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Purification and Identification of *Haemophilus ducreyi* Cytotoxin by Use of a Neutralizing Monoclonal Antibody

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Haemophilus ducreyi **produces a cytotoxin responsible for the killing of cultured human epithelial cells. Cytotoxin-neutralizing antibodies were detected in the majority of sera from patients with culture-proven chancroid, and a significantly higher level of such antibodies in patients than in blood donors was noted both in areas where the disease is endemic and those where it is not. We produced neutralizing monoclonal antibodies (MAbs) in mice with a crude osmotic preparation of the cytotoxin. These antibodies, with high capacity to neutralize cytotoxicity, were used for purification and identification of the cytotoxin. Purification was performed by a two-step procedure which included Sephacryl S-200 filtration followed by immunoaffinity chromatography. The purification resulted in poor cytotoxin protein recovery and contamination with MAbs from the affinity column. The results of the gel filtration experiments and immunoblotting indicate that the active cytotoxin consists of a single, small protein with an approximate molecular mass of 20 kDa. Cytotoxins from different strains seem to have the same or similar epitopes. The cytotoxin protein was not detected in preparations from nontoxic strains. The N-terminal amino acid sequence of the 20-kDa band was E-S-N-P-D-P-T-T-Y-P-D-V-E-L-S-P-P-P. This sequence does not resemble that of any currently known bacterial protein.**

Haemophilus ducreyi causes the sexually transmitted genital ulcer disease, chancroid (27). The organism is a major cause of genital ulcers in Africa (5, 17, 24, 29); however, locally restricted outbreaks have been reported in Canada, the United States, Latin America, and Europe (5, 7, 19, 23, 29). In developing countries, genital ulceration has been related to an increased risk for the spread of human immunodeficiency virus infection (9, 11, 23, 27).

The virulence factors and the pathogenic mechanisms responsible for the development of ulcers, as well as the bacterial antigens inducing a protective immunity to *H. ducreyi* infection, are unknown (13, 29). Some toxic virulence factors, however, have been postulated. Lipooligosaccharide produces skin abscesses in mice and rabbits after intradermal injection (4, 14). A hemolysin with activity on various mammalian erythrocytes has been identified and cloned, and the gene encoding a high-molecular-weight protein was identified (21, 22). Recently, it has been reported that the *H. ducreyi* hemolysin acts as a contact toxin that damages human foreskin fibroblasts in cell culture (1, 2, 10, 20). *H. ducreyi* elaborates another cytotoxin, which causes the death of cultured human epithelial cell lines and which is distinct from the hemolysin (25). This toxic activity was manifested in the majority of *H. ducreyi* strains (24). Evidence for *H. ducreyi* adherence to and destruction of human epithelial cell monolayers in vitro was also reported (16).

The aim of this study was (i) to detect the presence of serum-neutralizing antibodies in patients with culture-confirmed chancroid; (ii) to produce mouse monoclonal antibodies (MAbs) with neutralizing capacity; and (iii) to purify, identify, and characterize the chemical and immunological properties of the *H. ducreyi* cytotoxin.

Ten *H. ducreyi* strains were obtained from the Culture Collection of the University of Göteborg (CCUG): CCUG 4438 (CIP 542), CCUG 7470 (CIP 76118), CCUG 7311, CCUG 7781, CCUG 9276, CCUG 10045, CCUG 17713, CCUG 17675, CCUG 18804, and CCUG 20693. Four strains were obtained from the Institute of Tropical Medicine, Antwerp (ITMA): ITMA 3207, ITMA 3542, ITMA 4703, and ITMA 4747. Bacteria were cultivated on chocolate GVL-3 agar plates with vancomycin as described previously (25).

Crude cytotoxin preparations were prepared by osmotic or sonic treatment of *H. ducreyi* strains CCUG 4438 and CCUG 7470. For osmotic treatment, bacteria were collected from solid medium, washed, suspended in phosphate-buffered saline (PBS), pH 7.2, and centrifuged at 10,000 × *g* for 20 min at 4°C. The cell paste was then suspended in 0.2 M Tris-HCl buffer (pH 8.0) containing 1 M sucrose, 20 μl of 0.5 M (pH 8.0) EDTA/10 ml, 0.5 mM benzamidine, and 0.02 mg of soybean trypsin inhibitor/ml and gently mixed for 45 min at room temperature. Then, the suspension was centrifuged twice at 10,000 × *g* for 20 min at 4°C. The supernatant was concentrated by ultrafiltration, and sucrose was removed by repeated replacement of the buffer with PBS by gel filtration on a PD-10 column. This osmotic preparation (OP) was used for purification of the cytotoxin protein. Additionally, the sonic preparation of whole bacteria was prepared as previously described (15).

