin stratum corneum. The extent of epidermal differentiation and functional properties of KFCs are detailed elsewhere (13, 50, 51, 56, 63).

Bacterial cultures and inoculation procedures. *H. ducreyi* 35000 (ATCC 33922) was isolated in Winnipeg, Canada, in the 1970s (24). The virulence of this isolate has been demonstrated in several animal models of infection (27, 43, 53, 59). *Escherichia coli* CC118 (34) and an uncharacterized isolate of *Staphylococcus epidermidis* were used as controls in some experiments. Bacteria were grown on chocolate agar (Difco GC agar base, 1% [wt/vol] IsoVitaleX [Becton Dickinson, Cockeysville, Md.], and 1% hemoglobin) at 35°C in a humidified atmosphere with 5% [vol/vol] CO_2 and passaged daily up to three times from frozen stocks.

Inocula were prepared from log-phase cultures grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% IsoVitaleX and 50 μg of hemin per ml. Dilutions were made with filtered culture supernatants to maintain the concentration of potential bacterial virulence factors secreted during growth in vitro. Approximately 10^5 CFU in 10 μl were applied to the surface of KFCs. Filtered *H. ducreyi* culture supernatants and heat-killed inocula were applied to separate artificial foreskin squares to determine the relative contributions of secreted and heat-labile bacterial products in this model of infection. Controls for each experiment included BHI broth-inoculated and untreated but abraded samples. Infected and control cocultures were incubated at 35°C in a humidified atmosphere with 5% (vol/vol) CO_2.

Preliminary experiments to determine the degree of abrasion required for *H. ducreyi* infection and productive interaction with foreskin cells in this model were conducted. Inocula were delivered by painting KFCs with a pipette tip as 10 μl was dispensed evenly over the surface of the square. In some experiments, inocula were dropped onto squares without mechanically perturbing the surface. Ultimately, uniform abrasion was achieved by using a sterile cytology brush with the tip bent at a right angle to the handle. The bristles of the brush were tapped lightly across the entire surface of each square, moving first horizontally and then vertically and along both diagonals. The inoculum was dropped onto the center of each square immediately after surface abrasion. The results presented here are from two independent experiments, with different lots of KFCs, abraded with cytology brushes before inoculation. Uninoculated control KFCs were also abraded. For each experiment, three to seven individual squares were included in each treatment or control group.

Bacterial recovery and sample collection. At various times after inoculation, artificial foreskin samples were rinsed three times with 1 ml of phosphate-buffered saline (PBS) to remove loosely attached bacteria. To control for the effects of this procedure on subsequent microscopic and biochemical analyses, all samples, including uninoculated controls, were rinsed at the time of harvesting. Rinsed squares were quartered, and portions of each sample were fixed in 10% formalin for routine histology and 2% paraformaldehyde with 0.05% glutaraldehyde for electron microscopy, and these were frozen for future immunohistochemical analyses. The remaining quarter of samples infected with live *H. ducreyi* was incubated for 30 min in swelling buffer (1 mM EDTA, 10 mM Tris, 0.25 M sucrose) to render the eukaryotic cells osmotically fragile. Samples were then subjected to two 10-s bursts in a sonicating water bath, serially diluted in PBS, and plated on chocolate agar in duplicate. This procedure did not affect recovery from control *H. ducreyi* suspensions and resulted in lysis of the foreskin cells as judged by microscopic examination. Thus, potentially adherent and invasive bacteria would be recovered from these samples. Cell-associated counts recovered from one-fourth of a square (9 by 9 mm) were multiplied by 4 to normalize recovery to the entire sample. Likewise, the numbers of CFU per milliliter in PBS washes were multiplied by 3 for comparison with cell-associated counts.

At each sampling time, the 1 ml of culture medium beneath cell culture inserts was removed from all samples and replaced with fresh medium. Samples were aliquoted and stored at −70°C.

Cytokine assays. Human TNF-α, IL-1α, IL-6, and IL-8 Quantikine kits (R&D Systems, Minneapolis, Minn.) were used with a slight modification of the manufacturer's directions to determine cytokine concentrations in culture medium collected from KFCs. Sample and standard volumes (50 versus 100 μl) used were smaller than those suggested in product inserts for TNF-α and IL-1α. The minimum detectable concentrations were 4.4 and 0.5 pg/ml for TNF-α and IL-1α, respectively. Samples were diluted 1:100 and 1:1,000 to determine IL-6 and IL-8 concentrations, respectively. These assays were performed exactly as recommended by the manufacturer. The minimum detectable concentrations were 0.7 and 18 pg/ml for IL-6 and IL-8, respectively. Cytokine concentrations were expressed as picograms per milliliter for TNF-α and IL-1α and as nanograms per milliliter for IL-6 and IL-8.

