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Localization of Two Epitopes Recognized by Monoclonal Antibody PCG-4 on *Clostridium difficile* Toxin A

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The toxin A gene of *Clostridium difficile* contains a 2.5-kb region encoding a series of contiguous repeating units located at the COOH terminus of the molecule. We previously showed that the monoclonal antibody (MAb) PCG-4, which neutralizes the enterotoxic activity of toxin A, binds to epitopes located within these repeating units. In the present study, we subcloned a series of fragments from this portion of the gene. The recombinant peptides expressed from the gene fragments were examined for reactivity with MAb PCG-4 to identify the epitopes involved in binding. Our results showed that MAb PCG-4 recognizes epitopes in amino acid residues 2097 through 2141 and amino acid residues 2355 through 2398.

Clostridium difficile causes antibiotic-associated pseudomembranous colitis and diarrhea in humans because of the two toxins, toxin A and toxin B, that it produces (4, 11, 15, 19). Toxin A is a potent enterotoxin that elicits a positive hemorrhagic fluid response in the rabbit ileal loop assay and is weakly cytotoxic, causing rounding of tissue culture cells (1, 2, 16, 20, 29, 30). Toxin B causes the same type of rounding as toxin A, but toxin B is much more potent against most cells. Toxin A is thought to be responsible for the diarrhea and initial tissue damage in patients with pseudomembranous colitis because of its ability to bind to receptors in the colon (7, 14, 19). In addition to its toxic activity, toxin A has hemagglutinating activity and specifically agglutinates rabbit erythrocytes (14). This activity is due to the binding of toxin A to the trisaccharide Galα1-3Galβ1-4GlcNAc on rabbit erythrocytes. This trisaccharide likely serves as the receptor in hamsters and in certain tissue culture cells, such as F9 mouse cells.

The genes for the toxins have been cloned and sequenced (3, 10). Toxin A has a deduced molecular weight of 308,000 and consists of 2,710 amino acids. Toxin B has a deduced molecular weight of 279,000 and consists of 2,366 amino acids. An unusual feature of the toxin A gene is a 2.5-kb region encoding a series of 38 contiguous repeating units at the 3′ end of the gene. The repeating units have been divided by Dove et al. (10) into class 1 units, which are 90 nucleotides in length, and class 2 units, which are 60 nucleotides in length. The class 2 units are further divided into subclasses A, B, C, and D. Each class 1 repeat is followed by three to five of the smaller class 2 repeats.

The results of earlier studies from our laboratory have shown that a nontoxic recombinant peptide composed of the repeating units retains the hemagglutinating activity of the molecule (18, 26). In addition, we have found that the monoclonal antibody (MAb) PCG-4, which neutralizes the enterotoxic activity of toxin A and precipitates the toxin (21), binds to the recombinant peptide. These observations suggest that the antibody neutralizes the enterotoxic activity by blocking the binding of the toxin to its receptor. In the present study, we have identified the regions of the gene that encode the epitopes recognized by MAb PCG-4.

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MATERIALS AND METHODS

Bacteria and plasmids. Plasmids pBR322, pUC9, pUC18, and pUC19 and *Escherichia coli* JM109 were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Plasmids pCD11 and pCD11.06 were kindly provided by J. L. Johnson (Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University).

Affinity-purified toxin A antibodies and MAb PCG-4. Affinity-purified goat antibody against toxin A was prepared as previously described by using immunoaffinity chromatography on immobilized toxin A (23). Ascites containing high levels of MAb PCG-4 were prepared as previously described (22).

Enzymes and radiolabeled compounds. Restriction endonucleases were purchased from Bethesda Research Laboratories, Promega Biotec (Madison, Wis.), and New England BioLabs, Inc. (Beverly, Mass.). Exonuclease III and VII, T4 DNA ligase, and the large fragment of DNA polymerase I (Klenow) were obtained from Bethesda Research Laboratories. Calf alkaline phosphatase was purchased from Promega Biotec. Enzymes were used according to the instructions provided by the manufacturers. Labeled nucleotide triphosphate [α-^{35}S]dATP was obtained from DuPont, NEN Research Products (Boston, Mass.).

