Haemophilus ducreyi Elicits a Cutaneous Infiltrate of CD4 Cells during Experimental Human Infection

Stanley M. Spinola, Attilio Orazi, Janet N. Arno, Kate Fortney, Patricia Kotylo, Cheng-Yen Chen, Anthony A. Campagnari, and Antoinette F. Hood

Human subjects were experimentally infected with Haemophilus ducreyi for up to 2 weeks. Bacterial suspensions were delivered into the epidermis and dermis through puncture wounds made by an allergy-testing device. Subjects developed papular lesions that evolved into pustules resembling natural disease. Some papular lesions resolved spontaneously, indicating that host responses may clear infection. Bacteria were shed intermittently from lesions, suggesting that H. ducreyi may be transmissible before ulceration. Host responses to infection consisted primarily of cutaneous infiltrate of polymorphonuclear leukocytes, Langerhans cells, macrophages, and CD4 T cells of αβ lineage. Expression of HLA-DR by keratinocytes was associated with the presence of interferon-γ mRNA in the skin. There was little evidence for humoral or peripheral blood mononuclear cell responses to bacterial antigens. The cutaneous infiltrate of CD4 cells and macrophages provides a mechanism that facilitates transmission of human immunodeficiency virus by H. ducreyi.

Haemophilus ducreyi causes chancroid, a genital ulcer disease that facilitates transmission of human immunodeficiency virus (HIV) among populations in which both infections are endemic [1–4]. H. ducreyi infection may enhance HIV transmission by providing a more accessible portal for entry, by promoting viral shedding, and by recruiting lymphocytes and macrophages into the skin.

H. ducreyi is thought to enter the skin through breaks in the epithelium that occur during sexual intercourse [5]. After an incubation period of 4–7 days, an erythematous papule forms, which evolves into a pustule in 2–3 days and then ulcerates [5]. The papular and pustular stages of chancroid frequently are not noticed by patients, who usually seek medical care only after they have painful ulcers for several weeks [5, 6]. Few longitudinally collected clinical specimens are available for study. In patients with established ulcers, the epidermis is replaced by necrotic tissue, and superinfection or colonization of the ulcer with other bacteria is common [7–10]. Thus, analysis of clinical samples has yielded limited information about the location of H. ducreyi in the skin, virulence determinants, and host responses to infection. These parameters are critical to understanding the relationship between chancroid and HIV transmission.

To study H. ducreyi pathogenesis and host responses, we developed an experimental human model of infection [11]. Subjects were infected on the upper arm by applying suspensions containing 10^5–10^6 cfu of H. ducreyi to the skin with an allergy-testing device. Doses of 10^5–10^6 cfu caused pustule formation within 24 h, while 10^4 cfu caused erythematous papules. Because complications, such as superinfection and suppurative regional lymphadenitis, occur during the ulcerative stage of chancroid [3, 5], the trial was terminated with antibiotic therapy after 3 days. All doses elicited an infiltrate of polymorphonuclear leukocytes (PMNL), T cells, and macrophages in the skin; no humoral immune responses to H. ducreyi were detected. The rapid course of infection and antibiotic treatment may have altered host responses.

Here we report refinements in the experimental challenge model [11], which allowed us to infect subjects for up to 2 weeks. We estimated the dose of bacteria needed to initiate infection, examined whether bacteria were shed from the skin prior to ulceration, and characterized systemic and local host responses to infection.

Material and Methods

Human volunteers. Three men and 6 women (2 black, 7 white; age range, 23–47 years) with no history of chancroid volunteered for the study. Normal skin was obtained from the upper arm of an uninfected white man (age, 42).

Experimental challenge protocol. The protocol for preparation of the bacteria, inoculation, and clinical observation is described in detail elsewhere [11]. H. ducreyi 35000 was grown in broth to
mid-log phase. Live bacteria were processed exactly as described, while heat-killed bacteria were incubated at 56°C for 30 min. Groups of 3 subjects were challenged in 3 separate iterations. Each subject was challenged with three suspensions of live bacteria and one dose of heat-killed bacteria prepared from the suspension containing the highest number of live bacteria.