Statistical analyses. Data are presented as means ± standard deviations. *H. ducreyi* recovery data (Fig. 1) are from two separate experiments with a total of two to seven individual infected skin squares per time point, plated in duplicate. Cytokine concentrations (see Fig. 4) are from two separate experiments with a total of five to nine individual skin squares per time point, determined in duplicate. Since the data exhibited normal distributions, comparisons between treatment groups and controls were made by using the paired or unpaired Student's *t* test, as indicated in the text.

Microscopy. For light microscopy, formalin-fixed samples were embedded in paraffin, processed for routine histology, and stained with hematoxylin and eosin. *H. ducreyi* were identified in these samples by visual comparison with pure cultures prepared in the same way. Fixed samples of KFCs inoculated with live or heat-killed *H. ducreyi* were embedded in Epon or Lowicryl resin as previously described (64) for transmission electron microscopy (TEM). Specimens were examined in a Zeiss electron microscope operating at 60 kV. For immunoelectron microscopy, grids containing sections of Lowicryl-embedded samples were probed with a 1:100 dilution of rabbit antiserum that had been raised against formalin-fixed, whole *H. ducreyi* 35000. Nonspecific antibody binding was blocked with 1% ovalbumin in PBS containing 0.01 M glycine. The secondary antibody was goat anti-rabbit immunoglobulin G conjugated to 15-nm gold particles. As a control, pure *H. ducreyi* cultures embedded in Lowicryl and labeled in this way routinely bound three to eight gold particles/bacterium (data not shown).

RESULTS

***H. ducreyi* replicate and persist in KFCs for 1 to 2 weeks.** We infected human foreskin KFCs with virulent *H. ducreyi* 35000. The kinetics of bacterial growth and recovery in the model are shown in Fig. 1. Approximately 5.0 $\log_{10}$ CFU of log-phase bacteria were inoculated onto the abraded surface of squares (9 by 9 mm) of artificial skin. *H. ducreyi* achieved a density of approximately 5.9 $\log_{10}$ CFU/square over the first 6 h. This culture was maintained with daily medium changes beneath the cell culture inserts for 7 days in these experiments (Fig. 1, circles). In a single experiment using an artificial skin square (11 by 11 mm) with a similar inoculum, 6.1 $\log_{10}$ CFU of *H. ducreyi* were recovered 14 days after inoculation. When 5.8 $\log_{10}$ CFU of *H. ducreyi* were inoculated onto nylon mesh squares without skin cells, 1.8 $\log_{10}$ CFU were recovered from the bare substrate at day 4 (Fig. 1, squares). Although bacteria were never immersed in fluid in the artificial skin system, we examined the growth of *H. ducreyi* in culture medium for comparison with growth in the intact system. In culture medium alone, without foreskin cells, *H. ducreyi* viability declined to 3.7 $\log_{10}$ CFU over 4 days (Fig. 1, triangles). Thus, human KFCs provided an environment that supported a steady-state bacterial culture that was not maintained in the absence of eukaryotic cells. *H. ducreyi* replicated and persisted in vitro in KFCs for up to 2 weeks, consistent with bacterial recovery from naturally occurring (37, 45) and experimental (52, 53) chancroid lesions of several days' to several weeks' duration.

In contrast to the moderate growth of *H. ducreyi* in artificial skin, *E. coli* grew vigorously, achieving a density of 8.2 $\log_{10}$ CFU/square 4 days after inoculation with 5.5 $\log_{10}$ CFU. Light microscopic inspection of *E. coli*-infected skin squares revealed massive cellular destruction with no intact foreskin cells re-

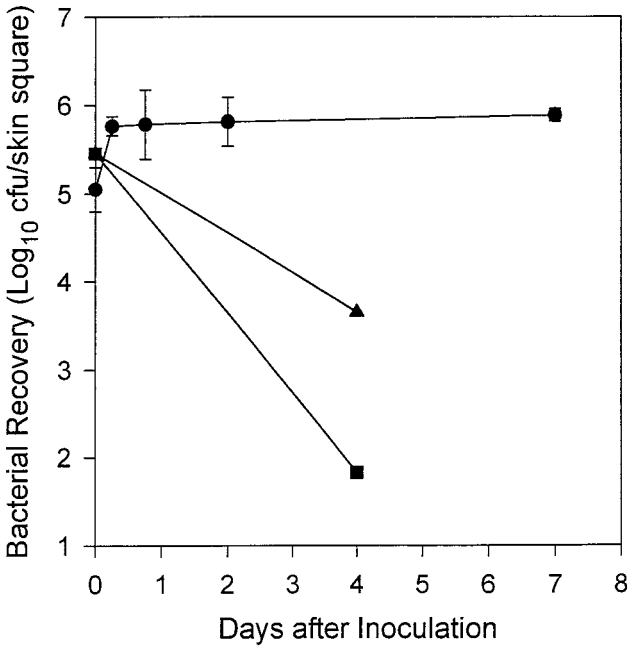

FIG. 1. Time course of *H. ducreyi* recovery from infected artificial skin. Approximately 10^5 CFU of strain 35000 were inoculated onto the abraded surfaces of skin squares (9 by 9 mm). Total recovery from skin squares was calculated from quantitative cultures of rinsed plus adherent bacteria (circles). Data are from two independent experiments with a total of two to seven individual infected skin squares per time point, plated in duplicate. Data for *H. ducreyi* cells recovered from single samples of blank nylon mesh (squares) and cell culture medium without eukaryotic cells (triangles) are shown for comparison.

