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Monoclonal Antibody to Shiga Toxin 2 Which Blocks Receptor Binding and Neutralizes Cytotoxicity

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A monoclonal antibody (MAb) was raised against Shiga toxin 2 (Stx2) of *Escherichia coli* O157:H7. MAb VTm1.1 belonged to the immunoglobulin G1 subclass and had a κ light chain, and it could neutralize the cytotoxic activity of Stx2 and variants derived from patient strains but not that of variants derived from animals. MAb VTm1.1 was shown to bind to the B subunit of these neutralized Stx2s by Western blotting. Comparison of B-subunit amino acid sequences and reactivities to these Stxs suggested six amino acids (Ser30, Ser53, Glu56, Gln65, Asn68, and Asp69) that were candidates for the MAb VTm1.1 epitope. Consequently, five Stx2 mutants (S30N, S53N, E56H, Q65K, and N68Ter) were prepared by site-directed mutagenesis to determine which residue is essential for the epitope. All of these mutants showed cytotoxicity almost equal to that of the wild-type Stx2. Of the five Stx2 mutants, only E56H could not be neutralized by MAb VTm1.1. Western blot analysis also showed that MAb VTm1.1 could not bind to the E56H B subunit. These results indicated that Glu56 is an important residue recognized by MAb VTm1.1. Immunofluorescence analysis further indicated that MAb VTm1.1 inhibits the binding of Stx2 to its receptors. MAb VTm1.1 could be a useful therapeutic agent for Shiga toxin-producing *E. coli* infection.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) has been recognized as an emerging food-borne pathogen that causes bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), mostly in industrialized countries (11). STEC secretes Stxs, which mediate STEC virulence (20). Stxs consist of an A-subunit monomer, which contains enzymatic RNA *N*-glycosidase activity that hydrolyzes the *N*-glycoside bond of adenosine of the 28S rRNA of 60S ribosomes and hence inhibits protein synthesis, and a B-subunit pentamer, which is involved in receptor binding (7, 26, 27). There are two major types of Stx, termed Stx1 and Stx2. Stx1 differs at a single amino acid in the A subunit from the Stx of *Shigella dysenteriae* 1 (15). Stx2 has approximately 55% amino acid homology with Stx1 and consists of several variants, such as Stx2vha and Stx2vp1 (20). STEC isolates produce Stx1, Stx2 (or its variants), or both of these toxins. Although the mechanisms of action of Stxs are thought to be the same, the cytotoxicity of Stx2 may be stronger than that of Stx1; the 50% lethal dose of purified Stx2 is 1 ng, whereas Stx1 has a 50% lethal dose of 30 ng (21, 34). Additionally, epidemiological data indicate that Stx2-producing strains are more frequently related to severe illnesses such as HUS than are Stx1-producing strains (2, 22). Although antibodies that neutralize Stxs are most likely to play a role in passive immunity, a minority of patients may develop rising levels of Stx-neutralizing antibodies following STEC infection (1, 5, 14).

There is a need to establish specific drugs to prevent severe disease caused by STEC, especially by the Stx2-producing strains. To facilitate the development of specific therapy and to investigate the role of Stx2 in the pathogenesis of HUS and hemorrhagic colitis, we have generated a monoclonal antibody (MAb) against Stx2 which neutralizes Stx2 cytotoxicity and have mapped the epitope of this MAb on Stx2 by using site-directed mutagenesis.

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MATERIALS AND METHODS

Materials. Recombinant *E. coli* VS-1 and *E. coli* MC1061(pITY1) (16, 33) were used for the purification of Stx1 and Stx2. Each recombinant was cultured in 10 liters of Luria-Bertani (LB) broth containing 100 μg of ampicillin per ml (for Stx1) or in 10 liters of Mueller-Hinton broth containing 5 μg of trimethoprim per ml at 37°C for 2 days with vigorous shaking. Stx1 and Stx2 were purified by DEAE-Sephacel (Stx1) or DEAE-Sepharose (Stx2) column chromatography, chromatofocusing chromatography on a column of a PBE94 (Pharmacia, Uppsala, Sweden), and high-performance liquid chromatography on a TSK-gel G-2000 SW (Tosoh, Tokyo, Japan) as described previously by Noda et al. (21) and Yutsudo et al. (34).

