INFECTION AND IMMUNITY, Jan. 1993, p. 289–294
0019-9567/93/010289-06$02.00/0

Vol. 61, No. 1

Epitope Mapping and Characterization of Antigenic Determinants of Heat-Stable Enterotoxin (STh) of Enterotoxigenic *Escherichia coli* by Using Monoclonal Antibodies

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Received 24 July 1992/Accepted 4 November 1992

A panel of monoclonal antibodies (MAbs) specific for the heat-stable enterotoxin (STh) of enterotoxigenic *Escherichia coli* was produced. All four MAbs (8G7, 53-4, 11C, and SH1) bound to native STh in an enzyme-linked immunosorbent assay to various degrees, with clone SH1 showing the best affinity. The MAbs were screened for neutralizing and guanylate cyclase-inhibiting activities by the suckling mouse assay and the cyclic GMP assay using T84 cells, respectively. The contact amino acid residues governing the reactivity of the four MAbs were precisely determined by using several chemically synthesized analogs of the various heat-stable enterotoxins (STa's). Three distinct antigenic sites of STh sufficiently removed from each other, one near the N terminus, another in the core functional region of the toxin, and the third in the C-terminal region, were recognized by the different MAbs. MAb SH1, which recognized Asn at position 4 and Tyr at position 5 from the N terminus was 100 times more potent in neutralizing the bioactivity of STh in the suckling mouse assay than was MAb 11C, which recognized Thr at position 16 and Tyr at position 19 from the N terminus of the STh molecule. The MAbs which recognized Leu at position 9 from the N terminus (MAb 53-4) and Tyr at position 19 from the N terminus (MAb 8G7) showed intermediate activities in the neutralization assay. The guanylate cyclase-inhibiting activities of SH1 and 11C essentially paralleled the results for the neutralization of bioactivity, while MAbs 53-4 and 8G7 exhibited reverse activity. These results indicate that MAbs that recognize the N-terminal residues which have been shown not to be essential for toxic activity have a potent protective capacity. None of the MAbs reacted with reduced and carboxy-methylated native STh. This suggests that all of the MAbs mediate their effect by reacting with conformation-dependent antigenic determinants.

Heat-stable enterotoxins of enterotoxigenic *Escherichia coli* are classified into two structurally, functionally, and immunologically unrelated types, namely, STa or STI and STb or STII (6, 29). STa includes methanol-soluble infant mouse-active peptide toxins, while STb is methanol insoluble and active in weaned pigs but inactive in infant mice. The STa's constitute a family of nonimmunogenic, low-molecular-weight peptide toxins that mediate a secretory type of diarrhea by activating the guanylate cyclase-cyclic GMP system, resulting in inhibition of sodium and chloride absorption, along with stimulation of anion and water secretion (8). The mode of biological action of STb is still unclear (14).

It has been established that several enteric bacteria produce structurally homologous and antigenically cross-reacting STa's. The STa family consists of at least seven distinct types which include two different STa's from strains of *E. coli* of human (STh) and porcine (STp) origins (1, 19), NAG-ST from *Vibrio cholerae* non-O1 (4, 32), H-ST from Hakata strains of *V. cholerae* non-O1 (2), M-ST from *V. mimicus* (3), Y-ST from *Yersinia enterocolitica* (20, 21), and C-ST from *Citrobacter freundii* (10). A more recent inclusion in this family is the O1-ST detected from a cholera toxin gene-positive strain of *V. cholerae* O1 (25). The cloned gene of O1-ST was expressed in *E. coli* K-12, and the amino acid sequence of mature O1-ST was found to be very similar to that of NAG-ST, except for an additional amino acid (Leu) at the N terminus (24), and identical to that of H-ST (2). The biochemical, physicochemical, and immunological properties and amino acid compositions of the various STa's characterized so far are remarkably similar. The STa's have common highly conserved regions with 10 amino acid residues, including 6 cysteine residues, that are located in the same relative positions and linked intramolecularly by three disulfide bonds, suggesting that these enterotoxins have similar tertiary structures (17). Furthermore, the secretory potency and heat stability of the STa's have been found to be dictated by this core conserved sequence (17, 30, 31).