DNA manipulations and construction of subclones. Plasmid DNA was isolated by the alkaline lysis procedure of Birnboim (6). Specific restriction endonuclease fragments for ligation were isolated by electrophoresis in low-melting-temperature agarose, and individual bands were cut from the gel or eluted by electroelution (5). When required, DNA fragments were blunt ended by using the large fragment of DNA polymerase I (Klenow). DNA ligation of fragments in low-melting-temperature agarose was performed as described previously (24, 28). Progressive exonuclease digestion of pCD290 for the determination of the 3′ end was performed by using the procedure of Yanisch-Perron et al. (31). Transformation of *E. coli* JM109 was by the method of Hanahan (12).

Sequencing and DNA analysis. The dideoxy chain termination method of Sanger et al. (27) was used to confirm the correct orientation and reading frame of the recombinant fragments. Sequencing also was used to determine the deduced size of the insert following progressive exonuclease digestion of the clone pCD290. Double-stranded plasmid

sequencing was performed with the Sequenase kit, version 2.0 (U.S. Biochemicals, Cleveland, Ohio), with synthetic oligonucleotide primers by the alkaline denaturation method recommended by the manufacturer.

Expression of recombinant proteins. *E. coli* JM109 containing the recombinant plasmids was grown at 37°C in T broth (13) supplemented with ampicillin (100 μg/ml) with shaking until early stationary phase and then induced for 3 h with a final concentration of 2 mM isopropyl-β-D-thiogalactoside. Cells were collected by centrifugation, suspended in 1/10 volume of 0.05 M Tris-HCl buffer, pH 7.2, containing 0.15 M NaCl (TBS), and lysed by passage through a French pressure cell at 16,000 lb/in^2. Cellular debris was removed by centrifugation, and the supernatant fluid was passed through a 0.45-μm-pore-size membrane filter and stored at 4°C.

Dot immunoassay. Immunoassays similar to previously described methods were used to screen clones for the production of peptides (13). Briefly, cell lysates were adjusted to a protein concentration of 20 mg/ml by the addition of TBS, and 2 μl of the sample was spotted onto a nitrocellulose membrane (Bio-Rad Laboratories) and air dried. Membranes were blocked with 0.5% sodium caseinate in TBS for 30 min at room temperature. The membranes were rinsed three times with TBS (10 min per rinse) and reacted with either MAb PCG-4 diluted 1/500 in TBS or affinity-purified toxin A antibody (0.5 μg/ml) diluted 1/250 in TBS. After incubation for 4 h at room temperature, the membranes were rinsed three times in TBS. Dot immunoassays with MAb PCG-4 as the primary antibody were reacted with a 1/500 dilution (in TBS) of anti-mouse immunoglobulin G–horseradish peroxidase conjugate (Sigma Chemical Co.). Dot immunoassays utilizing affinity-purified goat antibody against toxin A as the primary antibody were reacted with a 1/500 dilution (in TBS) of anti-goat immunoglobulin G–horseradish peroxidase (Sigma Chemical Co.) for 18 h at room temperature. Membranes were rinsed three times in TBS and developed with 4-chloro-1-naphthol (Sigma Chemical Co.). The reactions were stopped by rinsing the membranes in warm water. Results were graded according to the following scheme: +++, very intense reaction; ++, easily visible reaction; +, weakly visible reaction; −, no visible reaction. Controls consisted of the following: (i) nitrocellulose membranes coated with lysate from identical host and vector systems but without any gene fragment, (ii) membranes coated with buffer instead of lysate, and (iii) buffer instead of detecting antibody, to rule out any interaction of lysate and conjugate.

For the determination of epitope stability, the dot blots were performed as described above except that samples contained 2.5% (wt/vol) sodium dodecyl sulfate (SDS) and were heated at 100°C for 5 min.

Computer analysis. Calculations for protein secondary structure predictions were performed on an IBM-XT (IBM Corp., Armonk, N.Y.) with the MSEQ 1.17 computer program (University of Michigan, Ann Arbor) on the basis of the methods of Chou and Fasman (9).

Protein determinations. Protein concentrations were estimated by using the Pierce Coomassie protein assay reagent (Rockford, Ill.) by the Bradford procedure (8). Bovine gamma globulin (Bio-Rad Laboratories) was used as the standard.

