INFECTION AND IMMUNITY, Nov. 1995, p. 4476–4480 Vol. 63, No. 11
0019-9567/95/$04.00+0

Helicobacter pylori Cytotoxin: Importance of Native Conformation for Induction of Neutralizing Antibodies

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Received 19 April 1995/Returned for modification 27 July 1995/Accepted 22 August 1995

We have attempted to express the *Helicobacter pylori* vacuolating cytotoxin in *Escherichia coli*. Although the 95-kDa VacA polypeptide was expressed abundantly, it completely lacked any biological activity. In addition, this material failed to induce neutralizing antibodies after immunization of rabbits. In contrast, highly purified high-molecular-mass cytotoxin from the supernatant of *H. pylori* cultures was active in a HeLa cell assay and effectively induced a neutralizing response in rabbits. Neutralizing sera were shown to contain a high proportion of antibodies which recognized conformational epitopes found only on the native toxin. The data indicate that toxin-neutralizing epitopes are conformational and that potential vaccines based on the cytotoxin may benefit from the use of the intact molecule.

Helicobacter pylori infection is now recognized as the cause of most gastroduodenal disease, including chronic active gastritis, atrophic gastritis, and peptic ulcer (1). Recent data indicate that a subgroup of *H. pylori*, referred to as type I strains (17), which produce a potent cell-vacuolating cytotoxin (5) may be responsible for the more severe forms of disease (2, 15). In support of this hypothesis, extracts of cytotoxic (type I) strains but not noncytotoxic (type II) strains cause epithelial damage and ulceration when administered orally to mice. Furthermore, the highly purified toxin causes lesions similar to those observed after administration of whole-cell extracts (15). More recently, in a mouse model of *H. pylori* infection, cytotoxic strains but not noncytotoxic strains caused gastric lesions similar to those seen in severe disease in humans (6). Hence, study of the cytotoxin will be important to our understanding of *H. pylori*-induced disease, and the cytotoxin may be an important candidate for therapeutic or prophylactic vaccines.

The toxin is synthesized in *H. pylori* as a 140-kDa precursor protein with a 33-amino-acid amino-terminal signal peptide, which is presumably responsible for secretion across the plasma membrane (16). The carboxy-terminal 45-kDa segment of the precursor is structurally similar to the outer membrane transporter region of the immunoglobulin A protease family of exotoxins (13). This region is cleaved from the molecule during export across the outer membrane and remains associated with the bacterial cell (16). The 95-kDa mature portion of the toxin is released slowly from the bacteria and is found in the culture medium as a high-molecular-mass oligomer (3). The 95-kDa monomer is further processed in the culture medium by specific cleavage to produce an amino-terminal subunit with a size of 37 kDa and a carboxy-terminal subunit with a size of 58 kDa (16). The subunits copurify on gel filtration with the intact molecule, indicating that they remain associated after cleavage. The physiological relevance of this further processing is not yet clear, but it may be important for activation of the toxin, as is the case for several other bacterial toxins.

Large-scale production of the toxin is essential for studies of function and for assessment of its potential as a vaccine. For this reason, we have expressed the 95-kDa mature portion of the toxin and each of the two subunits separately in *Escherichia coli* and compared these products with highly purified native toxin in activity and ability to induce neutralizing antibodies in rabbits. The antisera raised against the recombinant products recognized the native toxin in enzyme-linked immunosorbent assay (ELISA) but failed to neutralize the toxic activity. Highly purified native toxin could be produced in milligram quantities and was highly potent in inducing neutralizing sera.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* M15 (pRep4) (Qiagen Inc., Chatsworth, Calif.) was used for cloning experiments. Recombinant bacteria were grown in Luria-Bertani broth or agar plates supplemented with ampicillin (100 μg/ml) and kanamycin (25 μg/ml). *H. pylori* reference strain NCTC 11637 and strain 60190 (kindly supplied by M. J. Blaser) were grown on Columbia agar with 5% horse blood, 0.2% cyclodextrin, and Dent or Skirrows antibiotic supplement (Oxoid, Basingstoke, United Kingdom). Liquid cultures were grown in 5-liter bioreactors in brucella broth supplemented with 2% cyclodextrin (7).

