MICROBIAL PATHOGENICITY

Characterisation of monoclonal antibodies against haemagglutinin associated with *Clostridium botulinum* type C neurotoxin

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Of 11 monoclonal antibodies (MAbs) prepared against the non-toxic component of type C *Clostridium botulinum* 16S toxin to clarify the function of the non-toxic component, seven recognised HA1, three recognised HA3b and one recognised HA2. Results of epitope mapping indicated that three of the seven anti-HA1 MAbs recognised the region between amino acid residues 121 and 140 and four recognised the three-dimensional structure of HA1. Three anti-HA3b MAbs recognised different regions between (approximately) amino acids 405–430, 180–270 and 275–297. The ability of these MAbs to interfere with binding of 16S toxin or non-toxic component, HA1 or HA3b to erythrocytes and to intestine tissue sections of guinea-pig was observed. MAbs against HA3b and HA2 did not inhibit 16S toxin binding to either erythrocytes or epithelial cells, whereas some MAbs against HA1 did inhibit binding. The seven anti-HA1 MAbs can be classified into four groups based on their binding inhibition activities. The anti-HA1 MAbs that inhibited the binding of 16S toxin to the epithelial cells also neutralised or reduced the oral toxicity in mice, indicating that HA may play an important role in the absorption of the 16S toxin from the small intestine.

Introduction

The gram-positive spore-forming bacterium *Clostridium botulinum* produces seven serologically distinct neurotoxins (NTX, types A–G) that are known to be deadly food poisons to man and animals. The neurotoxins exist as a complex in foods or in cultures by associating with non-toxic proteins. The complex, designated progenitor toxin, is found in three forms – 12S toxin, 16S toxin and 19S toxin [1]. Type C strains produce two forms of progenitor toxins, 12S and 16S. The 12S toxin consists of NTX and a non-toxic component showing no haemagglutinin (HA) activity designated non-toxic non-HA (NTNH). The 16S toxin is formed by conjugation of 12S toxin with HA. Type C HA is made up of four different subcomponents – HA1, HA2, HA3a and HA3b – with molecular masses of *c.* 33, 17, 22–23 and 53 kDa, respectively [2, 3].

After ingestion of contaminated food, the progenitor toxins are absorbed from the upper small intestine. The progenitor toxins dissociate into the NTX and non-toxic component, and finally the NTX enters into the bloodstream [4]. Toxin action at the neuromuscular junction causes flaccid paralysis, characteristic of the disease botulism. The non-toxic components are known to play an important role in the development of food poisoning. Experiments show that the progenitor toxins possess greater potential for oral toxicity than NTX alone, as non-toxic components protect the neurotoxin from adverse conditions such as exposure to proteases and acidity in the digestive tract [4, 5]. An earlier study reported that the HA of type C 16S toxin is also important in the binding and absorption of the toxin in the small intestine. Only the 16S toxin effectively binds to the epithelial cells and, therefore, a large amount of 16S toxin can be absorbed from the small intestine compared with HA-negative 12S toxin [6]. The latter study also demonstrated that the type A subcomponents HA1 and HA3b expressed as glutathione S transferase (GST) fusion proteins bind to human erythrocytes and microvilli of epithelial cells of guinea-pig upper small intestine as well as type A HA-positive progenitor toxin

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[7]. GST-HA2 and GST-HA3a showed no obvious binding.

The findings of other workers have suggested that HA is not involved in binding and absorption of toxin from the small intestine [8], indicating the need for further investigation. In an attempt to clarify the functional role of each subcomponent of the non-toxic component, the present study examined the characteristics of 11 monoclonal antibodies (MAbs) which reacted with the HA subcomponents of type C.

