

## Antibody Mapping to Domains of Botulinum Neurotoxin Serotype A in the Complexed and Uncomplexed Forms

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**The domain organization of the botulinum neurotoxin serotype A was studied by using antibody mapping of 44 monoclonal single-chain variable fragments. The analysis was carried out on (i) the individual domains of botulinum neurotoxin holotoxin (binding, translocation, and catalytic), (ii) botulinum neurotoxin holotoxin, (iii) the botulinum neurotoxin holotoxin in complex with the nontoxic portion, and (iv) botulinum neurotoxin holotoxin and nontoxic portion of the complex recombined in vitro. All 44 antibodies mapped to individual domains of botulinum neurotoxin. Forty of the 44 single-chain variable fragments bound the botulinum neurotoxin holotoxin relative to the isolated domains, suggesting that 4 epitopes are covered when the individual domains are in the holotoxin form. Only 20 of the antibodies showed a positive reaction to the toxin while in complex with the nontoxic portion. All of the covered epitopes were mapped to the binding domain of botulinum neurotoxin, which suggested that the binding domain is in direct contact with the nontoxic portion in the complex. Based on the antibody mapping to the different domains of the botulinum neurotoxin holotoxin and the entire complex, a model of the botulinum neurotoxin complex is proposed.**

The anaerobic bacterium *Clostridium botulinum* produces seven serotypes of neurotoxin, classified A through G (26). The neurotoxin, serotype A, can be purified as a 900-kDa complex consisting of a 150-kDa toxic component (botulinum neurotoxin [BT]), and a 750-kDa nontoxic component (hemagglutinin [HA]) (3). The BT inhibits cholinergic vesicle docking at the neuromuscular junction, resulting in flaccid paralysis (23), and most commonly intoxicates by oral ingestion. The three 50-kDa functional domains of BT—binding (13), translocation (2), and catalytic (1a)—allow the toxin to bind to a cell surface receptor, pass across the membrane (23), and cleave a protein involved in vesicle docking, respectively (9). Sugii and coworkers (24) have shown that the HA-BT complex has a higher oral toxicity in rats than the BT alone. The 750-kDa HA has been shown to have an agglutination ability (15) and is thought to protect the toxin from the extreme pH and proteases in the gut (25).

Little structural information is known about BT, HA-BT, or the interaction between BT and HA. The literature contains examples of antibodies used to detect various serotypes of BT (7, 12, 14, 27), but no mapping of the BT domains in the HA-BT complex has been carried out. Sugiyama and coworkers (27) showed that polyclonal antibodies to type A HA-BT complex recognized epitopes predominantly from the HA and not the BT. Monoclonal antibodies developed by Kozaki and coworkers recognized the light chain of the BT that causes infant botulism and the light chain of BT serotype A (14). However, the antibodies to the infant botulism BT heavy chain did not recognize the BT serotype A heavy chain. Studies using

polyclonal antibodies to the toxin detected BT at very low concentrations but did not provide specific information about the relationship between the BT and HA (7, 12). We probed the structure of the HA-BT complex by using a panel of 44 unique monoclonal single-chain variable fragments (scFv) derived from combinatorial phage antibody libraries (1, 28).

We used enzyme-linked immunosorbent assays (ELISAs) to identify 44 scFv that bind to different domains of botulinum neurotoxin serotype A. ELISAs were performed on purified BT, the purified BT domains, HA, HA-BT complex, and recombined HA-BT in vitro. Based on our results, we propose a model to illustrate the interaction between BT and HA. The model could act as a guide for the design of neutralizing antibodies and may explain how the HA protects the BT from proteolytic and pH attack.

### MATERIALS AND METHODS

**Purification of HA-BT complex, BT, and BT domains.** The HA-BT complex (Hal strain) was obtained as an ammonium sulfate precipitate from purified bacterial supernatant at a concentration of 3.3 mg/ml in 50 mM sodium citrate (pH 5.5) (5). Before use, the HA-BT complex was centrifuged at  $26,890 \times g$  (Dupont Sorvall RC-5B centrifuge) for 15 min and dialyzed against saline (0.68 M sodium citrate, 0.145 M NaCl [pH 7.4]) with three buffer exchanges within an hour. Concentration was determined by  $A_{278}$  measurements (1.66 arbitrary units/mg ml<sup>-1</sup>), using a Shimadzu UV-160 spectrophotometer (11).

