Identification and Immunological Characterization of the Domain of Actinobacillus actinomycetemcomitans Leukotoxin That Determines Its Specificity for Human Target Cells*

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Although extensive amino acid homology exists among the various repeats in ToxIn (RTX) family of bacterial cytolysins, the cellular and species specificities remain unique for individual toxins (i.e. Actinobacillus actinomycetemcomitans leukotoxin (LtxA) kills human mononuclear cells while a related toxin, Pasteurella hemolytica leukotoxin (LtxA) kills bovine lymphoid cells). To determine the Ltx domain responsible for species specificity, LtxA/LtxA chimeric toxin genes were expressed in tandem with the LtxC gene under control of the Ptx promoter. The activity of lysates to kill either HL-60 (human) or BL-3 (bovine) cells was assessed by trypan blue exclusion. The critical area required for the chimeric toxins to recognize human target cells is a 253-amino acid fragment (residue 688-941) that contains the GGGXDX(I/F)VWY/FWX repeats. A panel of 12 neutralizing anti-LtxA monoclonal antibodies also recognized specificities within the 253-amino acid fragment. Epitope mapping of the monoclonal antibody panel showed that all antibodies bound to one of three sites on the LtxA molecule. One monoclonal recognized epitope A which was composed of LtxA residues 698-709 (KLDYYYYNKGFK), six antibodies recognized epitope B, a peptide composed of residues 746-757 (LiyGYGD-DRLY), whereas the remaining five monoclonals recognized epitope C, which is composed of residues 926-937 (DRALRKQFELQ).

RTX1 (Repeats in ToxIn) (1) are a family of pore-forming hemolysins/leukotoxins (2-6) that are synthesized in a diverse group of Gram-negative pathogens. RTX share similar genes organization and are expressed from an operon consisting of at least four genes that are designated A, C, B, and D in transcriptional order (7, 8). The structural gene is encoded by the A gene and the three remaining RTX genes (B, C, and D) flanking the A gene are required for activating and transporting the RTX. In Escherichia coli, the a-hemolysin structural toxin gene product, is modified by fatty acid acylation, which requires an additional acyl carrier protein in addition to the hlyC gene product (9, 10). Although it has not yet been shown conclusively, it is likely that LtxC carries out a similar reaction in Actinobacillus actinomycetemcomitans. Leukotoxin is transported to the cell surface by a signal peptide-independent mechanism requiring the products of the ltxB and ltxD genes (11), which appear to be similar to the B and D genes of other RTX operons (12-14).

Although homologous clustering of hydrophobic and hydrophilic residues is found when RTX A genes are compared, the individual toxins of this group exhibit unique species and cell type specificity for killing. These findings suggest that although the mechanism of RTX toxin-mediated lysis may be similar, differences in host cell specificity indicate that certain regions of the RTX have undergone significant divergence from each other. Some members of the RTX toxin family are cytolytic against a narrow range of target cell types. For example, the leukotoxins from Pasteurella hemolytica and A. actinomycetemcomitans are lytic only to eukaryotic cells from ruminants (1, 15-17) and granulocytes from certain primates (16, 18), respectively. In contrast, the E. coli a-hemolysin is toxic to a wide range of cell types from humans and other animal species (19). The extensive amino acid sequence similarity that is shared by RTX provides an approach for locating the species-specific functional domains of the leukotoxin molecule.

Mutational analysis has been an important tool for ascribing characteristics and functions to specific regions of the RTX molecule. Truncation mutants were useful in demonstrating RTX carboxyl-terminal secretion functions (20, 21), whereas deleting regions of the various toxins has helped to define domains responsible for Ca2+ and erythrocyte binding (22, 23) and pore formation (21, 22, 24, 25). However, in many instances it is difficult to determine if an observed loss of cytotoxicity encountered with specific mutants is due to the deletion of the functional element or simply the inability of the mutant toxin to achieve the proper tertiary structure. Chimeric toxins, on the other hand, would be expected to contain all of the functional domains of the native toxin, exhibit normal or near normal protein folding, and maintain their ability to kill either bovine or human target cells as long as the respective "species recognition unit" is present. Toward this end, investigation of the differences in target cell specificity among RTX toxins has revealed discrete regions of the broadly reactive hemolysins which can confer specificity for new cell types and host species to the target cell-limited leukotoxins (26, 27). Studies of the structure of the regions responsible for species specificity will not only provide insight into the phylogenetic evolution of these molecules but will also provide important preliminary data on the mechanism of target cell recognition by RTX.