Protein concentrations were measured by the method of Bradford (protein assay kit; Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin as the standard (3).

Serum samples from 86 patients with culture-confirmed chancroid were obtained from the Institute of Tropical Medicine, Antwerp, Belgium. The samples were collected at the time of infection. The majority of these patients ($n = 76$) were from Africa; the remaining 10 were from Thailand. Sera from 20 healthy Thai blood donors and from 30 healthy adults living in Mwanza, Tanzania, kindly donated by Bencha Petchclai, Department of Pathology, Bangkok, Thailand, and R. Gabone, National Institute for Medical Research, Mwanza, Tanzania, were used as controls from areas where chancroid is endemic. In addition, 100 sera from Swedish blood donors were used as controls from an area where chancroid is not endemic.

The unpaired Student *t* test was used in order to compare

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TABLE 1. *H. ducreyi* cytotoxin-neutralizing antibodies in sera of patients with chancroid and in sera of healthy individuals

Serum source	No. of patients	No. of patients (%) with neutralizing antibody titer[a] of:				GM (±SD)[b]
		<10	10–39	40–159	160–640	
Patients with chancroid	86	29 (34)	15 (17)	18 (21)	24 (28)	20 (±5)
Healthy individuals from:						
Tanzania and Thailand	50	27 (54)	15 (30)	6 (12)	2 (4)	11 (±2)
Sweden	100	96 (96)	4 (4)	0 (0)	0 (0)	<10 (±1)

[a] Expressed as reciprocal serum dilutions.
[b] GM, geometric mean. All pairwise comparisons of neutralizing-antibody frequencies between groups yielded *P* values of <0.001.

differences between the groups with regard to the presence of neutralizing antibodies.

Rabbit hyperimmune sera were prepared as described elsewhere (15).

Mouse MAbs were produced as previously described (6). Briefly, 7-week-old BALB/c mice were injected intraperitoneally with an OP of cytotoxin from strain CCUG 7470 (20 μg of protein). Triple injections on days 1, 3, and 5 were given four times at intervals of 4 weeks. Mice with the highest levels of neutralizing antibodies were boosted intravenously with 50 μg of protein before fusion. Hybridoma cell culture media were tested for the presence of neutralizing antibodies by using a sonic preparation from strain CCUG 7470. The clone showing the highest neutralization activity was chosen. This MAb (designated M4D4) was also able to neutralize cytotoxic activity in sonic preparations from five toxic strains. Nanogram amounts of MAb could neutralize one cytopathic unit (CPU) of the cytotoxin (15). The antibody class and subclass of the MAbs were estimated by enzyme-linked immunosorbent assay (8). Because the MAbs were of the immunoglobulin G1 (IgG1) subclass, the purification from culture medium was performed by affinity chromatography with immobilized protein A (Prosep-A; high-capacity; Bioprocessing, Princeton, N.J.).

The cytotoxin neutralization assay was performed as previously described (15). The OP, the cytotoxic fraction after S-200 Sephacryl gel filtration, and the immunoaffinity-purified cytotoxin from strain CCUG 7470 were used in concentrations corresponding to about 4 CPU. The neutralization endpoint titer was defined as the dilution of serum giving a number of living HEp-2 cells that was 50% that of control wells without antiserum (100% growth). The results were expressed as reciprocal serum dilutions (15).

To measure cytotoxic activity in preparations, a cell viability colorimetric assay with HEp-2 cells was performed as previously reported (18, 24). The cytotoxic endpoint titer was defined as the dilution giving a 50% decrease in absorbancy (i.e., 50% cell death) compared with that of control wells.

For purification of cytotoxin the osmotic cytotoxin preparations were applied to the Sephacryl S-200 (Pharmacia, Uppsala, Sweden) column, equilibrated with PBS, and eluted with the same buffer. A fast-protein liquid chromatography (FPLC) system with a Super-Dex 75 10730 column (Pharmacia) equilibrated with Tris-EDTA buffer, pH 7.5, was used for molecular weight approximation. Vitamin B_{12} (1.35 kDa), equine myoglobulin (17 kDa), chicken ovalbumin (44 kDa), bovine gamma globulin (158 kDa), and thyroglobulin (670 kDa) were used as standards (Bio-Rad Laboratories). The purified MAb M4D4 was coupled to an Affi-Gel Hz hydrazide gel (Affi-Gel Hz immunoaffinity kit; Bio-Rad Laboratories) as described by the manufacturer.