maining by day 4 (data not shown). *S. epidermidis*, often present on human skin, also grew well in artificial skin, reaching 8.7 $\log_{10}$ CFU 2 days after inoculation with 5.6 $\log_{10}$ CFU. Unlike KFCs infected with *E. coli*, foreskin cells appeared to remain grossly intact during infection with *S. epidermidis* (data not shown). Thus, *H. ducreyi* replication in the artificial skin model is modest by comparison with the other bacteria tested.

The data shown in Fig. 1 are total numbers of *H. ducreyi* cells recovered from infected KFCs. To examine bacterial adherence to artificial skin squares, each sample was rinsed three times with PBS, and *H. ducreyi* in the wash and cell-associated fractions were quantitated separately (see Materials and Methods). Six hours after inoculation, there were more cell-associated bacteria than were recovered in the wash (mean $\log_{10}$ CFU/square $\pm$ standard deviations = 5.7 ± 0.1 versus 5.05 ± 0.1; $P < 0.001$ [paired Student's t test]). At all later times, recoveries from cell-associated and wash fractions were equivalent (data not shown).

***H. ducreyi* can invade suprabasal keratinocytes and cause basal cell cytotoxicity.** Portions of artificial skin squares were prepared for light EM or 18 h after abrasion and inoculation with live or heat-killed *H. ducreyi* 35000. Using light microscopy, we saw aggregates of bacteria among the top layers of the stratum corneum in samples infected with live but not heat-killed bacteria (Fig. 2A and B). To further localize *H. ducreyi* within infected KFCs, we used TEM to examine the keratinocytes from the stratum corneum down to the basal layer and fibroblasts in the uppermost portion of the dermis. In samples embedded in Epon resin, occasional gram-negative bacteria were observed in suprabasal keratinocytes (Fig. 2C). The morphology of these bacteria was virtually identical to the morphology of control cultures of log-phase *H. ducreyi* processed and examined by EM (data not shown). In sections embedded in Lowicryl for immunoelectron microscopy, intracellular bacteria were labeled with anti-*H. ducreyi* rabbit antiserum (Fig. 2D) but not with normal rabbit serum (data not shown). Unlike the aggregates of bacteria observed in the uppermost layers of KFCs, intracellular *H. ducreyi* appeared as single cells. These organisms were rare (one or two organisms/tissue section) and did not appear to be contained in membrane-bound vacuoles.

In these studies, we did not identify *H. ducreyi* in or among basal keratinocytes, fibroblasts, or the extracellular material of the dermis 18 h after inoculation of abraded artificial skin. However, the architecture of the dermal-epidermal junction was dramatically altered as a result of infection with *H. ducreyi* 35000. We observed significant keratinocyte cytotoxicity in the basal cell region and in the lower layers of squamous epithelial cells 18 h after abraded KFCs were inoculated with live *H. ducreyi*, but not after abrasion and inoculation with heat-killed bacteria (Fig. 3).

Human foreskin cells secrete a unique pattern of cytokines in response to *H. ducreyi* infection in vitro. We replaced the 1 ml of medium beneath cell culture inserts at various times after inoculation and determined the concentrations of TNF-α, IL-1α, IL-6, and IL-8 released by KFCs in response to *H. ducreyi* infection. Samples were collected from abraded artificial skin squares that had received live or heat-killed *H. ducreyi*, filtered supernatant from log-phase bacterial cultures, or BHI broth or were left untreated.

KFCs responded to *H. ducreyi* infection with increased secretion of IL-6 and IL-8 (Fig. 4A and B). The levels of secreted IL-6 were elevated in control and experimental samples 6 h after inoculation. IL-6 levels remained high in samples infected with live *H. ducreyi*, while they returned to baseline by 48 h in samples from KFCs that had received heat-killed *H. ducreyi*, filtered bacterial culture fluid, or BHI broth or from untreated controls (Fig. 4A). Forty-eight hours after inoculation, infected samples secreted at least 6.9 times more IL-6 than any of the controls ($P < 0.01$ [unpaired Student's t test]).