Expression of recombinant toxin. DNA cloning procedures were carried out according to standard protocols (12). *H. pylori* DNA fragments were generated either by restriction enzyme digestion of the cloned gene (16) or by PCR amplification with specific primers. pTOX140 was constructed in two steps as follows. (i) An approximately 5-kbp fragment from the *Eco*RI site at position 491 of the published sequence (16) to a *Bam*HI site approximately 1.5 kbp after the stop codon was cloned into the respective sites in pQE30 (Qiagen). This construct thus lacked the sequences coding for the ribosome binding site, initiator methionine, and histidine tag of the pQE30 plasmid. (ii) A short fragment synthesized by PCR from a synthetic *Eco*RI site at position 201 of the published sequence to the *Eco*RI site at position 491 was inserted into the plasmid from step i. Thus, the construct contains the pQE30 promoter followed by the ribosome binding site and the entire gene encoding the cytotoxin. A similar strategy was used for the construction of pTOX100 except that a *Bam*HI site was added immediately preceding base 316 of the published sequence and the insert terminated at the *Hin*dIII site at position 3215 of the published sequence. Thus, the region coding for amino acids 34 to 1000 was cloned in frame with the sequences coding for the histidine tag in the *Bam*HI-*Hin*dIII sites of pQE30. pTOX37 and pTOX58 were constructed by cloning the sequences coding for amino acids 34 to 352 and 353 to 995 in the *Bam*HI-*Sal*I sites of pQE30 with synthetic oligonucleotides with the appropriate restriction enzyme recognition sites.

Recombinant protein was purified on Ni-nitrilotriacetic acid resin (Qiagen), according to the manufacturer's instructions. Recombinant protein was bound to the column in 8 M urea–0.1 M sodium phosphate–0.01 M Tris (pH 8.0) and eluted with 6 M guanidinium hydrochloride–0.2 M acetic acid. Protein refolding was carried out by dialysis against 50 mM β-alanine (Sigma Chemical Co., St. Louis, Mo.) (pH 3.8)–10% glycerol–5 mM glutathione (reduced)–0.5 mM glu-

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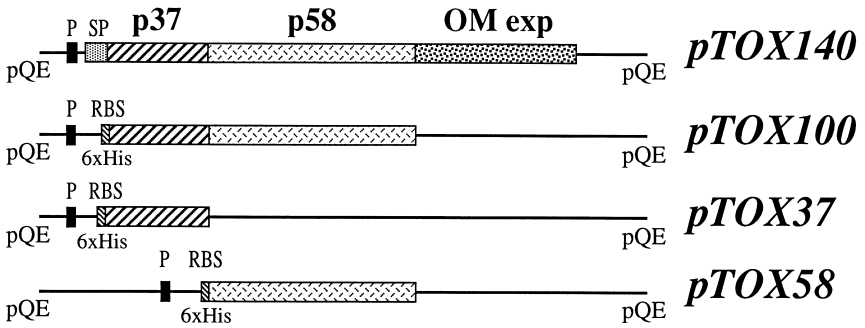

FIG. 1. Schematic representation of plasmid constructs used for expression of cytotoxin in *E. coli*. P, promoter; RBS, ribosome binding site; SP, signal peptide; OM exp, putative outer membrane exporter; 6xHis, tag of six histidine residues.

tathione (oxidized) and then dialysis stepwise against 50 mM β-alanine–10% glycerol at pH 4.5, 5.5, and 7.0. Rabbits were immunized intradermally with five doses, spaced 7 days apart, of 100 μg of recombinant proteins in Freund's adjuvant. Immunoblotting was carried out by using a chemiluminescence detection kit (ECL; Amersham) and horseradish peroxidase-labeled anti-rabbit immunoglobulin antibodies.