Crude Stx2 variants were prepared from *E. coli* O157 V50 (Stx2vh), *E. coli* O157 V354 (Stx2vh), and *E. coli* O157 V601 (novel Stx variant), which were isolated from patients at our laboratory in 1996, and from *E. coli* O157:H7 TK040 (Stx2 and Stx2vx1), *E. coli* O157:H7 TK051 (Stx2vx1), and *E. coli* O91:H21 TK080 (Stx2vha, Stx2vhb) as described previously (32). Crude Stx2 variants originating from animals were prepared from *E. coli* O22:H− KY019 (cow; Stx2vhb and Stx2vx2) and *E. coli* OUT:H21 TK096 (pig; Stx2vhb and Stx2vx3) and have been described previously (32). Recombinant *E. coli* VS-4 and *E. coli* HB101(pKTN817) were used for the preparation of crude Stx1v and Stx2vp1, respectively (4). These clinical and recombinant strains are listed in Table 1. Each strain was grown in 200 ml of LB broth at 37°C for 2 days, and crude toxins were extracted from the culture supernatant by precipitation with 80% saturated ammonium sulfate at 4°C. The resulting precipitate was collected by centrifugation, redissolved in approximately 3 ml of phosphate-buffered saline (PBS), and dialyzed three times at 4°C against 150 volumes of PBS.

The ACHN (human renal adenocarcinoma; ATCC CRL1611), Ramos (human Burkitt's lymphoma; ATCC CRL 1596), and 11E10 (mouse hybridoma which produces anti-Stx2 A subunit MAb; ATCC CRL 1907) cell lines were obtained from the American Type Culture Collection.

Preparation of MAb against Stx2. A hybridoma cell line secreting antibody to Stx2 was isolated from the fusion of P3U1 mouse myeloma cells (5×10^6 cells) with spleen cells from BALB/c mice immunized with Stx2 toxoid at the Pharmaceutical Discovery Research Laboratory II, Teijin Limited (Hino, Japan). Toxoid used for immunizations was produced by formaldehyde treatment of purified Stx2. Purified Stx2 containing 1 mg of protein was treated for 7 days at 37°C with 0.1 M phosphate buffer (pH 7.6) containing 0.4% formaldehyde and 0.2 M glycine. The resultant toxoid contained no residual toxicity for ACHN cells and was not lethal for mice.

TABLE 1. Origins, toxin types, and cross-reactivities with MAb VTm1.1 of various STEC strains

E. coli strain	Origin	Toxin type(s)	Neutralization ED_{50}[a] (ng/ml)
O157 V50	Human	Stx2vh	60
O157 V354	Human	Stx2vh	55
O157 V601	Human	Not determined[b]	160
O157:H7 TK040	Human	Stx2, Stx2vx1	170
O157:H7 TK051	Human	Stx2vx1	55
O91:H21TK080	Human	Stx2vha, Stx2vhb	60
O22:H− KY019	Cow	Stx2vhb, Stx2vx2	—[c]
OUT:H21 TK096	Pig	Stx2vhb, Stx2vx3	—
VS-1	Recombinant	Stx1	—
VS-4	Recombinant	Stx1v	—
MC1061(pITY1)	Recombinant	Stx2	55
HB101(pKTN817)	Recombinant	Stx2vp1	—

[a] ED_{50}, MAb VTm1.1 concentration necessary to increase cell viability to 50%.
[b] Other than Stx2, Stx2vh, Stx2vp, and Stx2v.
[c] —, >1,300 ng/ml.

MAbs were prepared from the culture supernatant of VTm1.1 cells by separation on an MAbTrap GII protein G affinity column (Amersham Pharmacia, Uppsala, Sweden) and dialysis against PBS (pH 7.0) and were stored at −20°C until use. The subisotypes of the MAbs were determined with a mouse MAb isotyping kit (Amersham Pharmacia).

Cytotoxicity assay. Cell viability was measured by use of a modification of the procedure of Riddell et al. (25). ACHN cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco BRL, Rockville, Md.). Cytotoxicity was assayed in wells of a microplate (Iwaki Glass, Chiba, Japan). Approximately 7×10^4 cells in 90 μl of growth medium were seeded into each well. A 10-μl volume of test sample was added to each well and incubated under 5% CO_2 in air at 37°C for 3 days. Cytotoxic effect was visualized by neutral red vital staining. After incubation at 37°C for 75 min with 100 μl of 0.014% neutral red per well, the supernatant was removed and cells were rinsed twice with PBS. Addition of an equal volume of 0.5 N HCl–35% ethanol released the dye from the lysosomes of viable cells. The absorbance of the dye at 540 nm was measured with a microplate reader and was directly related to the number of viable cells in each well. Results were expressed as percent viability compared with control culture viability (100%) from assays performed in the absence of Stxs. The cytotoxicity assay was done at least two times, and the average was used in the results.

Cytotoxin neutralization assay. The cytotoxin-neutralizing ability of MAb VTm1.1 was assayed on ACHN cells. A 10-μl volume of toxin solution containing 5 times the 50% cytotoxic dose (CD_{50}) of toxin was preincubated with 25 μl of diluted MAb VTm1.1 solution for 1 h, and then remaining unbound toxin cytotoxicity was measured by cytotoxicity assay as described above. Results were expressed as percent viability compared with control culture viability from assays performed with MAb VTm1.1 without Stxs (100% viability) and with only Stxs (0% viability).

