Haemophilus ducreyi Hemolysin Acts as a Contact Cytotoxin and Damages Human Foreskin Fibroblasts in Cell Culture

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***Haemophilus ducreyi*, which causes the sexually transmitted disease chancroid, produces several factors that damage human cells. We used isogenic mutants of *H. ducreyi* 35000 to demonstrate that the hemolytic activity and the cytotoxic effect of *H. ducreyi* on human foreskin fibroblasts are due to the same toxin.**

Haemophilus ducreyi is the etiologic agent of chancroid, a sexually transmitted genital ulcer disease. Chancroid is one of the most common sexually transmitted diseases in developing countries, and there are sporadic outbreaks of the disease in developed countries. It has been associated with an increased risk for the acquisition and transmission of AIDS in those countries in which both diseases are prevalent (4, 10, 20). Recent concerns about the underreporting of chancroid (13) and the link between chancroid and the exchange of sex for drugs in developed countries (7) have resulted in an increased focus on understanding the molecular mechanisms of the disease process.

Several potential virulence factors of *H. ducreyi*, including pili, lipopolysaccharide, iron-regulated proteins, heat shock proteins, outer membrane proteins, cytotoxins, and hemolysin, have been identified (19). In recent years, both in vitro (1, 16) and in vivo (5, 11, 14, 17) model systems that have contributed to our understanding of cellular damage (2, 3, 6, 12, 16), attachment (1–3, 16), and lesion formation (5, 11, 14, 17) have been described. Alfa et al. (3) compared microcolony formation, cytoadherence kinetics, and cytopathic effects (CPEs) on human foreskin fibroblast (HFF) cells with ulcer formation in the temperature-dependent rabbit model. However, the analysis of virulence factors has been hampered by the lack of isogenic mutants defective in the expression of specific virulence factors.

Recently, Totten et al. (18) and Palmer and Munson (9) cloned the hemolysin gene of *H. ducreyi* and described isogenic mutants of *H. ducreyi* that were defective in hemolysis. Other *H. ducreyi* cytotoxins, including a toxin that produces a CPE on HFF cells (1, 2) and cytotoxins active on several other human cell types (6, 12), have been described. Because many hemolysins are active on other cell types and because both hemolysin and the toxin that produces the CPE on HFFs are cell associated (1, 8, 18), we tested the *H. ducreyi* hemolysin mutants for their effects on HFFs. Our results demonstrate that hemolysin is responsible for the CPE on HFF cells.

The *H. ducreyi* strains and their hemolytic phenotypes are given in Table 1. Strain 35000 and its nonhemolytic isogenic derivatives, 35000-KmA and 35000-TcA, have been described previously (18). Hemolysin expression in the latter two strains is completely abolished by the insertion of transposons Tn1545-Δ3 and Tn916, respectively. Although we do not know

the exact transposon insertion sites, hybridization studies indicate that the transposons are inserted within a restriction fragment containing the hemolysin structural gene (18). Plasmid pPT384-Km contains the *H. ducreyi* hemolysin gene cloned into shuttle vector pLS88 as described previously (18) and restores the hemolytic phenotype to strain 35000-TcA. The ability of this hemolysin gene to restore the hemolytic phenotype to 35000-TcA verifies that a second transposon insertion in this strain has no effect in this system. The complementation studies also support the conclusion that the insertion of one of the transposons occurred within the hemolysin structural gene. Strain 35000-TcA(pUC19-Km) containing the vector sequences (without the hemolysin gene) served as a control in our assays.

Additional controls for cytopathic activity were constructed as follows. A random Tn916 insertion in strain 35000 produced a strain designated 35000-TcX, which was constructed by transforming pAM120 into strain 35000 by electroporation as described previously (18) and selecting for tetracycline-resistant hemolytic colonies on horse blood agar plates (HBAPs) containing tetracycline (18). Next, plasmid pUC19-Km was transformed into 35000-TcX by electroporation, and the resulting transformants were selected on HBAPs containing kanamycin (18). The resulting strain, 35000-TcX(pUC19-Km), served as a hemolytic kanamycin-resistant control containing Tn916.

The characterization of the hemolytic activity, preparation of bilayer HBAPs, and hemolysin assay have all been reported on previously (18). The strains were stored at –70°C in L broth with 25% glycerol. All *H. ducreyi* strains were grown on HBAPs to assess hemolysis before and after assays for CPEs on HFF cells and always had the appropriate phenotype. Hemolysis was observed as a zone of clearing around the colonies on HBAPs after a 48-h incubation at 35°C in a humidified atmosphere of 95% air–5% CO₂.