The HA was purified in two steps by using a modification of a published procedure (4). Forty-five milligrams of ammonium sulfate precipitate of HA (0.42 g/ml) was centrifuged at  $26,890 \times g$ . The pellet was dissolved in 20 ml of 70 mM Tris-HCl (pH 7.2) and dialyzed overnight. The dialyzed solution was centrifuged at  $26,890 \times g$  for 15 min and applied onto a DEAE-Sepharose column (1.5 by 24 cm; Pharmacia, Uppsala, Sweden) that was equilibrated with 70 mM Tris-HCl (pH 7.2). The column was washed with 100 ml of 70 mM Tris-HCl (pH 7.2) and the HA was eluted with 70 mM Tris-HCl-0.2 M NaCl (pH 7.2). The fractions containing HA were combined and run on an SP-Sepharose column (1.5 by 21 cm) (Pharmacia, Uppsala, Sweden) equilibrated with 70 mM Tris-HCl (pH 7.2). Since residual BT adheres to the column matrix at this pH, the HA was collected in the flowthrough. The concentration of protein was determined by  $A_{278}$  measurements (11).

The BT was purified as described previously (5) and stored as a 10-mg/ml solution in 10 mM HEPES (pH 7.0)-0.1 M KCl-2 mM sodium azide. The binding domain of BT type A, expressed in *Escherichia coli* and purified by immobilized metal affinity chromatography using a C-terminal His<sub>6</sub> tag, was

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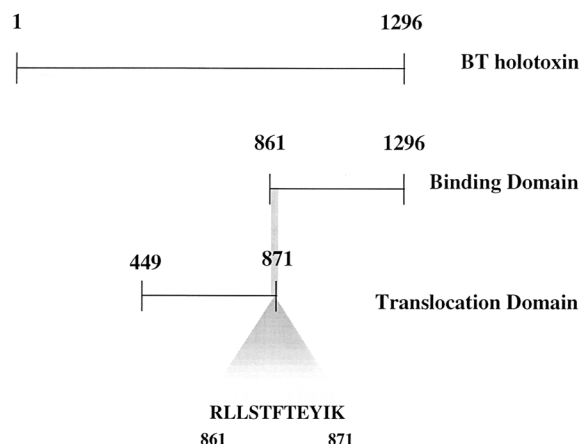


FIG. 1. Diagram illustrating the sequence overlap between the translocation and binding domain constructs. The top line represents the sequence of BT from the N terminus (residue 1) to the C terminus (residue 1296). The bottom two lines depict the C-terminal 11 residues from the translocation domain construct overlapping the N-terminal 11 residues of the binding domain (18, 19).

purchased from Ophidian Pharmaceuticals, Inc. (Madison, Wis.). The translocation domain (residues 449 to 872) of BT type A was expressed in *E. coli* and purified by immobilized metal affinity chromatography using a C-terminal His<sub>6</sub> tag (14a). The 11 C-terminal residues of the translocation domain overlap the 11 N-terminal residues of binding domain (18, 19) (Fig. 1). The polypeptide composition of each of the different batches of purified protein was analyzed on 12% polyacrylamide gels as described by Fling and Gregerson (8).