Suspensions were inoculated into the skin with a Multi-Test Applicator (Lincoln Diagnostics, Decatur, IL), an allergy-testing device. The applicator’s test-head configuration is identical to the Mono-Vacc tuberculin test device (Connaught Laboratories, Swiftwater, PA) and consists of nine pins mounted on a circular platform. Bacterial suspensions (12.5 µL) were placed on the tines, and the device was pressed on the skin so that an imprint of the puncture pattern of the points and the circular platform was clearly visible at each injection site. Some suspension remained on the device or on the surface of the skin after inoculation. The suspension remaining on the skin was allowed to dry for 15 min and then was covered with clothing. Although we could not determine the amount of the suspension delivered into the skin, tests done by the manufacturer show that the tuberculin test device reproducibly delivers 5–10 TU to the skin when loaded with 20 µL of a solution containing 300,000 radiolabeled TU/mL (Hein GL, personal communication). In other words, the device delivers ~1:1000 (0.08%–0.16%) of solutions loaded on its pins into both the epidermis and dermis to a depth of 1.9 mm.

Subjects were infected for up to 2 weeks or until they developed a painful or pruritic pustular lesion or a pustule that had an umbilical center. When a study end point was reached, lesions were anesthetized with 1% lidocaine containing epinephrine and methylparaben (Elkins-Sinn, Cherry Hill, NJ), and 6-mm punch biopsies were obtained. Subjects were treated with ciprofloxacin after biopsy or on day 14, even if they had no clinical evidence of infection.

Surface cultures. Cotton swabs moistened with PBS were rolled across the sites of inoculation and used to inoculate chocolate agar plates for culture. The plates were incubated for 5 days.

Culture and histologic analysis of biopsies. Biopsy specimens were divided in quarters. One section was homogenized in freezing medium and quantitatively cultured as described previously [11]. Results were expressed as colony-forming units per gram of tissue.

The remaining sections were fixed in formalin and embedded in paraffin or frozen in liquid nitrogen and embedded in optimal cutting temperature medium (Miles, Elkhart, IN) for light microscopy and immunohistochemistry. The tissue was stained with antibodies that bind to B cells (CD20), macrophages (CD68), T cells (CD3, CD4, CD8, γ-TCR, and β-TCR [supplied by G. Cattoretti, Columbia University, NY]), NK cells (CD57), and Langerhans cells (CD1a). Except as noted for β-TCR, antibodies were from Dako (Carpinteria, CA), Becton Dickinson (Mountain View, CA), and Biotest (Denville, NJ). HLA-DR expression was detected with antibody L31 (Biotest). Antibody binding to tissue was detected using biotinylated anti-rabbit or anti-mouse immunoglobulins (Kirkegaard & Perry, Gaithersburg, MD), peroxidase-conjugated streptavidin (Kirkegaard & Perry), and 3,3’-diaminobenzidine (Sigma, St. Louis). For controls, we reacted normal skin and tonsil with antibodies or omitted the primary antibody from probing.

Cells were enumerated under standard light microscopy using a square-millimeter grid. The ratio of Langerhans cells present in infected specimens to those in uninfected skin was estimated by counting the number of dendritic cells staining with CD1a per square millimeter of epidermis. The percentage of a lymphocyte cell subset in the dermal infiltrate was estimated by dividing the number of cells that stained with an antibody (×100) by the number of cells (300) examined. For each specimen, a similar level of each section centered around a dermal capillary was scored.

Reverse transcription polymerase chain reaction (PCR). Cytokine mRNA was detected as described by Yamamura et al. [12] with the following modifications. Forty to 60 5-µm sections of frozen tissue were lysed with TRI reagent (Molecular Research Center, Cincinnati) according to the manufacturer’s instructions. cDNA was synthesized from equivalent amounts of total RNA using the 1st Strand cDNA synthesis kit (CLONTECH Laboratories, Palo Alto CA). Samples were amplified by PCR with Taq polymerase, according to the manufacturer’s instructions, using primers specific for β-actin, interferon-γ (IFN-γ) (CLONTECH Amplimer sets), or cDNA cDNAs prepared from phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) and from normal skin, positive control cDNA fragments (CLONTECH) containing appropriate cytokine sequences, and samples lacking cDNA. PCR product was analyzed by agarose gel electrophoresis and ethidium bromide staining. The identity of the PCR products was confirmed by Southern blot with the labeled positive control cDNA fragments.