Monoclonal antibodies (MAbs) against *E. coli* STa's (5, 11, 18, 22) and against NAG-ST/Y-ST (23) have been developed, and these have proved to be valuable and sensitive reagents in the development of immunoassays (7, 18, 27). Although much is known about the structure-activity relationship of the various STa's, there is a conspicuous dearth of information on the antigenic region(s) and on the confor-

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mation of STa's necessary for antibody recognition. Therefore, this study was undertaken to identify epitopes within the STh molecule by using a panel of MAbs of different specificities and thereby determine the antigenic topology of this intriguing peptide toxin.

MATERIALS AND METHODS

Purification of native STh. STh was purified to homogeneity from *E. coli* SK1 by chromatography on cation and anion exchangers and on a preparative Lichrosorb RP-8 column by high-performance liquid chromatography as detailed elsewhere (1). The minimum effective dose of the purified toxin necessary to cause fluid accumulation in suckling mice, defined as 1 mouse unit, was 2.5 ng.

MAbs used for epitope mapping. The MAbs used in this study were 8G7, 11C, 53-4, and SH1. Production of MAb 8G7 was previously reported by Hemelhof et al. (11). MAb 53-4 was produced for use in an enzyme-linked immunosorbent assay (ELISA) kit to detect STa's, and the kit has been comprehensively evaluated (7, 16). MAbs 11C and SH1 were produced in this study. The procedure used to produce these two MAbs was essentially similar to that described previously (23). In brief, these MAbs were produced by immunizing BALB/c mice with polymerized STh (28). The immunoglobulin of the MAb was purified from ascites, and its concentration was calculated from the A_{280} of the samples. The immunoglobulin class of each MAb was determined by the Ouchterlony double-diffusion test with commercially available specific anti-mouse isotyping sera (ICN Immunobiologicals, Lisle, Ill.).

Titration and neutralizing activities of the MAbs. Titration of the binding of MAbs to the solid-phase STh and the neutralization test were performed as described previously (23). Briefly, a 100-μl portion of each severalfold serially diluted MAb was added to each well previously coated with native STh (1 μg/ml) and 0.5% bovine serum albumin. The immune reaction was performed as described earlier (23). The capacity of each MAb to neutralize the biological activity of native STh was evaluated by the suckling mouse assay (26). The concentration of purified STh was adjusted to 40 mouse units (100 ng) in 1 ml of phosphate-buffered saline (PBS), pH 7.2. For the neutralization assay, equal volumes of the STh solution were incubated with various dilutions of anti-STh MAbs for 15 min at 37°C. The sample (100 μl) was then introduced intragastrically into a suckling mouse. After 3 h, the mouse was sacrificed and the fluid accumulation ratio was calculated as the ratio of the weight of the entire intestine to that of the rest of the body. A fluid accumulation ration of ≥0.09 was considered to indicate a positive response. Each experiment was performed in duplicate, and the fluid accumulation ratio was determined as the mean value of at least three experiments.

Inhibition of guanylate cyclase activity. The capacity of the MAbs to inhibit the guanylate cyclase activity of purified STh was assessed by using T84 human colonic epithelial cells as described by Guarino et al. (9). Confluent monolayers of T84 cells were prepared in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4), 1.2 g of $NaHCO_3$, 40 mg of penicillin, 8 mg of ampicillin, and 90 mg of streptomycin per liter (HAM-DM) plus 5% fetal bovine serum in 24-well plates. The growth medium was aspirated and replaced with 400 μl of HAM-DM without fetal bovine serum. For determination of inhibiting activity, 50 μl of the indicated concentration of a MAb with 50 μl of STh (25 ng in 1 ml of PBS, pH 7.2) was added to duplicate wells. The cells were returned to the CO_2 incubator to allow 1 h of exposure to the toxin-antitoxin mixture at 37°C. The test was terminated by addition of 1 ml of cold 10% trichloroacetic acid, and the cells were subsequently kept at 0°C for 30 min. The supernatant was collected after centrifugation (1,000 × *g* for 10 min) and extracted three times with 4 to 5 volumes of diethyl ether. The samples were subsequently lyophilized, and cyclic GMP production was quantified after reconstitution of the samples in 100 μl of distilled water by using a cyclic GMP assay kit (Yamasa Corp., Tokyo, Japan). Appropriate toxin-positive and -negative controls were used, and each result was the average of duplicate samples.

