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Expression of Cytolethal Distending Toxin and Hemolysin Is Not Required for Pustule Formation by *Haemophilus ducreyi* in Human Volunteers

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***Haemophilus ducreyi* makes cytolethal distending toxin (CDT) and hemolysin. In a previous human challenge trial, an isogenic hemolysin-deficient mutant caused pustules with a rate similar to that of its parent. To test whether CDT was required for pustule formation, six human subjects were inoculated with a CDT mutant and parent at multiple sites. The pustule formation rates were similar at both parent and mutant sites. A CDT and hemolysin double mutant was constructed and tested in five additional subjects. The pustule formation rates were similar for the parent and double mutant. These results indicate that neither the expression of CDT, nor that of hemolysin, nor both are required for pustule formation by *H. ducreyi* in humans.**

Haemophilus ducreyi, the etiologic agent of the genital ulcer disease chancroid, makes at least two protein toxins: hemolysin and cytolethal distending toxin (CDT). Both toxins have potent cytotoxic functions in vitro, but their roles in human disease are unclear.

The *H. ducreyi* hemolysin is a member of a family of pore-forming toxins found in several bacterial genera, including *Proteus, Serratia*, and *Edwardsiella* (22, 24, 26, 31). The hemolysin is a 125-kDa protein encoded by two genes. *hhdB* encodes the secretion-activation protein (HhdB), while *hhdA* encodes the toxin HhdA (12, 23, 24, 26). The hemolysin is very labile, and its activity can only be detected in association with live bacterial cells (2, 22–24). We recently reported the characterization of an isogenic hemolysin-deficient mutant (35000HP-RSM1) in which *hhdB* is insertionally inactivated by the ΩKm-2 cassette (22). In vitro, the parent had cytopathic effect for human foreskin fibroblasts and keratinocytes whereas the mutant lacked cytotoxicity (22). Despite the dramatic activity of the hemolysin in vitro, inoculation of the isogenic hemolysin-deficient mutant caused pustules at a rate similar to that of its parent in human volunteers (26). Similarly, an *hhdA* deletion mutant caused ulcers as frequently as its parent in the temperature-dependent rabbit model (12).

H. ducreyi CDT is a soluble protein encoded by a gene cluster designated *cdtABC* (16, 30). CDT is also produced by several other gram-negative organisms, such as *Escherichia coli, Shigella* spp., and *Campylobacter* spp. and causes irreversible G_2 arrest and subsequent cell death of epithelial cells and apoptosis of T cells (11, 16, 30). CDT is secreted, and its activity can be detected in frozen cell culture supernatants after 1 month (10, 27). In vitro, CDT has cytopathic effect for fibroblasts, keratinocytes, and HeLa cells, and expression of all three gene products is essential for cytotoxic activity in cell culture supernatants (11, 27, 30). CdtB shares significant homology with type I mammalian DNases and has intrinsic DNase activity that may explain its role in eukaryotic cell cycle arrest (13). We recently constructed a mutant (35000.303) in the structural gene encoding one component of CDT, designated *cdtC* (30). In contrast to the parent, cell culture supernatants obtained from 35000.303 or whole cells of 35000.303 lack cytopathic effect on keratinocytes, fibroblasts, and HeLa cells and do not cause apoptosis of T cells (16, 30). However, the *cdtC* mutant was as virulent as the parent in the temperature-dependent rabbit model (30).

Here we tested the hypothesis that expression of CDT is required for the virulence of *H. ducreyi* in humans. The virulence of the isogenic CDT-deficient mutant (35000.303) and its parent was first tested in a double-blinded, escalating dose-response study. Since 35000.303 could still produce intact hemolysin, we also compared a CDT and hemolysin double mutant (35000.304) and its parent in a second trial. We compared the papule and pustule formation rates, the cellular infiltrate, and recovery of bacteria from lesions inoculated with the mutant and the parent in each trial.