Serum and whole blood. Serum was collected from subjects C-5 and C-8 prior to inoculation and 21 days after challenge. Whole blood and serum were collected from the other subjects at the above times and on the day of biopsy or antibiotic treatment.

Lymphocyte subset analysis. Cells were obtained from peripheral blood by the whole blood lysis method (Q-Prep; Coulter Diagnostics, Hialeah, FL) and stained with monoclonal antibodies to leukocyte common antigen (CD45), monocytes (CD14), total T cells (CD2), and CD3, CD4, CD8, B (CD19), and NK cells (CD16 and CD56). Percentages of lymphocyte subsets were determined by flow cytometry using an EPICS profile (Coulter). Absolute numbers of cells were calculated from the percentage of each subset and absolute lymphocyte count.

Blastogenesis. PBMC were isolated by ficoll-hypaque gradient centrifugation and diluted in RPMI supplemented with gentamicin, L-glutamine, and heat-inactivated AB serum as described previously [13]. Quadruplicate wells were seeded with 10^6 cells and 10-fold serial dilutions of strain 35000 that were killed by incubation at 56°C for 30 min. Cells were cultured for 5 days, incubated with 1 µCi of [3H]thymidine for 18 h, and harvested to measure [3H]thymidine uptake. As a control, PBMC was incubated with tetanus toxoid (0.09 µg/mL; Connaught) or with media. Stimulation indices were calculated as follows: (counts per minute of antigen-stimulated PBMC)/(counts per minute of PBMC incubated with media).

Immunodot assays, Western blot assays, and ELISA. Immunodot assays were done by applying 2 µg (total protein) of solubilized and nondenatured whole cell lysates of strain 35000 to nitrocellulose. The dots were probed overnight with 2-fold serial dilutions of serum (range, 1:400–1:25,600) with horseradish peroxidase-conjugated goat antibodies to human IgG, IgM, and IgA (Kirkegaard & Perry) and with horseradish peroxidase color developer (Bio-Rad Laboratories, Richmond, CA). Western blots of whole
cell lysates were probed with 1:125 dilutions of serum overnight with protein A-peroxidase or horseradish peroxidase–conjugated goat antibody to human IgM (Kirkegaard & Perry) and with horseradish peroxidase color developer [11]. Antibodies to lipooligosaccharide (LOS) were measured by ELISA as described previously [1], except that antigen-antibody complexes were detected with horseradish-labeled goat antibody to human IgM and IgG (Kirkegaard & Perry).

ELISA with absorbed serum was done by a modification of previously described methods [14, 15]. In brief, serum was absorbed with a soluble antigen mixture prepared from Haemophilus influenzae, Haemophilus parainfluenzae, and Haemophilus parahaemolyticus. Absorbed serum was diluted 1:200 and 1:400 and used to probe microtiter wells coated with soluble antigen prepared from strain 35000. Antigen-antibody complexes were detected with anti-human IgG or IgM as described [14, 15]. As controls, wells were probed with serum obtained prior to infection, pooled normal human serum, and serum from a patient with documented chancroid. For each subject, an optical density that was twice that of the prechallenge sample was considered positive in this assay [15].

Results

Dose response studies. Nine adult volunteers were challenged with H. ducreyi 35000. Suspensions containing live and heat-killed bacteria were inoculated into the skin of the upper arm with an allergy-testing device that delivers 1–1000 of the volume of solutions loaded on its pins to the epidermis and dermis. Consistent with the delivery characteristics of the device, no lesions occurred with doses containing <10³ cfu of H. ducreyi (table I). No lesions occurred at sites inoculated with heat-killed bacteria. Erythematous papules developed at 6 of 12 sites challenged with 1.5 × 10³ to 7.5 × 10³ cfu; 2 of the papules resolved, and 4 became pustules. Papules occurred at 7 of 9 sites inoculated with 1.5 × 10⁴ to 3.5 × 10⁴ cfu; pustules developed at 4 of these sites, and the remaining lesions resolved. Pustular lesions developed in 3–9 days, similar to the natural course of disease [5]. Subject C-9 developed a pustule at one site and resolved a second lesion (figure 1). Thus, inoculation of suspensions containing 10⁴ cfu caused disease in most subjects. These data also suggest that local host responses led to resolution of infection.