Peptide synthesis. To characterize the antigenic determinants that define the MAbs, we used several chemically synthesized analogs of STh, STp, NAG-ST, and Y-ST. Peptides containing single-position substitutions (see Table 1) were also synthesized to determine which contact residues were directly involved in the epitope recognized by the MAbs on the STh molecule. Peptide synthesis was performed manually by the solid-phase method described in detail elsewhere (17, 31). The synthesized peptides were purified by high-performance liquid chromatography on a reversed-phase column (4.6 by 250 mm; YMC-ODS, S-5; Yamamura Chemical Laboratories, Kyoto, Japan). The amino acid compositions and molecular masses of the purified peptides were examined, and the peptides were confirmed to have the same toxic activity as native STa's by the suckling mouse assay.

Analysis of antigenic determinants by competitive ELISA. Competitive ELISA was performed to determine the fine specificity of the MAbs. Competition between various synthetic analogs of STh, STp, NAG-ST, and Y-ST, including the peptides containing single-position substitutions for MAb binding, was tested by comparing the concentrations of the synthetic analogs required to achieve 50% inhibition of the reaction between native STh coated on a microtiter plate and the MAb. From this, it was possible to deduce the relative affinities of the MAb for the different synthetic peptides. The reactivity of the MAbs with reduced and carboxymethylated native STh, prepared by the procedure outlined by Takao et al. (19), was also examined by the competitive ELISA. Briefly, the method involved incubation of 50 μl of the appropriate concentrations of a MAb, in duplicate, with equal volumes of PBS containing the various synthetic peptides in the STh (1 μg/ml)-coated microtiter wells. Competitive incubation was allowed to proceed for 1 h at 37°C, and the plate was then processed as in the ELISA procedure described above. Inhibition of binding to the solid phase was plotted against toxin concentration to indicate reactivity. Inhibition of binding by the various synthetic analogs was graded and expressed as the reactivity of the analogs. When the inhibition curve of the binding of an analog was similar to that of the homologous native STh, the grade was marked +; when no inhibition was observed, the grade was marked −; and when the inhibition curve was between + and −, the grade was marked ±.

RESULTS

Fusion of spleen cells obtained from BALB/c mice immunized with native STh yielded many clones that were viable in hypoxanthine-aminopterin-thymidine medium. By using the screening ELISA, we were able to detect four stable hybrid cell lines (8G7, 53-4, 11C, and SH1) obtained in independent fusion experiments that produced significant

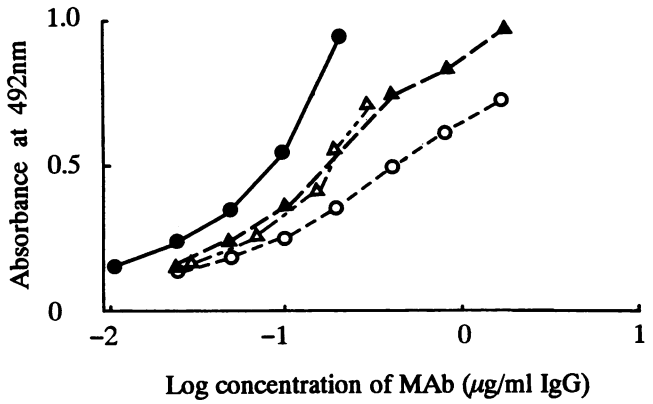

FIG. 1. ELISA titration of the four anti-STh MAbs. Experimental conditions were as described in the text. Symbols: ●, MAb SH1; ○, MAb 11C; ▲, MAb 53-4; △, MAb 8G7.

levels of anti-STh antibodies. When reacted with antisera specific for various mouse immunoglobulin classes or subclasses, the supernatant fluid from all four of the clones produced precipitin lines only against anti-immunoglobulin G1 (IgG1). After the hybrid cell lines were recloned by limiting dilution, a portion of the cells was injected into pristane-primed mice for ascites production. The relative antibody titer of the purified IgG from the ascites was determined in a plate coat ELISA. As shown in Fig. 1, all four of the MAbs bound to native STh in the ELISA to various degrees, with clone SH1 showing the greatest affinity, followed by 53-4, 8G7, and 11C, in that order.

