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NOTES

Production of Antisera against the Enterotoxin of *Bacteroides fragilis* and Their Use in a Cytotoxicity Neutralization Assay of HT-29 Cells

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To study the enterotoxin of *Bacteroides fragilis*, the colon carcinoma cell line HT-29 was used in a standard cytotoxicity assay. We produced high-titer neutralizing antisera in rabbits and goats against both crude and purified toxin and developed a cytotoxicity neutralization assay for use in confirming enterotoxin activity in culture filtrates and stools. The neutralization titers of the antisera on the colon carcinoma cell line HT-29 ranged from 1,600 to 2,400. In an antibody screening enzyme-linked immunosorbent assay, titers ranged from 10^4 to 10^5. The antisera produced against the highly purified toxin also neutralized the enterotoxic activity of the toxin and were monospecific by immunoelectrophoresis.

Bacteroides fragilis is a normal inhabitant of the human colonic flora, making up about 1 to 2% of the normal flora (5), and is the anaerobic species most commonly isolated from soft tissue infections in humans (16). Reports in the mid-1980s described a variety of strains of *B. fragilis*, isolated from diarrheic animals, which caused fluid accumulation in ligated ileal loops. The enterotoxigenic strains were subsequently associated with diarrhea in neonatal and infant domestic animals including lambs (7), calves (1, 12), piglets (8), and foals (9). More recently, enterotoxigenic *B. fragilis* was associated with diarrhea in humans, primarily infants (13, 14), and several animal models were developed for studying these organisms. Two of these animal models involve the induction of lethal diarrhea in infant rabbits and germfree pigs upon oral challenge with live organisms (10, 11).

In 1992, Weikel et al. (18) showed that culture filtrates from enterotoxigenic strains of *B. fragilis* caused a cytotoxic (rounding) response on the human colon carcinoma cell line HT-29. To date, this is the only cell line on which a cytotoxic activity has been observed. Using this cell line in a cytotoxicity assay, Van Tassell and coworkers (17) determined the optimal conditions for production of the enterotoxin in vitro, purified the toxin to homogeneity, and characterized many of its biological and physicochemical properties. However, there remained a lack of antisera and antibodies for use in a specific cytotoxicity neutralization format, which is required for confirmation of the enterotoxin in cultures and fecal specimens. In addition, such reagents would be useful for the development of immunoassays specific for the enterotoxin. We report here the production and characterization of polyvalent crude and monospecific goat and rabbit antisera against the enterotoxin of *B. fragilis* and the use of these antisera to develop a cytotoxicity neutralization assay for screening bacterial cultures and feces for toxin production.

HT-29 cells were obtained from the American Type Culture Collection (Bethesda, Md.) and were grown and maintained in McCoy's 5A medium supplemented with 10% fetal calf serum by standard tissue culture procedures. Thirteen enterotoxigenic *B. fragilis* strains, including VPI strain 13784, were obtained from our Virginia Polytechnic Institute (VPI) Anaerobe Laboratory culture collection with the permission of L. L. Myers, who originally deposited them for identification. The identities of all strains as *B. fragilis* were confirmed by using standard analyses involving gas chromatography of volatile fatty acids (3) and cellular fatty acids (15) and polyacrylamide gel electrophoresis of soluble proteins (6). Three other enterotoxigenic strains were obtained from the American Type Culture Collection. Culture filtrates of the *B. fragilis* strains were prepared by growing the organisms in brain heart infusion broth to maximum turbidity (early stationary phase) at 37°C (~16 to 20 h). The cells were removed by microcentrifugation (12,000 × *g*, 5 min), the culture supernatants were filtered (pore diameter, 0.45 μm), and the filtrates were stored at −20°C.

Antisera were made in rabbits and goats against crude and purified toxin prepared from VPI strain 13784 as described previously (17). In rabbits, the purified toxin vaccine consisted of highly purified toxin coupled to tanned erythrocytes (25 μg/ml) as described previously (2). In goats, the purified toxin vaccine consisted of highly purified toxin (25 μg/ml) in Tris-HCl buffer (50 mM, pH 7.2). The crude vaccine for both animals was the 70% ammonium sulfate precipitate in phosphate-buffered saline (PBS; 25 mg/ml). Two adult male New Zealand rabbits and one adult male Nubian goat were injected subcutaneously (2.0 and 1.0 ml, respectively) with the crude or pure toxin vaccine. For the first injection, vaccine was emulsified with complete Freund's adjuvant (Sigma Scientific Co., St. Louis, Mo.); incomplete adjuvant was used for all subsequent injections. After 8 weeks of injections, the goats were rested for 4 weeks and were then boosted weekly for 4 more weeks.

