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Neutralizing Antibodies to *Haemophilus ducreyi* Cytotoxin

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Neutralizing antibodies against cytotoxin produced by *Haemophilus ducreyi* bacteria were studied in rabbits by an assay employing HEp-2 cells and diluted crude cytotoxin preparations from the organism. Antisera to 12 different *H. ducreyi* strains were prepared by immunization of rabbits with bacterial sonicates combined with Freund's adjuvant. The antibody response during infection with *H. ducreyi* was studied in two groups of rabbits which were infected with five live strains by either single or multiple intradermal injections. Neutralizing antibodies in hyperimmune sera to sonicates from 12 *H. ducreyi* strains tested against their homologous cytotoxin preparations were present with titers ranging from 1:80 to 1:640. Similar antibody titers against heterologous cytotoxin preparations were recorded, indicating immunological similarity or identity between cytotoxins from the various *H. ducreyi* strains. Three strains did not produce cytotoxin, and these strains did not induce toxin-neutralizing antibodies. Hyperimmune sera to other gram-negative bacteria had no detectable neutralizing capacity, indicating species specificity of the *H. ducreyi* cytotoxin. Cytotoxin-neutralizing antibodies were not detectable in rabbit sera before infection with *H. ducreyi*. Repeated single injections with live bacteria resulted in development of low levels of neutralizing antibodies. Multiple primary injection of live bacteria of the cytotoxin-producing strain CCUG 7470 resulted in a low immune response to the cytotoxin preparation from the same strain. A booster infection resulted in the development of neutralizing antibodies in all rabbits infected with cytotoxin-producing strains. The antibody titers determined against the homologous cytotoxin preparation were similar to those recorded for two heterologous cytotoxin preparations.

Haemophilus ducreyi causes chancroid (soft chancre), a sexually transmitted disease with characteristic genital ulceration. *H. ducreyi* is a major cause of genital ulcers in Africa and Asia (2, 5, 6, 8, 12, 13, 15, 18). Locally restricted outbreaks have been reported in Canada, the United States, and Europe (6). In developing countries, genital ulceration has been related to an increased risk for the spread of human immunodeficiency virus infection (2, 8, 15). The pathogenesis of and protective immune mechanisms against *H. ducreyi* are unknown (3, 9, 15).

We have shown that cytotoxin produced by *H. ducreyi* causes death of cultured HEp-2 and HeLa cells and that antisera raised against homologous bacterial sonicates have the ability to neutralize this effect (20).

The antibody response to *H. ducreyi* in rabbits after one or more intradermal (i.d.) injections with bacteria has been estimated by enzyme-linked immunosorbent assay (ELISA) and immunoblotting, utilizing bacterial sonicates (9, 15, 17, 21). The results showed that rabbits respond with immunoglobulin G and M antibodies, persisting for up to 9 weeks after immunization (9, 10, 15, 17). Sera from uninfected animals were also shown to react with *H. ducreyi* antigens in immunoblotting and ELISA (9, 17, 21). Serum antibodies in patients with chancroid have been determined by dot immunoblotting and an enzyme immunoassay that uses whole-cell sonicates (5, 16). Serum immunoglobulin G antibodies were present in 89 and 55% of men with chancroid in Nairobi and Bangkok, respectively, compared with 2 to 17% in control groups (16).

The aims of this work were to develop a micro-cell culture method for the study of cytotoxin-neutralizing antibodies, to investigate the neutralizing capacity of immune sera to homologous and heterologous cytotoxin preparations from different *H. ducreyi* strains, and to measure the antibody responses in rabbits after single and multiple experimental infections with live bacteria.

Twelve *H. ducreyi* strains were used in the present study. The following nine strains were obtained from the Culture Collection, University of Göteborg, Göteborg, Sweden (CCUG): 4438 (=Cip 542), 7470, 7781, 9276, 10045, 17675, 17713, 18804, and 20693. Three strains originated from the Institute of Tropical Medicine, Antwerp, Belgium (ITMA): 3207, 3542, and 4703. The strains originated from different parts of the world. All strains except three (3542, 4438, and 20693) have been shown to produce cytotoxin (20).