Materials and methods

Toxin and non-toxic component

The 16S toxin and its non-toxic component (conjugation of non-toxic non-HA and HA) were obtained from the culture fluid of *C. botulinum* type C strain, C-Stockholm (C-ST), as reported previously [3, 6]. The organisms were cultured by a cellophane tube procedure and toxins were precipitated by 60% saturation with ammonium sulphate. The harvested precipitates were dialysed against 50 mM sodium acetate buffer (pH 4.2) and then applied to a SP-Toyopearl 650 M column (Tosoh, Tokyo, Japan) equilibrated with the same buffer. From this column, four main protein peaks were eluted by an exponential gradient of NaCl (0–0.8 M). Each fraction was analysed by SDS-PAGE and by mouse bioassay, and the fractions (peak 4) containing only 16S toxin were pooled. The 16S toxin separated in this way was dialysed against 50 mM Tris-HCl (pH 8.0), and then applied to a DEAE-Toyopearl column equilibrated with the same buffer to separate the NTX and non-toxic components. Two protein peaks were eluted by increasing the concentration of NaCl to 0.5 M. SDS-PAGE analysis revealed that the first peak was NTX, and the second peak was the non-toxic component.

Antisera

Rabbit antisera against type C non-toxic component which had been prepared previously [9] were used in this study.

Expression and purification of recombinant HA subcomponents

Type C HA subcomponents were prepared by PCR cloning and were expressed in *Escherichia coli* (DH5 α) as GST-fusion proteins as described previously [7]. The expressed GST-fusion proteins were affinity purified with Glutathione Sepharose 4B. The HA subcomponents were isolated from the GST moiety with a Restriction Protease Factor Xa Cleavage and Removal Kit (biotin-labelled factor Xa and streptavidin gel; Boehringer, Ingelheim, Germany). Finally, the purified HA subcomponents were obtained with a Glutathione Sepharose 4B column according to the

manufacturer's recommendations (Amersham Pharmacia Biotech, Little Chalfont, Bucks).

Construction and purification of deletion mutants of GST-HA3b

Three deletion mutants designated CDN1, CDN6 and CDC2 were generated by PCR with purified DNA from strain C-ST [2] as a template. The following primers were used: CG3, 5'-GTTAAAGggatccCCAATTCAAA TAATAATATAAA-3'; 53d1F, 5'-TTTggatccCCACTA GAAATAATCAAGGTA-3'; 53d6F, 5'-CTAggatccCC AGTCAACTAATTTTTTAAGA-3'; CT10, 5'-TTAgtcg acTTAACTATTACGATATGCATCA-3'; 53d2R, 5'-TA TgtcgacTTAACCATTTAATAATGATATTG-3'. Restriction sites introduced into the primers are indicated by lower-case letters: ggatcc, *Bam*HI; gtcgac, *Sal*I. Sequences encoding HA3bCDN1, CDN6 and CDC2 were obtained with the primer pairs 53d1F/CT10, 53d6F/CT10 and CG3/53d2R. All PCR fragments were digested with *Bam*HI-*Sal*I and cloned into pGEX-5X-3 (Amersham Pharmacia Biotech) digested with the same enzymes as described previously [7]. The sequences of the constructs were verified by cycle sequencing on a 373 DNA sequencer (Perkin-Elmer). The resulting plasmids were introduced into *E. coli*, and the GST-fusion proteins were expressed and affinity purified as described above.

Immunisation of mice and production of MAbs

The MAbs against type C non-toxic component were prepared as reported by Oguma *et al.* with slight modification [10]. Female BALB/c mice (8 weeks old) were inoculated subcutaneously twice at 4-week intervals with 100 μ g of type C non-toxic component mixed with Freund's incomplete adjuvant. Four weeks after the second injection, a booster dose of 3 μ g of type C non-toxic component was injected intravenously. Three days later, the spleen was removed aseptically and dissociated into a single-cell suspension. Lymphocytes (10^8 cells) obtained were fused with P1U3 myeloma cells (10^7 cells) with polyethylene glycol 4000 (Merck, Darmstadt, Germany) 50%. The fused cells were plated into 96-well tissue culture plates (Cellstar, Frickenhausen, Germany) and grown in HAT-20 medium (RPMI 1640 containing 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine) at 37°C. When colonies became visible macroscopically, culture supernates were screened for the production of anti-non-toxic component antibodies by an enzyme-linked immunosorbent assay (ELISA) with plates coated with non-toxic component. Antibody-producing hybridomas were subcloned in 96-well tissue culture plates by limiting dilution. RPMI 1640 supplemented with fetal calf serum 15% and Hybridoma Cloning Medium Briclon (Dainippon Pharmaceutical, Osaka, Japan) 5% was used throughout the experiment. Antibody production was again checked by ELISA and positive clones were then expanded in large-scale cultures. Cells