Construction and characterization of a *cdtC* and *hhdB* double mutant. *H. ducreyi* 35000 is a wild-type strain (30). *H. ducreyi* 35000.303, an isogenic CDT-deficient mutant that contains a chloramphenicol acetyl transferase (*cat*) cassette insertion in *cdtC*, was described previously (30). The plasmid pKLP107 contains an ΩKm-2 cassette inserted in *hhdB* (22). The plasmid pRSM1791 utilizes *lacZ* as a counterselectable marker to facilitate allele exchange (9). The interrupted hemolysin gene fragment from pKLP107 was ligated into pRSM1791, and the resulting plasmid, pRSM1920 (9) was electroporated into 35000.303. Colonies were selected on

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TABLE 1. CDT mutant trial responses to inoculation of live *H. ducreyi* strains[a]

Subject no.	Days of observation	Isolate	Initial no. of papules	Final outcome of initial papules		
				No. of papules	No. of pustules	No. resolved
121	6	35000	2	0	2	0
		35000.303	3	0	3	0
125	14	35000	2	0	1	1
		35000.303	2	0	0	2
126	8	35000	2	0	2	0
		35000.303	2	0	1	1
127	9	35000	2	0	1	1
		35000.303	3	0	2	1
128	8	35000	2	0	1	1
		35000.303	2	0	1	1
129	8	35000	2	0	1	1
		35000.303	3	0	2	1

[a] Note: each volunteer was inoculated at two sites with the parent (35000) and at three sites with the CDT mutant (35000.303).

chloramphenicol- and kanamycin-containing plates, and cointegrates were resolved in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as previously described (9). A CDT and hemolysin double mutant was recovered and designated 35000.304.

Lipooligosaccharide (LOS) and outer membrane proteins (OMPs) were prepared from 35000, 35000.303 and 35000.304 as described previously (26, 33). OMPs were subjected to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12.5% acrylamide gels as described previously (26, 33). LOS was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 14% acrylamide gels and silver staining as previously described (33). To examine the double mutant for CDT activity, a Jurkat T-cell proliferation assay was performed exactly as described previously (16). Hemolytic activity was measured in a liquid-suspension hemolysis assay, as described elsewhere (23, 26).

Human inoculation experiments. Healthy adult male and female volunteers over 18 years of age were recruited for the study. Informed consent was obtained from the subjects for participation and for human immunodeficiency virus serology, in accordance with the human experimentation guidelines of the Institutional Review Board of Indiana University Purdue University Indianapolis and the U.S. Department of Health and Human Services. The experimental challenge protocol, preparation and inoculation of the bacteria, determination of estimated delivered dose (EDD), and clinical observations were done as described previously (5, 26, 28, 29).

A modification of an escalating dose-response study was used to compare the virulence of 35000 and 35000.303 and that of 35000 and 35000.304 exactly as described previously (3, 6, 26). Briefly, each subject was infected at six sites. On one arm, three sites were inoculated with twofold serial dilutions of the mutant. On the other arm, two sites were inoculated with the parent, and one site was inoculated with the highest dose of the heat-killed mutant. To blind the study, the six suspensions containing bacteria were randomized, given a code number, and inoculated at identical sites on each subject in each iteration. The clinicians who evaluated the subjects were unaware of the identity of the suspensions. Subjects were observed until they reached a clinical endpoint, defined as either 14 days after inoculation, development of a painful pustule, or resolution of infection at all sites. When a clinical endpoint was achieved, the code was broken and up to two sites with active disease (one inoculated with the parent and one with the mutant), if present, were biopsied with a punch forceps. The subjects were then treated with antibiotics as described previously.

Each biopsy specimen was cut into portions. One portion was semiquantitatively cultured as described previously (28, 29). One portion was formalin fixed and used for immunohistological studies as previously described (25, 28, 29). The slides were coded and read by a dermatopathologist, who was unaware of the code.

Individual colonies from the inocula, surface cultures, and biopsy specimens were picked, suspended in freezing medium, and frozen in 96-well plates. All colonies were scored for susceptibility to chloramphenicol or chloramphenicol and kanamycin on agar plates.

Evaluation of *cdtC* mutant in human subjects. We previously reported construction of a mutant (35000.3) that contains a *cat* insertion in *cdtC* (30). Five men and two women (one black and six white; age range, 20 to 47 years, age [mean ± standard deviation], 35.3 ± 10.2 years) enrolled in the study. One male subject withdrew prior to inoculation. Three subjects (121, 125, and 126) were challenged in the first iteration, and three subjects (127, 128, and 129) were challenged in the second iteration (Table 1).