Subject C-14 did not develop disease from doses >10⁴ cfu, which in this and previous studies [11] caused infection in 8 of 8 subjects. Eight days after initial challenge, C-14 was challenged at new sites with 4 × 10³, 2 × 10⁴, and 4 × 10⁴ cfu. Disease did not develop during a 2-week observation period. Evaluation of sera and subsequent history obtained from this subject suggested that infection may have been prevented by an antibiotic taken 21–29 days before challenge.

None of the volunteers developed fever, chills, or lymphadenopathy during the course of infection. None of the subjects had local symptoms until 1–2 days before biopsy, when pustules were described as mildly tender, pruritic, or burning. At times indicated in table 1, selected lesions were biopsied, and volunteers were treated with ciprofloxacin [11]. Erythema resolved within 12–24 h, and all lesions healed. Thus, experimental infection resulted in minimal clinical symptoms and was safe.

Recovery of H. ducreyi from experimental lesions. Culture samples were obtained daily from the surface of the lesions of subjects C-12 and C-13 for 1 week after inoculation. H. ducreyi (range, 1–150 cfu) were recovered from 4 of 6 lesions (7 of 42 cultures). Three of C-12’s lesions were culture positive 1 day after inoculation; 1, 4, and 5 days after inoculation; and 6 and 7 days after inoculation, respectively. One of C-13’s lesions was culture positive 3 days after inoculation. Culture samples (n = 6) obtained 2 days after biopsy and antibiotic treatment were sterile, as were culture samples (n = 42) obtained from C-14 for 2 weeks after repeat challenge and from sites inoculated with heat-killed bacteria (n = 28). Thus, bacteria were shed intermittently from papules and pustules and not from sites where disease was clinically absent.

Each biopsy specimen was divided into quarters, homogenized in 1 mL of freezing medium, and quantitatively cultured. A pure growth of H. ducreyi was recovered from 5 of 6 pustules. One lesion (C-10) that had resolved was sterile. A mean of 2.0 × 10⁴ cfu/g was recovered from C-7, C-9, and C-11, whose courses most closely resembled naturally occurring disease. These biopsies were estimated to contain a total of only 2.8 × 10² to 5.9 × 10³ cfu. Incubation of H. ducreyi for 1 h in freezing medium mixed with an equal volume of lidocaine anesthetic had no effect on bacterial viability (data not shown).

Histologic analysis of lesions. The histopathology of the pustules was similar in all subjects (figure 2). Micro pus tules containing PMNL were present in the epidermis. In the dermis, there was a perivascular and interstitial mononuclear cell and PMNL infiltrate, and the venules were lined by reactive endothelial cells. Fivefold more dendritic cells bound CD1a antibody in the epidermis of infected skin than in uninfected skin. Dendritic cells that stained with CD1a antibody were also present in the dermis of infected skin. A lesion (C-10) that had clinically resolved was histologically normal.

We estimated the percentage of each type of mononuclear cells in the infiltrate by examining 300 cells in each specimen. Assuming that the true prevalence of a certain cell subtype was 80%, the estimates had an underlying accuracy (95% confidence interval) of ±4.5%. Approximately 80% of the mononuclear cells stained with CD3 antibody, 10% reacted with CD68 antibody, and 10% stained with CD20 antibody. In subjects infected for 13 or 14 days, 80% of the cells stained with CD4 antibody and 20% bound CD8 antibody. Lymphocytes reacted with β-TCR antibody but not with γ-TCR antibody. Few (<1%) cells bound CD57 antibody. Thus, the cellular immune response to experimental infection consisted primarily of PMNL, Langerhans cells, and CD4 cells of the αβ lineage.
Table 1. Response to live *H. ducreyi* on punctured skin.

