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Haemophilus ducreyi, a Cytotoxin-Producing Bacterium

MARIA PURVÉN AND TERESA LAGERGÅRD*

Department of Medical Microbiology and Immunology, University of Göteborg, Guldhedsgatan 10A, S-413 46 Göteborg, Sweden

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An extra- and an intracellular product from the bacterium *Haemophilus ducreyi* were shown to have a cytotoxic effect on cell lines of epithelial origin, e.g., HEp-2 and HeLa cells. The cytotoxic effect appeared on cells within 24 h and resulted in cell death and morphologically changed cells. The cytotoxic activity was heat and pronase sensitive. The activity could be removed by incubation with the target cells, suggesting attachment of the agent to the cells. The cytotoxic products were secreted into the environment during exponential growth of bacteria and produced by most of the *H. ducreyi* strains tested. The activity was neutralized by homologous rabbit immune serum but not by the corresponding preimmune serum. The results indicate that strains of *H. ducreyi* produce a cytotoxin which may be of importance to the pathogenesis of chancroid.

The gram-negative bacterium *Haemophilus ducreyi* causes chancroid (soft chancre), a sexually transmitted disease which is endemic in many developing countries (5, 18, 21), particularly in Africa (4, 16, 21, 23) and Southeast Asia (27). Locally restricted outbreaks of chancroid have also been reported in North America and Europe (5). The disease is characterized by painful genital ulcerations and, in more than 50% of the cases, frequent regional lymphadenopathy.

Chancroid has received renewed attention as a significant predisposing factor in the sexual transmission of human immunodeficiency virus. Heterosexual individuals with genital ulcers have been reported to be at a significantly higher risk of subsequent human immunodeficiency virus infection (3, 10, 15).

H. ducreyi, the etiological agent of chancroid, was first described by Auguste Ducrey in 1889 (7). Little is known about its essential virulence factors and about the pathogenesis of chancroid. Intradermal inoculation of bacteria in rabbits has been used to differentiate between virulent and avirulent strains (6). It has also been suggested that differences between the lipopolysaccharide compositions of avirulent and virulent *H. ducreyi* strains exist (24). No reports on any toxin other than endotoxin (lipopolysaccharide) have been published.

One aim of the present study was to investigate whether *H. ducreyi* products have a cytotoxic effect on cells in cell culture. We found that both extra- and intracellular substances produced by *H. ducreyi* had a strong toxic action on cells (causing cell death), and consequently another aim was to investigate whether this cytotoxic effect was due to production of a cytotoxin. We studied some chemical and biological properties attributed to bacterial toxins, such as (i) protein nature, (ii) attachment to target cells, (iii) production and secretion during bacterial growth, and (iv) antigenicity.

MATERIALS AND METHODS

Bacteria. Ten *H. ducreyi* strains were obtained from the Culture Collection of the University of Göteborg (CCUG), i.e., CCUG 4438 (=Cip 542), 7470, 7781, 9276, 10045, 17713, and 20693, and from Institute of Tropical Medicine Antwerp (ITMA), i.e., ITMA 3207, 3542, and 4747.

Other *Haemophilus* strains tested were *H. influenzae* CCUG 7566 and 12769 and *H. aegypticus* CCUG 629.

Cultivation of bacteria. *H. ducreyi* strains were cultivated on chocolate GVL-3 agar plates containing 5% brain heart infusion (BHI) agar, 1% horse blood, 1.5% horse serum, 0.05% yeast autolysate, 0.01% JAS (yeast autolysate; Department of Bacteriology, Sahlgrenska Hospital, Göteborg, Sweden), 0.015% IsoVitaleX, and 0.03 μl of vancomycin (11). The plates were incubated at 33°C for 48 h in high humidity in an anaerobic jar with Anaerocult C (Merck, Darmstadt, Germany) for generation of an oxygen-depleted and CO_2-enriched atmosphere.

The liquid medium for cultivation of *H. ducreyi* was BHI broth supplemented with 1% hemin histidine solution (BHI-hemin; Sigma Chemical Company, St. Louis, Mo.), L-histidine (Fluka Chemie AG, Buch, Switzerland), 10% fetal calf serum, 1% IsoVitaleX, and 0.03 μg of vancomycin (Department of Bacteriology, Sahlgrenska Hospital, Göteborg, Sweden) per ml. The bacteria were gently rotated at 75 rpm and 33°C for up to 70 h in an anaerobic jar as described above.