Preparation of Stx2B mutants. The nucleic acid sequence of the complete Stx2 gene from bacteriophage 933W of *E. coli* has been reported by Jackson et al. (13) and was used as the basis for all sequence manipulations and alterations. For epitope analysis of MAb VTm1.1, several mutants were prepared by using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) according to the manufacturer's manual. As the template plasmid, pITY1, which has a 4.6-kb *Eco*RI fragment containing the coding sequence for the Stx2 (33), was used. The following double-stranded oligonucleotide primers, which incorporate substitutions of the codons for serine 30, serine 53, glutamic acid 56, glutamine 65, and asparagine 68 of the Stx2 B subunit to those for asparagine, asparagine, histidine, and a termination codon, respectively, were used: S30N-F (GAATACTGGACCAATCGCTGGAATCTGCAACC) and S30N-R (GGTTGCAGATTCCAGCGATTGGTCCAGTATTC), S53N-F (CTGTCACAATCAAATCCAATACCTGTGAATCAGG) and S53N-R (CCTGATTCACAGGTATTGGATTTGATTGTGACAG), E56H-F (CCAGTACCTGTCATTCAGGCTCCGGATTTG) and E56H-R (CAAATCCGGAGCCTGAATGACAGGTACTGG), Q65K-F (CCGGATTTGCTGAAGTGAAGTTTAATAATGACTGAGG) and Q65K-R (CCTCAGTCATTATTAAACTTCACTTCAGCAAATCCGG), and N68Ter-F (GTGCAGTTTAATTAGGACTGAGGCATAACC) and N68Ter-R (GGTTATGCCTCAGTCCTAATTAAACTGCAC). The resulting plasmids were transformed into Epicurian Coli XL1-Blue. Transformants were selected with trimethoprim, and their toxin production was assayed with the reversed passive latex agglutination (RPLA) assay kit VTEC-RPLA (Denka, Tokyo, Japan) and cytotoxicity assay. Positive transformants were isolated, and plasmid DNA was extracted and sequenced on an ABI 310 Genetic Analyzer (Perkin-Elmer, Foster City, Calif.) to confirm the presence of the desired mutation. The resultant mutated plasmids were termed pSX2B1, pSX2B2, pSX2B3, pSX2B4, and pSX2B5 and had single amino acid substitutions at amino acids 30 (Ser30 to Asn), 53 (Ser53 to Asn), 56 (Glu56 to His), 65 (Gln65 to Lys), and 68 (Asn68 to Ter), respectively. Recombinant *E. coli* strains and *E. coli* strains harboring mutated plasmids were grown at 37°C for 24 h with vigorous shaking in LB medium containing 100 μg of ampicillin per ml. The cells collected were sonicated in an Ultrasonic Disrupter UR-200p apparatus (Tomy Seiko, Tokyo, Japan) five times for 30 s. The mixture was centrifuged at 13,000 × *g* for 20 min, and the resulting supernatant was collected and used as crude mutant toxin.

Western blot analysis. The subunit specificities and epitope of the anti-Stx2 MAb VTm1.1 were analyzed by Western blotting. Purified Stx1 and Stx2 (625 ng each) and crude variant or mutant toxins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10 to 20% polyacrylamide gradient slab gels (ATTO, Tokyo, Japan). The dissociated toxin subunits were electrophoretically transferred (30 min at 250 mA) to an Immobilon-P membrane (Millipore, Bedford, Mass.) which was then incubated at 4°C overnight in PBS containing 3% bovine serum albumin. The membrane was incubated for 1 h at 37°C with PBS containing 3% bovine serum albumin and 10 μg of MAb VTm1.1 per ml and then washed five times in PBS containing 0.05% Tween 20 for 10 min. The blot was then incubated at room temperature with a 1:1,000 dilution of alkaline phosphatase-conjugated anti-mouse immunoglobulin serum (BioSource, Camarillo, Calif.) and washed three times as described above. Alkaline phosphatase activity was detected colorimetrically by adding color substrate containing 330 μg of nitroblue tetrazolium per ml and 165 μg of 5-bromo-4-chloro-3-indolylphosphate per ml.

Immunofluorescence study. To analyze the mechanism of neutralization of MAb VTm1.1, 2.5 ng of Stx2 was incubated with or without 2 μg of MAb VTm1.1 for 30 min at 4°C. Stx2 was also incubated with 2 μg of isotype-matched control immunoglobulin (12C4) under the same conditions as a control. Ramos cell suspensions (0.5×10^6 cells/100 μl) were prepared by treating the cells with PBS containing 0.1% NaN_3 for 5 min followed by pipetting. The cells were incubated with Stx2 or the Stx2-immunoglobulin mixture described above for 30 min at 4°C, washed repeatedly with PBS, and incubated with either isotype-matched control immunoglobulin (12C4) or anti-Stx2 A-subunit MAb 11E10 for 30 min at 4°C as the first antibody. The Stx2-bound antibodies on Ramos cells were then stained with fluorescein-conjugated second antibody and analyzed by flow cytometry (EPICS Profile and EPICS-XL; Coulter) as described previously (30).