**Antibodies.** ScFv antibody fragments were selected from four different combinatorial phage antibody libraries (1, 28). Briefly, scFv phage antibody libraries were constructed from the immunoglobulin heavy (V<sub>H</sub>)- and light (V<sub>L</sub>)-chain variable regions of mice immunized with purified holotoxin (library 1); mice immunized with binding domain (library 2); humans immunized with pentavalent botulinum toxoid (Centers for Disease Control and Prevention) (library 3); nonimmunized human volunteers (library 4). Libraries 1 and 3 were constructed in the vector pCANTAB5E (Pharmacia), and library 2 was constructed in pHEN-1 (10). Specific scFv were isolated by selecting the libraries on either holotoxin or binding domain immobilized on polystyrene or in solution. The specificity of the isolated antibodies for the holotoxin or the binding domain was confirmed by ELISA on the relevant antigen and a panel of irrelevant antigens (1, 28). The number of unique scFv was determined by *Bst*N1 fingerprinting, followed by DNA sequencing of the V<sub>H</sub> and V<sub>L</sub> genes. Additional unique scFv were isolated from a 9.7 × 10<sup>9</sup>-member nonimmune library in pHEN-1 (10) constructed from human V<sub>H</sub> and V<sub>L</sub> genes.

For structural mapping of the HA-BT complex, BT, and BT domains by ELISA, native scFv was expressed from the appropriate phagemid in *E. coli* HB2151 (10). The amber codon between the scFv gene and gene 3 permits expression of native scFv in a nonsuppressor *E. coli* strain (HB2151). scFv binding was detected by using the epitope tag at the C terminus of the scFv (E tag for scFv in pCANTAB5E and Myc tag for scFv in pHEN-1). Since both the scFv in pHEN-1 and the translocation domain have a C-terminal Myc tag, ELISA on the translocation domain was performed with scFv fused to phage, and detection was achieved by using HRP/anti-M13 conjugate (Pharmacia).

Expression of native scFv (6) in the phagemid vectors (pHEN-1 and pCANTAB5E) was performed in 96-well microtiter plates as described previously (16), with the following exception: after overnight growth and expression at 25°C, 50 μl of 0.5% Tween 20 was added to each well and the plates were incubated for 4 h at 37°C with shaking to induce bacterial lysis and increase the concentration of scFv in the bacterial supernatant. Supernatants containing native scFv were used for ELISA. To prepare phage for ELISA, single ampicillin-resistant colonies were transferred into microtiter plate wells containing 100 μl of 2×YT medium (16 g of Bacto Tryptone, 10 g of Bacto Yeast Extract, 5 g of NaCl, 1 liter of deionized H<sub>2</sub>O) supplemented with 1 mM ampicillin (Sigma, St. Louis, Mo.) and 0.1% glucose. After 3 h of growth at 37°C to an A<sub>600</sub> of approximately 0.5 arbitrary unit, VCSM13 helper phage (2.5 × 10<sup>8</sup> phage particles) was added, and the cells were incubated for 1 h at 37°C. Subsequently, kanamycin was added to a final concentration of 25 μg/ml, and the bacteria were grown overnight at 37°C. Supernatants containing phage were used for ELISA.

**ELISA.** Microtiter plates (Falcon 3912) were incubated with 50 μl of antigen (10 μg/ml except for the binding domain, in which case 5 μg/ml was used) in phosphate-buffered saline (PBS; 25 mM NaH<sub>2</sub>PO<sub>4</sub>, 125 mM NaCl [pH 7.0]) at 4°C overnight. After being washed once with PBS, wells were incubated with 50 μl of bacterial supernatant containing either native scFv or scFv fused to phage. Myc-tagged scFv were detected with mouse monoclonal antibody 9E10 (1 μg/ml;

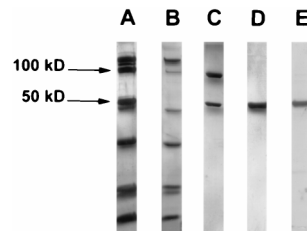


FIG. 2. SDS-PAGE analyses of column chromatography. Lanes: A, HA-BT complex; B, purified HA; C, purified BT; D, purified binding domain; E, purified translocation domain.

Santa Cruz Biotechnology) (17), and E-tagged scFv were detected by using anti-E tag antibody (1 mg/ml; Pharmacia), followed by peroxidase-conjugated anti-mouse Fc antibody (Sigma), with 2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid) as the substrate as described previously (21). Binding of scFv phage to antigens was detected with peroxidase-conjugated anti-M13 antibody (Pharmacia) as described elsewhere (20).