The HFF cell line (a nontransformed cell line originating from pooled human children foreskins) was used for all assessments of CPEs. The cultivation medium for HFF cells consisted of RPMI 1640 medium (ICN Biomedicals) supplemented with 1 mM sodium pyruvate (ICN Biomedicals), 2 mM L-glutamine (ICN Biomedicals), and 10% (vol/vol) fetal bovine serum (Gibco BRL, Grand Island, N.Y.). The HFF cells were incubated at 35°C in a humidified atmosphere of 95% air–5% CO₂. The monolayers were passaged every 72 h, with a solution of 0.05% trypsin and 0.53 mM EDTA (Gibco) being used to detach the cells. Viability determinations (by the trypan blue exclusion assay) made prior to inoculations of 96-well trays indicated that ≥96% of the HFF cells were viable. Each well of the 96-well tissue culture tray was seeded with 10⁴ HFF cells.

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TABLE 1. Hemolytic characteristics and CPEs of isogenic mutants of *H. ducreyi* 35000

Strain	Description	Source or reference	Kanamycin resistance	Hemolytic phenotype ^a	CPE on HFF cells	Growth index ^b
35000	Parental strain (Winnipeg isolate)	A. Ronald (University of Manitoba)	No	+	+	13.3
35000-KmA	Tn1545-Δ3 insertional mutant of hemolysin gene	17	No	—	—	Not tested
35000-TcA	Tn916 insertional mutant of hemolysin gene	17	No	—	—	8.9
35000-TcA (pPT384-Km) ^c	35000-TcA with pPT384-Km, which contains the hemolysin gene	17	Yes	+	+	21.3
35000-TcA (pUC19-Km) ^d	Plasmid control for pPT384-Km; carries plasmid but no insert	17	Yes	—	—	Not tested
35000-TcX (pUC19-Km) ^d	Tn916 insertional mutant of 35000 that does not affect hemolysin gene; also carries pUC19-Km	This paper	Yes	+	+	Not tested

^a Hemolytic phenotype of strains before and after the CPE assay. Organisms from the wells were subcultured onto HBAPs to determine if they retained the hemolytic phenotype after a 24-h incubation with HFF cells.

^b Ratio of total CFU in the HFF monolayer in a well at 6 h postinoculation and total CFU in a well at 0.5 h postinoculation.

^c pPT384-Km contains the entire plasmid, pPT384, cloned into the *Sst*I site of shuttle vector pLS88. Thus, it contains the structural gene for *H. ducreyi* hemolysin, pUC19, and pLS88. Kanamycin resistance is coded for by the pLS88 vector, and ampicillin resistance is coded for by pUC19.

^d pUC19-Km contains plasmid pUC19 cloned into shuttle vector pLS88 at the *Sst*I site. Kanamycin resistance is coded for by the pLS88 vector, and ampicillin resistance is coded for by pUC19.

A suspension of *H. ducreyi* cells (grown for 24 h on chocolate agar) in RPMI was prepared, and clumps were allowed to settle. The supernatant was removed and adjusted to a McFarland turbidity standard of 1. This bacterial stock was diluted 1:100 in RPMI, and 100 μ l was used to inoculate each well (the 100 μ l of medium that was previously added to each well of the 96-well tray was removed prior to the inoculation with bacteria). In instances in which the *H. ducreyi* strain was kanamycin resistant (i.e., it contained pUC19-Km or pPT384-Km), RPMI supplemented with 20 μ g of kanamycin per ml was used to prepare the bacterial inoculum. Viable cell counts of the inoculating suspension were performed by the spread plate technique. These determinations indicated that for all experiments, about 1×10^4 to 10×10^4 CFU of each bacterial strain was inoculated into each well (for a multiplicity of infection of 1 to 10). The inoculated trays were incubated at 35°C for 24 h. The supernatant fluid from all wells was subcultured to HBAPs with and without kanamycin to determine whether the organisms in the wells retained their hemolytic and/or kanamycin resistance characteristics. The monolayers in the 96-well trays were then washed four times with RPMI to remove unbound bacteria, and the washed monolayer was stained for 20 min with crystal violet stain consisting of 0.25% (wt/vol) crystal violet, 20% (vol/vol) methanol, 0.9% (wt/vol) NaCl, and 0.02 M Tris-HCl (pH 7.5). The trays were gently washed under running tap water until dye was no longer eluted. The monolayers were viewed by light microscopy with a Nikon Diaphot Phase Contrast-2 microscope-camera instrument. These experiments were repeated six times.

The effects of different bacterial strains on the HFF cell culture model have previously been well described (1–3). *H. ducreyi* 35000 but not *Haemophilus influenzae* causes CPEs in this system. Alfa et al. (3) have shown that microcolony formation, cytoadherence, and CPEs are separable characteristics in different strains. Thus, strain 35000 forms microcolonies, adheres rapidly, and causes CPEs, whereas strain A77 does not form microcolonies, adheres poorly, and causes CPEs at 24 h only if the A77 cells are not washed off the monolayer after 4 h of contact. The *H. influenzae* and A77 control strains produced the expected results on the HFF cells in the current investigation (data not shown).