*Rm, repeats in toxin; bp, base pair(s); PCR, polymerase chain reaction; mAb, monoclonal antibody.
plasmid cloning vehicle is derived from pKCS0 (30-32) (a gift from Drs. Allan Shatzman and Martin Rosenberg, SmithKline Beechnut Laboratories, Swedeland, PA) and utilizes the bacteriophage λ promoter P0 and carried in the lysogenic host AR120. In addition to providing a strong tightly controlled promoter, the system also ensures that P0-directed transcription efficiently traverses either ltxA/IktA or ltxA/IktA gbes by providing the phe λ antitermination function, N, and the rho-independent termination site (Nut) within P0. coli translation unit. E. coli translation (Nut site) within the P0 transcription unit. E. coli translation unit.

**Materials and Methods**

**Bacterial Strains—A. actinomycetemcomitans, strain J2P2, used in this study was obtained from Dr. N. Taichman (Department of Pathology, University of Pennsylvania, Philadelphia). The strain was grown in PYG medium (5 g of Bacto-peptone, 5 g of Trypticase-peptone, 10 g of yeast extract, 10 g of dextrose, 8 mg of CaCl2, 8 mg of MgSO4, 40 mg of KH2PO4, 40 mg of K2HPO4, 400 mg of NaClO4, 60 mg of NaCl) in 1 liter of dh2O for 24 h at 37 °C in an atmosphere of 5% CO2. E. coli strain AR120 was grown in LB medium (1% Tryptone, 0.5% yeast extract, 1.0% NaCl) with aeration at 37 °C. P. hemeolytica A1, strain 112, was obtained from Dr. Gordon Gheilen, University of California (Davis, CA) grown in PYG media and frozen in PYG, 20% glycerol as stocks.

**Construction of ltxC/IktA and ltxC/IktA Gene Cassettes—Oligonucleotide primers used in this study were synthesized in our laboratory by a modification of the phosphite method (28). Initially these primers were utilized to amplify the ltxC and the ltxA genes from genomic DNA in the following manner. Primers a and b were utilized to amplify a 594-bp fragment which contained the ltxC gene, the ltxC/IktA intragenic space, and 2 residues of the ltxA gene. NdeI and KpnI restriction sites were engineered into the 5' end and 3' end of this fragment, respectively. Ndel site was engineered into the restriction sites in the 5' and 3' ends of this fragment. Placement of the primer a, underlined which was used as a translation start signal for ltxC. Primers c and d were utilized to amplify a 3183-bp piece of DNA which included the ltxC gene (from bp 10), termination codon, and 12 bp of the ltxA/IktB intragenic space. Placement of the KpnI site in the ltxA gene was achieved by insertion of a glycine residue between residues 2 and 3 and the placement of a silent mutation in residue 3 of the ltxA gene. Addition of glycine residues had no effect on leukotoxicity (data not shown).

**Primer a 5'-CATATGGGAAAAATTAAATTATTTAGGAT3'**

**Primer b 5'-GGATCCGGCGCATACTTATCT-3'**

**Primer c 5'-GGTACCACCATCTGCTGCCAAT-3'**

**Primer d 5'-GGCTGCAGCTACACTGCTGCCAATG-3'**

**Fragments containing the P. hemeolytica ltxA gene were amplified from genomic DNA by PCR using oligonucleotide primers "e" and "f." A PvuII site (primer e, underlined) was engineered into the coding sequences of residues 2 and 3 of the ltxA gene. Primers e and f amplify a 2865-bp product that contains coding sequences for residues 4-953 of the ltxA gene and a transcription termination codon (primer f, italics). An SstI site (primer f, underlined) was added to the 3' end of the PCR fragment.

**Primer e 5'-GATCCACGACTTTACACACCTAAGCAAT-3'**

**Primer f 5'-GGCGCTCTTACTGCGTCTGCTGAAAAT-3'**

**Amplification was carried out on a Perkin-Elmer 9600 Thermocycler using TaqI polymerase according to the protocol supplied by the manufacturer.**

**Expression of ltx and Ikt Leukotoxin Genes—PCR-generated ltx and Ikt genes were cloned into the pMG1 (29) expression vector (Fig. 1). This**
tion site added to the 5' end, and primer d was used as the reverse primer. After PCR amplification these fragment were cloned into pMGl plasmid that contained ltxC and lktA plasmid by restricting with ClaI and SstI.