The magnitude and kinetics of IL-8 secretion by infected KFCs were different from those of IL-6 secretion. IL-8 levels secreted at baseline were higher than IL-6 levels, and infected foreskin cells secreted three to five times more IL-8 than IL-6 at times $\geq$18 h after inoculation (notice the different y axis scales in Fig. 4A and B). IL-8 concentrations remained constant for all treatment groups and controls over the first 6 h and nearly doubled by 18 h after inoculation (Fig. 4B). IL-8 levels continued to increase in samples from KFCs infected with live *H. ducreyi* but returned to baseline by 48 h for all other samples. Infected foreskin cells secreted at least 3.2 times more IL-8 than any of the controls ($P < 0.01$ [unpaired Student's t test]) 48 h after inoculation. Because the culture fluid beneath KFCs was removed and replaced with fresh medium at each time point, cytokine concentrations represent active secretion over the course of these experiments rather than accumulation and persistence of material released at early time points.

TNF-α levels in samples collected before inoculation and at 6, 18, or 48 h after inoculation ranged from $\leq$4.4 to 11.8 pg/ml, with no differences between experimental treatment groups and untreated abraded controls at any time point ($n = 17$). That KFCs were capable of TNF-α secretion was demonstrated by high levels of TNF-α in samples collected 24 h after inoculation with *E. coli* (273.2 pg/ml) and 48 h after inoculation with *S. epidermidis* (246.5 pg/ml).

KFCs released IL-1α over the first 6 h after inoculation in response to the abrasion technique; no experimental treatment elicited increased IL-1α secretion compared to untreated (but