Bacteria were cultivated on chocolate–GVL-3 plates containing brain heart infusion agar and vancomycin (Media Department, Bacteriological Laboratories, Sahlgrenska Hospital, Göteborg, Sweden) in an oxygen-depleted, CO_2-enriched, and humid atmosphere for 2 to 3 days at 33°C in an anaerobic jar with aerocult C (Merck, Darmstadt, Germany), as described previously (20).

For each strain, bacterial cells from 20 to 30 plates were harvested and suspended in about 15 ml of distilled water. The bacterial suspensions were ultrasonicated two times for 1 min each at 20 kc/s (MSE sonifier) at 30-s intervals on ice. After sonication, the suspension was centrifuged twice at 12,000 × *g* for 30 min and subjected to sterile filtration. The supernatant was aliquoted and stored at −20°C (9, 20). The preparations were used as crude cytotoxin preparations for the neutralization test. Protein concentrations, as measured by the method of Bradford (Bio-Rad protein assay kit) with bovine serum albumin as a standard (1), ranged from 2 to 4 mg/ml.

To obtain hyperimmune sera, rabbits were immunized subcutaneously with bacterial sonicates combined with Freund's adjuvant. To study the development of neutralizing antibodies during experimental infection, rabbits were in-

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jected i.d. with live bacteria. Single or multiple injections were given.

Twelve New Zealand White rabbits (2 to 3 kg) were hyperimmunized with cell sonicates from 12 *H. ducreyi* strains. One rabbit per strain was injected subcutaneously at four injection sites with 0.1 ml of cell sonicate containing 200 to 400 μg of protein incorporated in Freund's complete adjuvant. A second injection of sonicate from the same strain mixed with Freund's incomplete adjuvant was given 3 weeks later. One to three booster injections with the same bacterial sonicates at 2-week intervals were started 3 weeks after injection of the sonicate in incomplete Freund's adjuvant. The rabbits were bled 14 days after the last injection. Rabbit hyperimmune sera to other gram-negative bacteria were employed as controls. Antisera to *Haemophilus influenzae* type b and to four noncapsulated strains, *Yersinia entercolitica* types 3 and 9, *Salmonella typhi*, and *Salmonella paratyphi*, were obtained by immunization with whole bacteria, as described elsewhere (4). Antisera to *Bordetella pertussis*, *B. parapertussis*, *B. bronchiseptica*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Neisseria gonorrhoeae* were obtained by immunization with bacterial sonicates as described for *H. ducreyi* sonicates. Booster immunizations, however, were given at weekly intervals.

To study the antibody response to *H. ducreyi* infection, New Zealand White rabbits (2 to 3 kg) were each injected i.d. with one of the following strains: CCUG 4438, 7470, or 10045 or ITMA 3207 or 3542. One group of five rabbits was injected with six single doses of these five strains. Each rabbit received a dose of 0.1 ml containing approximately 10^8 viable cells in phosphate-buffered saline (PBS) as a primary dose (single infection). A second single dose was given 3 weeks later in the same manner, and then four single doses were given every 3 weeks.

Another group of five rabbits received 0.1 ml containing approximately 10^8 viable cells in PBS injected into four sites as a primary dose (multiple infection). Three weeks later, a second infective dose was given in the same manner. In addition, one of these rabbits (infected with CCUG 7470 bacteria) received three multiple infections. The third dose was given 4 weeks after the second injection.

Dermal lesions started to develop about 2 days after the infection, with pustular, abscess-like ulcers noted for all strains. Some lesions ulcerated by day 3, and upon squeezing white pus was extruded. After 6 days the ulcers started to dry and heal spontaneously, and after about 3 weeks only a thin residual scar remained. The lesions were of the same type as described previously by us and others (3, 9, 19).

Blood samples were taken prior to infection and weekly during the course of the immunization. Final bleedings were performed 4 weeks after the last infection. All sera were stored at −20°C until analyzed.

