Production of a Mouse Antiserum to Bacteroides fragilis Enterotoxin Using a Recombinant Enterotoxin Precursor

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The precursor of the Bacteroides fragilis metalloprotease enterotoxin was cloned and expressed in Escherichia coli, which was not able to process the precursor into the biologically active enterotoxin. Mouse antiserum elicited to the recombinant precursor reacted with the purified enterotoxin and with a crude enterotoxin preparation from an enterotoxigenic strain. The antiserum neutralized the cytotoxic activity of the enterotoxin in HT-29 cells.

In recent years, enterotoxigenic Bacteroides fragilis (ETBF) strains were isolated from the stools of children and adults and were found to be significantly associated with diarrheal disease (8, 10, 16).

ETBF strains characteristically produce a 20-kDa enterotoxin (12) that possesses a signature zinc-binding consensus motif characteristic of the metalloprotease family termed metalzincins (4). The enterotoxin causes pathological modification in animal models in vivo (5) and in a cultured carcinoma intestinal cell line, HT-29, in vitro (13). Its mechanism of action is mediated by cleavage of the extracellular domain of the zonula adherens protein E-cadherin (14).

Following the cloning and sequencing of the bft gene, the enterotoxin was recognized as the maturation product of a precursor protein of 397 amino acids (aa) with a molecular mass of 45 kDa, comprising a leader sequence, a pro-region of 193 aa, and the mature toxin of 186 aa (1, 3). The maturation process that leads from the precursor to the mature toxin is unknown.

In order to clone and express the precursor protein in Escherichia coli, the 1,700-bp bft gene was amplified by PCR from the genomic DNA of B. fragilis VPI 13784. The forward primer overlapped the translation start codon and contained a BamHI restriction site (5’-CCCGGATCCATGCTAGGAACCGCG CGG-3’), and the backward primer mapped downstream from the stop codon and contained a HindIII restriction site (5’-GGAAGCTTCAGTCGCAGATCAG-3’). The PCR product, digested with BamHI and HindIII, was cloned into the corresponding sites of pDS56/RBSII, 6X His/E’ vector, to generate pRLV128, which was transformed into E. coli M15 (2). Expression of the recombinant protein was obtained by inducing the recombinant E. coli with isopropyl-β-D-thiogalactopyranoside (IPTG; Roche Diagnostics, Milan, Italy) (9). The recombinant 6X His-tagged protein was purified by nickel-chelate affinity chromatography under denaturing conditions in the presence of 6 M guanidine hydrochloride, according to the manufacturer’s recommendations (Diagen, Hilden, Germany). Samples of induced and noninduced M15 cells and the purified recombinant protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% polyacrylamide gel) and stained with Coomassie blue.

CD2F1 mice (weight, 18 to 21 g) were immunized with three intraperitoneal injections of recombinant protein (20 μg per dose) at 10-day intervals. The mice were bled 10 days after completion of the immunization cycle.

A crude preparation of B. fragilis enterotoxin was obtained by the procedure described by Van Tassel et al. (12). Briefly, VPI 13784 was grown in 1 liter of brain heart infusion medium, the supernatant was precipitated with 70% ammonium sulfate, and the precipitate was dissolved in 25 ml of Tris buffer (50 mM), stabilized with the protease inhibitor Nα-p-tosyl-l-lysine chloromethyl ketone (Sigma-Aldrich, Milan, Italy), and dialyzed. As a negative control, the supernatant of the nonotoxigenic strain B. fragilis NCTC 9343 was processed in the same way.

For Western blotting experiments, samples (10 μl each) were separated by SDS-PAGE and electrophoreted onto nitrocellulose membranes, which were incubated with mouse anti-serum diluted 1:2,000. Phosphatase-conjugated anti-mouse immunoglobulin G antibodies were applied, and the reaction was revealed with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (Sigma-Aldrich).

To test the biological activity of the precursor, a cytotoxicity assay for HT-29 cells was performed as described previously (6). The ability of the mouse antiserum to neutralize the enterotoxin was tested by the same assay (6). The neutralization titer was defined as the highest dilution able to neutralize 8 cytotoxic units. A pool of sera from nonimmune CD2F1 mice was used as a control.

A protein of approximately 45 kDa, consistent with the predicted size of the 6X His-tagged recombinant protein (Fig. 1), was expressed by pRLV128-containing E. coli M15. The recombinant protein was purified to homogeneity, as assessed by SDS-PAGE (Fig. 1), with a yield of approximately 500 μg per liter of E. coli broth culture.

The purified recombinant protein did not induce any toxic modifications when applied to HT-29 cells up to a concentration of 5 μg/ml. In order to verify whether E. coli was able to process the enterotoxin precursor into the biologically active form, the broth culture supernatant of M15/pRLV128A and

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was found to be unable to process the precursor into the biologically active form and secrete it into the culture medium. Other metalloproteases from different microorganisms have been cloned as precursors in *E. coli*, with different outcomes. For instance, while the collagenase from *Vibrio parahaemolyticus* is secreted in active form in the supernatant (15), the metalloprotease from *Serratia marcescens* is expressed and secreted only if it is complemented with genetic determinants of transport systems such as those of the α-hemolysin (11).

The recombinant enterotoxin precursor, although devoid of biological activity, was able to elicit antibodies that recognized the mature enterotoxin by Western blotting and neutralized its cytotoxic activity in HT-29 cells. A few years ago, monoclonal antibodies to *B. fragilis* enterotoxin were used for detection of the enterotoxin in stool samples by a sandwich enzyme-linked immunosorbent assay that also included a polyclonal antienterotoxin antiserum (7). The antiserum produced to the recombinant precursor will be useful in further studies aimed both at the biological characterization of the enterotoxin and at the development of diagnostic assays.

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