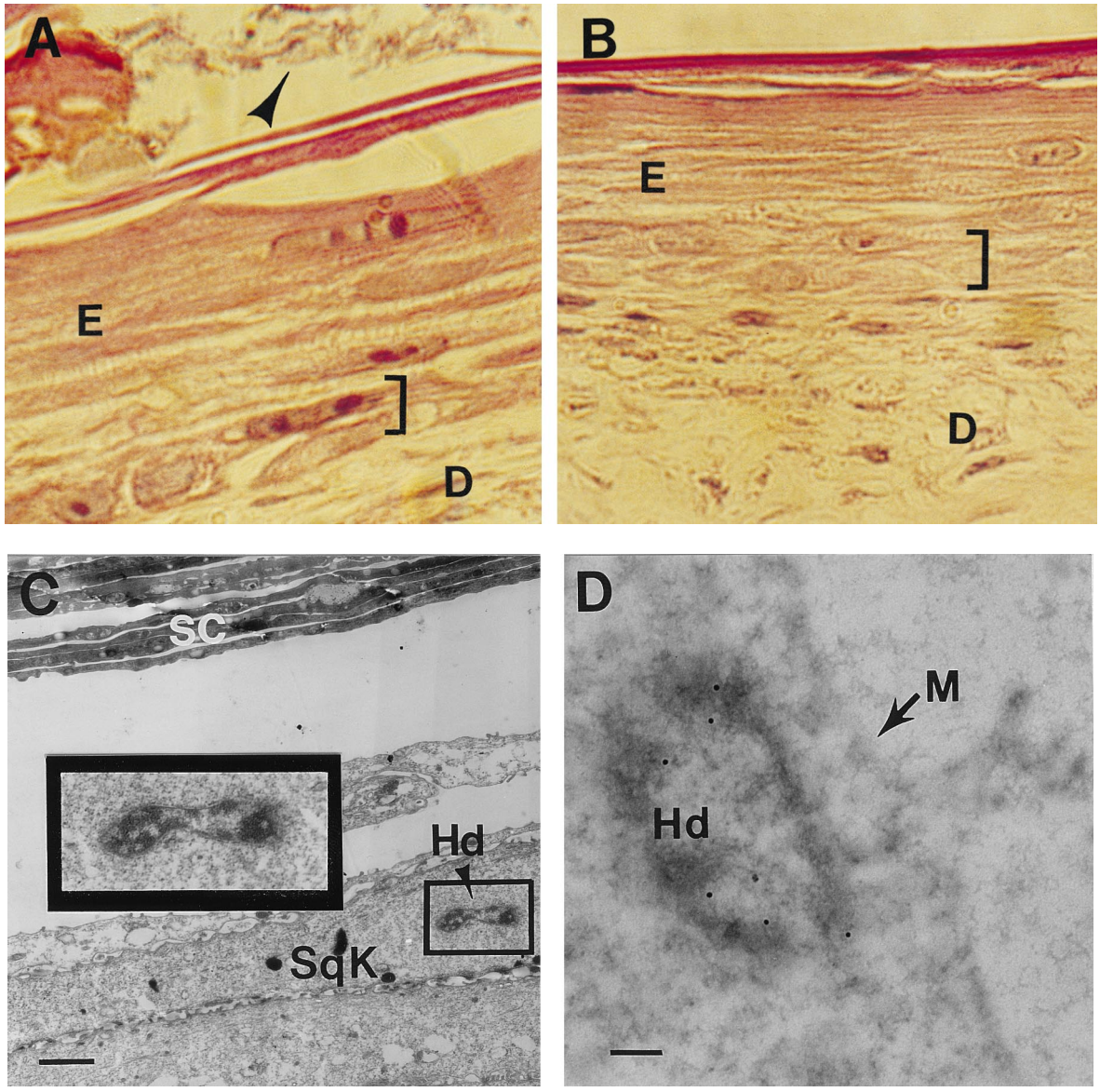

FIG. 2. Localization of *H. ducreyi* in infected artificial skin; light micrographs of hematoxylin-and-eosin-stained artificial skin squares 18 h after abrasion and inoculation with live (A) or heat-killed (B) *H. ducreyi* 35000. E, epidermis; D, dermis; bracket, basal keratinocyte region; arrowhead in panel A, *H. ducreyi* aggregates. (C and D) TEM of *H. ducreyi*-infected artificial skin 18 h after inoculation. SC, terminally differentiated keratinocytes of the stratum corneum; SqK, squamous keratinocyte; Hd, *H. ducreyi*; rectangle in panel C, area enlarged in inset. Sample in panel D was probed with anti-*H. ducreyi* antiserum, followed by gold-labeled secondary antibody. M, membrane of a squamous keratinocyte, two to three layers deep in the epidermis. Original magnifications: ×400 (A and B), ×3,000 (C), and ×12,900 (D); bars, 2.5 mm (C) and 0.19 mm (D).

abraded) controls (Fig. 4C). By 18 h after inoculation, secreted IL-1α levels returned to baseline for all samples. The capacity for high level IL-1α secretion by foreskin cells in this model was demonstrated by their response to *E. coli* with 848.3 pg of IL-1α/ml 24 h after inoculation. Thus, KFCs released neither TNF-α nor IL-1α in response to *H. ducreyi* infection in vitro. However, the trauma of abrasion immediately prior to inoculation caused a peak of IL-1α secretion 6 h later.

DISCUSSION

Understanding the molecular mechanisms responsible for diseases caused by strict human pathogens such as *H. ducreyi* requires relevant in vitro models of infection. The development of increasingly sophisticated in vitro cell culture techniques has improved the approximation of human epithelial surfaces over standard cell monolayer cultures. We have used an artificial skin model, composed of normal human foreskin keratinocytes and fibroblasts, as a substrate for *H. ducreyi* infection. Artificial skin is a complex tissue with epidermal and dermal compartments separated by a well-developed basal lamina (13). Morphological and biochemical characterization indicate that this model recreates many of the structural and functional features of skin in vivo, including maintenance of a selective permeability barrier and cutaneous metabolic activity (50). Thus, the host cell cytokine responses to *H. ducreyi* infection in artificial skin and the behavior of the bacteria in this model may parallel critical steps in the pathogenesis of chancroid.