RESULTS

Characterization of MAb VTm1.1. MAb VTm1.1 belonged to the immunoglobulin G1 subclass and had a κ light chain (results not shown). It bound in a dose-dependent manner to enzyme-linked immunosorbent assay (ELISA) plates coated with 1 μg of Stx2 per ml, whereas no binding was observed with Stx1, even when 100 μg of MAb VTm1.1 per ml was used. The MAb VTm1.1 concentration required to obtain 50% of the maximal binding to Stx2 was about 0.3 μg/ml, and that for maximal binding was about 10 μg/ml. The subunit specificity of MAb VTm1.1 was analyzed by Western blotting. MAb VTm1.1 bound only the Stx2 B subunit and showed no binding to the Stx2 A subunit or to Stx1 (Fig. 1, lanes 1 and 2).

A neutralization study was performed with MAb VTm1.1 to evaluate the reactivity with Stx1 and -2. MAb VTm1.1 neutralized the cytotoxicity of purified Stx2 (Fig. 2). The antibody concentration required to obtain 50% neutralization of 125 pg of purified Stx2 per ml was about 55 ng/ml. In contrast, MAb VTm1.1 could not neutralize Stx1 activity even when 1.25 μg of antibody per ml was used. These results were consistent with ELISA and Western blotting results and indicated that the epitope of MAb VTm1.1 was in the B subunit of Stx2.

Epitope analysis by site-directed mutagenesis. To characterize the MAb VTm1.1 epitope, we first compared the reactivities of the MAb with various Stx variants derived from humans and animals. The cytotoxicity of all clinical strains tested (*E. coli* O157 V50, *E. coli* O157 V354, *E. coli* O157 V601, *E. coli* O157:H7 TK040, *E. coli* O157:H7 TK051, and *E. coli* O91:H21 TK080) was neutralized by MAb VTm1.1. In contrast, MAb VTm1.1 could not neutralize the cytotoxicity of strains derived from animals (*E. coli* O22:H− KY019 [cow], *E. coli* OUT:H21 TK96 [pig], and Stx2vp1), Stx1, and its mutant Stx1v. These results are summarized in Table 1. On Western blotting, only

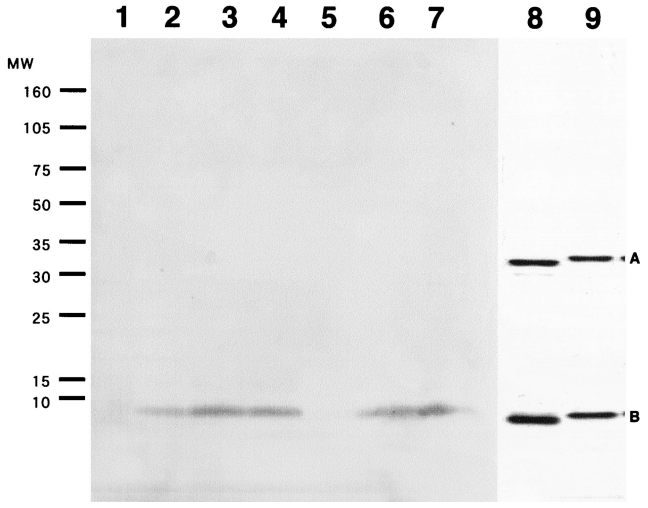

FIG. 1. Western blot analysis of binding of MAb VTm1.1 with Stxs. Purified Stx1 and Stx2 and crude extracts of Stx2 mutants were subjected to electrophoresis and blotted with MAb VTm1.1. Lanes: 1, purified Stx1 (625 ng); 2, purified Stx2 (625 ng); 3, S30N; 4, S53N; 5, E56H; 6, Q65K; 7, N68Ter. Coomassie brilliant blue R-250-stained purified Stx1 (lane 8) and Stx2 (lane 9) are also shown. MW, molecular weight (in thousands).

