A globotriaosylceramide (Gb_3Cer) mimic peptide isolated from phage display library expressed strong neutralization to Shiga toxins

Yoshiko Miura,[a] Akio Sakaki,[a] Masamichi Kamihira,[b]*

Shinji Iijima,[b] and Kazukiyo Kobayashi[a]

[a]Departments of Molecular Design & Engineering, and [b]Biotechnology, Graduate School of Engineering, Nagoya University, Chikusa-ku, Nagoya 464-8603, Japan.

*Corresponding Author

School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292, Japan

FAX: +81-761-51-1149

TEL: +81-761-51-1641

E-mail: miuray@jaist.ac.jp

Abstract

A Gb_3-trisaccharide mimic peptide was selected with biopanning from a phage display library against anti-Gb_3 antibody to neutralize Shiga toxins (Stxs). Biopanning was carried out on a microplate immobilized with a Fab fragment of anti-Gb_3 antibody and a subtraction procedure screening was applied to enhance specificity. The selected phage clones showed strong affinity to anti-Gb_3 antibody and to Stxs. Among these clones, a 9-mer sequence **WHWTWLSEY** was determined as the strongest Gb_3 mimic peptide and chemically synthesized. The peptide bound strongly to Stx-1 and Stx-2, though the binding was inhibited with Gb_3Cer. Surface plasmon resonance (SPR) and fluorescent spectroscopy determined that the affinity of the peptide to both Stxs was strong. Neutralization activity was confirmed by *in vitro* assay with HeLa cells. The Gb_3 mimic peptide potentially has great promise for use against Stxs.

Keywords: Carbohydrate mimic peptide, Gb_3, Phage display library, Shiga toxin

Abbreviations

ABTS, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); BOP, benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; BSA, bovine serum albumin; *B. simplicifolia*, *Bandeiraea simplicifolia*; CBB, Coomassie Brilliant Blue; Cer, ceramide; Con A, concanavalin A; DMA, *N*, *N*-dimethylacetamide; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanote; Fmoc, fluorenylmethoxycarbonyl; Gb_3, globotriaosylceramide Gal(α1-4)Gal(β1-4)GlcβCer; HBS-EP, HEPES-buffered saline with EDTA and polysorbate added; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; Ig, immunglobulin; WST-8, 2-(2-methoxy-5-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2*H* tetrazoluin monosodium salt; MALDI, matrix-assisted laser deposition ionization; RCA_{120}, *Ricinus communis agglutinin* 120; RMS, root mean square; SPR, surface plasmon resonance; Stx, Shiga toxin; TBS, Tris buffered saline; TOF, time of flight.

1. Introduction

Because bioactive saccharides play important roles in the molecular recognition of living processes such as cell adhesion, tumor metastasis and infection, they have attracted much interest for their medicinal and pharmaceutical applications [1,2] However, there are problems in utilizing the carbohydrates as drugs and epitopes, such as the difficulty obtaining sufficient quantities of pure oligosaccharide from either natural sources or synthetic procedures, and the weak carbohydrate-protein interaction.

The utilization of carbohydrate-mimetic compounds is a promising method for overcoming these difficulties [3-5]. Among the mimetic compounds, carbohydrate-mimic peptides have come in for much attention [6-20]. Carbohydrate-mimic peptides often show greater affinity for antibodies, enzymes and lectins than native carbohydrates. Carbohydrate mimic peptides can elicit an immune response that can be utilized for vaccine development, for example against diseases as cancers. In addition, peptides can be cloned within a short time from a phage display peptide library and can be synthesized by a simple solid-phase strategy that is superior to the more tedious process of saccharide synthesis. Carbohydrate-mimic peptides have been analyzed in terms of both structure and function, and investigations of them have increased remarkably.

Here, we report the production of Gb_3 mimic peptides by phage display library to

develop a drug to neutralize Shiga toxins (Stxs: Stx-1 and Stx-2). Stxs produced by *Eschericia coli* O157:H7 cause serious clinical complications [21]. Stxs are multisubunit proteins consisting of one toxic A-subunit and five carbohydrate-binding B-subunits [22]. The functional ligand for Stxs is Gb_3Cer glycolipid (α-D-Gal(1-4)β-D-Gal(1-4)β-D-Glc-Ceramide), which mediates the internalization of Stxs into host cells [23]. There have been extensive studies on the molecular design of artificial Stxs ligands using globotriose (a trisaccharide unit of Gb_3Cer), including our own reports on globotriose-carrying polymers with strong neutralization activities [24]. Since the interactions between carbohydrate mimic peptides and lectins are usually stronger than those between carbohydrates and lectins, the Gb_3 mimic peptides have the potential to be developed for new medicinal applications.