H. ducreyi infection of human foreskin KFCs stimulated

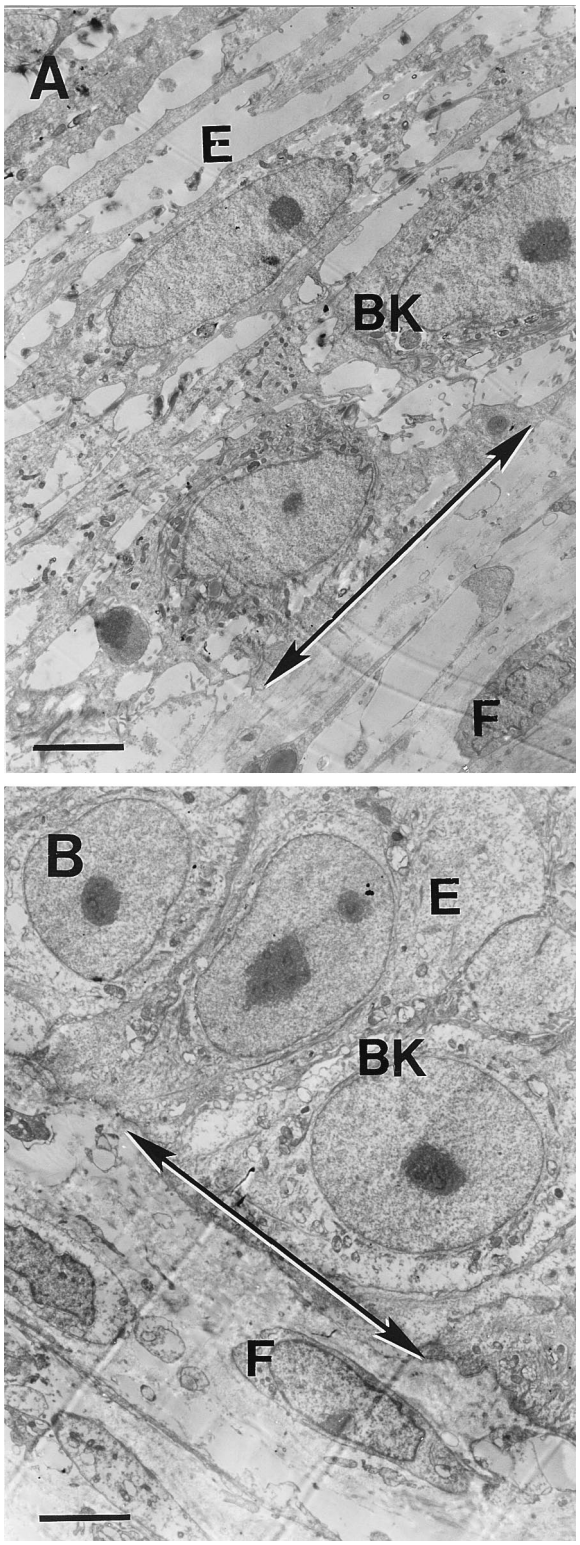

FIG. 3. Dermal-epidermal junctions (arrows) of artificial skin 18 h after abrasion and inoculation with live (A) or heat-killed (B) *H. ducreyi* 35000. E, epidermis; BK, basal keratinocyte; F, fibroblast in dermis. Original magnification, ×2,550; bars, 3.9 mm.

increased secretion of the proinflammatory cytokines IL-6 and IL-8, but not IL-1α and TNF-α. The potent PMN chemoattractant activity of IL-8 is thought to be largely responsible for the local accumulation of inflammatory neutrophils, a hallmark of chancroid ulcers caused by *H. ducreyi* (20, 29). In psoriasis, an inflammatory skin disease, IL-8 production by keratinocytes is elevated in lesions in which it contributes to hyperproliferation of these cells (21). Similarly, acanthosis is a characteristic feature of the epidermis surrounding chancroid lesions (49). Our studies suggest that the chemoattractant and mitogenic properties of IL-8 produced by skin cells in response to *H. ducreyi* infection may be involved in chancroid ulcer development.

IL-6 is a pleiotropic cytokine produced by a variety of cells including keratinocytes and fibroblasts. Like IL-8, IL-6 stimulates keratinocyte proliferation, and it induces IL-2 and IL-2 receptor expression in T cells (33, 62). IL-2 expression is associated with a T-helper cell 1 (TH1)-type CD4 T-cell response; preliminary evidence from clinical observations and experimental *H. ducreyi* infection suggests that TH1 responses may predominate during the initial stages of chancroid. Soluble IL-2 receptors are elevated in the urine of chancroid patients (1), and gamma interferon mRNA, also a TH1-type cytokine, has been observed in experimental lesions in humans (52). Although the immune cells that produce these cytokines are absent from the artificial skin model used in the present studies, IL-6 and IL-8 released by keratinocytes and/or fibroblasts in response to *H. ducreyi* infection could be early signals resulting in the recruitment of PMNs and CD4 T cells to chancroid lesions.

The observation that differences between cytokines induced by live *H. ducreyi* and control preparations did not appear until 48 h after inoculation suggests that there may be environmental control of the *H. ducreyi* factors required for the cytokine induction seen. Future experiments comparing bacterial gene expression in the presence and absence of skin cells may identify important virulence factors required for chancroid ulcer production.

The relative lack of TNF-α and IL-1α responses in our studies was unexpected, since these two cytokines play a central role in initiating inflammatory responses to lipopolysaccharide (LPS) and microbial infection. IL-1α and TNF-α are potent inducers of IL-8, and increased production of both cytokines in keratinocyte-fibroblast cocultures in response to other stimuli (17) suggested that they could have been involved in skin cell responses to *H. ducreyi* infection. We did not observe TNF-α levels above baseline in response to inoculation with live or heat-killed *H. ducreyi*, bacterial culture supernatant, or BHI broth. Suppression of TNF-α expression in macrophages has recently been attributed to YopB, a plasmid-encoded outer membrane protein of *Yersinia enterocolitica* (7). *H. ducreyi* may utilize a similar evasion strategy against this component of the mucosal epithelial defense system. Alternatively, *H. ducreyi* may simply fail to trigger a TNF-α response in foreskin keratinocytes or fibroblasts. The *H. ducreyi* outer membrane contains LOS lacking the extensive saccharide side chains found in the LPS of gram-negative enteric bacteria (46). This structural difference may account for the lack of TNF-α secretion in our experiments. Indeed, *E. coli* dramatically stimulated TNF-α secretion in KFCs. Future studies with purified *H. ducreyi* LOS as a stimulus in the artificial skin model may address this issue.