**Recombination.** The purified BT and HA were incubated at a 1:1 molar ratio for at least 24 h in 70 mM Tris-HCl (pH 7.2) at a protein concentration of 0.1 mg/ml. The mixture was stored at 4°C until diluted in PBS for coating ELISA plates.

## RESULTS

**Purification of antigens.** After column chromatography, the purities of HA-BT complex, BT, and HA were ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to be relatively free of contaminants (Fig. 2). The BT appeared as two bands, separated due to the reducing environment of the gel loading buffer—a 50-kDa light chain consisting of catalytic domain and a 100-kDa heavy chain consisting of translocation and binding domains. The HA-BT complex runs as nine bands that make up the BT and large HA. Purified HA appears identical to the HA-BT complex minus the 50-kDa polypeptide from the light chain of BT. A small amount of 100-kDa band from the heavy chain of BT was present in the HA. This residual BT was not enough to produce a positive signal for HA on ELISA. Binding and translocation domains, expressed in *E. coli* were also tested for purity by SDS-PAGE.

**Antibody isolation and initial characterization.** scFv were isolated by selection of phage libraries on immobilized purified BT or purified recombinant binding domain. The antibodies recognize only native protein and not denatured protein. Thus, the antibodies bound according to structure and not sequence libraries (1, 28). After three rounds of selection, initial scFv characterization by ELISA on BT and DNA sequencing of the V<sub>H</sub> and V<sub>L</sub> genes yielded 44 unique scFv (Table 1).

**Structural mapping of BT, HA-BT complex, and BT domains.** The forty-four scFv recognized the individual domains of BT, and none bound to HA alone (Table 1). Of the 44 scFv, 24 mapped to the binding domain and 3 mapped to the translocation domain (Table 1). In addition, two antibodies mapped to both the binding and translocation domains. These two scFv presumably bound to the 11-amino-acid overlap at the C terminus of the translocation domain and the N terminus of the binding domain (Fig. 1). Alternatively, cross-reactivity could result from binding to a four-amino-acid sequence (-KYVD-) that is homologous for residues 855 to 858 of the translocation domain and 1121 to 1124 of the binding domain. Thus, 26 scFv recognized the binding domain and 5 bound the translocation domain construct. The remaining 15 scFv presumably bound to the catalytic domain, though this was not tested directly due to lack of purified catalytic domain. Some of these 15 may recognize epitopes that are shared between domains.