RESULTS

To determine the location of the MAb PCG-4 epitope, we subcloned a series of fragments from a 4.7-kb toxin A gene fragment (pCD11) encoding the repeating units. The cloned gene fragments, which were obtained by utilizing various restriction endonuclease sites identified from the toxin A gene sequence, are listed in Fig. 1. Clone pCD11 was expressed by using the vector pBR322. Clones pCD2.6, pCD11.06, pCD1287, pCD692, and pCD404 were expressed by using pUC9. Clone pCD341 was expressed by using pUC18, and clones pCD347, pCD290, pCD290C(17), pCD290C(14), and pCD227 were expressed by using pUC19. The larger fragments (pCD11.06 through pCD290) and pCD227 were obtained by using various restriction endonuclease cut sites in the gene. Clones pCD290C(17) and pCD290C(14) were obtained by progressive exonuclease digestions. The expression of the fragments was determined immunologically by using either MAb PCG-4 or affinity-purified polyclonal antibody against toxin A as the detecting antibody. MAb PCG-4 served to identify peptides containing the PCG-4 epitope, and the polyclonal antibody was used to confirm the expression of peptides that did not react with the monoclonal antibody. The specificity of the dot immunoassay was shown by demonstrating that neither of the antibody preparations reacted with host-vector lysates in the absence of the gene fragments listed in Fig. 1. We obtained additional clones that were composed of gene fragments other than those listed in Fig. 1, but the peptides expressed by these clones did not react with either of the antibody preparations. The clones listed in Fig. 1 consist only of those that expressed a peptide that reacted with one or both of the antibody preparations.

The portions of the gene from which the various clones were derived are illustrated in Fig. 1. In the initial cloning, pCD11.06, containing most of the repeating units, was derived from pCD11. Clones pCD692 and pCD1287 were subsequently derived from pCD11.06. Peptides expressed from both of these fragments reacted with MAb PCG-4, confirming the presence of more than one PCG-4 epitope. Clones pCD404 and pCD339 were derived from pCD1287. The peptide expressed by pCD404 reacted with MAb PCG-4. The peptide from pCD339 did not react with MAb PCG-4 but did react with the polyclonal antibody. Clones pCD340 and pCD290 were sequentially derived from pCD692, and peptides expressed by both of these clones reacted with MAb PCG-4.

Because of its smaller size, the pCD290 fragment was selected for further study and subjected to progressive exonuclease digestion from the 3′ end. By using this procedure, two additional clones, pCD290C(17) and pCD290C (14), were obtained. The peptide expressed by clone pCD 290C(17), which encoded a 44-mer, reacted weakly with MAb PCG-4. Clone pCD290C(14) encoded a 42-mer that was only 2 amino acids shorter than the 44-mer. The 42-mer reacted weakly with the polyclonal antibody but did not react with MAb PCG-4. The sequence of the 44-mer is shown in Fig. 2. The peptide consists of two nearly identical repeating units of 21 amino acids each. The only difference is the presence of a glutamate residue in the first 21-amino-acid unit rather than an isoleucine residue in the second unit. Further analysis of the sequence showed that the peptide contains high numbers of alanine, glycine, lysine, asparagine, threonine, and tyrosine residues.

Another clone, pCD227, was obtained by using a restriction endonuclease site within pCD290. This clone encoded a peptide that lacked the first 21 amino acids present in the 44-mer but retained the second set of 21 amino acids. Analysis of this peptide showed that it reacted weakly with the polyclonal antibody but not with MAb PCG-4.