Purification of native VacA. The biomass from a nominal 5-liter culture of *H. pylori* CCUG was removed by centrifugation at 11,000 × *g* for 20 min, and the supernatant liquid (approximately 4 liters) was brought to 50% saturation by the addition of solid ammonium sulfate. The suspension was centrifuged at 11,000 × *g* for 20 min, and the precipitated proteins were resuspended in 100 mM NaCl–20 mM phosphate (pH 6.5) and dialyzed extensively against the same buffer. The dialysate was adjusted to a volume of 250 ml and applied, at a flow rate of 2.5 ml/min, to a column (2.5 by 11 cm, Econo column; Bio-Rad, Hercules, Calif.) containing Matrex Cellufine Sulfate (Amicon, Danver, Mass.). The proteins were washed extensively with loading buffer, eluted from the column with a gradient of 0.1 to 1.5 M NaCl in 20 mM phosphate buffer (pH 6.5), and monitored by A_{280}. The eluate between 0.5 and 0.8 M NaCl containing the VacA protein was collected and brought to 50% saturation with ammonium sulfate. The suspension was centrifuged at 11,000 × *g* for 20 min, and the pelleted proteins were resuspended in phosphate-buffered saline (PBS) (300 mM NaCl). Insoluble material was removed by centrifugation at 25,000 × *g* for 5 min, and the cleared solution was applied to a column (16 by 81 mm) packed with Superose 6 (Pharmacia, Uppsala, Sweden). Proteins were eluted with PBS and monitored by A_{280}. Fractions containing highly purified VacA, as assessed by Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were pooled. The protein concentration in the purified fractions was measured by using the Dc protein assay reagent kit (Bio-Rad) with bovine serum albumin as a standard.

Immunization of rabbits and neutralization of toxin. New Zealand White rabbits approximately 2.5 kg in size were immunized by intradermal injections of 25 μg each of purified toxin in a solution of 1 mg of aluminium hydroxide per ml as the adjuvant on days 0, 14, 28, and 56. Immunoglobulins were purified by using protein G-Sepharose (Pharmacia) and tested for their ability to neutralize VacA cytotoxic activity in an in vitro vacuolation assay (9) in which serial dilutions of the purified immunoglobulins were incubated with 1.85 μg of purified toxin in 40 μl of PBS added to the cells in 160 μl of culture medium containing 5 mM ammonium chloride.

ELISA. Flat-bottomed 96-well plates were coated by incubation overnight at 4°C with native VacA (2 μg/ml) or recombinant TOX100 (10 μg/ml) in PBS. After thorough washing with PBS–0.05% Tween 20, the plates were blocked by incubation for 2 h at 37°C with a solution of polyvinylpyrrolidone 360000 (2.7%) (Sigma Chemical Co.) and then washed again. Serial dilutions of purified immunoglobulins from anti-VacA and anti-TOX100 rabbit antisera in PBS–0.05% Tween 20 were incubated in the coated plates for 1.5 h at 37°C. The plates were washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin antibodies for 1.5 h at 37°C. After the plates were washed, the reaction was developed by using the ELISA *In vitro* Test (Sclavo Diagnostics, Siena, Italy).

RESULTS

Expression of vacuolating cytotoxin in *E. coli*. Plasmid pTox140 contains the entire *H. pylori* toxin gene including sequences coding for the signal peptide and putative outer membrane exporter cloned downstream of a synthetic inducible promoter in the plasmid vector pQE30 (shown schematically in Fig. 1). This construct was introduced into *E. coli* M15, and the expression of the toxin gene was induced by treatment with IPTG (isopropyl-β-D-thiogalactopyranoside). Despite the fact that the synthetic promoter in pQE30 is very strong, only trace quantities of a 140-kDa protein which reacted in Western blots (immunoblots) with antitoxin antisera could be detected in the cell extracts (Fig. 2A). No processed 95-kDa protein could be detected either in the cell extracts or in the culture supernatant. *E. coli* would thus appear to be incapable of correct synthesis, processing, and export of the full-length cytotoxin precursor.

To overcome this problem, we attempted to express the region of the gene coding only for the mature 95-kDa portion of the protein either intact or in two pieces corresponding to the 37- and 58-kDa subunits. To facilitate purification of these proteins, they were expressed with six amino-terminal histidines, which enable purification by nickel chelation chromatography. The plasmid constructs used are shown schematically in Fig. 1. Each of these proteins was expressed to high levels after induction in *E. coli* (Fig. 2B); however, the proteins precipitated inside the bacteria and were found in the insoluble fraction of the cells after rupture by sonication.