TABLE 1. ELISA absorbances of scFv to type A neurotoxin<sup>a</sup>

| Antibody | OD <sub>405</sub> (avg ± SD) |               |                |                      |               |
|----------|------------------------------|---------------|----------------|----------------------|---------------|
|          | BT                           | HA-BT         | Binding domain | Translocation domain | RR            |
| 3d12     | 2.303 ± 0.682                | 0.659 ± 0.072 | 2.260 ± 0.489  |                      | 1.181 ± 0.303 |
| 3a6      | 2.053 ± 0.768                | 0.660 ± 0.083 | 1.775 ± 0.263  |                      | 1.160 ± 0.322 |
| 3d4      | 0.964 ± 0.192                | 0.913 ± 0.192 |                |                      | 1.091 ± 0.108 |
| 4a4      | 0.950 ± 0.135                | 0.905 ± 0.045 |                |                      | 1.195 ± 0.071 |
| 3a2      | 1.091 ± 0.192                | 0.936 ± 0.138 |                |                      | 1.204 ± 0.234 |
| 3e3      | 1.106 ± 0.170                | 0.987 ± 0.158 |                |                      | 1.229 ± 0.255 |
| 3e8      | 1.058 ± 0.358                | 0.941 ± 0.097 |                |                      | 1.145 ± 0.245 |
| 3a11     | 1.036 ± 0.298                | 0.934 ± 0.108 |                |                      | 1.248 ± 0.157 |
| 3e7      | 1.022 ± 0.262                | 0.945 ± 0.156 |                |                      | 1.218 ± 0.153 |
| 3h3      | 1.143 ± 0.396                | 0.838 ± 0.380 |                |                      | 1.037 ± 0.316 |
| 3a1      | 1.019 ± 0.300                | 0.943 ± 0.148 |                |                      | 1.206 ± 0.167 |
| w3       | 2.083 ± 0.523                | 1.544 ± 0.192 |                |                      | 1.925 ± 0.267 |
| w42      | 1.796 ± 0.409                | 1.400 ± 0.223 |                |                      | 1.721 ± 0.270 |
| g23      | 1.875 ± 0.403                | 1.460 ± 0.194 |                |                      | 1.795 ± 0.183 |
| g3       | 1.287 ± 0.329                | 0.890 ± 0.298 |                |                      | 1.290 ± 0.174 |
| g11      | 1.200 ± 0.326                | 0.956 ± 0.192 |                |                      | 1.285 ± 0.100 |
| w7       | 1.552 ± 0.269                | 1.199 ± 0.273 |                | 1.182 ± 0.121        | 1.606 ± 0.393 |
| w9       | 1.492 ± 0.376                | 1.239 ± 0.247 |                | 1.201 ± 0.152        | 1.510 ± 0.194 |
| g7       | 1.072 ± 0.481                | 1.106 ± 0.327 |                | 0.590 ± 0.105        | 1.422 ± 0.261 |
| w20      | 1.557 ± 0.530                |               | 1.713 ± 0.349  |                      | 1.089 ± 0.053 |
| w36      | 1.302 ± 0.418                |               | 1.362 ± 0.300  |                      | 0.680 ± 0.026 |
| w43      | 1.421 ± 0.253                | 1.002 ± 0.109 |                |                      | 1.283 ± 0.334 |
| g53      | 1.193 ± 0.321                |               | 1.700 ± 0.242  |                      | 0.685 ± 0.095 |
| g57      | 1.647 ± 0.409                |               | 1.423 ± 0.521  |                      | 0.761 ± 0.117 |
| c9       | 1.560 ± 0.460                |               | 1.690 ± 0.229  |                      | 0.889 ± 0.213 |
| c15      | 1.936 ± 0.535                |               | 1.988 ± 0.458  |                      | 1.079 ± 0.358 |
| s25      | 1.395 ± 0.363                |               | 1.369 ± 0.435  |                      |               |
| 3f6      | 1.332 ± 0.338                |               | 1.553 ± 0.272  |                      | 0.811 ± 0.142 |
| 2a2      | 0.816 ± 0.297                |               | 1.172 ± 0.262  |                      | 0.838 ± 0.033 |
| 2b10     | 0.858 ± 0.332                |               | 1.204 ± 0.408  |                      | 0.661 ± 0.143 |
| 2b1      | 0.836 ± 0.121                |               | 1.247 ± 0.278  | 1.227 ± 0.505        | 0.566 ± 0.010 |
| 3e6      | 0.909 ± 0.109                |               | 1.190 ± 0.135  |                      | 0.605 ± 0.165 |
| 2e6      | 0.838 ± 0.181                |               | 1.046 ± 0.171  |                      |               |
| 3d1      | 0.908 ± 0.192                |               | 1.009 ± 0.385  |                      | 0.752 ± 0.072 |
| 2b6      | 0.652 ± 0.298                |               | 0.711 ± 0.213  |                      |               |
| 2h6      | 1.010 ± 0.326                |               | 1.261 ± 0.346  |                      | 0.928 ± 0.004 |
| 2a8      | 0.994 ± 0.275                |               | 1.069 ± 0.398  |                      | 0.908 ± 0.226 |
| id5      | 0.738 ± 0.289                |               | 1.273 ± 0.228  |                      | 0.716 ± 0.085 |
| ie8      | 0.556 ± 0.339                |               | 0.921 ± 0.513  | 1.000 ± 0.405        | 0.654 ± 0.057 |
| ig7      | 0.834 ± 0.174                |               | 1.113 ± 0.313  |                      | 0.688 ± 0.077 |
| 3c3      |                              |               | 0.621 ± 0.026  |                      |               |
| 2c3      |                              |               | 0.740 ± 0.065  |                      |               |
| 3e2      |                              |               | 0.427 ± 0.089  |                      |               |
| 3c5      |                              |               | 0.743 ± 0.204  |                      |               |