All four of the MAbs were screened for their relative abilities to neutralize the biological activity of native STh in the suckling mouse assay. Of the four MAbs, the greatest neutralization capacity was exhibited by SH1, with as little as 10 μg of IgG per ml needed to completely neutralize the biological activity of 40 mouse units of native STh per ml, which worked out to an SH1 IgG to STh molar ratio of approximately 1:1. In contrast, MAbs 11C and 53-4, in that order, were not as effective in neutralizing the biological activity of similar amount of STh and required about 1,000 μg of IgG per ml. The amount of IgG of MAb 8G7 required to neutralize the activity of STh was about 50 μg/ml (Fig. 2). The abilities of SH1, 53-4, 8G7, and 11C to inhibit the cyclic GMP accumulation activity of native STh in T84 human colonic epithelial cells are shown in Fig. 3. Fifty percent inhibition of STh (25 ng/ml)-catalyzed accumulation of cyclic GMP was brought about by 0.3 μg of SH1 per ml, 0.72 μg of 53-4 per ml, 1.2 μg of 8G7 per ml, or 6 μg of 11C per ml. The molar ratio of SH1 IgG to STh necessary to bring about 50% inhibition of STh-mediated cyclic GMP accumulation activity was approximately 1:6. The enzyme-inhibiting activities of SH1 and 11C essentially paralleled the results obtained for neutralization of the bioactivity of STh in the suckling mouse assay. On the other hand, 53-4 and 8G7 showed reverse activity, with the former showing lower neutralization capacity in the suckling mouse assay but higher activity in the enzyme-inhibiting assay and 8G7 showing higher neutralizing capacity but lower enzyme-inhibiting activity.

The synthetic peptides enabled us to study very precisely the epitopes that define all four of the MAbs, and the results are summarized Table 1. MAb 8G7 was found to recognize an epitope that includes the C-terminal Tyr residue because the MAb reacted with analogs of STh and STp containing the

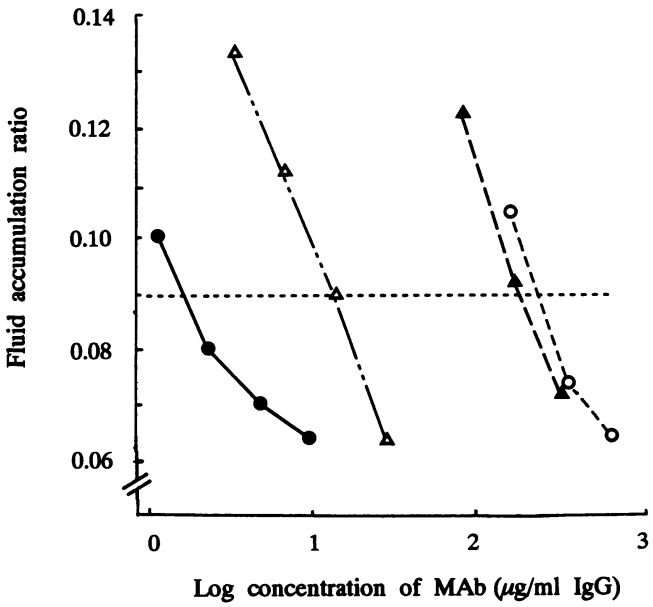

FIG. 2. Neutralizing activities of the four MAbs in the suckling mouse assay. The concentration of STh was 40 mouse units (100 ng) in 1 ml of PBS (pH 7.2). Experimental conditions were as described in the text. Symbols: ●, MAb SH1; ○, MAb 11C; ▲, MAb 53-4; △, MAb 8G7.

Tyr residue. Binding of 8G7 to analogs of STh and STp was aborted by removal of only a single amino acid residue, Tyr, indicating that Tyr constitutes the contact residue that defines the MAb. The five N-terminal amino acid residues (Asn-Ser-Ser-Asn-Tyr) did not affect the reactivity of MAb 8G7, as the synthetic analogs STh(6-19) and STp(5-18) reacted with the MAb.

The reactivity of MAb 11C was also governed by the C-terminal Tyr residue, since removal of this residue from STh resulted in nonreactivity of the MAb. MAb 11C consistently did not react with Y-ST(18-30) and NAG-ST(1-17). However, despite the presence of the C-terminal Tyr residue in the STp molecule at the same position as in the STh molecule, 11C showed only partial reactivity with STp. This reactivity profile indicates that the Thr residue at position 16