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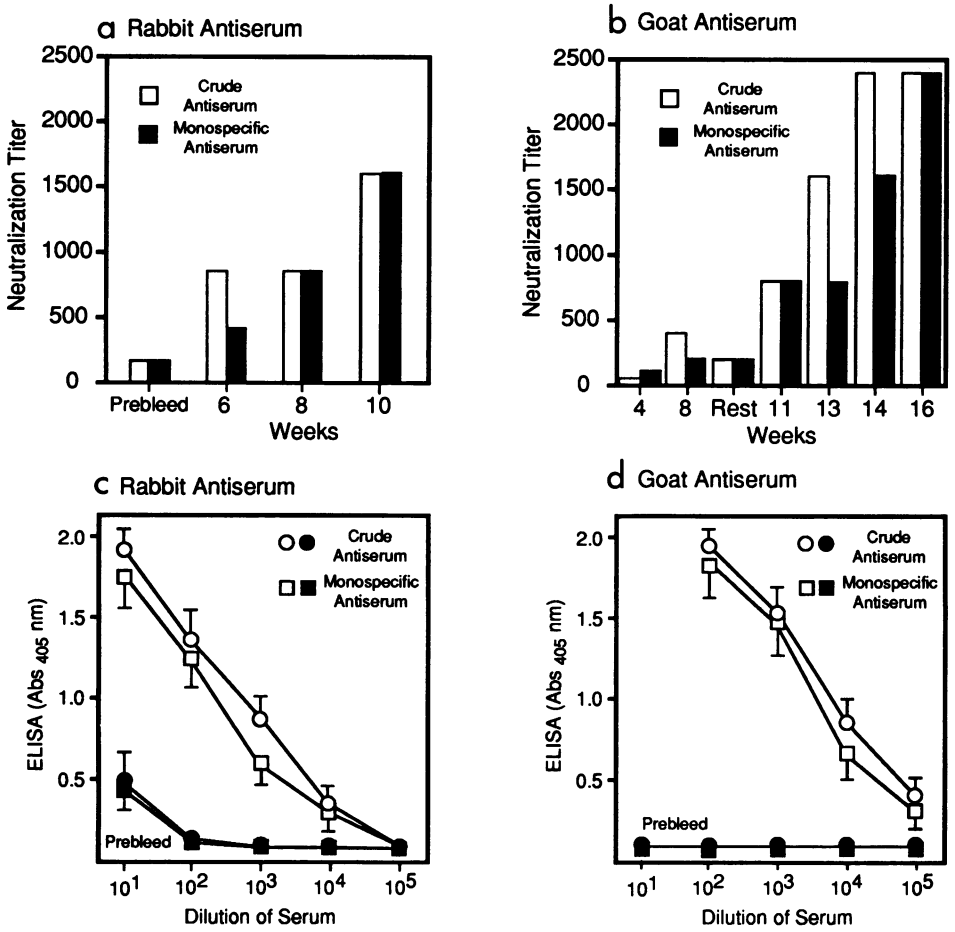

FIG. 1. Production of antisera in rabbits and goats against the *B. fragilis* enterotoxin. (a and b) Neutralization titers of the crude and monospecific antisera from the rabbits and goats vaccinated with the ammonium sulfate precipitate and purified toxin, respectively. Bars represent the neutralization titers of sera against 5 to 10 CU of purified toxin. (c and d) Responses of the same rabbit and goat antisera used for panels a and b in the antibody screening ELISA. Each value represents the average ± standard deviation for duplicate serum samples assayed in three separate ELISAs. ○ and □, ELISA titers in rabbits at 10 weeks and in goats at 16 weeks, respectively; ● and ■, prebleed ELISA titers for each rabbit and goat, respectively.

Weekly test bleeds were obtained, and neutralization titers were determined by the cytotoxicity assay and an enzyme-linked immunosorbent assay (ELISA) specific for antibodies against the toxin.

The cytotoxicity assay was performed in 96-well plates as described previously (17). Cytotoxicity titers were expressed as the reciprocal of the highest dilution of toxin that caused greater than 50% cell rounding. A cytotoxic unit (CU) was the smallest amount of toxin that caused greater than 50% cell rounding. All toxin samples and antisera were diluted in PBS. To screen sera for neutralizing titers, duplicate dilutions of antisera were mixed with equal volumes of purified toxin and were incubated at 37°C for 1 h. The mixtures were then assayed for residual cytotoxic activity. The neutralization titers of each antiserum sample were expressed as the reciprocal of the lowest dilution of antiserum that neutralized 5 to 10 CU of purified toxin. PBS and neutral rabbit and goat sera were used as controls.