A HEp-2 cell line (ATCC CCL23; American Type Culture Collection, Rockville, Md.), sensitive to *H. ducreyi* cytotoxin (20), was employed in a neutralizing assay. Cells were cultivated in Eagle's medium with 8% fetal calf serum (FCS) at 37°C in a 6% CO_2-enriched and 92% humidified atmosphere for 2 to 3 days. The cells were detached from the plastic surface by treatment with 0.1% trypsin–0.02% EDTA in PBS and suspended in Eagle's medium containing 2% FCS.

The cytotoxin neutralization assay was developed by the principles described for pertussis and diphtheria toxin neutralization assays (7, 11, 14). The optimal working dilution of the crude cytotoxin preparation as well as optimal cytotoxin-antiserum incubation times were determined in pilot studies as follows.

A crude cytotoxin preparation was titrated in cell culture medium with 2% FCS in serial twofold dilutions to estimate the cytotoxic activity of the preparations, as described previously (20). Cells with addition of PBS instead of cytotoxin preparation were used as controls. One cytopathogenic unit was defined as the amount of toxin giving 50% cell death or morphologically changed cells or both (7). The relationship between the cytotoxin concentration and the neutralizing endpoint titers in sera was inversely proportional, giving higher titers with more diluted preparations. High concentrations of cytotoxin diminished the detection of low levels of antibody, while low concentrations gave less distinct endpoint titers. A cytotoxin concentration corresponding to about 4 cytopathogenic units was chosen as optimal for the assay. This concentration was obtained by dilution of the different cell sonicate preparations from 1:4,000 to 1:100,000.

It was also found that increases in the cytotoxin-serum incubation time resulted in increased neutralization titers. However, after 3 h of incubation, the cytotoxin activity was reduced by one titer step and endpoint titers were less distinct. An incubation time of 2 h was therefore chosen as optimal for the neutralization test.

On the basis of these results, the neutralization test was performed as follows. Test sera in duplicates were twofold diluted eight times in 96-well tissue culture plates, using 50 μl of culture medium containing 2% FCS. Then, 50 μl of the crude toxin preparation containing 4 cytopathogenic units was added. The mixture was incubated at 37°C for 2 h, which was followed by addition of 100 μl of freshly trypsinized HEp-2 cells, diluted to a concentration of 4×10^4 to 7×10^4 cells per ml in culture medium with 8% FCS. This amount of cells was estimated to be optimal for cytotoxic assay (20). Two hours later, when the cells were attached to the bottom of the wells, the medium was replaced by fresh medium with 8% FCS.

Cytotoxin titrations as well as control cells were included in each test. After 2 to 3 days, the plates were examined with an inverted microscope and re-examined after staining with 10% Giemsa solution (Merck). The neutralization endpoint titer was defined as the dilution of serum giving 50% cell growth compared with the controls. The results were expressed as reciprocal serum dilutions.

The reproducibility of the assay, expressed as coefficient of variation (= standard deviation/mean × 100), was calculated from reciprocal titers of one rabbit immune serum. The intra- and interassay coefficients of variation were 36 and 47%, respectively.

Levels of neutralizing antibodies in hyperimmune sera to 12 different *H. ducreyi* strains tested against homologous and heterologous cytotoxin preparations are summarized in Table 1. Homologous as well as heterologous titers in the hyperimmune sera against nine cytotoxin-producing strains varied from 1:80 to 1:320. In three rabbit sera raised against non-cytotoxin-producing strains, no neutralizing antibodies to any of the cytotoxins were detected (Table 1). No neutralizing activity was detected in rabbit antisera to *H. influenzae* (Table 1) or to other gram-negative bacteria tested with the cytotoxins from strains CCUG 7470 and 10045 (not shown in Table 1).

The development of neutralizing antibodies to five *H. ducreyi* strains after repeated single i.d. injections of live bacteria in rabbits is presented in Fig. 1a. Neutralizing antibodies were not detectable in any preimmune serum.