H. influenzae and *H. aegypticus* were cultivated on chocolate Grand Lux (GL) agar plates for 24 h at 37°C in a CO_2-enriched environment.

Cell-free culture medium preparation. Bacteria were cultivated in 50 ml of BHI-hemin broth for 48 h. After cultivation, the bacterial suspensions were centrifuged at 12,000 × *g* for 20 min at 4°C. The pH of the supernatants was adjusted to 7.2 with 2 M NaOH and then sterilely filtered through a 0.22-μm-pore-size filter (Millipore Corp., Bedford, Mass.). The preparations were stored at −20°C.

To study production of cytotoxic activity in relation to bacterial growth, strains CCUG 7470 and 10045 were cultivated for up to 70 h. Samples were taken continuously during growth. Optical density at 600 nm (OD_{600}) and viable counts were measured. Each cell-free culture medium sample was sterilely filtered. Bacteria were also washed, suspended in phosphate-buffered saline (PBS) to the original volume, sonicated, sterilely filtered, and stored at −20°C until analyzed.

Sonic preparation. Bacteria were cultivated on GVL-3 and GL chocolate agar plates for 48 (*H. ducreyi*) and 24 (*H. influenzae* and *H. aegypticus*) h, respectively. After cultivation, the bacteria were harvested, washed with PBS and/or

* Corresponding author.

directly suspended in PBS to an OD_{600} of 1.0, and ultrasonicated on ice twice for 1 min each time at 20 kHz (MSE sonifier) with a 30-s interval. After sonication, the bacterial suspensions were centrifuged at 12,000 × *g* for 20 min at 4°C and the supernatants were sterilely filtered. The preparations were stored at −20°C until analyzed.

Osmotic preparation. Bacteria were cultivated as described for sonic preparation. After cultivation, bacteria were harvested and suspended in sterile distilled water, adjusted to an OD_{600} of 1.0, and kept for 10 min at room temperature (17). After treatment, the bacterial suspensions were centrifuged at 12,000 × *g* for 20 min at 4°C. This procedure caused no visible destruction of cell morphology, as judged on gram-stained preparations. The supernatants were sterilely filtered and stored at −20°C until analyzed. This preparation was referred to as the osmotic preparation, and the protein concentration was <1 mg/ml.

Other sonic and/or osmotic preparations with a protein concentration of >1 mg/ml were prepared from strains CCUG 7470, 7781, 9276, 10045, and 17713 and ITMA 3207. Bacteria from 20 to 30 plates were suspended in either 20 ml of distilled water or 20 ml of PBS. The bacteria were treated as described above.

The osmotic and sonic preparations from *H. influenzae* and *H. aegypticus* were further concentrated 10-fold with a MINICON B-15 (Amicon, Beverly, Mass.) before use in the cytotoxicity test.

Protein assay. The protein concentrations of the preparations were measured by using the method of Bradford (2) with bovine serum albumin as the standard (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, Calif.).

Heat and protease treatment. Cell-free culture medium and sonic and osmotic preparations from strains CCUG 7470 and 10045 were incubated for 30 min at 23, 56, 70, 85, and 100°C. After treatment, the preparations were sterilely filtered, stored on ice, and analyzed immediately for cytotoxic activity on HEp-2 cells.

One milliliter of immobilized Pronase-CB (Pierce) was packed into 5-ml syringes. The gels were washed 10 times with sterile enzyme buffer (0.05 M Tris/HCl, 2 mM $CaCl_2 \cdot 6H_2O$, pH 8.0). Sonic preparations from strains CCUG 7470 and 10045 were loaded on the gels and incubated at 37°C for 20 h. After pronase treatment, the preparations were collected, immediately sterilely filtered, and analyzed for cytotoxic activity on Hep-2 cells. As controls, the same sonic preparations were incubated over the same period of time with enzyme buffer.

Cultivation of cell lines. HEp-2 (ATCC CCL 23), HeLa (ATCC CCL 2), A549 (human) (ATCC CCL 185), primary human fetus fibroblast (Hum) (Department of Virology, Göteborg University), Vero (ATCC CCL 81), GMK (Department of Virology, Göteborg University), MDCK (NBL-29) (ATCC CCL 34), and McCoy (ATCC CRL 1696) cells were cultivated in Eagle minimal essential medium with a 5 to 8% concentration of calf serum. For BHK-21 (C-13) (ATCC CCL 10) cells, Glasgow medium with 8% calf serum, 8% tryptose phosphate broth, and 1% L-glutamine was used for cultivation. All cells were incubated at 37°C in a 6% CO_2-enriched and 93% humidified atmosphere.