<table>
<thead>
<tr>
<th>Subject, dose</th>
<th>Erythema, pustule formation on postinoculation day</th>
<th>Day of biopsy</th>
<th><em>H. ducreyi</em> recovered from biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>C-5</td>
<td>1.5 ± 0.48 × 10^3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>× 10^3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>× 10^4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>C-7</td>
<td>1.5 ± 0.48 × 10^3</td>
<td>3 × 3 (-)</td>
<td>2 × 2 (-)</td>
</tr>
<tr>
<td></td>
<td>× 10^3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>× 10^4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>C-8</td>
<td>1.5 ± 0.48 × 10^3</td>
<td>2 × 2 (-)</td>
<td>2 × 2 (-)</td>
</tr>
<tr>
<td></td>
<td>× 10^3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>× 10^4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>C-9</td>
<td>1.5 ± 0.31 × 10^3</td>
<td>4 × 5 (-)</td>
<td>4 × 4 (-)</td>
</tr>
<tr>
<td>7.5 ± 0.16 × 10^3</td>
<td>5 × 5 (-)</td>
<td>4 × 3 (-)</td>
<td>3 × 3 (-)</td>
</tr>
<tr>
<td>1.5 ± 0.31 × 10^3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>C-10</td>
<td>1.5 ± 0.31 × 10^4</td>
<td>5 × 5 (-)</td>
<td>4 × 3 (-)</td>
</tr>
<tr>
<td>7.5 ± 0.16 × 10^4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1.5 ± 0.31 × 10^4</td>
<td>3 × 3 (-)</td>
<td>3 × 2 (-)</td>
<td>LR</td>
</tr>
<tr>
<td>C-11</td>
<td>1.5 ± 0.31 × 10^4</td>
<td>5 × 5 (-)</td>
<td>5 × 4 (-)</td>
</tr>
<tr>
<td>7.5 ± 0.16 × 10^4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1.5 ± 0.31 × 10^4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>C-12</td>
<td>3.5 ± 0.40 × 10^4</td>
<td>5 × 5 (-)</td>
<td>5 × 5 (-)</td>
</tr>
<tr>
<td>1.7 ± 0.20 × 10^4</td>
<td>5 × 5 (-)</td>
<td>4 × 4 (-)</td>
<td>8 × 8 (+)</td>
</tr>
<tr>
<td>3.5 ± 0.40 × 10^3</td>
<td>3 × 3 (-)</td>
<td>3 × 3 (-)</td>
<td>5 × 4 (+)</td>
</tr>
<tr>
<td>C-13</td>
<td>3.5 ± 0.40 × 10^4</td>
<td>5 × 5 (-)</td>
<td>7 × 7 (+)</td>
</tr>
<tr>
<td>1.7 ± 0.20 × 10^4</td>
<td>5 × 5 (-)</td>
<td>4 × 4 (-)</td>
<td>3 × 2 (+)</td>
</tr>
<tr>
<td>3.5 ± 0.40 × 10^4</td>
<td>6 × 6 (-)</td>
<td>4 × 4 (+)</td>
<td>8 × 6 (+)</td>
</tr>
<tr>
<td>C-14</td>
<td>3.5 ± 0.40 × 10^4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1.7 ± 0.20 × 10^4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3.5 ± 0.40 × 10^4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

NOTE. Dose, mean cfu ± SD loaded on application device. Erythema, diameter in millimeters; pustules present (+) or absent (−). *H. ducreyi* recovery, cfu/g of tissue. ND, not done; LR, lesion resolved; --, no lesion developed.

Association between HLA-DR expression and IFN-γ mRNA. Mononuclear cells, endothelial cells, and keratinocytes expressed HLA-DR in 5 of 7 biopsies (figure 2). Because HLA-DR expression by keratinocytes is induced in vivo and in vitro by stimulation with IFN-γ [16–19], 2 specimens (C-13) that were HLA-DR negative and 2 specimens (C-12) that were HLA-DR positive were tested for the presence of IFN-γ mRNA by reverse transcription PCR. The amount of cDNA amplified by IFN-γ primers was normalized to the amount of CD3δ cDNA or β-actin cDNA present in the specimen. β-actin mRNA was detected in all specimens (data not shown). CD3δ mRNA was present in all infected specimens but absent in normal skin (figure 3). IFN-γ mRNA was detected in the HLA-DR–positive specimens and PHA-stimulated PBMC (data not shown) but was absent in HLA-DR–negative specimens and control skin. These preliminary observations indicate that HLA-DR expression was associated with the presence of IFN-γ mRNA in the lesions.