To determine the ability of the antisera to neutralize toxin in fecal extracts, we performed experiments using spiked normal feces. Fecal samples from five healthy adults (ages, 24 to 50 years) were collected individually in high-density plastic bags which were then sealed under argon, mixed thoroughly by kneading, pressed flat, and frozen at −20°C until they were processed. A portion of each frozen fecal sample was thawed, diluted 1:4 in PBS, microcentrifuged (12,000 × *g*, 5 min), and filtered (pore diameter, 0.45 μm). The fecal filtrates were spiked with 0.5 μg of purified toxin per ml and then assayed for residual cytotoxic activity with and without neutralization with the monospecific antiserum.

To determine the ability of the antisera to neutralize the fluid accumulation response, the lamb ligated ileal loop assay was performed as described previously (17). Ten micrograms of filter-sterilized purified toxin, which is enough to cause a strong positive fluid response, was mixed with the monospecific antisera diluted 1/20 in PBS, and the mixture was incubated at 37°C for 1 h prior to being injected into the animals. PBS and commercially available neutral rabbit and goat sera (Cocalico Biologicals, Inc., Reamstown, Pa.) were incubated with the toxin as controls.

For the antibody screening ELISA, wells of Immulon type 1 plates (Dynatech, Inc., Alexandria, Va.) were coated at 4°C for 16 to 18 h with 100 ng (0.1 ml) of purified *B. fragilis* enterotoxin diluted in carbonate buffer (50 mM, pH 9.6). Each well was

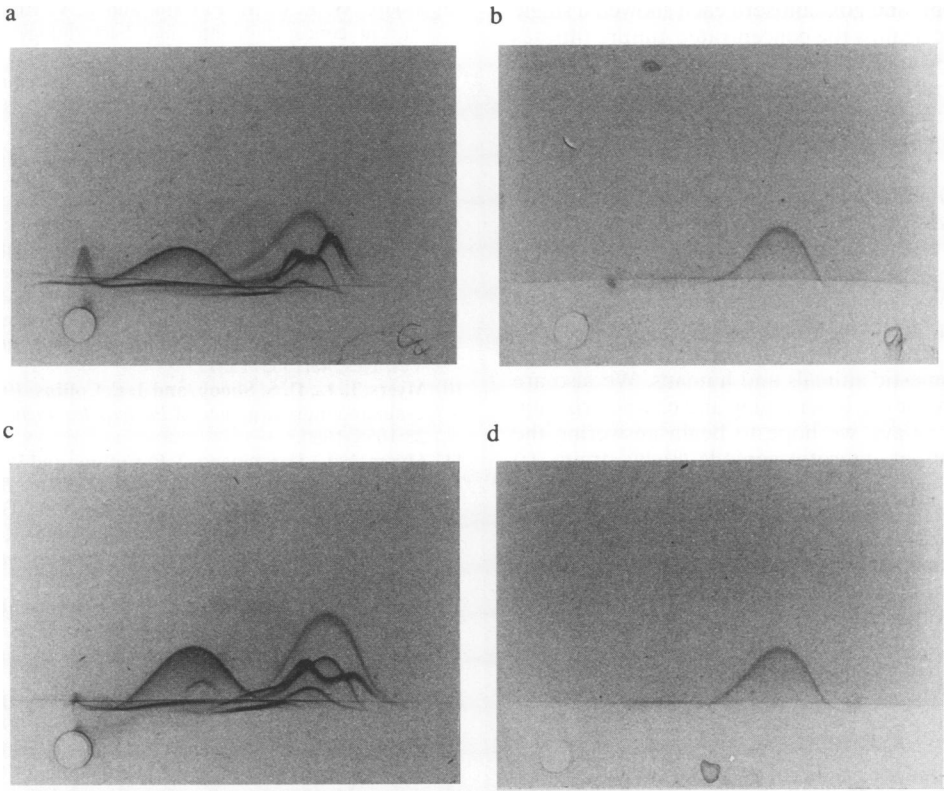

FIG. 2. Crossed immunoelectrophoresis analysis of rabbit and goat antisera. The well on each plate initially contained 10 μl of culture filtrate from VPI strain 13784 concentrated 20-fold with a Centricon-10 concentration device (Amicon, Beverly, Mass.). The upper portions of the gels contained 200 μl of crude rabbit antiserum (a), 200 μl of monospecific rabbit antiserum (b), 100 μl of crude goat antiserum (c), and 100 μl of monospecific goat antiserum (d). Electrophoresis of the sample in the first dimension at 20 to 30 V/cm was followed by electrophoresis in the second dimension at 2 to 3 V/cm.