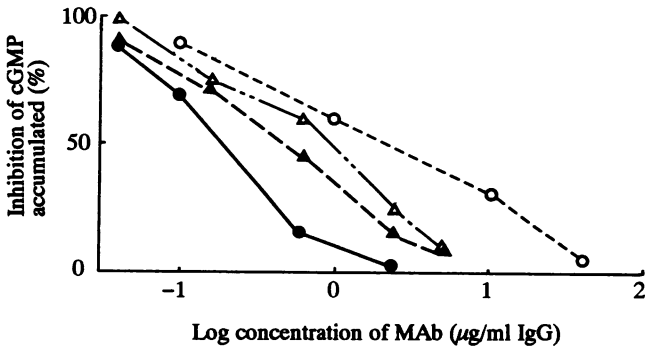

FIG. 3. Inhibition of guanylate cyclase activity by the four MAbs in T84 human colonic epithelial cells. The concentration of STh was 25 ng in 1 ml of PBS (pH 7.2). Experimental conditions were as described in the text. Symbols: ●, MAb SH1; ○, MAb 11C; ▲, MAb 53-4; △, MAb 8G7. cGMP, cyclic GMP.

TABLE 1. Reactivity profiles of the four MAbs in a competitive ELISA with various synthetic analogs of STa

MAb	Synthetic analog	Amino acid sequence	MED (ng)[a]	Reactivity[b]
8G7	STh(1-19)	NSSNYCCELCCNPACTGCY[c]	0.8	+
	STh(6-19)	CCELCCNPACTGCY	0.6	+
	STh(6-18)	CCELCCNPACTGC	0.6	−
	STp(1-18)	NTFYCCELCCNPACAGCY	1.0	+
	STp(5-18)	CCELCCNPACAGCY	1.0	+
	STp(5-17)	CCELCCNPACAGC	0.7	−
11C	STh(1-19)	NSSNYCCELCCNPACTGCY	0.8	+
	STh(6-19)	CCELCCNPACTGCY	0.6	+
	STh(6-18)	CCELCCNPACTGC	0.6	−
	STp(1-18)	NTFYCCELCCNPACAGCY	1.0	±
	STp(5-17)	CCELCCNPACAGC	0.7	−
	Y-ST(18-30)	CCDVCCNPACAGC	12.5[d]	−
	NAG-ST(1-17)	IDCCEICCNPACFGCLN	8[c]	−
53-4	STh(1-19)	NSSNYCCELCCNPACTGCY	0.8	+
	STh(6-19)	CCELCCNPACTGCY	0.6	+
	STh(6-18)	CCELCCNPACTGC	0.6	+
	STp(5-17)	CCELCCNPACAGC	0.7	+
	Y-ST(18-30)	CCDVCCNPACAGC	12.5[d]	−
	NAG-ST(3-17)	CCEICCNPACFGCLN	2[h]	−
	STh(6-18)I[9]	CCEICCNPACTGC[e]	1.4[f]	−
	STp(5-18)V[8]	CCEVCCNPACAGCY[g]	2.5[h]	−
	STp(5-18)D[7]	CCDLCCNPACAGCY[i]	6.25[h]	+
SH-1	STh(1-19)	NSSNYCCELCCNPACTGCY	0.8	+
	STh(4-19)	NYCCELCCNPACTGCY	1.1[j]	+
	STh(5-19)	YCCELCCNPACTGCY	0.8	±
	STh(6-19)	CCELCCNPACTGCY	0.6	−
	STp(1-18)	NTFYCCELCCNPACAGCY	1.0	±

[a] MED, minimum effective dose, defined as the minimum amount of toxin required to evoke a positive fluid accumulation ratio in the suckling mouse assay. Data are from reference 31, except as otherwise indicated.
[b] Reactivity is defined in the text.
[c] The underlined amino acid residue(s) is the contact residue(s) recognized by the MAb.
[d] Data from reference 30.
[e] Short analog of STh(6-18) substituted at position 9 with isoleucine.
[f] Data from reference 17a.
[g] Short analog of STh(5-18) substituted at position 8 with valine.
[h] Data from reference 32.
[i] Short analog of STp(5-18) substituted at position 7 with aspartic acid.
[j] Data from reference 12.

from the N terminus of the STh peptide also influenced the reactivity of 11C since Ala at position 15 from the N terminus was the residue present at the same position of STp and constitutes the only difference in the core region of the STh (residues 6 to 18) and STp (residues 5 to 17) molecules.

Nonreactivity of Y-ST(18-30) and NAG-ST(3-15) with MAb 53-4 suggested that the reactivity domain of MAb 53-4 was the Leu residue at position 9 from the N terminus of the STh molecule. This was verified by using short analogs containing single-position substitutions at positions 9 of STh and 8 of STp (Ile and Val, respectively) which resulted in nonreactivity. Substitution at position 7 of STp with Asp did not affect reactivity, indicating that Leu is the essential residue recognized by this MAb.