The preparation of cytotoxin partly purified on S-200 was added to the immunoaffinity column. The cytotoxin was eluted with 2 bed volumes of 3.5 M NaSCN. The protein-containing fractions were pooled and immediately desalted on a PD-10 column. Finally, the purified cytotoxin was concentrated by using a YM10 filter with a cutoff of 10 kDa (Amicon Inc. Danvers Mass.).

All samples were subjected to polyacrylamide gel electrophoresis (PAGE) on 1.0-mm-thick sodium dodecyl sulfate (SDS)–14% polyacrylamide gels with a 2.6% cross-linker, as described previously (12). Samples were separated under nonreduced conditions and without boiling. Polyacrylamide gels were stained with either Coomassie blue or silver stain (Bio-Rad). After separation by SDS-PAGE, proteins were electrotransferred to nitrocellulose sheets and incubated overnight with MAb. Bound MAbs were detected by goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad) (28).

The amino acid sequencing of the 20-kDa-molecular-mass cytotoxin band was performed at the Institute of Cell Research, Uppsala, Sweden. The immunoaffinity-purified toxin from strain CCUG 7470 was subjected to SDS-PAGE, transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, Mass.), and visualized with Ponceau S staining (Sigma). The N-terminal amino acid sequence of the membrane-bound protein was determined by Edman degradation with a pulsed liquid phase (model 476A; Applied Biosystems Inc., Foster City, Calif.) protein sequencer, as recommended by the manufacturer. An IBI Pustell sequence analysis program (version 1991) was used for comparison of the amino acid sequences.

The levels of cytotoxin-neutralizing antibodies in serum samples from patients with chancroid and from individuals from areas where chancroid is endemic and areas where it is not, as well as geometric means for each group, are presented in Table 1. Cytotoxin-neutralizing antibodies were detected in 66% of patients with chancroid, while only 4% of Swedish blood donors had detectable neutralizing antibodies (Table 1). There were significant differences in the neutralizing-antibody frequencies between groups ($P < 0.001$).

The next step of this study was to produce mouse MAbs with neutralizing capacity, which were the principal tool used in purification and identification of *H. ducreyi* cytotoxin by immunoaffinity chromatography and Western blotting. The cytotoxin was purified from the OP of CCUG 7470 strain by a two-step purification scheme.

The separation pattern of OPs on S-200 is shown in Fig. 1. The cytotoxic activity was mainly detected in a peak corresponding to about 20 kDa. Coomassie and silver staining of the pooled cytotoxic fractions resulted in a very weak staining of the toxin band, probably because of the insufficient amount of purified protein. Therefore, immunoblotting with MAb M4D4 was mainly used for detection of the cytotoxin protein. A strong band was detected with the MAb in the 20-kDa fractions (Fig. 2). OP from noncytotoxic strain CCUG 4438, tested as described above, showed no cytotoxic activity and no 20-kDa toxin band (data not shown).

To confirm the molecular mass of the cytotoxin obtained by

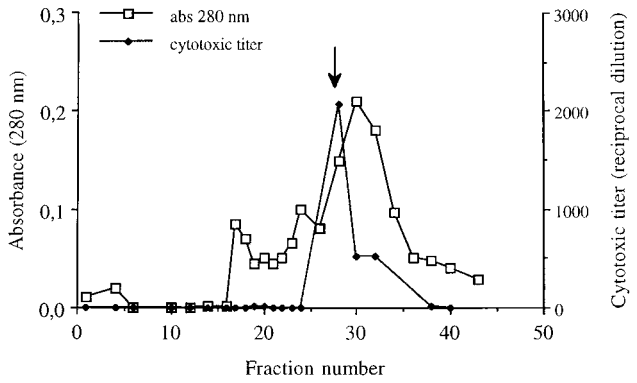

FIG. 1. Elution profile of osmotic preparation from strain CCUG 7470 on Sephacryl S-200. Cytotoxic fractions 28 to 32 were collected. The arrow indicates the cytotoxic peak. abs 280 nm, absorbance at 280 nm.

the osmotic treatment of bacteria, the OP was subjected to an FPLC Super-Dex column. A cytotoxic activity was detected mainly in fractions with molecular masses between 17 and 44 kDa (data not shown). The results from these two chromatography separation experiments indicated that the molecular mass of the native cytotoxin is about 20 kDa.

The preparation from the two-step purification was studied with an immunoblot probed with the MAb. The immunoblot membrane was incubated with either anti-mouse IgG conjugate or with MAb M4D4. With this method it was shown that the cytotoxin band with a molecular mass of 20 kDa was enriched. The cytotoxin was, however, contaminated with MAbs shed from the column (data not shown).

The N-terminal amino acid sequence of 18 residues of the 20-kDa protein obtained by purification on immunoaffinity gel was as follows: E-S-N-P-D-P-T-T-Y-P-D-V-E-L-S-P-P-P. A search using the computer program BLAST revealed that no other known amino acid sequence resembled that of this protein.