After 48 to 72 h of growth, the cells were harvested by using a solution of EDTA in PBS containing 4% trypsin for GMK, BHK, HEp-2, HeLa, A549, and MDCK cells or 4% trypsin in buffered NaCl for Vero, McCoy, and primary human cells. The cells were then suspended in fresh culture medium before being used in the cytotoxicity assay (see description below).

Cytotoxicity assay. The cytotoxicity assay employed was performed principally as described for pertussis (9) and diphtheria (20) toxin assays. However, the experimental conditions of the cytotoxicity assay with regard to cell concentration, cell seeding volume, and incubation period with cells were established in pilot experiments.

The assay was performed as follows. Flat-bottom microtiter plates (96 wells; Nunclon) were used. Fifty microliters of cell culture medium was placed into each well. Twenty-five or 50 μl of the preparations was added, and serial dilutions were made threefold and twofold, respectively. All preparations were tested in duplicate. Finally, 200 μl of freshly trypsinated cells at a concentration of 4×10^4 cells per ml in culture medium were added. The cells were incubated with the preparations for 1 to 2 h, and the medium was then replaced with 200 μl of fresh medium. The plates were incubated for 60 to 72 h. As a cell control, PBS or BHI-hemin medium was added instead of preparations.

Additionally, a confluent monolayer of cells was tested with sonic and osmotic preparations as described above.

The final evaluation was made after 60 to 72 h, before and after staining with 10% Giemsa solution (Merck Diagnostica, Darmstadt, Germany), by using inversion microscopy.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide and trypan blue treatments were used to confirm and evaluate death of cells in the cytotoxicity assay. Plates with cells incubated as described above were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma Chemical Company) at a final concentration of 0.5 mg/ml as previously described (22). Trypan blue (Flow Laboratories) was used in a final concentration of 0.025% (vol/vol). Cells were examined after 1 h of incubation in 37°C by using inversion microscopy. One hundred stained (trypan blue) or unstained [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] cells in each dilution were examined and counted.

The endpoint titer of the cytotoxic effect (cytotoxicity titer) was defined as the dilution giving ≥50% dead cells and/or morphologically changed cells compared with the cell controls.

The reproducibility of the assay was ± 1 two- or threefold dilution.

Absorption of cytotoxic activity with HEp-2 cells. A 100-μl volume of cell-free culture medium and sonic (diluted 1:10) and osmotic preparations from strains CCUG 7470 and 10045 were added to test tubes containing 200 μl of a freshly preparated suspension of Hep-2 cells (10^8 cells per ml). The samples were gently mixed and incubated at 37°C with a 6% CO_2 and 93% humid atmosphere for 1 h. After incubation, the tubes were centrifuged at 300 × *g* for 1 min at 23°C and the supernatants were incubated once more with new cells for 1 h. After centrifugation, the supernatants were analyzed for cytotoxic effect on Hep-2 cells. As a control, the preparations were incubated in the same manner but without cells, to investigate the possibility of cytotoxic inactivation due to 2 h of incubation. Additionally, as an absorption control, cells exposed to the different preparations were seeded into 96-microwell plates to verify the cytotoxic effect (cell death). The untreated preparations were also used as controls to establish the cytotoxicity titer.

Influence of exposure time on the cytotoxic effect. Cell-free culture medium and sonic and osmotic preparations from strain CCUG 7470 were diluted twofold, and HEp-2 cells were exposed for 5, 15, 30, 45, 60, and 90 min. After each incubation period, the plates were centrifuged, the medium

was changed, and the plates were further incubated for 60 to 72 h and then examined for a cytotoxic effect.

Immunization of rabbits. Six New Zealand White rabbits were immunized with sonic preparations from strains CCUG 7470, 10045, 9276, 7781, and 17713 and ITMA 3207 (about 1 mg of protein per ml). The rabbits were injected intradermally with four injections (0.1 ml each) of the bacterial sonicate incorporated in Freund's complete adjuvant (1:1). Injection of the bacterial sonicate mixed with Freund's incomplete adjuvant was performed 3 weeks later. After another 3 weeks, two or three booster injections with bacterial sonicate in PBS were made at 2-week intervals. Each rabbit received 200 μg of protein per immunization. Final bleedings were performed 14 days after the last injection. All sera were kept at −20°C until analyzed.