**Epitope Mapping of the ltxA Repeat Region**—Forty-six noncleavable 12-mer peptides consisted of the LtxA-deduced amino acid sequence (36, 37) beginning with residue 403 and extending through residue 955 were synthesized to permit mapping antibodies whose combining site might be at the juncture of two peptides in the first set. Additionally, a second series of 46 peptides that covered the same amino acid sequence, but with a 6 residue offset (residues 409-961) (peptides 47-92), was synthesized to permit mapping antibodies whose combining site might be at the juncture of two peptides in the first set.

The panel of anti-leukotoxin hybridomas (35) was grown to stationary phase in serum-free medium (50% Dulbecco's modified Eagle's medium/high glucose:50% Ham's F-12 supplemented with 5 μl/ml insulin, 5 μg/ml transferrin, 1.2 μg/ml ethanolamine, 0.44 ng/ml sodium selenite, 2 mM 1-glutamine, and 1 mM sodium pyruvate), then harvested by centrifugation and the supernatant frozen until assayed.

Between assays the pins were cleaned by sonicating for 10 min in 0.1 M Na,2HPO₄, 16.8 mM citrate acid monohydrate and read at 405 nm in a plate reader. Amino acid sequences of A. actinomycetemcomitans and P. haemolytica toxins are compared in the splice site region (residues that are encoded at the restriction site are underlined).
kemia line (Fig. 5). As expected, native toxins, P. hemeolytica toxin (PP4) and A. actinomycetemcomitans leukotoxin (CH1) killed BL-3 and HL-60 cells, respectively. Chimeric toxins containing progressively larger fragments of ltxA produced toxins that killed HL-60 and BL-3, respectively. A construct containing ltxC produced a toxin which lysed target cells (BL-3), indicating that ltxC could activate lktA. Expression of either the LtxA or LktA toxin without the ZtxC gene product failed to produce an LktC toxin for its ability to activate lktA genes produced toxins that killed HL-60 and BL-3, respectively. A chimeric toxin containing progressively larger fragments of DNA were then cloned into CH1 at a specific restriction site, and this dodecapeptide only contains three exact matches and three conservative substitutions out of 12 residues, whereas the sequences of epitopes A and C were more divergent. For example, LtxA epitope A contains 3 consecutive tyrosines which are not found in the other RTXs for which there is no homologous structure in the other proteins. In the LtxA region, only the E. coli α-hemolysin has a corresponding sequence, and this dodecapeptide only contains three exact matches and three conservative substitutions with respect to the LtxA sequence. It therefore seems likely that the unique functional specificity of the LtxA gene product resides, at least in part, in the unique sequences of epitopes A and C. Moreover, despite the high level of sequence similarity among the four toxins in epitope B, antibodies directed against LtxA epitope B react only with the homologous RTX, suggesting either that the epitope B region is not accessible to antibody in the other toxins or that these similar sequences can assume alternate conformations. Taken together, these analyses of homologous sequences to the LtxA epitopes A, B, and C show that the unique specificity of the LtxA toxin for human leukocytes probably results from both sequential and conformational elements.

**DISCUSSION**

*Actinobacillus* is a member of the Family Pasteurellaceae, a group of nonenteric, fermenting, Gram-negative rods, which are of considerable importance in both human and veterinary medicine. Although various *Actinobacillus* species are found in animals, only *A. actinomycetemcomitans* is routinely cultured.
A. actinomycetemcomitans Leukotoxin

![Image of a page from a scientific document showing a table and text discussing the characterization of anti-leukotoxin monoclonal antibodies and a comparison of the amino acid sequences of epitopes A, B, and C with homologous regions of other RTX. A. actinomycetemcomitans leukotoxin epitope sequences (ltx) are compared with corresponding sequences from three other RTX A genes. Shown above are: E. coli a-hemolysin (hyt) (49); A. pleuropneumoniae hemolysin (hpp) (50), and P. hemoelytica leukotoxin (lkt) (61).](image)