We attempted to solubilize and renature the recombinant proteins to produce functional toxin. The proteins were solubilized in 8 M urea, purified on nickel-chelating columns, and

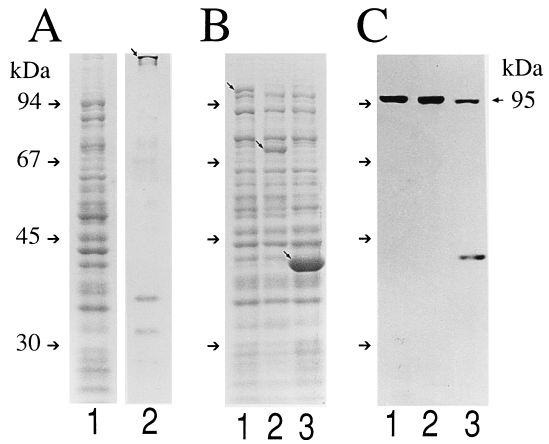

FIG. 2. Expression of cytotoxin polypeptides in *E. coli*. (A) Coomassie blue staining (lane 1) and immunoblot with anticytotoxin antibodies (lane 2) of TOX140 in *E. coli* extracts after SDS-PAGE. The angled arrow shows the position of the 140-kDa precursor. (B) Coomassie blue staining of TOX100 (lane 1), TOX58 (lane 2), and TOX37 (lane 3) in extracts of *E. coli* expressing the respective plasmids. The angled arrows indicate the recombinant proteins. (C) Immunoblot of extracts of *H. pylori* NCTC 11637 with rabbit antisera raised against TOX100 (lane 1), TOX58 (lane 2), and TOX37 (lane 3). The positions of protein molecular mass standards are indicated (horizontal arrows).

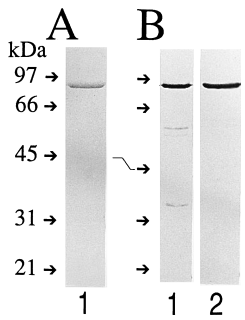

FIG. 3. SDS-PAGE and immunoblot of purified cytotoxin. (A) Coomassie blue staining of purified cytotoxin after SDS-PAGE; (B) immunoblot of the material in panel A (lane 2) and a similar preparation (lane 1) with antisera raised against TOX100. The migration of protein molecular mass standards is shown.

dialyzed stepwise to remove the urea. Although the proteins treated in this way remained in solution, no activity could be detected in a HeLa cell vacuolation assay (9). Rabbit antiserum raised against this material reacted well in a Western blot with the 95-kDa toxin protein in *H. pylori* extracts (Fig. 2C) and recognized the native protein at a high dilution in ELISA (see below); however, none of the serum samples was capable of neutralizing toxin activity in the in vitro vacuolation assay (data not shown).

Purification of native cytotoxin. The above results indicated that protective epitopes in the toxin molecule may be conformational and that the *E. coli*-expressed toxin was incapable of folding correctly. To test this and to establish whether the cytotoxin could be considered as a possible vaccine candidate, we needed to purify sufficient native active toxin to immunize rabbits. Published procedures for the purification of the cytotoxin have proven difficult for the preparation of large quantities. To overcome this problem, we developed a new, simplified procedure which gives reproducibly good yields of active cytotoxin. Supernatants from *H. pylori* NCTC 11637, cultured in bioreactors under conditions which ensure a high level of cytotoxin synthesis (7), were first concentrated, and then the toxin was purified by using a two-step procedure involving affinity chromatography and gel filtration (see Materials and Methods). The active cytotoxin eluted from the gel filtration in fractions corresponding to an apparent molecular mass of around 700 kDa. Coomassie blue staining of this material after SDS-PAGE revealed a single polypeptide with a molecular mass of 95 kDa which reacted with antiserum raised against the recombinant cytotoxin (Fig. 3). In some preparations, traces of the previously described 37- and 58-kDa processed products of the 95-kDa monomer (16) could be detected (Fig. 3B, lane 1).

With this procedure, milligram quantities of active cytotoxin were obtained from the culture supernatant. The estimated molecular mass of the toxin, although slightly lower, is within the limits of the technique which gave the value of 900 kDa previously reported (3).