(>10⁷) were collected and injected intraperitoneally into BALB/c mice that had been pretreated 7 days earlier with pristane 0.5 ml. Ascitic fluid was collected and the MAbs were purified by DE52 (Whatman, Maidstone, Kent) ion-exchange chromatography. The class and subclass of MAbs thus obtained were determined by Mouse MonoAB ID/SP KIT (Zymed, San Francisco, CA, USA).

ELISA

Microtitration plates (EIA/RIA Plate, Costar, Laguna Niguel, USA) were coated with 1 µg of 16S toxin, 0.5 µg of non-toxic component, HA1 or HA3b in 100 µl of 0.01 M phosphate buffer (pH 6.0 and pH 7.4 was used for 16S toxin and other proteins, respectively, throughout the experiments). After washing with phosphate-buffered saline (PBS, pH 6.0 or pH 7.4) containing Tween 20 0.05% (PBS-Tween) and blocking with skimmed milk 10% in PBS (skim milk-PBS), MAbs diluted in skim milk-PBS were added and incubated for 1 h at room temperature. Secondary antibody, peroxidase-conjugated rabbit immunoglobulins against mouse immunoglobulins or swine immunoglobulins against rabbit immunoglobulins (Dako, Copenhagen, Denmark), was used at a dilution of 1 in 1000. Colour was developed with *o*-phenylenediamine (Wako, Osaka, Japan) 0.04% and absorbance was read at 490 nm.

SDS-PAGE and immunoblot analysis

SDS-PAGE was performed by the method of Laemmli [11] with a 12.5% separating gel and protein bands were visualised by staining with CBB R-250 (Merck). For immunoblot analysis, the protein bands were electroblotted to PVDF membranes (ProBlott; Applied Biosystems, Tokyo, Japan) with a semi-dry blotting apparatus (Nippon Eido). Non-specific binding sites were blocked (skim milk-PBS) and antibodies were diluted 1 in 1000 in skim milk-PBS followed by peroxidase-labelled secondary antibodies. Immuno-reactive bands were visualised with the ECL detection system (Amersham Pharmacia Biotech).

Epitope mapping

The epitopes of HA1 and HA3b with which the MAbs reacted were determined by the use of protease-digested HA1 and HA3b fragments. Initially, the sensitivity of purified HA1 and HA3b to proteases was examined with a Residue-specific Proteases Kit (Takara, Kyoto, Japan) containing six proteases (asparaginylendopeptidase, arginylendopeptidase, *Achromobacter* protease I, trypsin, *Staphylococcus aureus* V8 protease and endoproteinase Asp-N) according to the manufacture's protocol. From this result, specific proteases were chosen for fragment isolation for HA1 and HA3b. HA3b was treated separately with asparaginylendopeptidase and arginylendopeptidase in SDS 0.03% at 37°C for 3 h, whereas HA1 was first boiled

for 5 min in SDS 1%, and then treated with asparaginylendopeptidase or endoproteinase Asp-N at 37°C for 8 h. Thereafter, each preparation was boiled for 5 min in SDS-PAGE sample buffer, and then electrophoresed on a 12.5% gel. The separated protein bands were transferred to PVDF membranes as described above. Part of the PVDF membrane was reacted with each MAb, while the remainder was stained with CBB R-250. Protein bands were excised and sequenced with a pulsed liquid phase protein sequencer (Model 477-A; Applied Biosystem) to determine the N-terminal amino acid sequence and hence identify the region recognised by the MAbs.