<sup>a</sup> Values obtained with antigen coated at 10 µg/ml except for the binding domain, which was coated at 5 µg/ml. OD<sub>405</sub>, optical density at 405 nm; RR, recombination of purified BT and HA. The antibodies were produced in the lab of James D. Marks. The values represent the averages of 13 plates coated with BT, 10 plates coated with HA-BT complex, 6 plates coated with binding domain, 6 plates coated with translocation domain, 5 plates coated with HA, and 4 plates coated with recombined BT and HA. The background was defined as the signal from plates containing antigen and primary and secondary antibodies. Values represent absorbances after background absorbance is subtracted. Blanks in columns represent absorbances below three times the background absorbance. These scFv were assumed not to bind the corresponding antigen. Since the ELISAs were performed using different batches of supernatant, the average absorbance of each plate was normalized to the average absorbance of the first plate for a particular antigen.

Forty scFv bound to the holotoxin (Table 1). Thus, four epitopes were covered when the individual domains came together to form the BT. Of these 40 scFv, 22 mapped to the binding domain and 3 mapped to the translocation domain. The remaining 15 scFv were deduced to recognize the catalytic domain (Table 1).

ELISA of scFv on the HA-BT complex permitted identification of BT epitopes which were inaccessible in the HA-BT complex. Twenty epitopes were covered when in the HA-BT complex. All of these covered epitopes were localized to the binding domain. Of the 22 scFv that mapped to the binding domain and BT, only 2 scFv bound to the HA-BT complex

(Table 1). The three scFv that bound to translocation domain and BT also bound to the HA-BT complex (Table 1). The 15 scFv that mapped to catalytic domain in BT likewise bound to the HA-BT complex (Table 1).

## DISCUSSION

Using antibodies to map the different domains of botulinum neurotoxin serotype A, we propose a model illustrating how the toxin may bind into the HA assembly. Forty-four scFv were produced to the BT and its domains. The antibodies specific to individual domains were used to map relative positions of

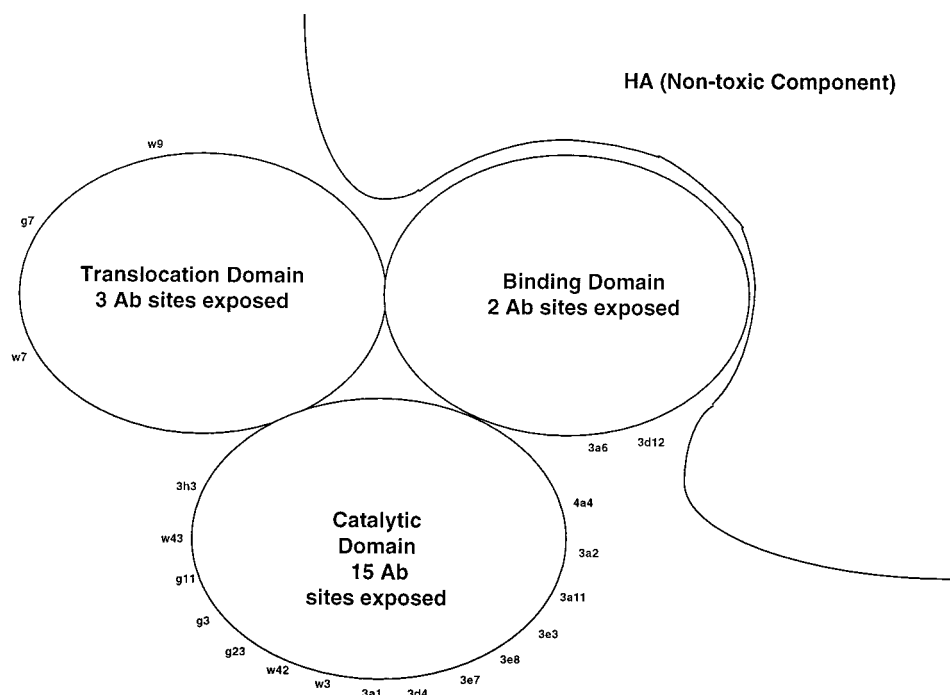


FIG. 3. Representation of a possible arrangement of BT with respect to HA. Only clones which bind the HA-BT complex are shown. The binding and translocation domains are covered partially by HA. Ab, antibody.