Induction of neutralizing antisera by native cytotoxin. The purified native toxin proved very efficient at inducing neutralizing antisera in rabbits. Figure 4 shows the dose response of neutralization of purified toxin in a HeLa cell vacuolation assay. Antisera from two different rabbits gave similar results of 50% inhibition at around 10 μg of purified immunoglobulins per ml. Hence, it appears that conformational epitopes important for neutralization and thus for toxic activity are formed only on assembly of the high-molecular-mass oligomeric structure of the native toxin.

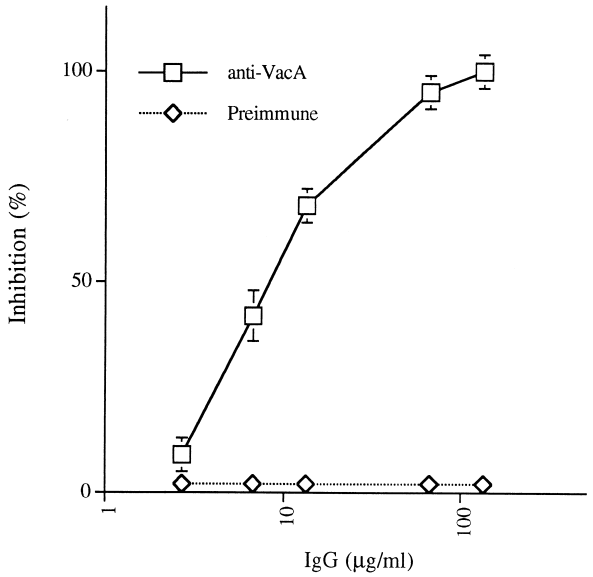

FIG. 4. Inhibition of cytotoxic activity by antibodies raised against purified native cytotoxin. Solid line, protein G-Sepharose-purified immunoglobulins from anticytotoxin serum; broken line, purified immunoglobulins from preimmune serum. The figure shows the mean and standard deviation of triplicate samples from representative experiments.

H. pylori isolates show a large degree of genetic variability. Nevertheless, cytotoxic activity extracted from an independent strain, *H. pylori* 60190 (3), isolated in a different geographic location was neutralized by similar concentrations of the rabbit antisera (data not shown). Even though the primary sequence of the toxin from this strain is only 93% identical to that of the protein used for immunization (4, 16), the protective epitopes are sufficiently conserved to allow effective cross-reaction.

Conformational epitopes in native toxin detected by ELISA. Figure 5 shows a comparison of antibody titers between rabbit antisera raised against native and recombinant cytotoxins. While sera raised against the different antigens had similar titers in ELISAs against the recombinant protein, they differed greatly when measured against the native protein. Antisera raised against the native toxin reacted with the native toxin at a considerably higher dilution than with the recombinant protein, indicating that a large part of the antibodies recognized conformational epitopes not present in the denatured molecule. In contrast, much higher concentrations of the antisera against the recombinant protein were required to react with the native protein, probably because some linear epitopes present in the denatured protein were masked in the correctly folded structure.

DISCUSSION

We have shown that native oligomeric toxin purified from cultures of *H. pylori* is capable of inducing high titers of neutralizing antibodies in rabbits whereas recombinant toxin proteins expressed in *E. coli* are not. The *E. coli*-expressed proteins were inactive in a HeLa cell assay of vacuolation, suggesting that the reason that they failed to induce a neutralizing response was because they failed to fold correctly. Hence, the neutralizing epitopes in the native toxin protein are likely to be conformational and may be formed only when the protein folds correctly into its native structure.