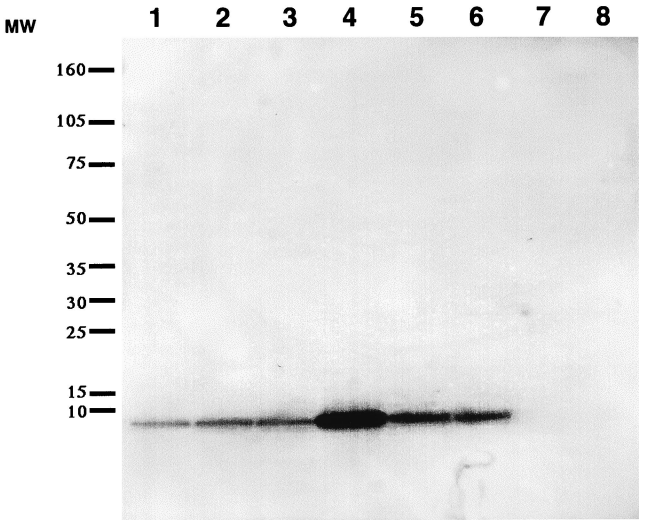

FIG. 3. Western blot analysis of binding of MAb VTm1.1 with Stx2 variants. Crude extracts of Stx2 variants were subjected to electrophoresis and blotted with MAb VTm1.1. Lanes: 1, *E. coli* O157 V50; 2, *E. coli* O157 V354; 3, *E. coli* O157 V601; 4, *E. coli* O157:H7 TK040; 5, *E. coli* O157:H7 TK051; 6, *E. coli* O19:H21TK080; 7, *E. coli* O22:H− KY019; 8, *E. coli* OUT:H21 TK096. MW, molecular weight (in thousands).

the B subunits of Stxs from strains which had neutralization activity could bind MAb VTm1.1 (Fig. 3).

By comparing the amino acid sequences of these Stx B subunits, six amino acids (Ser30, Ser53, Glu56, Gln65, Asn68, and Asp69) were chosen as candidates for the epitope of MAb VTm1.1, because they were common in Stx2, Stx2vha, and Stx2vhb but not in other Stxs which did not react with MAb VTm1.1 (Fig. 4). Based on these results, five mutants were designed and prepared by site-directed mutagenesis so as to have a single amino acid substitution for one of the five residues identified. Culture supernatants of these mutant strains were used for epitope analysis. To confirm that these mutations did not affect cytotoxicity, their cytotoxic activities in ACHN cells were compared with the results of RPLA. The titers of their cytotoxic activities were in proportion to the titers in RPLA, indicating that the mutations had no effect on specific activity (data not shown).

Stx2 and the five mutant toxins were used at a concentration of 5 times the CD_{50} for neutralization assays with various

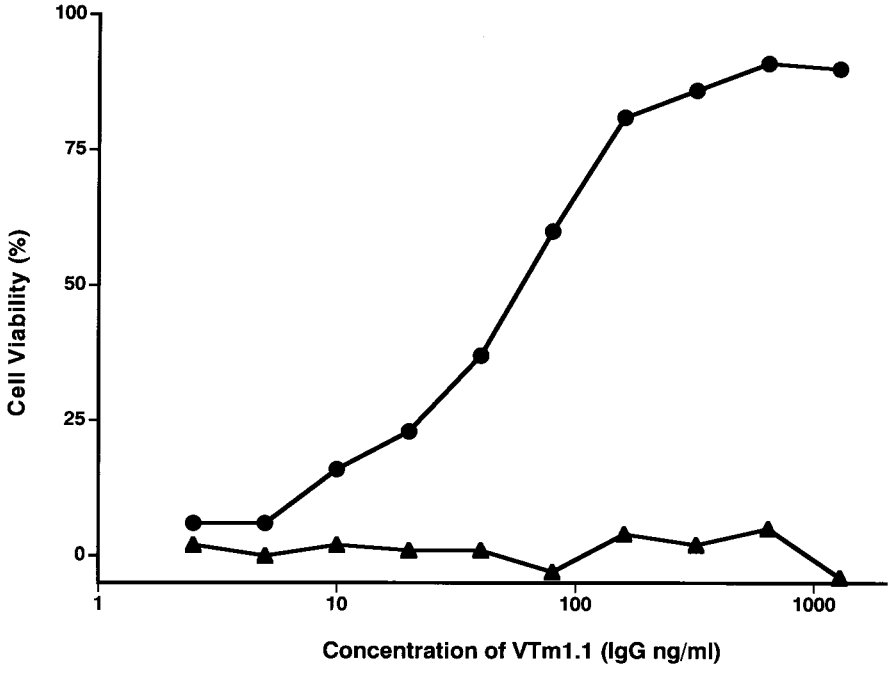

FIG. 2. Neutralizing activity of MAb VTm1.1 against Stx1 (▲) and Stx2 (●). Purified Stxs with a cytotoxicity of 5 times the CD_{50} were preincubated with diluted MAb VTm1.1 at 37°C for 1 h, and then the remaining unbound toxin cytotoxicity was measured by cytotoxicity assay as described in Materials and Methods. Cell viabilities were calculated according to the following formula: (A_{540} of sample − A_{540} obtained with only Stx)/(A_{540} with only MAb VTm1.1 − A_{540} obtained with only Stx) × 100%.