Inhibition of binding of antigens to human erythrocytes by MAbs

Inhibition of binding of the C-ST 16S toxin, non-toxic component, HA1 and HA3b to erythrocytes by MAbs was examined. Microtitration plates (EIA/RIA Plate, Costar) were coated with the C-ST 16S toxin (2.5 µg/well), non-toxic component (1 µg/well) or recombinant proteins (1 µg/well) overnight at 4°C. The plates were washed twice with 200 µl of PBS-Tween and free binding sites were blocked with 200 µl of bovine serum albumin 1% in PBS (PBS-BSA) at room temperature for 2 h. Plates were incubated with 100 µl of antibodies diluted in serial two-fold steps (0.001–64 units) with PBS-BSA for 1 h at room temperature. After washing twice with PBS-Tween, the plates were incubated with 100 µl of a 1% suspension of human type B erythrocytes in PBS-BSA for 30 min at room temperature. The plates were carefully washed five times with 200 µl of PBS-Tween. The adherent erythrocytes were lysed with 50 µl of distilled water and absorbance of the released haemoglobin was analysed at 405 nm.

Inhibition of binding of antigen to sections of guinea-pig upper small intestine by MAbs

Inhibition of binding of the 16S toxin to paraformaldehyde-fixed sections of guinea-pig upper small intestine by MAbs was examined as described previously [6]. The C-ST 16S toxin (2 µg/ml) was pre-incubated with an equal volume of purified MAbs (50 units or 100 µg/ml) for 1 h at 37°C. Mixtures were then applied to deparaffinised sections for 1 h at room temperature. The 16S toxin binding was assessed by successively treating with dilutions (1 in 4000) of rabbit anti-type C non-toxic component serum, biotinylated goat anti-rabbit antibody, avidin-biotin-peroxidase complexes (Vectastain Elite ABC kit; Vector Laboratories), and then DAB solution. Sections were finally counterstained with haematoxylin and examined by light microscopy.

Neutralisation of oral toxicity by MAbs

The neutralising activity of the MAbs was investigated.

The minimum oral lethal toxicity (oral MLD) of the C-ST 16S toxin was first determined. The toxin was serially diluted in phosphate buffer (pH 6.0) and 0.3 ml was inoculated directly into the mouse stomach *via* a metal probe. One oral MLD of C-ST 16S toxin corresponded to 1.2×10^4 ($0.3 \times 4 \times 10^4$) mouse intraperitoneal MLD (ip MLD); i.e., inoculation of 0.3 ml of a preparation showing 4×10^4 ip MLD/ml. Toxin preparations of 2 or 10 oral MLD were mixed with an equal volume of diluted MABs (100 or 10 μ g/ml) for 1 h at 37°C in a water-bath. The mixtures (0.3 ml) were then given to the mice orally and the mice were observed for 1 week. As controls, rabbit anti-type C non-toxic component (1 in 10, 1 in 100), or normal rabbit or mouse sera were used in place of the MABs.

Results

Establishment of MABs against type C non-toxic component

A total of 11 MABs was obtained. The class and subclass of each antibody was first determined (Table 1). The subcomponent recognised by the MABs was determined by ELISA or Western blot employing the non-toxic component and recombinant HA subcomponents (GST-HA1 and GST-HA3b), or both. Of the 11 MABs, seven recognised GST-HA1 and three recognised GST-HA3b in ELISA. In Western blotting, three antibodies reacted each with HA1 or HA3b, and one reacted with HA2 (Fig. 1). In contrast, the four MABs (CHA1-15, CHA1-23, CHA1-29 and CHA1-54) that recognised HA1 in ELISA did not react with any band on the Western blot.