exposed or hidden epitopes in the BT and in the HA-BT complex. All of the antibodies specific to the translocation domain also recognized the HA-BT, indicating that the translocation domain is as exposed to the solvent in the complex (Fig. 3) as in the holotoxin (Table 1).

Not all of the scFv which bound the individual domains bound the BT holotoxin. Twenty-two of the 26 antibodies that recognized binding domain also recognized the BT. Thus, 4 antibody sites on the binding domain are covered by the catalytic or translocation domain in the BT (Table 1). However, not all of these scFv bound when the BT was complexed with the HA. Of the 22 exposed sites of binding domain on BT, only 2 bound the HA-BT complex. Therefore, the majority of antibodies to the binding domain recognize epitopes that must be covered by HA in the HA-BT complex (Fig. 3).

Fifteen antibodies were deduced to bind the catalytic domain. These antibodies were inferred by subtracting the antibodies that bound the binding and translocation domains from the antibodies that bound BT, since it is assumed that all of the antibodies must bind a domain of BT. All of these 15 antibodies bind an epitope that is accessible in the HA-BT complex. These 15 antibodies may bind conformational epitopes shared between domains. Such epitopes may not exist in the separate domains.

The model illustrated in Fig. 3 is subject to several caveats. Since the sequences of the epitopes are unknown, it is not possible to know the distribution of antibody binding sites. Hence the epitopes could be distributed evenly on the exposed surfaces or could be concentrated in certain regions of the protein. If the epitopes are clustered together, the area of BT covered by HA may be overestimated. If the epitopes are spaced regularly, the area of BT covered by HA may be underestimated. In either case, verification requires mapping of these antibodies to specific sequences of BT. Finally, since the model is drawn in two dimensions, it may not depict accurately

the surface area of BT covered by HA. In addition, the arrangement of exposed and unexposed epitopes may be different from that diagrammed. The representation shows contiguous groups of exposed or unexposed epitopes that may be commingled.

The recombination experiments were performed to determine whether purified BT would interact with purified HA to reform the stable HA-BT complex at physiological pH. Since HA was undetectable by any of the antibody clones, the clones that bound to recombined HA-BT must bind exposed regions of BT. If no reconstitution of the complex occurred, recombined HA-BT should show the same number of clones as purified BT. If the HA-BT complex was formed, then recombined HA-BT should exhibit a positive reaction with the same clones as the purified HA-BT complex. The results from Table 1 indicate that an incomplete recombination took place. Whereas 40 clones recognized BT and 20 clones recognized the HA-BT complex, 37 clones bound recombined HA-BT. The three clones found in recombined HA-BT but not in the BT complex (s25, 2e6, and 2b6) are specific to the binding domain. Thus, under the recombination conditions, BT and HA do not fully reassemble. Some of the binding domain is left uncovered by HA.

Surprisingly, all of the epitopes that were covered in the HA-BT complex were mapped to the binding domain, strongly suggesting that the interactions between HA and BT are mediated by the binding domain. This idea is biologically relevant, since uncomplexed BT is susceptible to trypsin cleavage at the binding domain (22). Furthermore, the trypsinized BT could not bind to brain synaptosomes. When uncomplexed BT was incubated with trypsin, the translocation and catalytic domains showed no sign of proteolysis. Therefore, in the HA-BT complex, the HA may protect the binding domain of BT from proteolytic attack. These observations may guide in developing

antibodies for therapeutic design of neutralizing antibodies against botulism poisoning.

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