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        -20                -1+1       10         20         30         40         50         60
Stx1      ---TLLI-ASLSFF-AS-L-TP --VT--V-YT ---D------ --GD--LF-N --------L- --I------- -TNA-HN-G- -S--I-R++
          -20        ·        -1 +1       10         20         30         40         50         60
Stx2      MKKM+FMAVLFALASVNAMAA+ DCAKGKIEFS KYNEDDTFTV KVDGKEYWTS RWNLQPLLQS AQLTGMTVTI KSSTCESGSG FAEVQFNND
Stx2vha   ----+--------V-------+ ---------- ----N----- --A------- ---------- ---------- ---------- ---------
Stx2vhb   ----+--------V-----P-+ ---------- ----N----- --A------- ---------- ---------- ---------- ---------
Stx2vp1   ----+-I------V-------+ ---------- -----N---- --S-R----N ---------- ---------- I-N--S---- --Q-K--++
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FIG. 4. Comparison of amino acid sequences of B subunits of various Stxs. The solid arrow indicates an amino acid residue which is important for the neutralizing epitope recognized by MAb VTm1.1. The open arrows indicate the amino acids changed in this study.

concentrations of MAb VTm1.1. MAb VTm1.1 could neutralize the toxic activities of Stx2 and four Stx mutants but could not neutralize E56H activity, even at a MAb VTm1.1 concentration of 23.75 μg/ml (Fig. 5). The binding ability of MAb VTm1.1 was also examined by Western blotting analysis. Only the B subunit of E56H could not be bound with MAb VTm1.1, as predicted (Fig. 1).

Inhibition of Stx2 binding to Ramos cells by MAb VTm1.1. To investigate whether MAb VTm1.1 inhibits binding of Stx2 to its receptor or entry of the Stx2 A subunit into the cell, we performed an immunofluorescence study. When MAb 11E10 was used for the detection of bound Stx2, Ramos cells were negative for Stx2 after preincubation of Stx2 with MAb VTm1.1 (Fig. 6C) but were strongly positive after preincubation of Stx2 with isotype-matched control immunoglobulin or with Stx2 only (Fig. 6A and B). No positive results were obtained when control immunoglobulin was used instead of MAb 11E10.

DISCUSSION

This study characterized a highly specific neutralizing MAb against Stx2. MAb VTm1.1 was selected based on its strong binding to Stx2. There have been several MAb studies with Stx1 (10, 23, 28) and Stx2 (6, 23, 24), but none of these studies reported their epitopes. ELISA results showed that MAb VTm1.1 could bind to the Stx2, Stx2vh, Stx2vha, Stx2vhb, and Stx2vx1 variants, which were isolated from human patients in Japan, but could not bind to Stx1, Stx1v, Stx2vp1, Stx2vx2, or Stx2vx3. Neutralization assays further confirmed the wide spectrum of MAb VTm1.1 activity, which could neutralize all clinical variant toxins. Western blot analysis indicated that MAb VTm1.1 bound to the Stx2 B subunit. Previous studies of anti-Stx2 MAbs showed that most anti-Stx2 MAbs reacted with the Stx2 A subunit (6, 23, 24). Padhye et al. (23) suggested that denaturation of antigenic determinants on the B subunit might have occurred during the heat treatment of toxin used for immunizations, resulting in the production of MAbs predominantly against the A subunit of Stx. Perera et al. obtained MAbs by using crude Stx2 and reported that all of the neutralizing MAbs recognized the A subunit of Stx2; they suggested that the A subunit was more immunodominant than the B subunit (24). However, Downes et al. obtained MAbs which recognized the B subunit of Stx2 and neutralized its cytotoxicity (6). As both we and Downes et al. used purified Stx2 and