Epitope mapping

Epitope mapping of HA1 and HA3b was performed by Western blotting techniques with protease-digested peptide fragments. For HA3b only, an ELISA was also performed with deletion mutants. HA1 was very resistant to protease treatment, whereas HA3b was sensitive. The six fragments produced by cleavage of HA1 with

Fig. 1. Western blot analysis of each MAB and anti-non-toxic component polyclonal antibody with the purified type C non-toxic component. **1**, rabbit anti-non-toxic component polyclonal antibody; **2**, CHA1-28; **3**, CHA1-394; **4**, CHA1-489; **5**, CHA1-15; **6**, CHA1-23; **7**, CHA1-29; **8**, CHA1-54; **9**, CHA2-14; **10**, CHA3b-6; **11**, CHA3b-12; **12**, CHA3b-62.

asparaginylendopeptidase and endoprotease Asp-N, and also six fragments produced by cleavage of HA3b with asparaginylendopeptidase and arginylendopeptidase were selected, and their N-terminal amino acid sequences were determined. The C-terminus of the fragments was then deduced on the basis of their mol. wts. In the case of HA1, MABs CHA1-489, CHA1-394 and CHA1-28 showed the same reaction profiles. They recognised fragments 1, 2, 4 and 5 strongly and to a lesser extent fragments 3 and 6. These results indicate that the epitopes of these three MABs are located between amino acid residues 121 and 140 of HA1 (Fig. 2). As expected, the other four anti-CHA1 MABs that did not recognise any bands on the Western blot of whole 16S toxin failed to recognise any fragments. For the HA3b antibodies, CHA3b-6 recognised fragments 3 and 4 and deletion mutants CDN6 and CDN1. CHA3b-12 recognised all fragments except fragment 6 and also recognised deletion mutant CDC2. HA3b-62 recognised fragments 1, 3 and 4 and the deletion mutant CDC2. These results indicate that the three MABs that reacted with HA3b – CHA3b-6, CHA3b-12 and CHA3b-62 – appeared to react with different regions between (approximately) amino acids 405–430, 180–270 and 275–297, respectively (Fig. 3).

Table 1. Subclasses and reactivity of MABs

MABs	Subclasses of MABs	Western blotting with non-toxic component	ELISA		
			non-toxic component	GST-HA1	GST-HA3b
CHA1-28	IgG κ	HA1	+	+	–
CHA1-394	IgG2b κ	HA1	+	+	–
CHA1-489	IgG2a κ	HA1	+	+	–
CHA1-15	IgG1 κ	No band	+	+	–
CHA1-23	IgG1 κ	No band	+	+	–
CHA1-29	IgG2b κ	No band	+	+	–
CHA1-54	IgG1 κ	No band	+	+	–
CHA3b-6	IgG2b κ	HA3b	+	–	+
CHA3b-12	IgG1 κ	HA3b	+	–	+
CHA3b-62	IgG1 κ	HA3b	+	–	+
CHA2-14*	IgG1 κ	HA2	+	–	–

*No GST-HA2 was available for use as an antigen in ELISA.

Fig. 2. Reactivity of three anti-HA1 MAbs (CHA1-28, CAH1-394, CHA1-489) with enzyme-digested HA1 fragments. The reactivity of MAbs with peptide fragments was checked by Western blot analysis. Intensity of reaction: +, weak; ++, intense. ■, estimated epitope-containing region.

Reactivity of MAbs with 16S toxin, non-toxic component, GST-HA1 and GST-HA3b

The reactivity of anti-HA1 and anti-HA3b MAbs with type C 16S toxin, non-toxic component and either GST-HA1 or GST-HA3b was determined by ELISA. All the MAbs except CHA3b-62 showed a similar pattern of reactivity against the different antigens (Fig. 4). CHA3b-62 gave a low absorbance reading with the 16S toxin and non-toxic component, but similar absorbance reading with GST-HA3b as compared with those of CHA3b-6 and HA3b-12.