The reactivity of SH1 was governed by the Asn and Tyr residues at positions 4 and 5 from the N terminus of the STh molecule. Removal of Asn at position 4 resulted in partial loss of reactivity, and the MAb did not bind with short analogs which did not have these two residues. This was confirmed by the partial reactivity of SH1 with STp, which has Phe located at the site of Asn in the STh molecule.

None of the four MAbs reacted with native NAG-ST or Y-ST (data not shown). Likewise, none of the MAbs reacted with reduced and carboxymethylated native STh (data not shown).

Recent studies on the three-dimensional structure of the toxic domain of STp (residues 5 to 17), which is almost identical to that of STh, have revealed that the toxin has a right-handed spiral structure consisting of three β turns fixed by three disulfide linkages (15). The exact locations of the proposed antigenic determinants recognized by the four MAbs in a space-filling display of the ST molecule depicting the front and back faces of the toxin are shown in Fig. 4.

DISCUSSION

The primary objective of this work was to develop a panel of MAbs that would be useful in topographically mapping the antigenic regions of STh. By using four MAbs generated in independent fusions, we were able to determine, by a competitive ELISA using synthetic peptides of various STa's, contact residues which were directly involved in the epitope recognized by the MAbs on the STh molecule. At least three distinct antigenic sites of STh sufficiently removed from each other, one near the N terminus, another in the core functional region of the toxin, and the third in the C-terminal region, were recognized. However, even when the structure of the contact residues involved in MAb recognition remained unchanged, the native overall shape of the STh molecule was required to allow proper MAb bind-

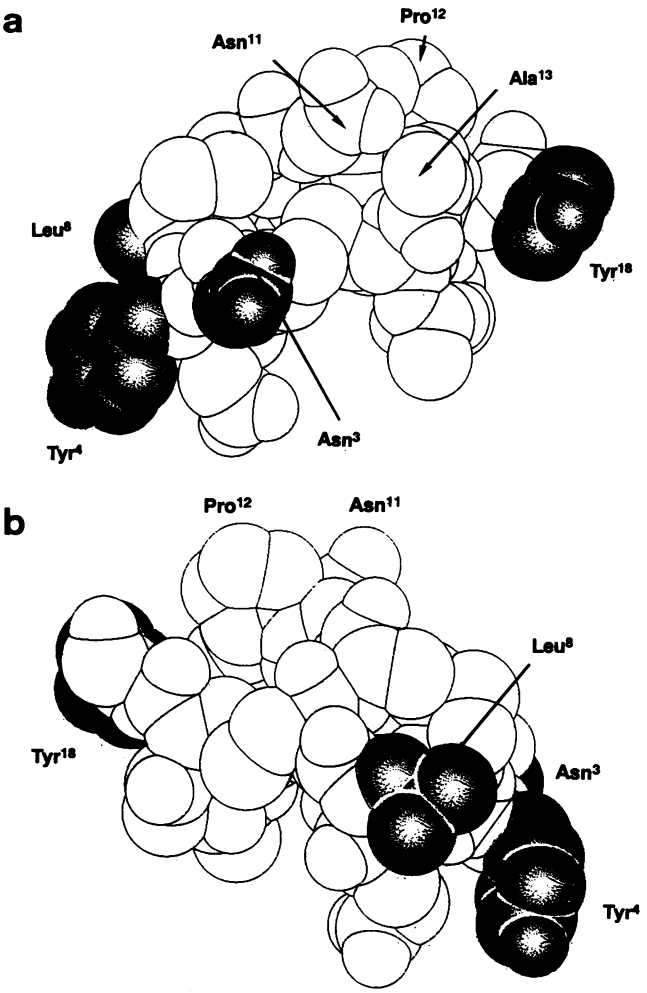

FIG. 4. Space-filling display of the ST molecule, based on the findings on STp reported by Ozaki et al. (15), depicting the front (a) and back (b) faces of the toxin molecule. The N-terminal two residues (Asn-3 and Tyr-4) and the C-terminal residue (Tyr-18) are placed at geometrically rational positions. Asn-3, Tyr-4, Leu-8, Asn-11, Pro-12, Ala-13, Thr-14, and Tyr-18 in this proposed model are Asn-4, Tyr-5, Leu-9, Asn-12, Pro-13, Ala-14, Thr-16, and Tyr-19, respectively, in STh. The shaded portions represent the proposed antigenic determinants of the four anti-STh MAbs.

ing, as revealed by the nonreactivity of the MAbs with reduced and carboxymethylated native STh. It is therefore clear that all of the MAbs generated in this study mediate their effect by reacting with conformation-dependent antigenic determinants.