Neutralization of cytotoxic activity by immune sera. We mixed 0.3-ml volumes of sonic preparations from strains CCUG 7470, 7781, 9276, 10045, and 17713 and ITMA 3207 with a protein concentration of >1 mg/ml with 0.3 ml of preimmune or homologous immune serum. The tubes were gently mixed at 37°C for 45 min and centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatants were tested for cytotoxic effect on Hep-2 cells.

RESULTS

Cytotoxic effect on HEp-2 cells. When three different preparations, cell-free culture medium and sonic and osmotic preparations from *H. ducreyi* CCUG 7470, were tested on freshly seeded Hep-2 cells, cytotoxic activity was registered for all three preparations, showing similar patterns. When cells were exposed to high concentrations of the *H. ducreyi* products, rounded cells (dead cells) were seen. With increased dilution, dead cells and cells with changed morphology, e.g., star-shaped, elongated, and enlarged (dying cells), appeared (Fig. 1A to C). In the highest dilutions, morphologically changed cells mixed with normally growing cells were seen. The range between 100% cell death and normal growth occurred within 3 to 4 threefold dilutions. The killing curve showed a sigmoidal shape.

Monolayered HEp-2 cells exposed to sonic preparations (Fig. 1D) were not as sensitive as freshly seeded cells. The cytotoxic endpoint titer was 40 times lower than the endpoint titer of freshly seeded cells.

The developmental kinetics of the cytotoxic effect on HEp-2 cells was studied over a period of 3 days. The cytotoxic effect was detectable within 24 h of incubation for all three preparations tested. After 48 and 72 h, the cytotoxic effect was extended to higher dilutions for all three preparations. An increase in cell death occurred with extended incubation time.

Sensitivities of different cell lines to *H. ducreyi* products. *H. ducreyi* products also had a cytotoxic effect on other cell lines. Table 1 shows the sensitivities of the different cell lines tested (expressed as the dilution giving ≥50% cell death and/or morphological changes). According to these criteria, HEp-2, HeLa, and A549 cells were the most sensitive. Vero, GMK, BHK, MDCK, and primary human cells showed poor sensitivity to the different preparations. Cell death was not seen in any dilution. However, morphologically changed cells were observed. The most sensitive cell line was HEp-2.

Heat and pronase sensitivity of the cytotoxic activity. Cell-free culture medium and sonic and osmotic preparations from *H. ducreyi* CCUG 7470 and 10045 were used to test the heat sensitivity of the cytotoxic activity (Table 2). The results for all of the preparations tested showed that the cytotoxic activity was partly inhibited at 56°C and not detectable at 70°C.

When sonic preparations from strains CCUG 7470 and 10045 were treated with pronase, the cytotoxic activity decreased 25 times for strain 10045, compared with the control, and was totally abolished for strain 7470.

Absorption of the cytotoxic effect by incubation with HEp-2 cells. When cell-free culture medium and sonic and osmotic preparations from strains CCUG 7470 and 10045 were incubated with HEp-2 cells, the cytotoxic activity for all three preparations from both strains was absorbed. Controls incubated with cell culture medium showed 1 to 2 dilution steps lower activity after 2 h of incubation than the native preparations that were not incubated. The incubated cells died after treatment with the different preparations, as verified after they were seeded into tissue culture plates, while the cell controls showed normal growth.

Influence of exposure time on the cytotoxic effect. HEp-2 cells were exposed to cell-free culture medium and sonic and osmotic preparations for 5 to 90 min. The cytotoxic effect reached the cytotoxic endpoint titer after 15 min of exposure. However, after 5 min of exposure a significantly high cytotoxic titer was already noted for all three preparations (Fig. 2).

Relationship between bacterial growth and cytotoxic activity. Two *H. ducreyi* strains, CCUG 7470 and 10045, were cultivated in liquid medium to investigate the relationship between growth and production of cytotoxic activity. Activity increased gradually during the log phase for both of the strains, reaching the maximal endpoint titers of 1:128 for strain 7470 and 1:512 for strain 10045 after 40 and 45 h of growth. Figure 3 shows the relationship between the growth of strain CCUG 10045 and the cytotoxic activity in medium detected on HeLa cells. The viable count correlated with the OD up to about 45 h of cultivation, but after this time no viable bacteria were detected.