To select a Gb_3 mimic peptide, we adopted biopanning using a phage display to monoclonal anti-Gb_3 antibody, and then examined the Gb_3 mimic peptide with a subtraction procedure screening. We synthesized Gb_3 mimic peptides and investigated their interactions with Stxs by enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR) and fluorescent spectroscopy. Gb_3 mimic peptides were also analyzed by computer simulations. Our detailed information expands the potential of carbohydrate mimic peptides for biomedical applications.

2. Materials and methods

2.1. Chemicals

The following substances were used as received: anti-Gb_3 antibody (rabbit, IgG2b) (Seikagaku, Tokyo, Japan); Shiga toxins (Stxs) and Gb_3Cer (Nacalai Tesque, Kyoto, Japan); concanavalin A (Con A) and *Ricinus communis* agglutinin 120 (RCA_{120}) (Honen Co. Ltd., Tokyo, Japan); *Bandeiraea simplicifolia*, papain agarose, iodoacetamide and Gly-HCl (Sigma-Aldrich, St. Louis, MO, USA); anti-M13 antibody and bovine serum albumin (BSA) (Amersham Bioscience, Piscataway, NJ, USA); 1-hydroxybenzotiazole hydrate (HOBt) and benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (Kokusan Chemical, Tokyo, Japan); fluorescein -4-isothiocyanate (FITC-I) and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium monosodium salt (WST-8) (Dojindo, Kumamoto, Japan); and Fmoc-amino acids (Peptide Institute, Osaka, Japan).

2.2. Antibody purification [25]

The Fab fragment of the anti-Gb_3 antibody was prepared as follows. Anti-Gb_3 antibody

(50 μg), 1 M cysteine (6 μl), 20 mM EDTA (6 μl) and papain agarose (1.6 μl) were dissolved in acetate buffer (pH 5.5, 50 μl) in a tube and mixed with a tube rotator for 45 min. Papain was deactivated with 1 M iodoacetamide (4 μl) and the resultant solution was centrifuged twice at 15000 rpm at 4 °C for 10 min. The production of Fab fragment was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10 % gels. Gels were stained with Coomassie Brilliant Blue (CBB). The supernatant was separated from precipitated papain. pH was adjusted to 8.0 with Tris buffer (pH 8.5). Protein A agarose was added to the solution and incubated at 4°C for 3 h to remove the Fc fragment. The solution was centrifuged at 15000 rpm at 4 °C for 10 min to obtain the Fab solution as supernatant. The concentration of Fab was determined by absorbance at 562 nm, comparing the absorbance of Fab of IgG2.

2.3. Biopanning with anti-Gb_3 antibody

Biopanning was carried out using a phage display library kit (New England Biolabs., Cambridge, MA, USA) based on a combinatorial library of random 12-mer peptides fused to a minor coat protein (pIII) of the filamentous coliphage M13. A 96-well plate was coated with 100 μg of anti-Gb_3 Fab at 4 °C overnight, treated with 200 μl of blocking solution (100 mM $NaHCO_3$, 5 mg/ml BSA, and 0.02 % NaN_3) at 4 °C for 2 h, and washed

six times with Tris buffered saline (TBS) containing 0.1 % Tween. The phage library (1.5 x 10^{11} pfu) was added to the plate and incubated at room temperature for 60 min. The plate was washed six times with TBS 0.5 % Tween, and the bound phages were eluted with 0.1 N Gly-HCl buffer (pH 2.2). The eluate was neutralized with 1 M Tris HCl buffer. The phage clones obtained were then amplified by infecting them into host bacterial cells (*E. coli* ER 2738).

2.4. Screening utilizing a phage display subtraction method [26]

Subtraction biopanning was independently carried out. A 96-well plate was coated with 100 μg of IgG2 in 100 μl of 0.1 M $NaHCO_3$ buffer (pH 8.6) at 4 °C overnight, treated with 200 μl of blocking solution at 4 °C for 2 h, and washed six times with TBS 0.1 % Tween. The phage library (1.5 x 10^{11} pfu) was added to the plate and incubated for 60 min. The supernatant containing unbound phage clones was collected and purified, and the unbound phage was subject to biopanning with anti-Gb_3 antibody by the above-detailed method.