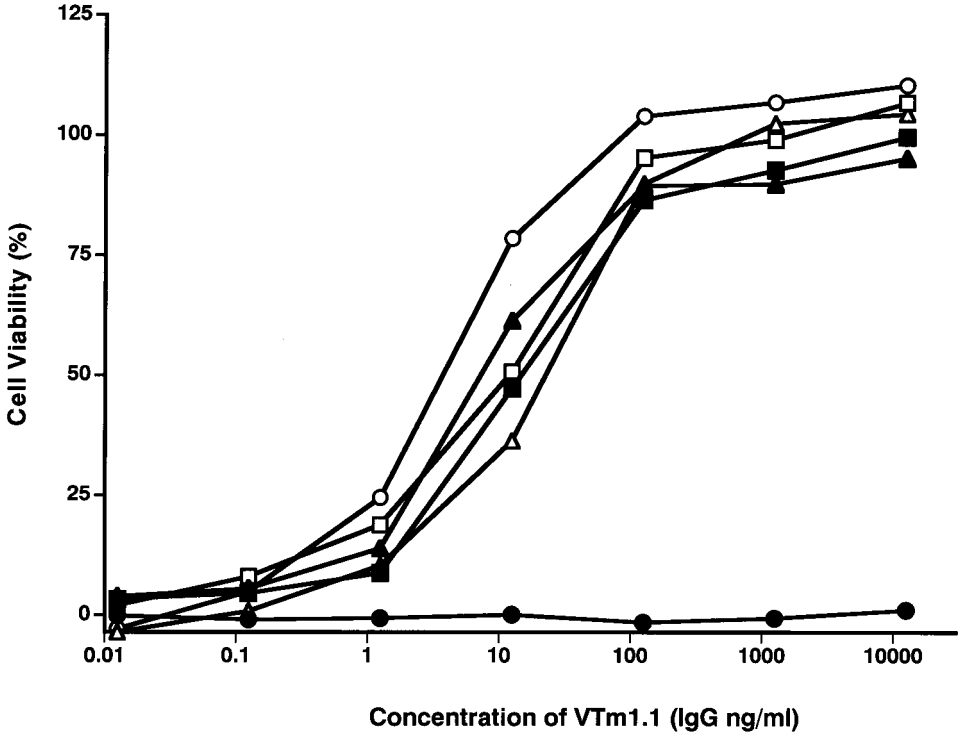

FIG. 5. Neutralizing activity of MAb VTm1.1 against several Stx2 mutants. Crude extracts of S30N (■), S53N (▲), E56H (●), Q65K (□), and N68Ter (△) and purified Stx2 (○) with a cytotoxicity of 5 times the CD_{50} were preincubated with diluted MAb VTm1.1 for 1 h, and then the remaining unbound toxin cytotoxicity was measured by cytotoxicity assay as described in Materials and Methods.

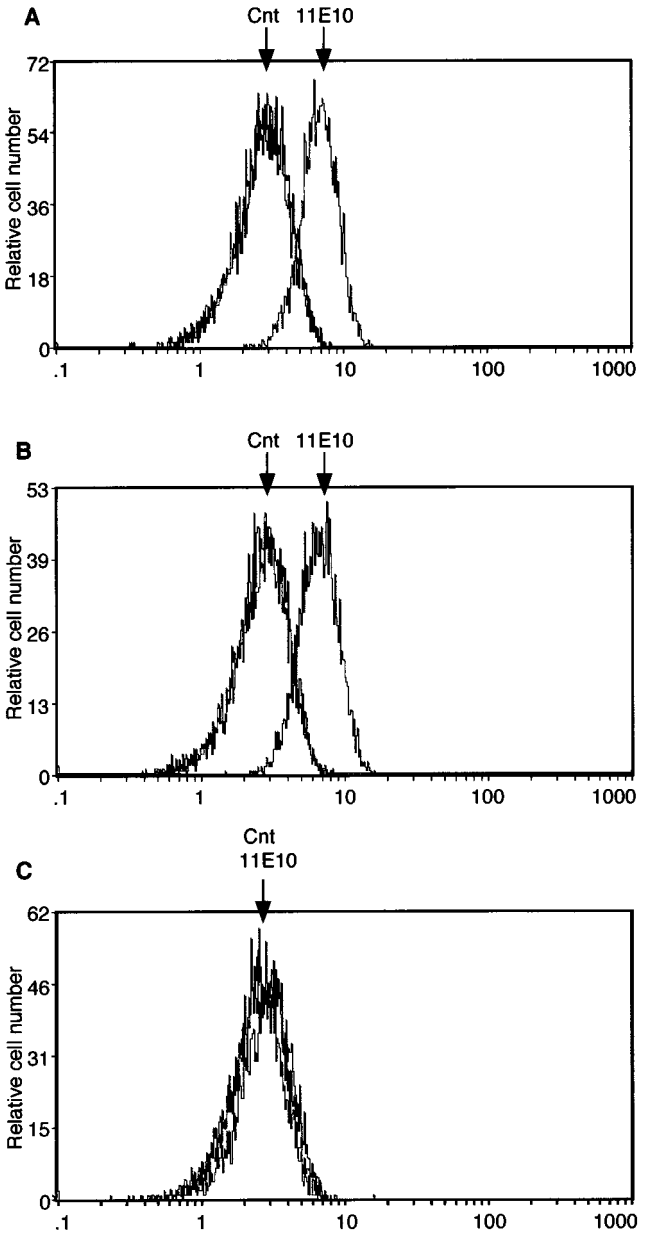

FIG. 6. Flow cytometry analysis of Stx2 binding on Ramos cells. Stx2 was preincubated without antibody (A), with isotype-matched control immunoglobulin 12C4 (B), or with MAb VTm1.1 (C) at 4°C for 30 min. Ramos cells were added to this mixture and incubated at 4°C for 30 min. The binding of Stx2 was analyzed by flow cytometry as described in Materials and Methods. MAb 11E10, which recognizes the Stx2 A subunit, was used for the detection of Stx2 on Ramos cells. MAb 12C4 was used for a control antibody in the detection reaction. The histogram obtained with MAb 11E10 was superimposed on that obtained with the control 12C4 (Cnt).