washed twice with 0.3 ml of PBS containing 0.05% Triton X-100 (PBS-T). Samples (0.1 ml) of preimmune and immune sera, diluted in PBS, were added to the wells, and the plates were incubated for 2 h at 37°C. The plates were washed five times with PBS-T, and 0.1 ml of the appropriate anti-immunoglobulin G-alkaline phosphate conjugate (Sigma), diluted 1/1,000 in PBS-T, was added to the wells. After 1 h of incubation at 37°C, the plates were washed five times with PBS-T, and 0.1 ml of a 1-mg/ml solution of Sigma 104 phosphatase substrate (in 50 mM diethanolamine buffer [pH 9.8]) was added to the wells. The plates were incubated for 30 min at room temperature, and the A_{405}s were read by using an automated ELISA plate reader (model E111; Dynatech). Background values typically ranged from 0.1 to 0.15. The ELISA titer was defined as the dilution of serum that gave an A_{405} of greater than 0.3.

After 4 weeks of injections with the crude and purified toxin vaccines, samples from weekly test bleeds from the goats and rabbits were screened for their ability to neutralize the cytotoxic activity of the purified toxin on HT-29 cells and to react in the ELISA. These results are shown in Fig. 1. The crude and monospecific rabbit antisera from all four rabbits had final neutralization titers of 1,600 and screening ELISA titers of 10^4. The crude and monospecific goat antisera had final cytotoxicity neutralization titers of 2,400 and screening ELISA titers of 10^5. These values were consistent upon repeated testing whether the sera were stored at 4°C or were repeatedly frozen and thawed. Although the preimmune goat sera did not show any neutralization activity, the preimmune rabbit sera had background neutralization titers of 40 to 80 and ELISA titers of 10^1.

Using the crude and monospecific antisera in the cytotoxicity neutralization format, we screened culture filtrates of 16 strains of *B. fragilis* known to be enterotoxigenic and 62 strains which had never been tested for their abilities to produce the enterotoxin. The cytotoxic activities of the culture filtrates of the enterotoxigenic strains ranged from 20 to 320 CU/ml, and all filtrates were neutralized by both the crude and monospecific rabbit and goat antisera at dilutions of 1/50. Of the 62 untested *B. fragilis* strains, culture filtrates of 4 strains exhibited cytotoxic activity ranging from 20 to 80 CU/ml, and all were similarly neutralized by the antisera. The fecal filtrates spiked with purified toxin had cytotoxic activities of 320 to 640 CU/ml and were neutralized by the 1/50 dilutions of all antisera.

In the lamb ligated ileal loop assay, the monospecific rabbit and goat antisera effectively neutralized the fluid accumulation. Toxin alone had a positive fluid accumulation ratio of 1.3, and the neutral rabbit and goat serum controls had no neutralizing effect.

To determine the specificities of the antisera, we performed crossed immunoelectrophoresis analysis as described previously (4). The crude rabbit and goat antisera yielded several immunoprecipitin arcs against both the concentrated culture filtrates of the enterotoxigenic *B. fragilis* strains (Fig. 2a and c) and the nonenterotoxigenic *B. fragilis* strains. Only one arc was observed with the purified toxin (data not shown). Conversely,

the monospecific rabbit and goat antisera each showed a single immunoprecipitin arc against the concentrated culture filtrates of the enterotoxigenic strains (Fig. 2b and d), but yielded no arcs with culture filtrates of 10 nonenterotoxigenic *B. fragilis* strains. As with the crude antisera, only one arc was observed with the purified toxin and the monospecific antisera (data not shown).

We now routinely use the high-titer goat antisera in the cytotoxicity neutralization assay to confirm the presence of enterotoxin in pure and mixed culture filtrates of *B. fragilis*. That the antisera also neutralized fecal filtrates spiked with toxin suggests that the cytotoxicity neutralization assay may be a reliable means for screening fecal samples. Thus, we are beginning screening studies for the *B. fragilis* enterotoxin in diarrheic feces of domestic animals and humans. We also are developing an ELISA for use in screening and characterization studies. Using these assays, we hope to begin answering the following questions about enterotoxigenic *B. fragilis* strains. (i) Do they proliferate, produce toxin, and induce diarrhea in neonates and infants before a normal protective flora can be established? (ii) Do these strains also cause diarrhea in normal adults or adults whose flora has been compromised? (iii) Is the enterotoxin of this most commonly isolated clinical pathogen somehow involved in the etiology of soft tissue infections?

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