2.5. ELISA

Isolated phage clone binding to anti-Gb_3 antibody and Stxs was analyzed by ELISA. A

96-well plate was incubated with anti-Gb_3 antibody (70 μl of 70 μg/ml in 0.1 M $NaHCO_3$ (pH 8.6)) at 4 °C for 14 h, or with Stxs (70 μl of 10 μg/ml in PBS buffer) at 4 °C for 16 h. The antibody or Stx solution was removed, and the plate was incubated with 200 μl blocking buffer at 4 °C for 2 h. The plate was washed six times with TBS-0.5% Tween, and the phage clones (1.6×10^9 virions for anti-Gb_3 antibody and 2.4×10^7 virions for Stxs) were added to the plate. After incubation for 1 h, the plate was washed six times with TBS-0.5% Tween. Horseradish peroxidase (HRP) anti-M13 conjugate in blocking buffer was added and the plate was incubated for 30 min. The assay was visualized by addition of H_2O_2 and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) followed by quenching with sodium citrate for 1 h. Absorbance at 405 nm was recorded as a function of the diluted phage with a microplate reader. All results were averages of at least four different measurements.

An aqueous solution of Gb_3Cer was prepared by vortex mixing of Gb_3Cer (100 μg) with PBS buffer (50 ml) for overnight. Anti-Gb_3 antibody or Stxs were incubated with the aqueous solution of Gb_3Cer (0.5 μl of 2 μg/ml) for 1 h, followed by assay of the mixture of phage and Gb_3Cer according to the above-detailed method.

2.6. Peptide synthesis

Peptides were synthesized by a solid phase method using standard Fmoc chemistry on Wang resin. Coupling of amino acids was carried out using HOBt and BOP in DMA. The Fmoc group was removed in 20 % piperidine/DMF. FITC labeled peptide was synthesized by incubation of peptide (**AcWHWTWLSEYK**) with fluorescein-4-isothiocyanate (FITC) [27]. Peptides were purified by reversed phase HPLC (JASCO, Tokyo, Japan) with a gradient of acetonitrile/0.1 % TFA; purity as estimated by HPLC was >95 %. MALDI-TOF-MS: **WHWTWLSEY** 1330.7 $[M+Na]^+$ (calculated 1330.4), **AcWHWTWLSEYK** 1478.1 $[M+H]^+$ (calculated 1478.6). **AcWHWTWLSEYK-FITC** 1822.9 $[M+H]^+$ (calculated 1822.7).

2.7. Peptide-protein interaction

Interactions of the Gb_3 mimic peptide with proteins were analyzed by SPR and fluorescence spectroscopy, with a Langmuir binding model assumed for both analyses. SPR was carried out with a BIAcore 2000 using CM5 chip (BIAcore, Uppsala, Sweden) on which a peptide having Lys at the C-terminal (**AcWHWTWLSEYK**) had been immobilized. Measurements were carried out in HBS-EP buffer (pH 7.4) at 4 °C at a flow rate of 20 μl/min. Proteins at various concentrations were injected to determine

their affinity with the peptides. The chip surface was regenerated to remove bound analyte by injection of 5 mM Tris-HCl (pH 9.0) for 1.5 min. Kinetic constants were calculated with BIAevaluation software.

The dissociation constants were also analyzed by fluorescence changes of FITC-labeled peptide (**AcWHWTWLSEYK-FITC**) (Ex. 490 nm, Em. 518 nm) with FP-6500 (JASCO, Tokyo, Japan). Proteins with various concentrations were added to the peptide solution in PBS buffer at 4 °C, and the interactions were analyzed by Scatchard plots [28].

2.8. Neutralization of Stxs with a Gb_3 mimic peptide [29]

Neutralization activity of the Gb_3 mimic peptide was examined *in vitro* using a HeLa cell cytotoxicity assay. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) at 37 °C for 24 h. An aliquot containing 5 x 10^3 cells was transferred to each well of a 96-well plate, and DMEM was removed. A solution of Stx (5 pg) and the Gb_3 mimic peptide (0.001 – 100 μg) in 100 μl DMEM, preincubated at room temperature for 2 h, was added to each well. The cells were incubated in a 5 % CO_2 atmosphere at 37 °C for 72 h. Cell indicator WST-8 (10 μl) was added to the plate, and the cell survival rate was calculated by absorbance at 480

nm. Results were calculated from four different wells.

2.9. Computer simulation

Molecular simulations of the binding of a Gb_3 mimic peptide (**WHWTW**) to a Stx-1 B subunit were carried out using Amber 6 (University of California, San Francisco, CA, USA) on the basis of the model in the Protein Data Bank (PDB 1BOS) [30]. The binding was first calculated by random modeling with a genetic algorithm and then, optimized under an Amber 1994 force field. The water was appended in the Generalized-Born model. The potential energy of the structures was minimized with a root mean square (RMS) gradient of 0.01 kcal mol^{-1} $Å^{-1}$ [31].