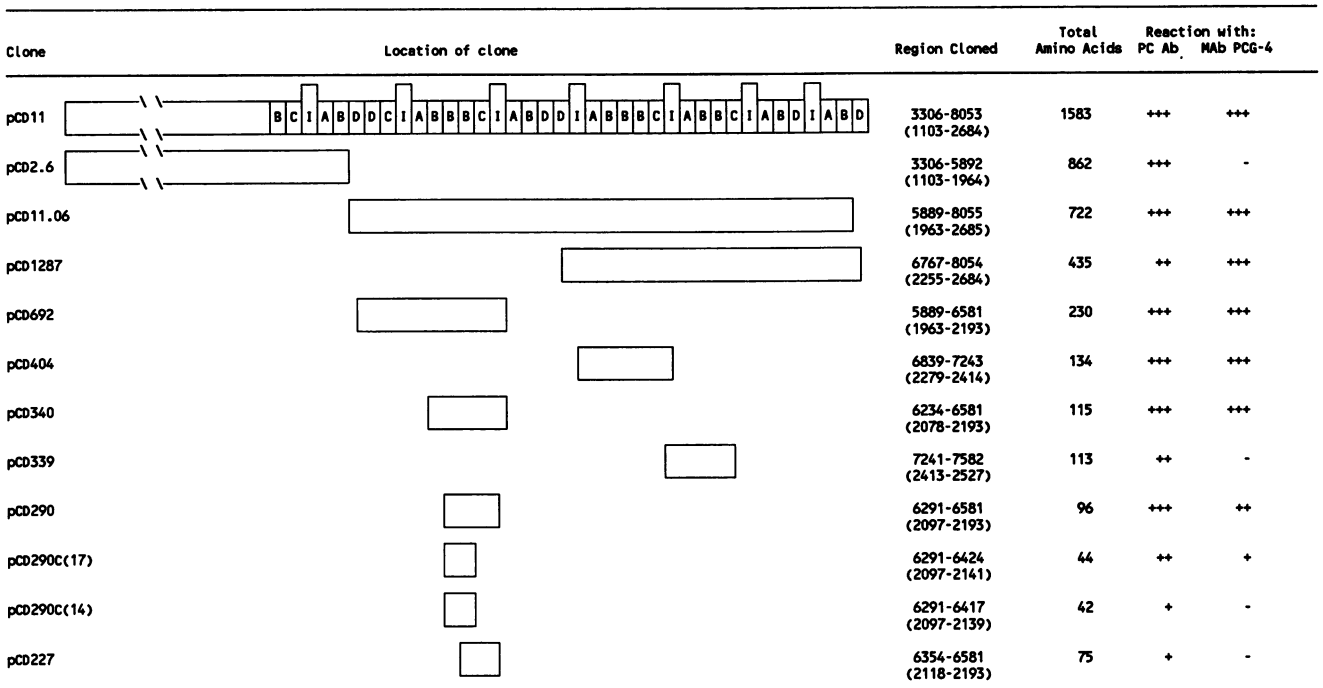

FIG. 1. Clones derived from the 4.7-kb *PstI* fragment encoding the repeating units of toxin A. Clones pCD11, pCD2.6, and pCD11.06 are *PstI* fragments that have been described previously (10, 26). The break in pCD11 and pCD2.6 indicates that a 1.5-kb region of the gene has been left out of the diagram. The repeating units of toxin A are classified into class I units, which are 30 amino acids in length, and class II units, which are 20 amino acids in length. The class II units are subdivided into A, B, C, and D units on the basis of their levels of homology. Under "Region Cloned," the top numbers (not in parentheses) represent the nucleotide sequence, and the bottom numbers (in parentheses) represent the amino acid sequence corresponding to the nucleotide sequence. The reactions with affinity-purified polyclonal antibody against toxin A (PC Ab) and monoclonal antibody against toxin A (MAb PCG-4) range from highly reactive (+++) to not detectable (−).

To examine the stability of the epitope, we subjected cell lysates from four clones expressing PCG-4-reactive peptides of various sizes (pCD11.06, pCD692, pCD340, and pCD290) to harsh denaturation conditions (2.5% SDS, 100°C for 5 min). The cell lysates were then spotted onto nitrocellulose and probed with MAb PCG-4. We found that as the size of the peptide containing the PCG-4-reactive epitope decreased, the epitope became more susceptible to denaturation by SDS. The pCD340 peptide gave only a weakly visible reaction, and the pCD290 peptide, which gave a weakly visible reaction in the native state, did not react when denatured.

We examined other regions of the repeating units for amino acid sequences that closely resembled the sequence of the 44-mer. Clone pCD404, which expressed a peptide that reacted with MAb PCG-4, and clone pCD339, which expressed a peptide that reacted with the polyclonal antibody but not with MAb PCG-4, encode similar amino acid sequences, and these are shown in Fig. 2. The amino acid sequence from pCD404 is identical to that of the 44-mer except for the substitution of a leucine residue in place of threonine at position 16 and that of a phenylalanine residue in place of alanine at position 40. A comparison of the predicted secondary structures for these sequences, shown in Fig. 2B, illustrates the close similarities. Both exhibit high degrees of β-turns and comparable α-helix and β-sheet structures. The amino acid sequence of the peptide from pCD339 (which did not react with MAb PCG-4) is shown in Fig. 2A and differs from the sequence of the 44-mer by 7 amino acid residues. These differences result in significant changes in the predicted secondary structure (Fig. 2B), including the absence of an α-helix structure and changes in the β-sheet and β-turn patterns.