Relationship between cell-bound and secreted cytotoxin. Another experiment was done to investigate the location of the cytotoxin in bacteria during growth. Strains 7470 and 10045 were cultivated in liquid medium, and the relationship between cell-bound cytotoxicity (washed, sonicated bacteria) and secreted cytotoxicity (culture supernatant) was tested on Hep-2 cells. After 48 h of growth, the cytotoxic effect for strain 10045 was 1:6,561 in medium and 1:27 in sonicated bacteria. The corresponding titers for strain 7470 were 1:512 and 1:4. These results showed that about 99% of the total activity was secreted into the medium. The same strains were also cultivated on solid medium to compare cytotoxicity between washed and unwashed bacteria. The results showed that there was no difference in cytotoxicity in the sonicate when bacteria were washed or unwashed. Moreover, the supernatants from the PBS wash had only 0.1% of the total activity in the sonicate.

Cytotoxic activity in cell culture medium and sonic preparations among different *Haemophilus* strains. Ten *H. ducreyi* strains, two strains of *H. influenzae*, and one strain of *H. aegypticus* were tested on two or three occasions for cytotoxic activity (Table 3). The same 6 of 10 *H. ducreyi* strains showed a high cytotoxic effect on HEp-2 cells in both preparations. Four strains showed low or no cytotoxic activity in both cell-free culture medium and sonic preparations. For most of the strains, a 10- to 1,000-fold higher cytotoxic activity was found in bacterial cells grown on solid medium, suspended to an OD of 1.0, than in culture medium from cells grown in liquid medium to an OD between 0.25 and 0.85.