Analysis of lymphocyte subsets in the circulation and blastogenesis assays. Blood was obtained prior to infection, on the day of antibiotic treatment or biopsy, and 21 days after challenge. Lymphocyte subsets were determined by flow cytometry to be positive for CD19, CD3, CD4, CD8, or CD56. Lymphocyte purity (CD45 CD14−) was >99% in all assays. In all subjects tested (C-9 to C-14), there were no changes in the percentage or number of lymphocyte subsets after challenge (data not shown). Thus, the influx of CD4 cells into the skin was not reflected by changes in lymphocyte subsets in the circulation.
Figure 1. Lesions on volunteer C-9, 1 (A), 6 (B), and 12 (C) days after inoculation of $1.5 \times 10^4$ (1), $7.5 \times 10^3$ (2), and $1.5 \times 10^3$ (3) live *H. ducreyi* and $1.5 \times 10^4$ (4) heat-killed *H. ducreyi*. D, Pustular lesion caused by $7.5 \times 10^3$ dose on day 14. Circles vary because areas were marked daily.

Figure 2. Histologic sections of specimen obtained from site (C-7) inoculated with $1.5 \times 10^3$ cfu of *H. ducreyi*. A, Perivascular mononuclear cell infiltrate in dermis ($\times 20$). B, Intraepidermal pustule (arrow) ($\times 100$). C-I are $\times 200$. C, Capillary lined with reactive endothelial cells (arrow) and perivascular mononuclear cell infiltrate that did not stain with CD57 antibody. D, Perivascular mononuclear cells stained with CD3 antibody. E, Staining with CD4 antibody. F, Staining with CD8 antibody. G, Expression of HLA-DR by keratinocytes and mononuclear cells; arrow shows dermal-epidermal junction. H, Staining with $\beta$-TCR antibody. I, Infiltrate of dendritic cells in epidermis stained with CD1a antibody.
PBMC were isolated from subjects C-9, C-12, C-13, and C-14 and were stimulated with media, various concentrations of heat-killed *H. ducreyi*, and tetanus toxoid. The concentration (0.004–4.0 µg/mL) of *H. ducreyi* antigen used in the assays corresponded to 10^4–10^7 cfu/mL and is within the optimal range of bacterial antigens reported to elicit PBMC responses [20]. Preliminary experiments showed that maximal stimulation occurred after 5 days of incubation with 0.4 µg/mL *H. ducreyi* (data not shown). Prior to inoculation, *H. ducreyi* stimulated low levels of blastogenesis. None of the subjects showed an increase in blastogenic responses to *H. ducreyi* after challenge (data not shown).

Lack of serologic response to experimental infection. Serum was obtained from subjects prior to infection, on the day of biopsy, and 21 days after challenge. By immunodot, there were no changes in the titer of serum IgG, IgM, and IgA antibodies that bound to whole cell lysates (data not shown). By Western blot, each subject had preexisting serum antibodies of the IgG or IgM class that reacted to whole cell lysates (figure 4). Serum antibodies did not change over time, even in volunteers infected for 2 weeks. As assessed by ELISA, there were no increases in the binding of serum immunoglobulins of the IgG or IgM class to LOS (data not shown).

To examine whether preexisting antibodies masked our ability to detect *H. ducreyi*-specific responses, serum was absorbed with a lysate of *H. influenzae*, *H. parainfluenzae*, and *H. parahaemolyticus* and used to probe *H. ducreyi* antigens by ELISA. No subjects had a rise in antibody titer as detected by Western blotting (data not shown). Thus, experimental infection with *H. ducreyi* for up to 2 weeks did not evoke antibody responses to bacterial proteins or LOS.

Discussion

*H. ducreyi* is a strict human pathogen and expresses cell surface components that are similar to human antigens [21]. *H. ducreyi* naturally infects genital skin and mucosal surfaces and may infect extragenital skin sites, presumably due to auto-inoculation [3, 5]. In our initial human challenge trial [11], the course of infection was rapid and did not resemble natural disease. By using lower doses of bacteria, we have safely infected subjects for up to 2 weeks. The subjects developed painless papular lesions that evolved into pustules between days 3 and 9. The clinical course of experimental infection now resembles the initial stages of human chancroid [5]. The limitations of the model are that we cannot infect genital skin, mucosal surfaces, or allow the subjects to develop ulcers or lymphadenitis. However, the model permits direct study of initial events in pathogenesis and host responses in a natural setting of *H. ducreyi* infection: human skin.