DISCUSSION

In previous studies in our laboratory, we showed that MAb PCG-4 neutralizes the enterotoxic activity of *C. difficile* toxin A by binding to the repeating units located at the COOH terminus (18, 21). The present study was undertaken to identify the regions within the repeating units that compose the PCG-4 epitope. Our approach consisted of subcloning fragments of decreasing sizes from a 4.7-kb *PstI* fragment encoding the repeating units and analyzing the recombinant peptides for their reactions with MAb PCG-4. By using this approach, we identified a small fragment of 290 bp (pCD290) that encoded a peptide that reacted with MAb PCG-4. A subclone, pCD290C(17), encoding a sequence of 44 amino acids was subsequently derived from pCD290 and expressed a peptide recognized by the monoclonal antibody. Interestingly, a clone expressing a 42-mer that differed from the 44-mer by the absence of 2 amino acids at the COOH terminus did not react with this monoclonal antibody. The epitope appears to be conformationally dependent, since denaturation with SDS and heating reduces the binding of the antibody to the recombinant peptide.

Analysis of the 44-mer revealed additional information about the MAb PCG-4 binding site. It consists of two nearly identical sequences of 21 amino acids each. Both of these sequences appear to be needed for recognition by MAb

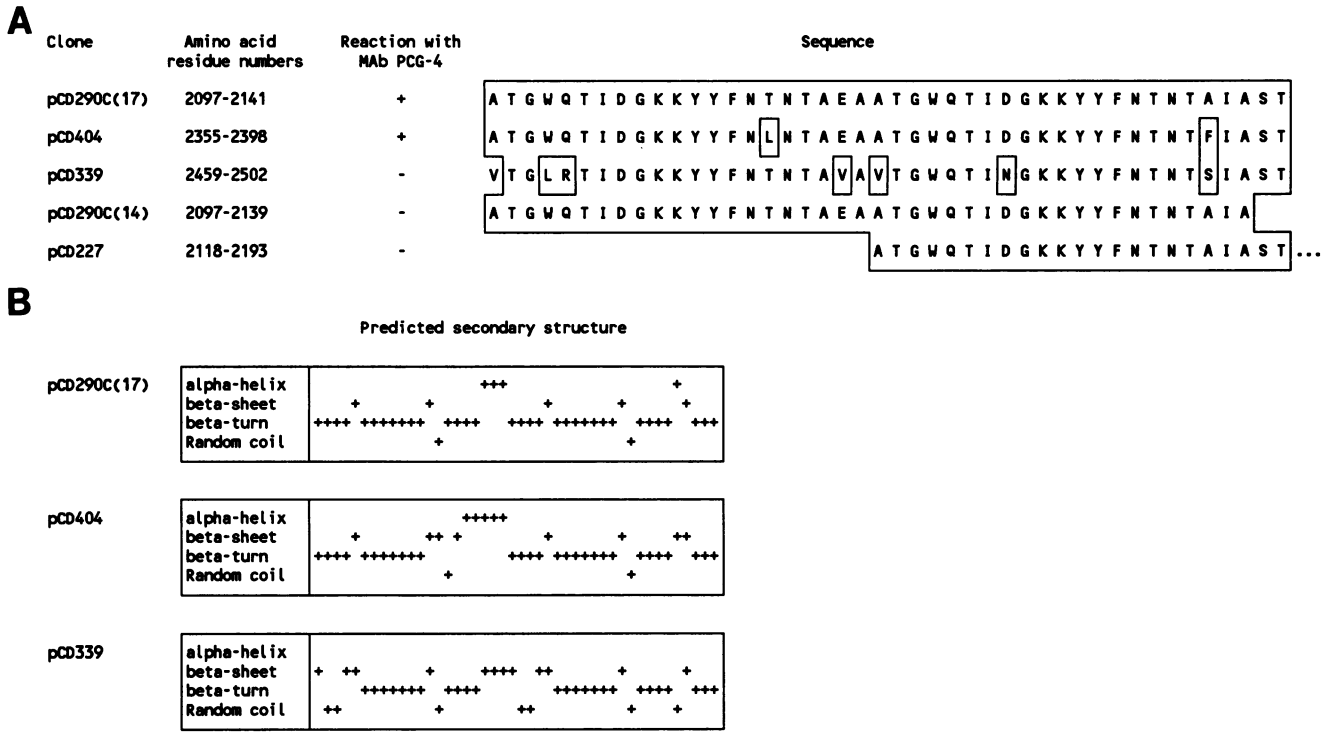

FIG. 2. Comparison of the deduced amino acid sequences of peptides expressed by clones pCD290C(17), pCD404, and pCD339. Clones pCD290C(17) and pCD404 express peptides that react with MAb PCG-4, whereas the others do not. Regions of homology (A) and the predicted secondary structures for pCD290C(17), pCD404, and pCD339 (B) are shown.

PCG-4. This is based on the finding that a clone (pCD227) expressing a peptide containing the second set of 21 amino acids did not react with the antibody. On the basis of the computer-generated secondary structure, this region contains a high percentage of β-turns. This region also contains high percentages of threonine (20.5%) and alanine (13.6%) residues compared with the percentages of threonine and alanine residues found in the intact molecule (6.4 and 5.2%, respectively). There also are a number of lysine residues present. Interestingly, the peptidyl bonds involving these lysine residues are not accessible to trypsin cleavage, since trypsin, even at very high levels, does not degrade the native toxin (17). This suggests either that the lysine residues are buried within the epitope or that the bulky side chains from the surrounding amino acids block the accessibility by trypsin. The 44-mer comprises the B4 and B5 class 2 repeating units, and an analysis of this portion of the protein by hydropathy plot showed it to be hydrophilic (10). The results of other studies in our laboratory indicated that the repeating units of toxin A contain immunodominant regions. This is based on findings that (i) the binding of the monoclonal antibody to the native toxin inhibits the binding of the polyclonal antibody about 75% (23a) and that (ii) antiserum against the repeating region and antiserum against the complete toxin exhibit a complete band of immunological identity when analyzed with native toxin A (18). The presence of hydrophilic amino acid residues and β-turns, which are often present in immunodominant regions, and the fact that these units repeat possibly explain why these regions on toxin A elicit high levels of antibodies.