![Image of a table showing the species specificity of LtxA/LktA chimeric toxins. Chimeric toxins constructed by splicing lka and ltx A genes at sites shown in Fig. 2 were compared for their ability to kill either HL-60 (human) or BL-3 (bovine) target cells. PP4 contains open areas which represent locations of major gaps in the lka sequence that occur when the lka and ltx A genes are aligned. Cytotoxicity was assessed by trypan blue exclusion.](image)

![Image of a table showing the inhibition of cytolytic activity of LtxA/LktA chimeric toxin, CH57, by anti-LtxA monoclonal antibodies.](image)

![Image of a figure showing the comparison of the amino acid sequences of epitopes A, B, and C with homologous regions of other RTX. A. actinomycetemcomitans leukotoxin epitope sequences (ltx) are compared with corresponding sequences from three other RTX A genes. Shown above are: E. coli a-hemolysin (hyt) (49); A. pleuropneumoniae hemolysin (hpp) (50), and P. hemoelytica leukotoxin (lkt) (61).](image)

The ability of a panel of monoclonal antibodies (30) to inhibit the cytolytic effects of native P. haemolytica (PP4) and A. actinomycetemcomitans (CH1) toxins and LtxA/LktA chimeric toxin (CH57). In these experiments an LD50 dose of toxin was incubated with anti-leukotoxin monoclonal antibody (30 min, 4°C) after incubation, the mixture was added to either 2 x 10^6 HL-60 cells (CH1 and CH57) or BL-3 (PP4) cells in 100 μL and incubated (45 min, 37°C). Cell viability was assessed by Trypan Blue exclusion, and results are reported as the mean and standard deviation of four assays. Results are expressed as percent kill of an untreated control.

**Table I**

| Antibody | CH57 | CH1 | PP4 |
|----------|------|-----|-----|
| 3        | 54.8 ± 1.1 | 49.2 ± 10.2 | 82.5 ± 4.8 |
| 13       | 57.0 ± 14.6 | 48.6 ± 12.6 | 46.5 ± 2.4 |
| 16       | 52.1 ± 8.2  | 30.3 ± 1.9  | 83.1 ± 7.0 |
| 28       | 46.9 ± 8.9  | 5.1 ± 3.1   | 84.4 ± 4.1 |
| 29       | 2.9 ± 0.5   | 21.2 ± 6.1  | 89.5 ± 3.7 |
| 31       | 0.5 ± 0.5   | 2.8 ± 2.7   | 87.2 ± 3.4 |
| 46       | 56.4 ± 14.5 | 5.0 ± 4.3   | 86.7 ± 11.1 |
| 53       | 66.8 ± 2.6  | 28.4 ± 12.6 | 83.9 ± 7.3 |
| 64       | 5.1 ± 5.1   | 23.0 ± 14.1 | 86.7 ± 7.0 |
| 82       | 16.4 ± 5.6  | 7.6 ± 7.6   | 86.2 ± 7.0 |
| 83       | 54.7 ± 5.6  | 16.8 ± 16.7 | 83.4 ± 4.7 |
| 107      | 15.9 ± 9.5  | 14.8 ± 13.3 | 84.7 ± 9.1 |

**Table II**

| Antibody | Isotype* | Peptide* | Epitope |
|----------|----------|----------|---------|
| 3        | IgG1,κ   | 75       | LTVLDYGDDRDLY |
| 13       | IgG1,κ   | 75       | LTVLDYGDDRDLY |
| 16       | IgG1,κ   | 75       | LTVLDYGDDRDLY |
| 28       | IgG1,κ   | 71       | KLYYYNTKGGK |
| 29       | IgG1,κ   | 90       | DRARKQRQFEQ |
| 31       | IgG1,κ   | 90       | DRARKQRQFEQ |
| 46       | IgG1,κ   | 75       | LTVLDYGDDRDLY |
| 53       | IgG1,κ   | 75       | LTVLDYGDDRDLY |
| 64       | IgG1,κ   | 90       | DRARKQRQFEQ |
| 82       | IgG1,κ   | 90       | DRARKQRQFEQ |
| 83       | IgG1,κ   | 90       | DRARKQRQFEQ |
| 107      | IgG1,κ   | 90       | DRARKQRQFEQ |

* Antibodies isotypes used by SBA Clototyping System I (Fisher Biotech, Pittsburgh, PA) according to manufacturer’s instructions.