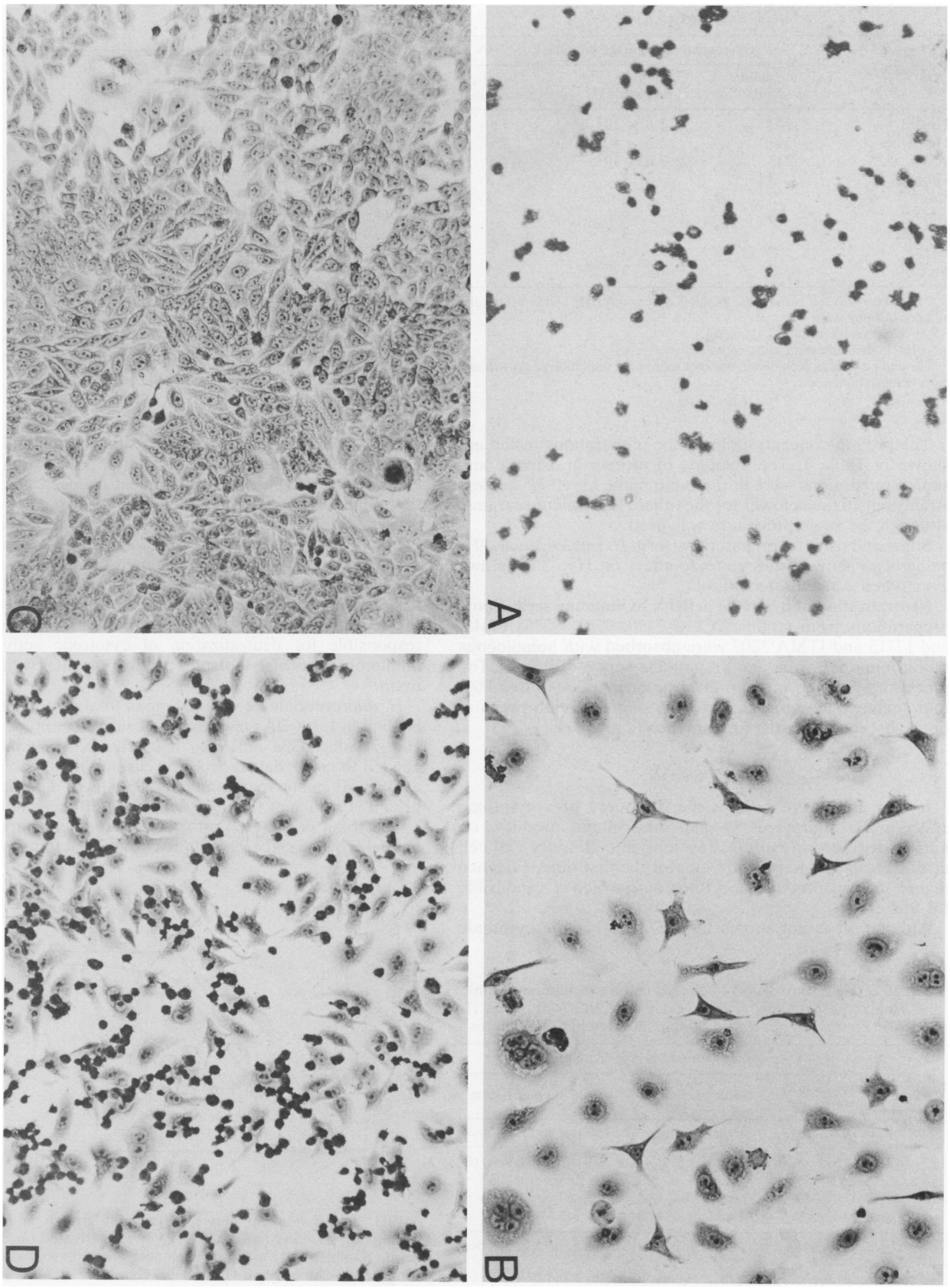

FIG. 1. HEp-2 cells were treated with the sonic preparation from strain CCUG 7470 for 60 h (protein concentration, 0.41 mg/ml). Panels: A, dead cells—dilution, 1:81; B, morphologically changed cells—dilution, 1:6,561; C, control (cells incubated in PBS); D, monolayered HEp-2 cells exposed to a sonic preparation—protein concentration, 1.2 mg/ml—dilution, 1:81. The cells were stained with Giemsa and photographed at a magnification of ×10.

TABLE 1. Sensitivities of different cell lines exposed to cell-free culture medium and sonic and osmotic preparations from *H. ducreyi* CCUG 7470

Cell line	Cytotoxicity (reciprocal dilution)[a]		
	Cell-free culture medium	Sonic preparation[b]	Osmotic preparation[c]
HEp-2	243	1.8×10^5	729
HeLa	243	3.6×10^3	729
A549	243	3.6×10^3	729
Hum[d]	3	3	3
Vero[d]	3	3	3
GMK[d]	3	9	3
MDCK[d]	3	3	3
BHK[d]	3	3	3
McCoy	<3	<3	<3

[a] The controls used were PBS, distilled water, and BHI-hemin broth, and all control titers were <3.
[b] Protein concentration, 0.41 mg/ml.
[c] Protein concentration, 0.28 mg/ml.
[d] Rounding of cells (cell death) was not seen, only inhibition of growth and morphological changes.

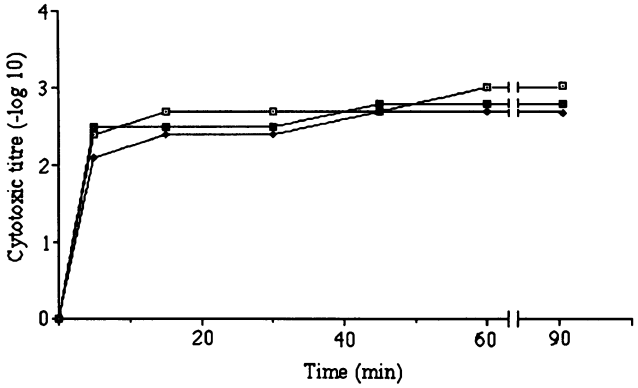

FIG. 2. Influence of time of incubation with preparations from *H. ducreyi* CCUG 7470 on Hep-2 cells. The cytotoxic effect was measured after 5 to 90 min of exposure. Protein concentrations for sonic and osmotic preparations were 0.41 and 0.28 mg/ml, respectively. Symbols: ⊡, cell-free culture medium; ◆, sonic preparation (1:100 dilution); ■, osmotic preparation (1:10 dilution).

The protein concentrations of the preparations tested are shown in Table 3. The amounts of protein in osmotic and sonic preparations were in the same range for all *H. ducreyi* strains but 10 times lower for the other *Haemophilus* strains, although the same procedure was used.

Sonic and osmotic preparations from *H. influenzae* and *H. aegypticus* showed no cytotoxic effect on Hep-2 cells, not even when concentrated 10-fold.

Neutralization of cytotoxic activity by immune sera. Sonic preparations from strains CCUG 7470, 7781, 9276, 10045, and 17713 and ITMA 3207 were absorbed with homologous rabbit immune serum and preimmune serum (Table 4). The procedure resulted in neutralization or in a 2,000- to 5,000-fold decrease in cytotoxic activity compared with preparations absorbed with the preimmune serum.