aldehyde-treated toxoid, the purity of the toxoid might affect the results of Stx2 immunization, in addition to toxoid preparation. Padhye et al. (23) reported that their MAb reacted with the A subunits of both Stx1 and Stx2, whereas other MAbs bound only either the A or B subunit of Stx2. Perera et al. also reported that one of their MAbs, 11E10, could partially neutralize the cytotoxic activity of Stx2e (formerly called SLT-IIv) (24). Stx2e was isolated from an *E. coli* strain responsible for edema disease of swine (19). The A-subunit amino acid sequences for Stx2e and Stx2 were highly homologous (93%), whereas the B subunit amino acid sequences were less homologous (84%) (31). As MAb 11E10 recognized the A subunit of Stx2, it might recognize a common region between Stx2 and Stx2e, but MAb 11E10 could not detect strains which produced only Stx2e in colony ELISA (24).

As several Stx2 variants have been isolated since the previous MAb studies, we used these variants for further analysis. As shown in Table 1, MAb VTm1.1 could neutralize all Stx2 variants of clinical strains. The amino acid sequences of these variants were compared, and six amino acids were chosen as candidate epitopes because they were conserved in neutralized toxins but not in other variants. As two of the six candidates were in the carboxyl-terminal region, five Stx2 mutants were prepared for further analysis by site-directed mutagenesis. All but one, E56H, were neutralized by MAb VTm1.1 (Fig. 5). Western blot analysis also indicated that MAb VTm1.1 could bind to the B subunits of the four mutants which were neutralized but not to that of E56H (Fig. 1). The crystal structure of Stx2 has not been reported, but the structures of Stx1 and the Stx from *S. dysenteriae* have been described (8, 18, 27). Based on the amino acid homology between Stx1 and Stx2 (55% for A subunit and 57% for B subunit) (20), their common receptor (Gb3), and the results of our mutation study showing that all Stx2 mutants retained cytotoxic activity, the structures of the B subunits of Stx1 and Stx2 might be similar. His58 of Stx1, which corresponds to Glu56 of Stx2, is exposed on the surface of the B-subunit pentamer. Recently, Ling et al. (18) reported the X-ray crystal structure of the Stx1 B-subunit pentamer complexed with an analogue of Gb_3, αGal(1-4)βGal(1-4)βGlc(1-8) methoxycarbonyloctyl (Pk-MCO). They showed that three potential Gb_3-binding sites existed on one B-subunit monomer, and all 15 Gb3-binding sites were located on the same flat face of the B-subunit pentamer, opposite the A subunit. They also described that the crystal structure of the double mutant of SLT-IIe Q65E/K67Q (designated GT3) and its Pk-MCO complex showed two binding sites on GT3 corresponding to sites 1 and 2 of Stx1. Judging from their results, His58 of Stx1, which corresponds to Glu56 of Stx2, may not contribute to the binding with Gb3, but the binding site may be located between sites 1 and 2. Our immunofluorescence study showed that MAb VTm1.1 inhibited Stx2 binding to the cell receptor. These data suggested that Glu56 of Stx2, which is thought to be the epitope of MAb VTm1.1, is on the surface of the receptor and that MAb VTm1.1 inhibits Stx2 cytotoxicity by covering the Stx2 B-subunit-binding site or by causing conformational changes in the Stx2 B subunit.

MAb VTm1.1 was able to detect denatured toxin proteins transferred to membranes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This finding suggests that MAb VTm1.1 recognizes a sequence-determined epitope rather than conformational epitope. Since the frequency of Stx2 in *E. coli* O157 is significantly higher than that of Stx1 (29), it would be expected that the frequency of antibodies to Stx2 would be higher than that of antibodies to Stx1. In a natural infection with STEC, the host immune system produces a low antibody response against Stxs, especially Stx2 (1, 9, 14). Several possible explanations for such a poor serologic response with *S. dysenteriae* Stx were suggested by Levine et al. (17). The neutralization assay showed that this sequence-determined epitope within the Stx2 B subunit was involved in antibody-mediated toxin neutralization and that the epitope region is a potential vaccine component. Synthetic peptides of *S. dysenteriae* Stx B subunit were shown to induce antibodies in rabbit which neutralize its cytotoxicity (12). Immunization with the peptide conjugates also protected mice against the lethal effect

of Stx. Boyd et al. also suggested that the Stx1 B subunit and its peptide fragments were Stx1 vaccine candidates and demonstrated that the linear region of the B subunit is able to generate a toxin-neutralizing immune response (3). There is no peptide vaccine to Stx2 at present, but the region containing Glu56 might be an Stx2 vaccine candidate. The neutralization ability of MAb VTm1.1 for Stx2 and Stx2 variants of clinical origin suggested that it may be a useful reagent for the development of passive immune therapy and prophylactic agents for STEC infection.

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