Inhibition of binding of antigens to human erythrocytes by MAbs

The ability of MAbs to interfere with the binding of erythrocytes to 16S toxin, non-toxic component, GST-HA1 and GST-HA3b coated on 96-well plates was determined (Fig. 5). To allow a comparison, a unit definition of the MAbs was established; this was taken as 300 times the concentration of MAbs giving an absorbance of 0.5 in the ELISA test (Fig. 4). All the anti-CHA1 MAbs and anti-non-toxic component polyclonal antibody significantly inhibited the binding of erythrocytes to non-toxic component and GST-HA1. However, in the case of 16S toxin, the inhibition activities of MAbs were different. CHA1-15, CHA1-23 and CHA1-29 noticeably inhibited the binding, whereas CHA1-28, CHA1-394 and CHA1-489 inhibited to a lesser degree, and CHA1-54 showed no significant inhibition. All the anti-HA3b MAbs inhibited the binding of erythrocytes to GST-HA3b. However, CHA3b-6 showed little inhibition activity in the case of both

non-toxic component and 16S toxin. CHA3b-12 inhibited the binding to non-toxic component, but did not inhibit the binding to 16S toxin. CHA3b-62 failed to inhibit the binding to 16S toxin and non-toxic component when *c.* 1 unit was employed. Because of the low reactivity of this antibody to 16S toxin and non-toxic component, the study was unable to use higher unit concentrations. CHA2-14 showed slight inhibition activity with non-toxic component, but no obvious inhibition with 16S toxin.

Inhibition of binding of antigens to intestinal tissue sections by MAbs

The ability of the MAbs to interfere with the binding of 16S toxin to guinea-pig intestinal tissue sections was also determined. In this experiment, MAbs (50 U and 100 µg/ml) were mixed with an equal volume of 16S toxin (2 µg/ml). CHA1-23, CHA1-29, CHA1-394 and CHA1-489 greatly inhibited the binding, whereas CHA1-54 showed less activity and CHA1-15 showed less activity at 100 µg/ml. CHA1-28, CHA3b-6, CHA3b-12, CHA3b-62 and CHA1-14 seemed to have no significant binding inhibition activity, although 50 U of CHA3b-12 and CHA3b-62 were not employed (Table 2).

Neutralisation of oral toxicity by MAbs

The neutralising activity of the MAbs was studied. When mice were inoculated orally with a mixture of 100 µg of MAbs and 2 oral MLD of toxin, some mice survived (Table 2). CHA1-23, CHA1-29, CHA1-54,

Fig. 3. Reactivity of three anti-HA3b MAbs (CHA3b-6, CAH3b-12, CHA3b-62) with enzyme-digested HA3b fragments and deletion mutants. The reactivity of MAbs with peptide fragments was checked by Western blot analysis and reactivity with deletion mutants was checked by ELISA. Intensity of reaction: -, absent; ±, trace; +, weak; ++, intense. ■, estimated epitope-containing region.

CHA1-394 and CHA1-489, which inhibited the binding of the toxin to epithelial cells of the small intestine, could neutralise toxicity. In those cases, although some mice died, their time to death was significantly longer than the controls; control mice died within 1 day, whereas the other mice survived >2 days. In contrast, MAbs CHA1-28, CHA3b-6, CHA3b-12 and CHA2-14, which did not inhibit toxin binding to the cells, did not neutralise the toxicity; all mice died within 1 day. 10 oral MLD of toxin was employed, all inoculated mice died. Also, when 10 µg of MAbs were mixed with 2 oral MLD of toxin, all inoculated mice died. In these cases, the survival time of mice inoculated with a mixture containing the MAbs which blocked toxin-binding to the cells was longer than that of the control mice. Ten times diluted anti-non-toxic component polyclonal rabbit serum could neutralise 2 oral MLD but not 10 oral MLD of toxin. One hundred times diluted serum did not neutralise 2 oral MLD toxin. The survival time of the mice was longer than the controls, as expected. The neutralisation test was also performed with mixtures of two or three different MAbs; however, this did not significantly increase their neutralising activities.

Discussion

Eleven MAbs against the non-toxic component of type C progenitor toxin were prepared to clarify the function of the non-toxic component. The reactivity of these MAbs to each antigen and the region containing their specific epitope were first determined. Thereafter, the ability of the MAbs to block the binding of 16S toxins or each non-toxic component to erythrocytes or epithelial cells and to neutralise oral toxicity of 16S toxin was analysed.

Seven MAbs reacted with HA1 on ELISA, but four of them formed no visible bands on Western blot, indicating that these four MAbs recognise a three-dimensional structure or that their epitopes are destroyed by SDS or heat treatment, or both.