In all nine sonicates from cytotoxic strains, tested by Western blotting, a band in the 20-kDa region was detected. This band could not be detected in the three non-cytotoxin-producing strains, further indicating that this protein is responsible for the cytotoxic activity on the epithelial cells.

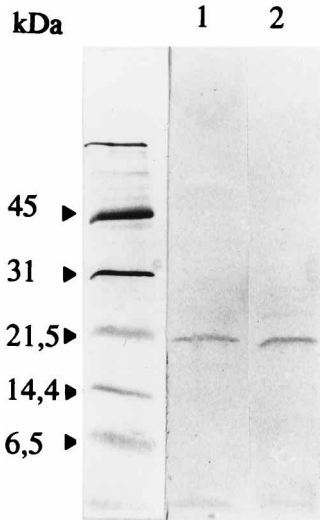

FIG. 2. Western immunoblot analysis of OP (lane 1) and Sephacryl S-200 separation fractions (28 to 32) (lane 2) immunostained with MAb M4D4 and showing the 20-kDa band. SDS-PAGE protein standards are indicated on the left.

The neutralizing pattern for 12 rabbit immune sera against different strains (nine toxigenic and three nontoxigenic) was investigated with the OP, the S-200 preparation, and the immunoaffinity-purified cytotoxin in the neutralization assay. The results showed similar neutralization titers for antisera against toxigenic strains, indicating identical or similar immunogenicities of cytotoxins in all toxigenic strains, whereas no activity was observed in sera tested with nontoxinogenic strains.

In this study, we found that the disease caused by *H. ducreyi* can result in the development of antibodies which neutralize the cytotoxic effect on epithelial cells, indicating the in vivo production of cytotoxin. The frequency of antibodies neutralizing *H. ducreyi* cytotoxin was significantly greater in sera from patients with culture-confirmed chancroid than in sera from healthy individuals. However, in sera from some patients no detectable levels of neutralizing antibodies were noted. The explanation for this could be that in order to evoke a significant antibody response to toxin, the disease must be present in the host for a certain time or that repeated infections are needed to evoke a detectable levels of neutralizing antibodies. It should also be observed that the majority of *H. ducreyi* strains have the ability to produce cytotoxin (24), though it is conceivable that some of the patients could be infected with low- or non-cytotoxin-producing strains. The presence of neutralizing antibodies to cytotoxin in sera from chancroid patients indicates that cytotoxin is produced in vivo and that infection with *H. ducreyi* can elicit neutralizing antibodies.

Our results show that the *H. ducreyi* active cytotoxin is a small protein with an approximate molecular mass of 20 kDa and that it is present only in preparations from cytotoxin-producing *H. ducreyi* strains. The complete neutralizing capacity of one MAb indicates that the recognized epitope is essential for biological activity on epithelial cells. The cytotoxic effect was detected mainly in fractions (S-200 and FPLC) with a molecular mass of about 20 kDa, supporting the finding that the active cytotoxin is probably one protein with a molecular mass of 20 kDa, as detected by SDS-PAGE and immunoblotting. However, the possibility that the 20-kDa protein is the toxic part of a larger structure cannot be excluded.

Moreover, all toxigenic strains produce a 20-kDa protein with similar properties with respect to the toxic epitope. We therefore conclude that the cytotoxin responsible for the killing of epithelial cells is a single toxin, with identical or related immunological properties in all *H. ducreyi* strains.

Our results indicate that the determined sequence E-S-N-P-D-P-T-T-Y-P-D-V-E-L-S-P-P-P represents the N-terminal amino acid sequence of the cytotoxin. The sequence was neither homologous with the sequences of any other known bacterial protein nor a part of a contaminated mouse MAb.

In order to limit the purification steps, which can subsequently result in a more active product, we used immunoaffinity chromatography with cytotoxin-neutralizing MAbs. However, when the biologically active product was obtained, a relatively poor recovery of cytotoxin was recorded and, moreover, the purification technique resulted in the contamination of cytotoxin by MAbs. The poor recovery of cytotoxin obtained in pilot experiments and in this study suggests that only small amounts of the toxin are produced by the bacterium. This finding could explain the observation that not all patients with culture-proven chancroid had a large antibody response. A new methodology for the purification of *H. ducreyi* epithelial cytotoxin is needed to obtain larger amounts of the cytotoxin. A reasonable approach is one to be based on recombinant

DNA technology. The identification of the cytotoxin gene and its cloning and overexpression, as reported for other exotoxin-producing bacteria, appears to be necessary for successful production and purification of this cytotoxin.

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