TABLE 1. Cytotoxin-neutralizing antibodies in rabbit hyperimmune sera to 12 different *H. ducreyi* strains

Antiserum to strain:	Neutralization titer (reciprocal dilution)[a] to cytotoxin prepn from strain:								
	7470	7781	9276	10045	17675	17713	18804	4703	3207
H. ducreyi									
CCUG 7470	320	320	320	160	160	160	320	160	320
CCUG 7781	640	640	640	320	320	640	640	640	640
CCUG 9276	320	160	320	160	320	640	640	640	640
CCUG 10045	160	320	320	160	160	160	320	320	320
CCUG 17675	80	160	80	320	160	160	80	320	320
CCUG 17713	320	160	160	160	640	320	320	320	320
CCUG 18804	160	640	320	320	80	640	640	160	640
ITMA 4703	320	160	640	320	320	160	320	320	640
ITMA 3207	160	160	160	160	160	160	320	320	320
CCUG 4438	<10	<10	<10	<10	<10	<10	<10	<10	<10
CCUG 20693	<10	<10	<10	<10	<10	<10	<10	<10	<10
ITMA 3542	<10	<10	<10	<10	<10	<10	<10	<10	<10
H. influenzae									
a–f	<10	<10	<10	<10	<10	<10	<10	<10	<10
Noncapsulated	<10	<10	<10	<10	<10	<10	<10	<10	<10

[a] Homologous titers are underlined. Four cytopathogenic units of cytotoxin was used.

The antibodies against homologous cytotoxin were detected after two single injections with strain CCUG 7470, but for two other cytotoxin-producing strains, CCUG 10045 and ITMA 3207, detectable antibodies developed after three and five single injections, respectively. The maximum titer obtained was 1:40. The antibody response tested against two other cytotoxins was similar to those tested against homologous cytotoxin preparations. No antibody response was noted in rabbits immunized with strains which do not produce cytotoxin. Figure 1b presents the development of neutralizing antibodies in sera to five different strains tested against cytotoxin preparation from strain CCUG 7470.

The development of neutralizing antibodies to the three cytotoxin-producing strains after multiple injections of live *H. ducreyi* resulted in a low primary response for the rabbit immunized with strain CCUG 7470 only. A five- to eight-fold-higher booster response was noticed 1 to 2 weeks after the second injection with bacteria. However, 3 weeks after the second infection the neutralizing antibodies started to decrease. The highest levels of neutralizing antibodies (titer of 1:320) were obtained after three injections (not shown in Fig. 1a). The neutralizing antibody response tested against cytotoxin preparations from two other cytotoxin-producing strains were generally observed after the second infection. No antibody response was noted in rabbits immunized with the strains which do not produce cytotoxin.

In this study we have developed a micro-cell culture method with HEp-2 cells for the determination of antibodies neutralizing *H. ducreyi* cytotoxin. This assay was roughly similar to the neutralization assays described for pertussis and diphtheria toxins (7, 11, 14). In the diphtheria neutralization test, the antitoxin titers estimated with crude and purified toxin preparations correlated well (14), indicating the possibility of using crude toxin preparations in the assay. Since the cytotoxic activity in the crude preparations used in this study was high, highly diluted preparations could be used to obtain a reproducible, specific, and sensitive assay.

Antibodies produced against various *H. ducreyi* strains neutralized the heterologous toxin preparations to roughly the same extent as the homologous cytotoxin. This observation indicates that *H. ducreyi* cytotoxins derived from different strains are immunologically identical or cross-reacting antigens. Antigenically, the cytotoxin seems to be specific to *H. ducreyi*, since no cross-reactivity could be detected when the antisera were tested against a wide range of other gram-negative bacteria.

Cytotoxin-neutralizing antibodies were not detected in rabbit sera prior to infection. However, "naturally" occurring antibodies to cell sonicates from *H. ducreyi* in rabbit sera were demonstrated in other studies by the immunoblotting assay (17, 21) and ELISA (9, 15, 17). Moreover, antibodies to, e.g., *H. influenzae* could be demonstrated in antisera obtained after immunization with *H. ducreyi* bacteria (9). These results indicate that rabbits could develop cross-reacting antibodies after previous contact with bacteria other than *H. ducreyi* (15, 17).