The EDD in the first iteration was 40 CFU for 35000 and 16, 32, and 64 CFU for 35000.303. Papules developed at six of six sites inoculated with the parent and at seven of nine sites inoculated with the mutant. At endpoint, pustules were present at five of six parent sites and four of nine mutant sites.

Since inoculation of both the mutant and the parent caused pustules at similar rates, we continued the experiment with

TABLE 2. CDT-Hemolysin double mutant trial responses to inoculation of live *H. ducreyi* strains[a]

Subject no.	Days of observation	Isolate	No. of initial papules	Final outcome of initial papules		
				No. of papules	No. of pustules	No. resolved
137	9	35000	1	0	1	0
		35000.304	3	0	1	2
148	8	35000	2	0	2	0
		35000.304	3	0	2	1
150	8	35000	2	0	2	0
		35000.304	3	0	2	1
153	5	35000	2	0	0	2
		35000.304	3	0	0	3
157	7	35000	1	0	0	1
		35000.304	2	0	0	2

[a] Note: each volunteer was inoculated at two sites with the parent (35000) and at three sites with the CDT and hemolysin double mutant (35000.304).

similar target doses. In the second iteration, three subjects were inoculated with an EDD of 50 CFU of 35000 and 30, 60, and 120 CFU of 35000.303. Papules developed at six of six sites inoculated with the parent, and at eight of nine sites inoculated with the mutant. At endpoint, three of six parent sites and five of nine mutant sites contained pustules.

For this trial, no lesions developed at sites inoculated with the heat-killed controls. The pustule formation rates were 66.7% (exact binomial 95% confidence interval [CI], 34.9 to 90.1%) at 12 sites for 35000 and 50% (exact binomial 95% CI, 26 to 74%) at 18 sites for 35000.303 (one-tailed Fisher's exact test; $P = 0.301$). Thus, expression of CDT was not required for pustule formation.

Characterization of the CDT and hemolysin double mutant. In a previous human challenge trial, an isogenic hemolysin-deficient mutant caused pustules at a rate similar to that of its parent (26). We constructed a CDT and hemolysin double mutant (35000.304) in order to exclude the possibilities that the single mutants were virulent because they expressed the other toxin.

In a Southern blot assay, genomic DNA from 35000 and 35000.304 were digested with *Pst*I and probed with the *hhdB* and *cdtC* open reading frames as well as the ΩKm-2 and *cat* cassettes. The *hhdB* probe bound to a 4.3-kb DNA fragment in the parent and a 6.1-kb DNA fragment in the double mutant. The *cdtC* probe bound to a 4.1-kb DNA fragment in the parent and a 5.5-kb DNA fragment in the double mutant. The ΩKm-2 and *cat* probes did not bind to 35000 DNA but did bind to 6.1- and 5.5-kb DNA fragments in 35000.304, respectively (data not shown).

35000 and 35000.304 had similar growth rates in broth (data not shown). OMPs and LOS prepared from 35000.304 and 35000 were analyzed by SDS-PAGE. Both isolates had similar LOS patterns (data not shown) and OMP profiles (data not shown).

The double mutant was evaluated for loss of CDT and hemolysin activity. Jurkat T-cell proliferation was not inhibited by 35000.304 but was inhibited by 35000 as described previously (data not shown) (30). As expected, broth-grown 35000 had activity while 35000.304 lacked activity in a liquid-suspension hemolysis assay (data not shown).

Evaluation of the CDT and hemolysin double mutant in human subjects. Four men and four women (one black and seven white; age range, 21 to 38 years; age [mean ± standard deviation], 28.3 ± 7.3 years) enrolled in the study. Three subjects (148, 150, and 153) were challenged in the first iteration, and two subjects (137 and 157) were challenged in the second iteration (Table 2). Two subjects (151 and 156) withdrew on the day of inoculation. Another subject (178) was excluded because he took azithromycin just prior to enrollment in the study.