DISCUSSION

In this study, we present the discovery of a cytotoxic effect in *H. ducreyi* extra- and intracellular products on human cell lines in culture, resulting in cell injury and cell death. Furthermore, we provide, for the first time, evidence that *H. ducreyi* produces a cytotoxin(s), which is responsible for this effect.

Although it is not shown by the data that the cytotoxic activity is due to a single protein, some results indicate the action of one toxin. Different bacterial preparations caused the same type of cell morphological changes, showed the same kinetics of action, and affected the same cell lines.

The evidence that the cytotoxic activity produced by *H. ducreyi* is a protein can be summarized as follows. The cytotoxic activity is protein mediated, since the cytotoxic effect is inhibited by heat and pronase treatment. The toxin has antigenic properties, since immunization of rabbits with the bacterial sonicate results in production of antibodies responsible for neutralization of cytotoxic activity. The immunogenicity also indicates a high molecular weight of the toxin.

H. ducreyi sonicate with 0.4 mg of total protein per ml can be diluted 1.8×10^5 times and still cause a cytotoxic effect on Hep-2 cells. This indicates that less than a nanogram is needed to cause damage to the cells and thus a potent toxic activity. It can be mentioned that 0.15 ng of diphtheria toxin per ml is sufficient to cause death of Vero cells (19).

The toxin is secreted into the environment during exponential growth, since 99% of the cytotoxic activity was found

TABLE 2. Heat sensitivity of cell-free culture medium and sonic and osmotic preparations from *H. ducreyi* CCUG 7470 (strain 1) and 10045 (strain 2) treated for 30 min at different temperatures

Temp (°C)	Cytotoxicity titer (reciprocal dilution)					
	Cell-free culture medium		Sonic preparation[a]		Osmotic preparation[b]	
	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2
23	243	6.6×10^3	5.3×10^5	5.9×10^4	1.9×10^4	1.9×10^3
56	<3	81	243	729	243	243
70	<3	<3	<3	<3	<3	<3
85	<3	<3	<3	<3	<3	<3

[a] Protein concentrations, 1.2 and 1.0 mg/ml (for strains 1 and 2, respectively).
[b] Protein concentrations, 1.4 and 1.7 mg/ml (for strains 1 and 2, respectively). Cytotoxic activity after treatment was examined on HEp-2 cells.

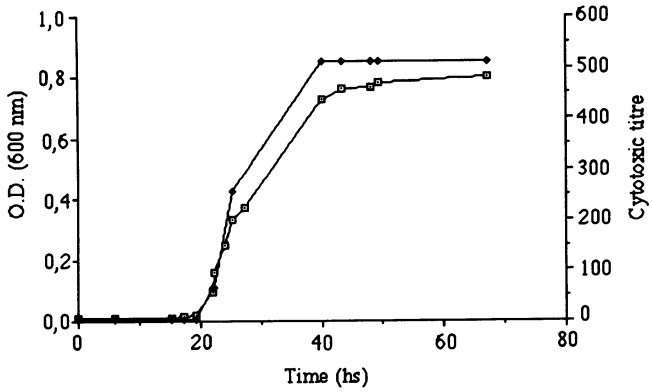

FIG. 3. Relationship between bacterial growth and cytotoxin production. *H. ducreyi* CCUG 10045 was cultivated on BHI-hemin broth. Samples were taken on each occasion indicated and analyzed for cytotoxic activity on HeLa cells. Symbols: ⊡, OD; ◆, cytotoxicity titer.

TABLE 3. Cytotoxic activities of different *Haemophilus* strains

Strain	Cytotoxicity titer (reciprocal dilution)[a]			Protein concn (mg/ml)
	Cell-free culture medium[b]	OD_{600}[c]	Sonic preparation[d]	
H. ducreyi				
CCUG 4438 (CIP 542)	≤3	0.30	≤9	0.27
CCUG 7470	243	0.51	1.8×10^5	0.41
CCUG 7781	729	0.60	1.8×10^5	0.53
CCUG 9276	1.9×10^4	0.18	1.8×10^5	0.51
CCUG 10045	6,561	0.69	1.8×10^5	0.44
CCUG 17713	6,561	0.85	1.9×10^4	0.41
CCUG 20693	<3	0.35	≤9	0.40
ITMA 3207	2,187	0.24	6,561	0.25
ITMA 3542	<3	0.69	≤9	0.45
ITMA 4747	<3	0.36	≤9	0.40
H. influenzae				
7566	ND	ND	<3	0.029
12769	ND	ND	<3	0.053
H. aegypticus 629	ND	ND	<3	0.053