Three MAbs reacting with HA3b and one reacting with HA2 were also obtained. Of the three HA3b MAbs, the reactivity of CHA3b-6 and CHA3b-12 to different antigens was similar, but that of CHA3b-62 was significantly different. The reaction of CHA3b-62 with GST-HA3b was strong, but the reaction with 16S toxin

Fig. 4. Reactivity of different concentrations of 11 MAbs to (a) 16S toxin, (b) non-toxic component, and to either (c) GST-HA3b or (d) GST-HA1 was determined by ELISA: ○, CHA1-28; ◇, CHA1-394; □, CHA1-489; ⊕, CHA1-15; ⊞, CHA1-23; ◆, CHA1-29; △, CHA1-54; ■, CHA3b-6; ♦, CHA3b-12; ●, CHA3b-62; *, CHA2-14.

and non-toxic component was weak. This indicates that the epitope reacting with CHA3b-62 is different from that reacting with CHA3b-6 and CHA3b-12, and is partially hidden in both 16S toxin and non-toxic component but becomes apparent in HA-3b. The epitope-containing regions of these MAbs were analysed by Western blot assay with enzyme-digested peptides and by ELISA with deletion mutants of HA3b. All three of the MAbs reacting on Western blot with HA1 (CHA1-28, CHA1-394 and CHA1-489) appeared to react to the region between 121 and 140 amino acid residues. In contrast, the three MAbs reacting with HA3b (CHA3b-6, CHA3b-12 and CHA3b-62) appeared to react with different regions between amino acids 405 and 430, 180 and 270, and 275 and 297, respectively. HA1 and HA3b were treated with six different proteases. HA3b was cleaved at many different sites whereas HA1 was cleaved at only a few sites, indicating that HA1 is much more resistant to proteases than HA3b, as reported previously [12].

The seven MAbs against HA1 showed similar reactivity on ELISA even when different antigens were used, and many of the MAbs possessed binding inhibition activity. However, the results tests of inhibition of binding to erythrocytes or to the cells of the small intestine were somewhat different. For instance, of three MAbs (CHA1-28, CHA1-394 and CHA1-489) that inhibited the binding of 16S toxin to erythrocytes, only the last two inhibited the binding of 16S toxin to the epithelial cells, but CHA1-28 did not. This may be due to differences in epitopes recognised by MAbs, or differences in receptors on human erythrocytes and guinea-pig epithelial cells, or both. On the basis of results presented in Tables 1 and 2, the seven MAbs seem to be classified into at least the following four groups; (1) CHA1-28, (2) CHA1-394 and CHA1-489, (3) CHA1-15, CHA1-23 and CHA1-29, (4) CHA1-54. The three HA-3b MAbs, inhibited the binding of GST-HA3b to erythrocytes but did not inhibit the binding of 16S

Fig. 5. Inhibition of binding of antigens to human erythrocytes by MAbs. Unit (U) definition of the MAbs was taken as 300 times the concentration of MAbs giving an absorbance 0.5 at ELISA in Fig. 4 (shown by dotted line). Different amounts of MAbs were added to (a) 16S toxin, (b) non-toxic component, (c) GST-HA3b and (d) GST-HA1 coated on microtitration plates. Erythrocytes were added. After washing, the bound erythrocytes were lysed by distilled water, and the absorbance at 405 nm was determined. Symbols: $\circ$, CHA1-28; $\diamond$, CHA1-394; $\square$, CHA1-489; $\oplus$, CHA1-15; $\boxplus$, CHA1-23; $\blacklozenge$, CHA1-29; $\triangle$, CHA1-54; $\blacksquare$, CHA3b-6; $\blacklozenge$, CHA3b-12; $\bullet$, CHA3b-62; $\ast$, CHA2-14.