The EDD in the first iteration was 44 CFU for 35000 and 19, 37, and 74 CFU for 35000.304. Papules developed at six of six parent sites and nine of nine mutant sites (Table 2). Papules resolved at two of six parent sites and five of nine mutant sites. At endpoint, four of six parent sites and four of nine mutant sites contained pustules.

In the second iteration, two subjects were inoculated with an EDD of 64 CFU of 35000 and 25, 50, and 100 CFU of 35000.304. Papules developed at two of four parent sites and five of six mutant sites (Table 2). Papules resolved at one of four parent sites and four of nine mutant sites. At endpoint, pustules were present at one of four parent sites and one of six mutant sites.

For this trial, three papules developed at five sites inoculated with the heat-killed control and resolved in 2 to 4 days. For sites inoculated with live bacteria, the pustule formation rates were 50% (exact binomial 95% CI, 18.7 to 81.3%) at 10 sites for 35000 and 33.3% (exact binomial 95% CI, 11.8 to 61.6%) at 15 sites for 35000.304 (one-tailed Fisher's exact test; $P = 0.34$). Thus, expression of neither CDT nor hemolysin was required for pustule formation.

Surface cultures were obtained from all inoculation sites at each follow-up visit. No bacteria were recovered from sites inoculated with the heat-killed control. In trial 1, *H. ducreyi* was recovered intermittently from parent and mutant sites in four of six subjects. The recovery rate was 4% from sites inoculated with the parent ($n = 73$) and 5% from sites inocu-

lated with the mutant ($n = 74$). In trial 2, bacteria were isolated from one of five subjects, and the recovery rate was 5% from the parent ($n = 39$) and 2% from the mutant ($n = 49$) sites. In both trials, the surface culture recovery rates of bacteria from mutant and parent sites were not statistically different. All biopsy specimens were semiquantitatively cultured. In the first trial, bacteria were recovered from five of six parent sites and five of five mutant sites. The yield for 35000 and 35000.303 from positive cultures ranged from 2.1×10^4 to 1.1×10^6 CFU/g of tissue and 1.7×10^3 to 3.0×10^5 CFU/g of tissue, respectively. In the second trial, bacteria were recovered from all three parent and all three mutant sites biopsied. The yield for 35000 and 35000.304 ranged from 1.8×10^4 to 8×10^5 CFU/g of tissue and 1.0×10^3 to 2.6×10^5 CFU/g of tissue, respectively. In both trials, the numbers of bacteria recovered from mutant and parent biopsy specimens were similar.

We examined the cellular infiltrate in nine parent and eight mutant sites that were present at endpoint. In biopsy specimens obtained from both the parent and mutant sites, micropustules with polymorphonuclear leukocytes (PMNs) were present in the epidermis. The dermis contained a perivascular infiltrate of mononuclear cells and some PMNs, and the venules were lined with reactive endothelial cells. In both the parent and the mutant specimens, the majority of the mononuclear cells were stained with a CD3 marker (data not shown).

To confirm that the inocula were correct and that we had inoculated the sites as intended, individual colonies were analyzed for antibiotic susceptibility as described previously (33). If available, 30 colonies per specimen were analyzed so that there was a high likelihood (89%) that the cultures were pure (33). For the two cultures used to prepare the inocula in the CDT mutant trial, all 78 parent colonies and 80 mutant colonies tested were phenotypically correct. Positive surface cultures were obtained from four subjects in this trial, and all 39 parent and all 46 mutant colonies tested from their respective sites were phenotypically correct. Of five parent site biopsy specimens and five mutant site biopsy specimens that were culture positive, all 185 parent colonies and 161 mutant colonies tested were phenotypically correct.

For the inocula in the CDT and hemolysin double mutant trial, all 96 parent colonies and 95 mutant colonies tested were phenotypically correct. Positive surface cultures were obtained only from one subject, and both parent colonies and 30 mutant colonies tested were phenotypically correct. Of three parent biopsy specimens and three mutant biopsy specimens that were culture positive, all 144 parent colonies and 100 mutant colonies tested were phenotypically correct. Thus, all colonies tested had the expected antibiotic susceptibility.