In our studies of *H. ducreyi* infection of artificial skin, we noted a greater-than-20-fold increase in IL-1α secretion by KFCs over the first 6 h after inoculation (Fig. 4). This elevation did not require live *H. ducreyi* and appeared to be in response

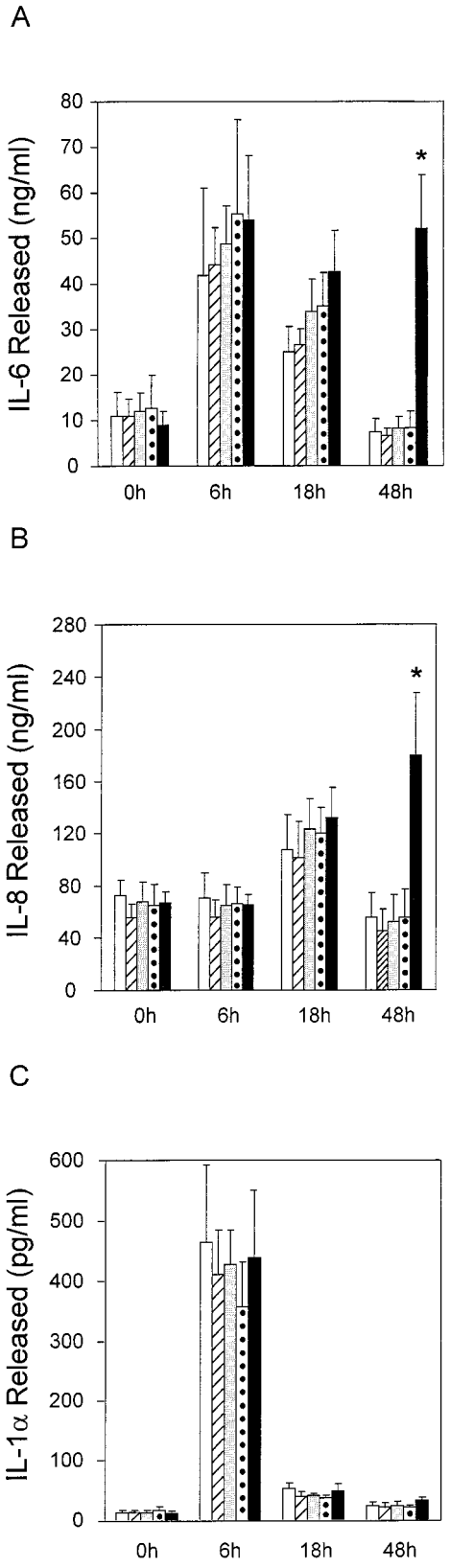

FIG. 4. Cytokine secretion by artificial skin in response to *H. ducreyi* infection; kinetics of IL-6 (A), IL-8 (B), and IL-1α (C) secretion. Open bars, abraded, uninoculated skin squares; other bars, skin squares inoculated with BHI broth (hatched bars), *H. ducreyi* culture supernatant (lightly shaded bars), heat-killed *H. ducreyi* (dotted bars), and live *H. ducreyi* (dark bars) following abrasion. Data are from two independent experiments. Results are means ± standard deviations determined from a total of five to nine individual samples per time point, each assayed in duplicate. *, $P < 0.01$ compared to all other samples at 48 h (unpaired Student's *t* test).

to the abrasion required to produce a productive infection in vitro. The nonspecific nature of increased IL-1α production in our studies does not rule out a role for this cytokine in the host response to chancroid. In naturally acquired and experimental chancroid, *H. ducreyi* requires breaks in the epidermis to enter the skin and cause infection (27, 38, 43, 53, 59).

Initially, we were uncertain of the barrier properties of artificial foreskin cell cultures with respect to *H. ducreyi* infection. In preliminary experiments, KFCs were painted with a pipette tip as the inoculum was dispensed evenly over the surface of the tissue square. Alternatively, inocula were allowed to drop onto squares without mechanically perturbing the surface. Cytokine responses of KFCs inoculated by the painting method were erratic, with discordant levels in replicate samples in the same treatment groups (data not shown). This result suggested that abrasion resulting from contact of the pipette tip with the surface of KFCs during inoculation was inadequate, since samples were not uniformly affected. That abrasion was required in the model was demonstrated by the relative lack of cytokine responses in KFCs inoculated by the drop method (data not shown).

The cytokine responses described herein resulted from uniform abrasion of the artificial skin surface immediately prior to inoculation, designed to approximate epidermal abrasions that occur during sexual intercourse. Our results suggest that relatively low levels of IL-1α may be present in genital skin as a result of such breaks in the epithelium. IL-1α induces physiologic responses at much lower concentrations (1 to 10 pg/ml) than other proinflammatory cytokines such as IL-8 (>10 ng/ml) (5). Thus, keratinocyte- or fibroblast-derived IL-1α may contribute to the cutaneous cytokine response to *H. ducreyi* infection in vivo.