toxin to either erythrocytes or epithelial cells. In binding tests with recombinant (GST fused) HA1 and HA3b and guinea-pig small intestine, both type C HA1 and HA3b were able to bind to the epithelial cells (unpublished data), as also reported for type A recombinant HA1 and HA3b [7]. The data showed that only some MAbs against HA1 obviously inhibited the binding of 16S toxin to the epithelial cells. This may be explained by the three-dimensional structure of 16S toxin, especially the non-toxic component including HA-1 and HA-3b. Some MAbs reacting with HA1 may simultaneously cover several binding sites on HA (HA1 and HA3b), and in this manner block the binding of 16S toxin to the cells. In neutralisation tests *in vivo*, some MAbs which inhibited the toxin binding to the epithelial cells were able to neutralise the toxicity when large amount of MAbs (100 $\mu\text{g}/\text{ml}$) and a small

amount of toxin (2 oral MLD) were employed. When 10 oral MLD was mixed with those MAbs, the time to death of mice inoculated with the mixtures became significantly longer than that of the controls. Anti-non-toxic component polyclonal antibody also showed a similar effect. From these results, it is concluded that HA may play an important role in the absorption of 16S toxin from the small intestine. When a large amount of 16S toxin is inoculated it may dissociate into NTX and non-toxic component, and NTX alone can be absorbed, leading to death.

Other workers have reported that the non-toxic component is not required for toxin absorption, and NTX alone can be absorbed from the stomach and the small intestine [8]. In an earlier study, when the same amount of 16S toxin, 12S toxin and NTX were directly

Table 2. Binding inhibition test and neutralisation test with MAbs

Antibody	Inhibition of binding to							Neutralisation test in mice; 16S toxin (2MLD; 100 µg/ml)
	erythrocytes				small intestine			
	16S toxin	NT-component	GST-HA1	GST-HA3b	16S toxin (2 µg/ml)			
					50 U	100 µg/ml		
CHA1-28	+	++	++	—	(1.5 mg/ml)*	—	—	0/5 [†]
CHA1-394	+	++	++	—	(1.5 mg/ml)	++	+	4/5
CHA1-489	+	++	++	—	(1.5 mg/ml)	++	+	4/5
CHA1-15	++	++	++	—		ND ¹	+	2/5
CHA1-23	++	++	++	—	(800 µg/ml)	++	++	4/5
CHA1-29	++	++	++	—	(300 µg/ml)	++	++	5/5
CHA1-54	—	+	+	—	(400 µg/ml)	+	+	3/5
CHA3b-6	—	—	—	++	(400 µg/ml)	—	—	0/5
CHA3b-12	—	+	—	++		ND ¹	—	0/5
CHA3b-62	ND ¹	ND ¹	—	++		ND ¹	—	1/5
CHA2-14	—	—	—	—	(400 µg/ml)	—	—	0/5
Anti-NT-component	++	++	++	++		ND	ND	5/5

Inhibition of binding of 16S toxin, non-toxic component, GST-HA1 and GST-HA3b to erythrocytes by different amounts of MAbs, and inhibition of binding of 16S toxin to the epithelial cells by MAbs 50 U and 100 µg/ml were performed. +, positive inhibition; ++, strongly positive inhibition; —, no inhibition; ND, not done; ND¹, not done because of the concentration required to attain 50 U. The neutralisation test was performed by orally injecting the mixture of 2 oral MLD of 16S toxin and MAbs 100 µg/ml into mice.

*Protein concentration, 50 U of each MAb.

[†]Number of mice that survived/number of mice tested.

inoculated into the small intestine of guinea-pigs without ligation, the guinea-pigs inoculated with 16S toxin died within 8 h, but those inoculated with 12S and NTX died after 12 h [6]. It was speculated that 12S toxin and NTX might be absorbed by a different mechanism from 16S toxin; one possibility is that 12S and NTX might be absorbed from M cells. The mechanism or the route of absorption of 16S toxin, 12S toxin and NTX from small intestine (or stomach) should be clarified in the near future. In the present study, it was not possible to conclude which region is the binding site of the toxin to the cells. Ongoing studies in this laboratory are attempting to determine the binding sites of the toxin by preparing more MAbs and deletion mutants of HA1 and HA3b.

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