**Fig. 6. Comparison of the amino acid sequences of epitopes A, B, and C with homologous regions of other RTX. A. actinomycetemcomitans leukotoxin epitope sequences (ltx) are compared with corresponding sequences from three other RTX A genes. Shown above are: E. coli a-hemolysin (hyt) (49); A. pleuropneumoniae hemolysin (hpp) (50), and P. hemoelytica leukotoxin (lkt) (61).**

and periodontal disease (13, 17, 41, 42). A. actinomycetemcomitans leukotoxin is of considerable interest, since it represents a potential virulence factor in infections caused by this organism and possesses a unique biological specificity, killing only cells of the monomyelocytic lineage of man and some higher non-human primates. Cloning and analysis of the leukotoxin operon represented an important first step toward explaining these unique properties (36). In the present study, we continued our investigations of this interesting molecule by constructing a series of A. actinomycetemcomitans/P. hemoelytica chimeric toxins to determine the region of the toxin that was responsible for the specificity of this molecule for human monomyelocytes.

Our studies provide novel information on the location and nature of the LtxA region that is necessary for recognition of the human target cells. The experimental data we have presented indicate that the region from residue 688 to residue 941 is required for recognition of the HL-60 target cells. The principal feature of the region is a series of tandemly repeated nonapeptides that have the consensus sequence GGXGXD-X(L/I)I/V/W/Y/F/X. Recently, the three-dimensional structure of another protein that contains these glycine-rich repeats, alkaline protease from Pseudomonas aeruginosa, has been solved (43), and analysis of the “repeats” has indicated that they form from humans. This organism has been associated with a variety of infectious disease processes in man including endocarditis, brain abscesses, osteomyelitis, subcutaneous abscesses,
a novel folded structure which has been named the "parallel β-roll." In the β-roll, the GGXXGXD motif forms a series of successive β-turns which are wound in continuous right-handed spirals. In addition to forming the β-turns, the GGXXGXD motif forms a series of Ca\(^{2+}\) binding sites. Individual X(L/D\(\rightarrow\)mally found in native clear; however, when one chimera was spliced within the repeat 673-726 of HylA. Since mAb D12 recognizes biologically active HL-60. The mechanism by which this is achieved is not yet the putative acylation site for LtxA. Rowe the most notable of which are a series of five metalloproteinases (13, 44-46). Second, the proteins which contain GGX-GXX(L;I/V;W;Y;F)X repeats also appear to utilize secretory proteins, such as the RTX B and D gene products for translocation to the bacterial cell surface rather than rely on the canonical signal sequence used by most bacterial proteins.

The current studies clearly indicate that the repeat region of LtxA is essential in forming a unique species recognition unit which is required for the P. hemolytica/Actinomyocetemcomitis Leukotoxin chimeric toxins to kill a human target cell line such as HL-60. The mechanism by which this is achieved is not yet clear; however, when one chimera was spliced within the repeat region (CH41), it failed to kill either target cell line. CH41 produced a recombinant toxin that contained nine GGXGXX(D[L;I/V;W;Y;F)]X repeats instead of the 14 repeats normally found in native Actinomyocetemcomitis leukotoxin. Far less drastic deletions have resulted in toxins that have different specificities or an increased requirement for Ca\(^{2+}\) in order to complete the lytic process (27).

Our epitope mapping studies have shown that a panel of 12 monoclonal antibodies has defined three distinct linear epitopes within residues 688-941. The three regions have been designated epitope A, epitope B, and epitope C. Epitope A (KGKLDDYNTKGFK)\(^{(69)}\) was recognized by one monoclonal antibody (mAb 28) from the panel and is located 12 residues from the initiation of the first GGXGXXDX(L;I/V;W;Y;F)X repeat. Studies with other RTX indicate that epitope A could be the putative acylation site for LtxA. Rowe et al. (47) have described an erythrogenic-neutralizing monoclonal antibody to HyLA (mAb D12) which recognizes an epitope within residues 673-726 of HyLA. Since mAb D12 recognizes biologically active HyLA, but not HyLA, produced in the absence of HyLC, these observations have led to the conclusion that the mAb D12 epitope contains the HyLA acylation site. Analysis of the D12 epitope with deletion analysis mutants suggests that it may be linear and formed by residues 684-701. Utilizing a panel of noncleavable leukotoxin peptides, we have shown that LtxA mAb 28 clearly is linear and overlaps with corresponding residues described by the D12 epitope. Studies are currently being done to determine the relationship of epitope A and the LtxA acylation site.