[a] Controls (PBS and BHI-hemin broth) showed no cytotoxic effect on HEp-2 cells.
[b] Bacteria were cultivated for 48 h in BHI-hemin broth.
[c] OD_{600} measures the bacterial cell suspension.
[d] Bacteria were cultivated for 48 h on GVL-3 plates and suspended to an OD of 1.0 before sonication. Cytotoxic activity was tested on HEp-2 cells.

in the culture medium. This is also characteristic for some other exotoxin-producing bacteria, e.g., *Clostridium tetani*, *C. perfringens*, *Vibrio cholerae*, and *Staphylococcus aureus* (1, 26).

When bacteria were cultivated on solid medium, a very high cytotoxic activity was found in the cell sonicate. This may indicate that cytotoxin can accumulate in cells or in the periplasmic space, since a short osmotic shock resulted in relatively high cytotoxic activity. Other exotoxin-producing gram-negative bacteria, such as *V. cholerae* and *Escherichia coli*, also accumulate toxin before secreting it into the extracellular milieu, *E. coli* to a greater extent than *V. cholerae* (1, 13, 19, 25). The difference between cytotoxic activity in bacterial cells due to different cultivation procedures needs further investigation.

The absorption study with HEp-2 cells indicated that the toxic effect can be removed by target cells, suggesting specific attachment of the toxin to the target cells. Moreover, this binding step seems to occur within 5 min, reaching its maximum within 15 min. The binding of the toxin is probably also irreversible, since removal of medium in the cytotoxicity assay did not affect the cytotoxicity titer. Binding of toxin to specific cell receptors, on cell membranes of sensitive cells, is known to be the first step in the mechanism of action for some cytotoxins. This step can also be irreversible after binding to the target cells (8, 19).

TABLE 4. Neutralization of the cytotoxic activity of *H. ducreyi* sonic preparations by homologous immune sera

Strain	Preparation protein concn (mg/ml)	Cytotoxicity titer[a] after absorption with:	
		Preimmune serum	Immune serum
CCUG 7470	1.9	3.2×10^4	<100
CCUG 7470	5.4	5.1×10^5	<100
CCUG 7781	3.1	2.0×10^6	800
CCUG 9276	1.5	2.5×10^4	<100
CCUG 9276	4.2	5.1×10^5	<100
CCUG 10045	1.3	5.1×10^5	<100
CCUG 10045	4.6	1.0×10^6	100
CCUG 17713	1.6	1.0×10^6	200
ITMA 3207	1.1	1.2×10^5	<100
ITMA 3207	2.8	5.1×10^5	100

[a] Cytotoxic activity was tested on HEp-2 cells.

Moreover, this binding step seems to occur within 5 min, reaching its maximum within 15 min. The binding of the toxin is probably also irreversible, since removal of medium in the cytotoxicity assay did not affect the cytotoxicity titer. Binding of toxin to specific cell receptors, on cell membranes of sensitive cells, is known to be the first step in the mechanism of action for some cytotoxins. This step can also be irreversible after binding to the target cells (8, 19).

H. ducreyi toxin seems to be active mainly on human cell lines. Except for human fibroblasts, all three human cell types (HEp-2, HeLa, and A549) showed the highest sensitivity. It is important, however, to point out that cell death was recorded only for human cell lines. This specificity of the toxin to human cell lines of epithelial origin may explain the fact that chancroid is a disease which probably affects only humans (14).

We demonstrated that 6 of the 10 *H. ducreyi* strains tested had the ability to produce a cytotoxin(s) with high cytotoxic activity. Furthermore, we found that 35 of 40 other *H. ducreyi* strains produced a cytotoxin (unpublished data). Some strains, e.g., *H. ducreyi* reference strain Cip 542, showed low or no cytotoxic activity. This strain has also been reported to be avirulent in rabbit intradermal tests (12).

These data indicate that the ability to produce a cytotoxin is characteristic of most *H. ducreyi* strains. Bacteria from the genus *Haemophilus* are not known to produce any toxins. *H. influenzae* and *H. aegypticus* sonicates had no cytotoxic effect on HEp-2 or HeLa cells, as expected.

The fact that *H. ducreyi* cytotoxin causes damage to human epithelial cells suggests that the toxin acts as a virulence factor and that it is responsible for the development of chancroid ulcers. However, the actual role of this cytotoxin in the pathogenesis of chancroid remains to be evaluated.

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