Inoculation of suspensions containing <10^3 cfu did not produce lesions, while inoculation of 1.5 × 10^1 to 3.5 × 10^4 cfu caused lesions in 7 of 9 subjects. The allergy-testing device used to deliver the suspensions delivers ~1:1000 of solutions of tuberculin loaded on its tines to the epidermis and dermis. Thus, experimental infection may have resulted from inoculation of as few as 1–35 cfu into the skin. This is the first estimate of the infectious dose of *H. ducreyi* and is consistent with the 70%–80% transmission rate reported after single sexual exposures [22, 23]. However, our estimates of the infectious dose could be affected by differences in the ability of the device to deliver bacterial suspensions and antigenic solutions. *H. ducreyi* clumps both in vivo and in vitro [5]; organisms grown in broth clump less than those grown on plates [24]. Although we grew the inoculum in broth, the cfu reported in this study represent minimal estimates of the number of bacteria in a dose.

*H. ducreyi* were recovered intermittently from the surface of papular and pustular lesions. Two of the lesions were initially culture negative and became culture positive 3 and 6 days after inoculation. The timing and intermittent nature of recovery suggests that the bacteria recovered from lesions were not bacteria that remained on the skin after the inoculation procedure. The data also suggest that *H. ducreyi* is shed from papules and pustules and may be transmissible before ulceration. In subjects C-7, C-9, and C-11, whose courses most closely resembled naturally occurring disease, a mean of 2 × 10^6 cfu/g were recovered from the biopsies. Entire biopsies were estimated to contain 2.8 × 10^5 to 5.9 × 10^5 recoverable cfu. If only 2–15 cfu were injected into the skin of these subjects, the data suggest that *H. ducreyi* were replicating in the skin. Attempts to visualize the bacteria by conventional light microscopy or immunostaining with *H. ducreyi*-specific antibodies [25] were unsuccessful (data not shown), probably due to the low number of bacteria in the lesions.

We previously reported that inoculation of 10^4 cfu caused erythematous papules. *H. ducreyi* were recovered from the papules, which were histologically similar to pustules biopsied 3 days after challenge [11]. In the present study, 7 subjects developed 13 papules, and 4 of the papules spontaneously resolved during the 2-week observation period. A biopsy obtained from...
a resolved-lesion site had no histologic abnormalities and did not grow \( H. ducreyi \). Simultaneous resolution of a papule and progression of a second papule to pustule occurred in subject C-9. In this subject, resolution occurred at a higher dose (1.5 \( \times 10^4 \) cfu) than pustule formation (7.5 \( \times 10^3 \) cfu). Although no \( H. ducreyi \) surface structures are known to phase or antigenically vary, resolution or progression of papules may be due to phase or antigenic variation of a bacterial structure, variations in the actual number of bacteria injected by the device, or variations in the local host response. However, these data suggest that the human host can sometimes mount an effective immune response to \( H. ducreyi \) at the papular stage of disease.

At the pustular stage of disease, the major histologic findings included the formation of intraepidermal pustules, a primary perivascular and interstitial dermal infiltrate of PMNL, Langerhans cells, macrophages, CD4 cells, and endothelial cell activation. Previous studies showed that mature ulcers contain a superficial zone of PMNL, dermal edema, endothelial cell proliferation, and an infiltrate of plasma cells [7, 8, 26, 27]. However, a recent analysis showed that culture-proven chancroid ulcers contain a predominant mononuclear cell infiltrate of macrophages and CD4 and CD8 T cells and a few B cells or plasma cells [28]. Recruitment of CD4 cells and macrophages into experimental and naturally occurring lesions is consistent with the observation that cell-mediated mechanisms play a role in \( H. ducreyi \) pathogenesis [29, 30] and may partly explain the association between chancroid and acquisition of HIV. The lack of response of HIV-infected persons to standard antibiotic regimens [2, 3, 31] also suggests that CD4 cells play an important role in host defenses against \( H. ducreyi \).