This interpretation is supported by the results of the ELISA

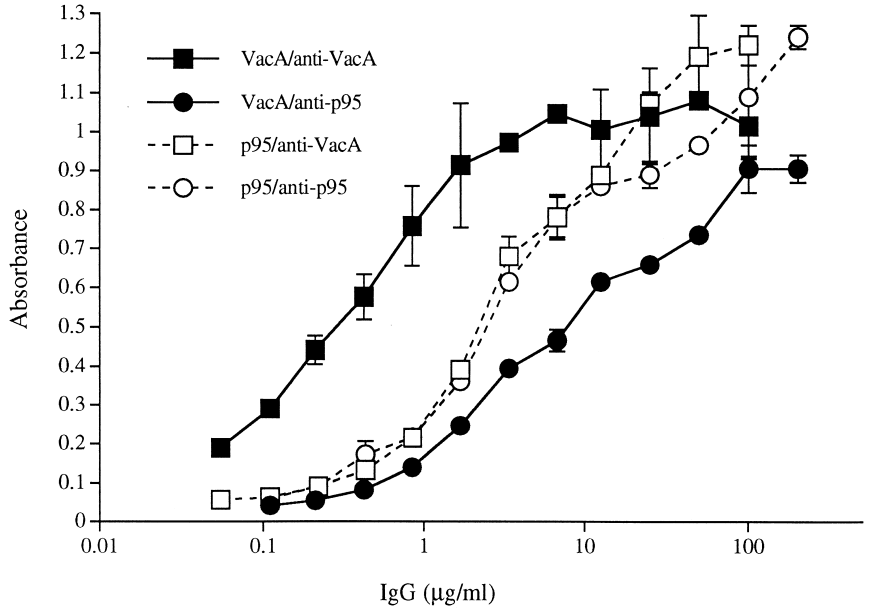

FIG. 5. ELISA determination of antibody titer in neutralizing rabbit antisera raised against native cytotoxin (squares) or recombinant TOX100 (circles) against the native antigen (solid lines, filled symbols) or the recombinant antigen (broken lines, open symbols).

using the native and recombinant proteins. Since both the responses of individual rabbits and the efficiency of binding of the antigens to the plate are likely to vary, the interpretation of these data relies on the crosswise assay of the titer of each antiserum with both the native and recombinant antigens. Antisera raised against the native antigen recognized the native protein at titers much higher than those of the recombinant protein. This difference in titers was not due to large differences in the quantities of antigens in the assay, as demonstrated by the fact that the antisera against the recombinant antigen reacted with the recombinant protein at a higher titer than with the native protein. The differential response of the two serum samples indicates that the native molecule is highly structured such that the immune response is due primarily to conformational epitopes. These data may be highly relevant to studies of serum antibodies against the toxin in *H. pylori*-infected individuals. It is likely that use of the recombinant protein will seriously underestimate the antibody titers and that only the native toxin will give accurate measurements of the host response during infection.

The native toxin has been reported to migrate in gel filtration with an apparent molecular mass of >900 kDa (3). Our estimate of the mass of the purified toxin is closer to 700 kDa, but this difference is within the limits of error of the preparative gel filtration used. Since only the purified protein was active and capable of inducing a neutralizing response, it is likely that the high-molecular-mass form of the toxin is not simply a nonspecific aggregate of toxin monomers but that it is an ordered structure required for activity. In support of this interpretation, visualization of the native toxin by electron microscopy has revealed a regular heptameric structure (unpublished data).

The data and conclusions are similar to those reported for the pertussis toxin, which is a heteropentameric protein. Antisera raised against the individual subunits failed to neutralize the toxin (8) whereas chemically inactivated or genetically detoxified holotoxins (10) are effective at inducing protective immunity (11). The pentameric structure of pertussis toxin has subsequently been characterized, and pertussis toxin, like several other multimeric bacterial toxins, has been shown to possess functional regions which span several subunits (14). The oligomeric structure of the *H. pylori* cytotoxin is likely to have similar properties.

The integrity of the native conformation of a vaccine can be extremely important for the induction of protective immunity. Even partial destruction of the conformational epitopes by chemical inactivation can result in lowering the effective immunogenicity. This variation in effectiveness has been amply demonstrated by inactivation of the pertussis toxin. Pertussis toxin genetically detoxified by substitution of two key amino acids in the enzymatically active site such that the structure is virtually unaltered has proven to be a considerably more effective immunogen than chemically inactivated forms (11). This possibility will have to be taken into consideration in the assessment of the cytotoxin as a possible vaccine candidate. A genetically detoxified molecule which retains the native structure will be an important goal.

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Editor: P. J. Sansonetti