Rabbits have been used as a model for *H. ducreyi* infection in previous studies (3, 9, 19). However, in this model, the organisms do not grow significantly in the lesions after i.d. injection of live bacteria. Moreover, dermal lesions have been reported to be mainly dependent on lipooligosaccharide activity, and possibly other heat-stable components of bacteria, but not on cytotoxin activity (3, 9). We found that the cytotoxin from *H. ducreyi* has the capacity to kill only human cell lines (9, 20). In the present study, rabbits were employed to investigate the antigenicity of cytotoxin from *H. ducreyi* and development of cytotoxin-neutralizing antibodies after injection of live bacteria. Our results show that infecting rabbits i.d. with live bacteria can induce cytotoxin-neutralizing antibodies. Repeated contact with bacteria or a high dose of bacteria is required to obtain a detectable antibody response, probably because bacteria do not multiply significantly in rabbit lesions.

The results of preliminary studies employing human sera in the described neutralization assay indicate that neutralizing antibodies are generally not detected in blood samples from Swedish blood donors. Such antibodies could, however, be detected at low levels in 50% of adults from Tanzania, where chancroid is occurring endemically. Furthermore, sera from patients with culture-proven chancroid

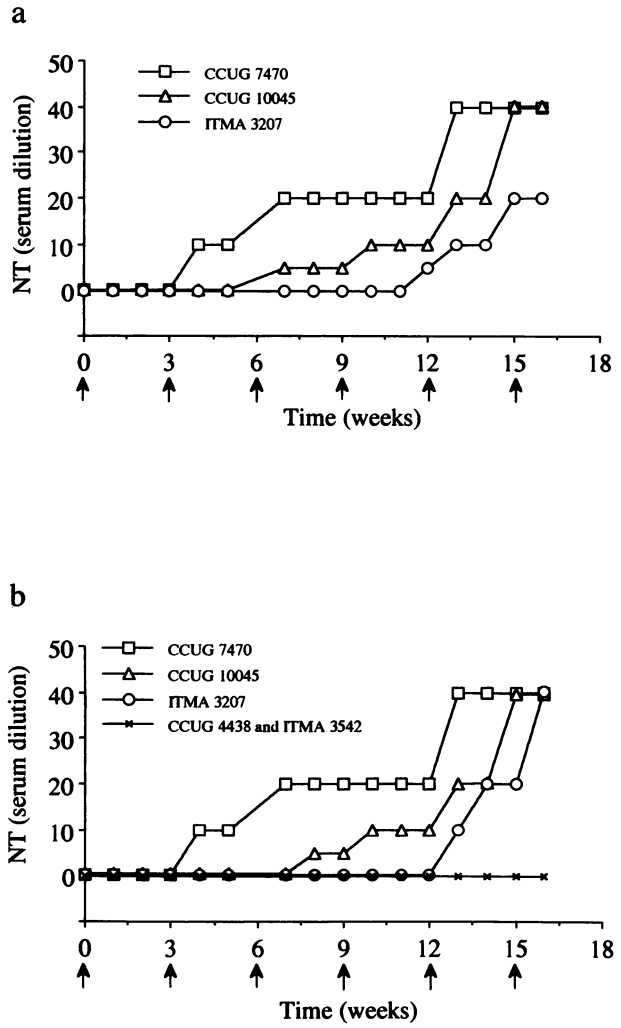

FIG. 1. Antibody responses in rabbits to repeated i.d. infections (single injections) with five *H. ducreyi* strains. (a) Neutralizing (NT) antibodies in sera to three cytotoxin-producing strains tested with homologous cytotoxin preparations. (b) Neutralizing antibodies in sera to five strains tested with the cytotoxin preparation from strain CCUG 7470. Arrows indicate immunizations.

had high levels of neutralizing antibodies (9). Further studies are in progress to characterize neutralizing antibody responses in patients with chancroid as well as to evaluate the role of such antibodies in disease.

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