In 17 of 19 biopsies obtained in this and previous studies [11], mononuclear cells, endothelial cells, and keratinocytes expressed HLA-DR. Preliminary experiments showed that HLA-DR expression was associated with the presence of IFN-\( \gamma \) mRNA in the lesions. These data are consistent with the observation that HLA-DR expression by keratinocytes is induced in vivo and in vitro by stimulation with IFN-\( \gamma \) [16–19]. We do not know the cellular origin of the IFN-\( \gamma \) mRNA. We speculate that after entering the skin, \( H. ducreyi \) may be engulfed by keratinocytes, Langerhans cells, or dermal dendritic cells. These cells may present \( H. ducreyi \) antigens and recruit and activate resting or naive T cells. The recruited T cells may secrete IFN-\( \gamma \) and stimulate the expression of major histocompatibility complex class II antigens. Activation of keratinocytes, Langerhans cells, and dermal dendritic cells may amplify recruitment of PMNL, macrophages, and lymphocytes to the site of infection.

In contrast to the recruitment of CD4 cells to the skin, there were no changes in circulating lymphocyte subsets during the course of infection. Similarly, in \( Mycobacterium leprae \) and herpes simplex infection, the phenotype of T cells in cutaneous lesions is not reflected by the phenotype of the T cells found in the peripheral blood [32, 33]. Preliminary experiments suggest that there were no changes in PBMC responses to \( H. ducreyi \) antigens after inoculation. These data are consistent with the observation that the number of T cells in cutaneous
lesions that specifically react with infectious agents are usually several orders of magnitude above the number of antigen-specific T cells found in the peripheral blood [34, 35].

Experimental infection for up to 14 days did not evoke serum antibody responses to *H. ducreyi* antigens. Few longitudinal data exist on serum antibody responses to natural infection. In seroprevalence studies, African patients with chancroid have significantly higher levels of antibody to *H. ducreyi* LOS and whole cells than do noninfected controls or patients with other sexually transmitted diseases [36–39]. The sensitivity of the ELISA for *H. ducreyi*–specific antibody increases with the duration of patient-reported symptoms [15], which probably reflects the duration of ulcerative disease. Our data suggest that antibody responses to *H. ducreyi* are not a major feature of the immune response during the first 2 weeks of experimental infection. The differences between the host responses to experimental and natural infection may reflect differences in the site or duration of disease.

Intradermal injection models of *H. ducreyi* infection have been developed in macaques and rabbits [40, 41]. The portal of entry in these models differs from that in humans and bypasses keratinocytes and Langerhans cells. The injected doses (10^7–10^8 cfu in macaques and 10^4–10^5 cfu in rabbits) are probably higher than the number of bacteria that cause human disease. The cellular infiltrate in these models consists predominantly of dermal heterophils and plasma cells. In a swine model of *H. ducreyi* infection, 10^7 cfu of strain 35000 were inoculated with the Multi-Test applicator and caused an inflammatory infiltrate of PMNL and T cells similar to that seen in the human model [42]. All species of infected animals develop serum IgG responses to *H. ducreyi* during the first 2 weeks of infection to *H. ducreyi*, as assayed by Western blots. Thus, doses and host responses differ among the human and animal models.

CD4 helper subpopulations were first defined in murine systems by cytokine production as involved in delayed-type hypersensitivity responses (Th1, expression of IFN-γ and interleukin [IL]-2) or in regulation of antibody production (Th2, expression of IL-4, IL-5, and IL-10) [43]. The outcome of infectious processes, such as leprosy, is influenced by the pattern of cytokines produced at the site of disease [12, 35, 44]. The presence of IFN-γ mRNA in the lesions and lack of antibody responses to *H. ducreyi* suggest that Th1 responses may predominate during the initial stages of infection. Whether T cells present in the experimental chancroid are Th1, Th2, or mixed is unknown. However, the outcome of experimental infection may depend on the equilibrium between Th1 and Th2 populations.

In future studies, we will standardize the model using a dose of bacteria that is likely to cause infection in >80% of subjects (1.5 × 10^4 to 2 × 10^4 cfu). Subjects will be challenged with 2 identical doses of live bacteria to examine whether lesion formation is concordant, and they will have biopsies at different times during the course of infection. Our goals are to study longitudinally the location of the bacteria, T cell subsets, and cytokine patterns in the lesions. We will also examine whether cellular responses vary as papules regress or progress to pustule. These studies should yield important information about pathogenesis and protective immunity.

Acknowledgments

We thank Byron Batteiger, Robert B. Jones, Barry Katz, and Robert Modlin for advice and Karla John, Daisy Thayyil, and Wendy Winkle for excellent technical assistance.

References