Epitope B (KGKLDDYNTKGFK)\(^{(69)}\) is located within the GGXGXXDX(L;I/V;W;Y;F)X repeat region and is recognized by six monoclonals from our panel (mAbs 3, 13, 16, 46, 53, 83). All of these antibodies bind an epitope that is composed of the β-strand (KGKLDDYNTKGFK)\(^{(69)}\) from the third repeat and the entire fourth repeat (GYYGDDRLY)\(^{(57)}\). It is not clear at the present time as to why this was the only repeat of the 14 repeats recognized by our monoclonal antibodies. An obvious explanation is that the fourth repeat is playing a crucial role in target cell recognition and for this reason we were able to identify six neutralizing monoclonal antibodies which reacted with it. An alternative explanation is that the fourth repeat is located on the surface of the leukotoxin molecule, whereas other repeats are found internally. We are currently examining this point by constructing a series of chimeric toxins where LtxA repeats will have been replaced with repeats from another RTX. If the chimeric toxins continue to kill HL-60 cells we can conclude that the fourth repeat is simply located on the surface of the molecule. On the other hand, loss of toxicity in chimeras with repeat substitutions could mean that this area of the repeats could be playing a role in target cell recognition.

Pellett et al. (48) have reported an epitope recognized by anti-HyLA mAb A10 which also lies within the GGXGXXDX(L;I/V;W;Y;F)X repeat region of HyLA. Utilizing HyLA deletion mutants, the epitope of this mutant is reported to be somewhere between residues 745 and 829 (repeats 4 and 11) of HyLA. However, several differences exist between mAb A10 and the six LtxA mAb presented above. First, the HyLA antibody is a "pan-reactive"antibody which recognizes several additional members of the RTX family. Anti-Ltx mAbs 3, 13, 16, 46, 53, and 83 do not recognize the P. hemolytica leukotoxin, nor do they recognize any of the other 13 repeats within LtxA in spite of a similarity in sequence. Furthermore, the HyLA antibody is non-neutralizing, whereas the anti-Ltx antibodies all neutralize the effects of the A. actinomyocetemcomitis leukotoxin. Until the epitope of E. coli α-hemolysin is more clearly defined, it is impossible to determine if these antibodies are recognizing the same or different epitopes. If in fact mAb A10 does recognize the fourth glycine-rich repeat of HyLA as its anti-Ltx counterparts do, it would indicate that this repeat does play a critical role in target cell recognition by A. actinomyocetemcomitis leukotoxin, but not E. coli α-hemolysin.

The final five monoclonal antibodies recognized a sequence mapped to KGKLDDYNTKGFK\(^{69}\). Epitope C begins 78 residues after the terminal (14th) repeat. Comparison of amino acid sequences from this epitope with corresponding sequences from other RTX with LtxA indicates that considerable diversity of structure exists in this region for various members of the RTX family. LtxA and AppA are gapped when compared with LtxA. Although the HyLA and LtxA sequences are homologous in 3 of 12 residues, deletion mutants of LtxA which terminate before this epitope (residue 910) will not kill target cells, whereas those which terminate at a Sulf site in the gene (residue 941) are toxic for HL-60 target cells. This indicates that the region encompassing this epitope is critical for the maintenance of cytolysis.\(^2\)

In this study, we have successfully utilized chimeric toxins to show that insertion of the A. actinomyocetemcomitis leukotoxin gene into the P. hemolytica leukotoxin gene will result in a switch of the toxin from killing bovine to human target cells. The portion of the gene which is critical for this determination of target cell specificity is a series of glycine-rich repeats and residues which flank the repeats on both the amino- and carboxyl-terminal ends. The mechanism by which the repeats affect the species specificity of RTX is not clear at the present time; however, some form of a species recognition unit must exist, as chimeras with splice sites within the repeat region do not kill either bovine or human target cells. Our epitope mapping studies have defined three epitopes which are necessary for toxigenic functions. The combination of our chimeric toxins and the characterization of our mAb panel have provided important preliminary data and critical reagents for further study of toxigenic function.

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\(^2\) E. T. Lally, unpublished observations.