Conclusions. Both hemolysin and CDT have potent in vitro cytopathic effect for human cell cultures and are postulated to be major virulence determinants for chancroid. In a previous study, an isogenic hemolysin mutant caused pustules at a rate similar to that caused by its parent (26). In this study, we constructed a CDT-deficient mutant (35000.303) and compared it with its parent (35000) in the human challenge model. Despite its lack of cytopathic activity in vitro, inoculation of 35000.303 caused papules and pustules at similar rates to those of its parent. A CDT and hemolysin double mutant (35000.304) was also constructed and caused papules and pustules at rates similar to its parent. Thus, expression of either CDT, hemolysin, or both is not required for pustule formation in humans.

For subject safety and practical considerations, we inoculate volunteers on their upper arms and allow the infection to proceed only until they develop painful pustules or for 14 days. Thus, a major limitation of the human model is that we cannot study disease beyond the pustular stage, at stages such as ulcers or lymphadenitis. Therefore, we cannot exclude the possibility that CDT or hemolysin contributes to pathogenesis beyond the pustular stage. Nevertheless, isogenic hemoglobin receptor (HgbA)-deficient, peptidoglycan-associated lipoprotein-deficient, and DsrA-deficient mutants are unable to form pustules even at doses 10-fold that of the parent in the model (2, 8a, 14). Thus, the model can be used to examine whether a putative virulence determinant has a role in pustule formation.

An explanation for the disparity between the in vitro experiments using cell monolayers and the human challenge experiments may be the differences in bacterial doses used in the respective models. For example, 10^5 to 10^7 CFU of bacteria were allowed to interact with 10^5 eukaryotic cells to study hemolysin activity (22). In the human model, subjects are inoculated with EDDs that range from 1 to 100 CFU. Thus, the in vitro models employ pharmacological doses of the organism relative to the physiological doses that cause infection.

Supernatants and whole cells of 35000 cause apoptosis of T cells, while preparations made from 35000.303 (16) and 35000.304 do not. We examined eight paired biopsy specimens of mutant and parent sites from both the CDT and CDT-hemolysin trial by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (In Situ Cell Death Detection Kit, Fluorescein; Roche Molecular Biochemicals, Mannheim, Germany). Few (<3%) of the lymphocytic nuclei stained with TUNEL in all of the biopsies (data not shown). TUNEL stains all cells with nicked DNA (15), and in some specimens it was difficult to distinguish necrotic cells from apoptotic bodies, especially in the pustule. In vivo, macrophages clear apoptotic bodies rapidly (15), and clearance may obscure differences between the mutants and parent in their ability to induce programmed cell death. Although we found no gross difference in the ability of the mutants and parent to cause apoptosis in vivo, these results should be interpreted with caution.

In these trials, isolates that did not make CDT and/or hemolysin elicited an inflammatory infiltrate that was similar to that elicited by the parent. The histopathology of natural and experimental infection consists of two major components: a PMN infiltrate that coalesces at the ulcer or pustule base and a dermal mononuclear infiltrate that has features of a delayed-type hypersensitivity or a homing response (1, 18–20, 25, 28). Confocal microscopy indicates that *H. ducreyi* colocalizes with PMNs and macrophages throughout experimental infection, but the organism remains extracellular and evades phagocytosis (7; unpublished observations). The PMN, macrophage, and T-cell responses apparently do not always clear the organism, and the elicited host response may damage the skin. In a previous study, we hypothesized that CDT interfered with T-cell responses to *H. ducreyi* by induction of apoptosis (7). Similarly, hemolysin causes lysis of T cells, B cells, and macrophages in vitro and may down regulate host responses (16, 32). If the immune response primarily damages the skin, the

inability of these mutants to interfere with the host response might not affect lesion formation or pathology.

In summary, neither hemolysin, CDT, nor both are required for pustule formation in human subjects. Although we cannot exclude the possibility that these toxins contribute to ulcer formation, chancroid may not be a toxin-mediated disease. The organism elicits and apparently evades a vigorous host response, which may damage the skin. Limited data from natural and experimental infection also suggest that infection with *H. ducreyi* does not reliably confer protective immunity (4, 8, 17, 21). Thus, future studies should focus on examining immunopathogenesis as a likely cause for lesion formation.

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