Although there were quantitative differences in antibody binding, all four of the MAbs bound to native STh coated onto plates, indicating that the epitopes recognized by these MAbs remain accessible under conditions in which STh is bound to the solid phase. The differences in binding affinity between the MAbs were, however, no greater than 10-fold. The differential neutralizing abilities and enzyme-inhibiting activities of the four MAbs provided interesting insights into the epitopes of the STh molecule. The MAb with the greatest neutralizing capacity (molar ratio, approximately 1:1) in this study was MAb SH1, which recognized Asn and Tyr at positions 4 and 5 from the N terminus of the STh molecule. Likewise, MAb 2F, raised by us against NAG-ST, has high neutralizing activity, and on retrospective analyses it appears that this high activity is probably related to the fact that the contact residue of MAb 2F was an aspartic acid located in the N-terminal region (23). These data suggest that the N-terminal residues (Asn-Ser-Ser-Asn-Thr) which have been demonstrated not to be essential for the biological activity of STh in the suckling mouse assay (1, 17, 30, 31) possess an important antigenic determinant which can generate a potent antibody to neutralize the biological and enzymatic activities of STh.

The neutralization capacity of the other three MAbs (8G7, 53-4, and 11C) was not as potent as that of SH1, indicating that epitopes resident at position 9 (Leu) from the N terminus or in the C-terminal region appear to be less antigenic than the N-terminal region. The lower neutralizing ability exhibited by these three MAbs could also reflect indirect steric hindrance of the active toxic domain of STh rather than direct involvement of the epitopes recognized by these MAbs in generating a neutralizing antibody. Between MAbs 8G7 and 11C, which recognized C-terminal epitopes of the STh molecule, there was a substantial difference in neutralization potency, and this needs to be investigated in more detail. In addition to Tyr at position 19, the activity of MAb 11C was also governed by Thr at position 16, while the contact residue of MAb 8G7 was Tyr at position 19. This suggests that even subtle differences in structure could make profound changes in the activity profile and obviously 8G7 and 11C recognized different dimensions of the C-terminal region. Similarly, Kazemi and Finkelstein (13) have demonstrated that epitope expression can be affected by amino acid residues which are sequentially distant from the dominant residue. For instance, they have shown that residue 46 of the chimeric B-subunit protein of the cholera toxin family plays a dominant role in the formation of a number of epitopes, but this was affected by residues which were sequentially far from it, upstream or down.

The relative guanylate cyclase-inhibiting activities of MAbs SH1 and 11C paralleled the results of the neutralization assay with suckling mice. This indicates that the bioactivity-neutralizing epitope of SH1 also participates in the enzyme-inhibiting activity of STh. Alternatively, Asn at position 4 and Tyr at position 5 may be spatially adjacent to the biologically and enzymatically active sites in the STh molecule because of folding of the molecule. According to Ozaki et al. (15), in STp, the β turn from Cys at position 10 (Cys at position 11 in STh) to Cys at position 14 (Cys at position 15 in STh) is most important for the biological function, and Ala at position 13 (Ala at position 14 in STh) is particularly essential for binding of the STh molecule to the receptor on rat intestinal cells. Previous studies (17, 31) have shown that the five N-terminal residues are not important for toxic activity. The epitope recognized by SH1 may include some part of the β turn (Asn at position 12, Pro at position 13, and Ala at position 14), because it is possible that Asn at position 5 and Tyr at position 6 are spacially adjacent to the β turn, owing to the disulfide linkage of Cys at position 6 and Cys at position 11 (15, 17).

The acquisition of these MAbs and the precise localization of their epitopes provide us with reagents and knowledge which can be used to map the topology of the STh-host membrane complex. In addition, these MAbs would be useful tools for defining detoxification conditions which would minimally alter the native configuration of native STh, which in turn would be useful in developing a suitable vaccine.

ACKNOWLEDGMENTS

We are indebted to T. Sato, Institute for Protein Research, Osaka University, Osaka, Japan, for permitting us to use the space-filling model of the ST molecule.

This work was supported by funds from the Japan Health Sciences Foundation.

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