In this study, isogenic mutants of *H. ducreyi* 35000 that lacked the ability to hemolyze horse erythrocytes were evaluated for their ability to produce CPEs in the HFF cell culture model system. Strain 35000 was both hemolytic (Table 1) and produced CPEs on HFFs (Fig. 1A and Table 1). In contrast, Fig. 1B demonstrates that *H. ducreyi* 35000-TcA, which is a Tn916 insertional mutant of *H. ducreyi* 35000 (Table 1), is nonhemolytic on HBAPs and does not cause CPEs on HFF cells despite exhibiting good growth and microcolony formation (Table 1). Another mutant that was independently derived, 35000-KmA, showed the same loss of CPE concurrent with the loss of hemolysis (Table 1). Insertional mutants 35000-KmA and 35000-TcA were both stable, and even if they were passaged multiple times in vitro, they did not regain hemolytic activity. The defect in the hemolysin gene of isogenic mutant 35000-TcA(pPT384-Km) is complemented with pPT384-Km, which contains an intact *H. ducreyi* hemolysin gene. This complemented mutant regained the wild-type hemolysis phenotype on HBAPs as well as the ability to cause CPEs on HFF cells (Fig. 1D). This result was not attributable to the plasmid vector alone, since *H. ducreyi* 35000(pUC19-Km) did not reestablish the wild-type phenotype (Fig. 1C). Isogenic mutant 35000-TcA(pPT384-km) was less stable than either 35000-KmA or 35000-TcA. However, after multiple selective passages on HBAPs (only hemolytic colonies were subcultured), a stable clone was established. This clone retained its hemolytic capacity and was used for the experiments described in this paper. Table 1 lists the various isogenic mutants tested and summarizes their hemolytic, cytopathic, and microcolony formation characteristics.

Previous reports have indicated that *H. ducreyi* 35000 produces a hemolysin (8, 18) and CPEs on HFF cells (1). We have now shown that these two activities are due to the same toxin. The results of the current study demonstrate that for the isogenic mutants described in Table 1, there is a direct correlation between the ability of *H. ducreyi* to lyse horse erythrocytes and its ability to damage HFF cells. Alfa et al. (3) have indicated that the inability to cause CPEs in HFF cells might be related to the reduced growth rates for some strains. However, the inability of strain 35000-TcA to cause CPEs on HFF cells was not due to a reduced growth rate (Table 1), since the 35000-

FIG. 1. CPEs of isogenic *H. ducreyi* mutants on HFFs. HFFs were inoculated with approximately 10^4 CFU of *H. ducreyi* per well and allowed to incubate for 24 h. The fibroblasts were then washed and stained with crystal violet (as described in Materials and Methods). The strains included *H. ducreyi* 35000 (A), *H. ducreyi* 35000-TcA (B), *H. ducreyi* 35000-TcA(pUC19-Km) (C), and *H. ducreyi* 35000-TcA(pPT384-Km) (D).

TcA growth index was 8.9 and an index of ≥ 1 is considered good growth (3). Table 1 indicates that bacterial cells retrieved from the HFF monolayer after the experiment still retained kanamycin resistance and hemolytic characteristics (i.e., the association with the HFF monolayer did not alter the expression of these components).

In this study, we have shown that *H. ducreyi* hemolysin mutants were unable to damage HFF cells. This result establishes the identity of the hemolysin and the contact-dependent toxic activity on fibroblasts. This activity is distinct from that of the diffusible cytotoxin described by Purven and Lagergard (12). As was reported by Purven and Lagergard (12), the diffusible cytotoxin does not damage human fibroblasts; rather, it affects human epithelial cells but does not damage Hum cells (a primary human fetus fibroblast cell line). We did not test whether the isogenic hemolysin mutants that do not damage HFF cells still produce the soluble cytotoxin that affects epithelial cells.

The significance of many hemolysins in virulence depends upon their effects on cell types other than erythrocytes. The target cell range of hemolysins has been most extensively studied with the RTX class of hemolysins, which includes the *Escherichia coli* alpha-hemolysin (21). The *E. coli* hemolysin has a lytic effect on many cell types at high doses but may alter cell functions at lower doses (21). The *H. ducreyi* hemolysin is homologous to the calcium-independent hemolysin of a *Proteus* sp. (17). While the *Proteus* hemolysin has been shown to affect other cell types, such as Daudi, Raji, T24, U937, and Vero (15), the range of potential target cells and effects of sublytic doses of this class of hemolysin have not been extensively studied.

We have shown in this paper that the *H. ducreyi* hemolysin damages human fibroblasts in cell culture. In vivo, the hemolysin may also cause direct damage to fibroblasts in the dermis, contributing to the formation of genital ulcers. The effect of

the hemolysin on other cell types, such as T cells, neutrophils, and macrophages, and the exact role it plays in ulcer formation require further evaluation.

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