By comparing the sequence of the 44-mer to those of other regions within the repeating units, we identified a second, very similar sequence (in pCD404) that differed by only 2 internal amino acids (Fig. 2). The peptide expressed by this clone also reacted with MAb PCG-4, demonstrating the presence of at least two PCG-4 epitopes in distinct regions of toxin A. This second region comprises the B8 and B9 class 2 repeating units, and like the B4 and B5 repeating units composing the 44-mer, it is composed of two 21-amino-acid sequences, is hydrophilic, and has about the same amino acid composition. The presence of two PCG-4 epitopes demonstrates that the toxin can be cross-linked by MAb PCG-4, thus explaining why the toxin is precipitated by the monoclonal antibody. There are a number of other class 2B regions that show various levels of homology with the B4-B5 and B8-B9 regions. An example is the region encoded by pCD339. Although this region, which contains the B10 and B11 units, shows homology with the 44-mer, the peptide expressed by pCD339 does not react with MAb PCG-4. Whether these regions of lower levels of homology represent low-affinity sites for MAb PCG-4 is not known, but it is possible that the antibody has low affinity for these sites.

MAb PCG-4 has been used previously in our laboratory to purify the hemorrhagic toxin, toxin HT, from culture filtrates of *Clostridium sordellii* by immunoaffinity chromatography (25). Therefore, toxin HT contains PCG-4 epitopes similar to those on toxin A. There is some indication that toxin HT binds less tightly to MAb PCG-4 than toxin A (25a), suggesting that the epitopes are different. Therefore, comparison of the gene sequences of these regions from toxins A and HT should give us additional information about the PCG-4 epitopes.

In conclusion, we have identified two regions on the toxin A molecule that are recognized by MAb PCG-4. These regions are important in the expression of the enterotoxic activity of the toxin, since the binding of the antibody to

these sites neutralizes the activity. Presumably, these regions are responsible for the binding of the toxin to its receptor, since the repeating units by themselves are not toxic and because they are the portion that binds to galactose-containing moieties. This is further supported by the finding that MAb PCG-4 blocks the binding of the toxin to these moieties. The identification of these regions may allow the examination of other properties of the PCG-4 epitope, including the epitope size, and the determination of the binding affinities to these sites.

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