The keratinocyte-fibroblast coculture system used in our studies recreates many of the structural and functional features of skin; however, it lacks key features of intact human skin that contribute to its immune functions in vivo. For example, Langerhans cells, potent antigen-presenting dendritic cells, are absent from the engineered epidermis and dermis. Langerhans cells play a critical role in cutaneous immune responses and have been shown to produce TNF, IL-1, IL-6, and IL-8 (12, 15, 18, 22, 54). The absence of peripheral blood-derived and resident skin leukocytes obviously reduces the complexity of the observable cytokine network, since PMNs, macrophages, and T cells are key components of the host immune response to *H. ducreyi* infection (38, 52). The cytokine responses documented here are only the potential contributions of foreskin keratinocytes and fibroblasts and thus represent a subset of the cutaneous immune response to *H. ducreyi* infection. Nevertheless, these are the predominant cell types in human skin, and keratinocytes are probably the first host cells with which *H. ducreyi* comes in contact during the initial stages of infection. Reconstitution of the current coculture system with dendritic cells, neutrophils, and/or lymphocytes may improve the model for use in defining and understanding the cytokine and ensuing cellular responses involved in chancroid ulcer formation.

The location of *H. ducreyi* within chancroid ulcers has not been well-characterized. Organisms can be recovered from clinical and experimental lesions of several days' to several weeks' duration (23, 43, 53) and are thus present in developing

and mature lesions. Consistent with these observations, we recovered *H. ducreyi* from infected artificial skin at times ranging from 6 h to 2 weeks after inoculation. Early microscopic studies of chancroid biopsies occasionally demonstrated chains of bacilli located extracellularly at the base of ulcers (20, 35). We observed chains of *H. ducreyi* among the keratin layers on the surface of infected keratinocyte-fibroblast cocultures 18 h after inoculation. Many of these bacteria were only loosely associated with artificial skin, since we consistently recovered equivalent numbers of bacteria in the wash and cell-associated fractions ≥18 h after inoculation. These tangles of *H. ducreyi* are reminiscent of the microcolonies described by Alfa and colleagues (3, 4) and Lammel et al. (32) on the surface of infected fibroblast monolayers; however, we did not observe bacteria in the dermis, where fibroblasts are located in this model.

In addition to the surface bacteria, we observed *H. ducreyi* within suprabasal keratinocytes 18 h after inoculation. These bacteria were consistent in size and shape with reported characteristics of Ducrey's bacillus. The range of variation in size is 0.5 to 2.5 mm long by 0.3 to 1.0 mm broad, and the organism is "frequently constricted in the middle" (26). The mottled interior of the organisms in artificial skin is typical of *H. ducreyi* (Fig. 2). Intracellular *H. ducreyi* cells were rare and were seen only as single longitudinal or cross-sections that appeared to be free in the keratinocyte cytoplasm (Fig. 2). The apparent paucity of *H. ducreyi* below the surface of infected artificial skin is consistent with the relatively low numbers of organisms recovered from lesions in experimental human infections (52, 53). Keratinocytes containing bacteria were typically three to four layers deep in the epidermis and two to three layers above the basal keratinocytes.

We did not see *H. ducreyi* in the basal keratinocyte region of infected artificial skin. Nevertheless, 18 h after abrasion and inoculation with live but not heat-killed bacteria, the architecture of the basal cell layer was disrupted and the keratinocytes appeared necrotic. This disruption was localized to the basal cell region, as suprabasal keratinocytes were grossly unaffected by infection and the overall architecture of the epidermal compartment remained intact (Fig. 2A and B). *H. ducreyi* elaborates several toxins that may be important virulence factors for ulcer formation. A bacterial cell-associated hemolysin belonging to the *Proteus-Serratia* family of pore-forming toxins has been characterized by Palmer et al. (41) and Totten et al. (60). Toxic activity of the *H. ducreyi* hemolysin requires bacterial contact with host target cells and is relatively specific for fibroblasts in vitro (41). The structural changes that we observed at the dermal-epidermal junction of *H. ducreyi*-infected artificial skin were probably not caused by the hemolysin, since bacteria were not evident in the zones of destruction. *H. ducreyi* also produces a secreted cytotoxin with homology to the cytolethal distending toxins (CDTs) of *E. coli* (47) and *Campylobacter* species (42). The *H. ducreyi* CDT is active against HeLa cells in vitro (14); its relative toxicity for keratinocytes and fibroblasts has not yet been described. Given its potential for activity at a distance from bacterial cells and the sensitivity of at least some epithelial cells to the toxin, the soluble *H. ducreyi* CDT may be responsible for the cytotoxicity that we noted at the basal keratinocyte layer of infected KFCs. Experiments with toxin-deficient mutants in the artificial foreskin model of infection should provide further information on the role of CDT in *H. ducreyi* virulence.

The artificial human skin model described here provides a relevant in vitro system for studying the pathogenesis of *H. ducreyi* infection. Using the in vitro skin model, we intend to characterize the pathogenic potential of defined *H. ducreyi* mutants to identify virulence factors important in the development of chancroid ulcers.

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