3. Results

3.1. Selection of anti-Gb_3 antibody binding phage clones.

We screened the peptides bound to anti-Gb_3 antibody. To efficiently select the Gb_3 mimic peptide, the antibody Fab fragment was liberated through hydrolysis of the hinge region with papain and purified. After three rounds of biopanning, 10 individually isolated bacterial clones were randomly selected, and the phage clones obtained from the host bacterium were subjected to DNA sequence analysis. The deduced peptide

sequences are summarized in Table 1a. The sequences of the 10 colonies were classified into seven clones. Clones **Gb3-1** from three colonies, **Gb3-3** and **Gb3-4** were found to carry a 6-mer consensus sequence **WXWTWTXL** (**X**=**H** or **Q**). **Gb3-1** and **Gb3-3** carried a similar 12-mer sequence **WHWTWLSXYPPP** (**X**=**E** or **D**). Biopanning to Stx-1 was also investigated but to no avail; a consensus sequence with Stx-1 affinity was not obtained.

3.2. Analyses of the selected clones by ELISA

The binding of the selected phage clones to anti-Gb_3 antibody was assayed by ELISA (Fig. 1a). Clones **Gb3-1**, **Gb3-3**, **Gb3-4** and **Gb3-7** were well bound to anti-Gb_3 antibody, which contrasted with the modest binding of **Gb3-5** and the low binding of **Gb3-2** and **Gb3-6**. ELISA was carried out in the presence of an excess amount of Gb_3Cer to confirm Gb_3 mimicry. Binding of **Gb3-1**, **Gb3-3** and **Gb3-7** was inhibited by Gb_3Cer, but that of **Gb3-4** was inhibited just a little.

The extent of phage clone binding to Stx-1 and Stx-2 was analyzed by ELISA (Fig. 1b). The phage clones having a consensus sequence of **WXWTWL** (**X**=**H** or **Q**) (**Gb3-1**, **Gb3-3** and **Gb3-4**) showed a lot of binding to both Stxs, and biding was inhibited with an excess amount of Gb_3Cer. **Gb3-1** had the greatest amount of binding. **Gb3-5** had a high

amount of binding to Stx-1, but this was modestly inhibited by Gb_3Cer. These phage clones were strongly bound to both Stxs. **Gb3-7** carrying a different sequence also showed Gb_3 mimicry.

3.3. Screening using a subtraction method

Using the above conventional biopanning procedure, selected phage clones were probably bound not only to the variable domain but also to the constant domain of the Fab fragment. Subtraction screening using IgG2 as a negative control was carried out to enhance the binding specificity to the Fab fragment variable domain. After four rounds of biopanning six different sequences were obtained from 10 colonies (Table 1b) and subjected to ELISA. **Gb3-S-1** was the only clone that showed a great extent of binding to anti-Gb_3 antibody. The **Gb3-S-1** clone obtained from three colonies was found to carry the 12-mer **WHWTWLSEYPPP** sequence identical to that of the **Gb3-1** clone in Table 1a. We believed that **Gb3-1** bound most selectively and strongly to the variable domain of anti-Gb_3 antibody.

3.4. Binding affinity of the Gb_3 mimic peptide

We have selected **WHWTWLSEYPPP** of the **Gb3-1** clone as the most important Gb_3

mimic sequence. Since C-terminal Pro-Pro-Pro was not concerned with interaction with Stxs and was a little difficult to synthesize, we synthesized 9-mer **WHWTWLSEY** as a Gb_3 mimic peptide based on **Gb3-1** clone. We also synthesized **AcWHWTWLSEYK** for the affinity measurements, in which Lys (K) at the C-terminal and an acetyl group at the N-terminal were designed to conjugate the peptide to the biochip of SPR and to FITC [32].

The affinity of the synthetic Gb_3 mimic peptide for Stxs was quantitatively evaluated by SPR (Fig. 2) [33]. The peptide had strong affinity for both Stx-1 and Stx-2. The binding response (RU) of Stx-1 was a little greater than that of Stx-2, but both were of the same order. The affinities for non-carbohydrate binding proteins such as trypsin and fibrinogen were very weak; each RU was less than 10 % of that for Stxs. The rate equilibrium constants of association and dissociation to Stxs were estimated based on assumptions of a heterogeneous binding model (Peptide + Stx → Peptide-Stx) (Table 2). The association rate constant k_a and dissociation rate constant k_d of the peptide for Stx-1 were larger and smaller, respectively than those for Stx-2. Consequently, the peptide showed stronger affinity to Stx-1 than to Stx-2.

Binding of the Gb_3 mimic peptide to Stxs was also analyzed by fluorescence spectroscopy with a FITC-labeled peptide. The dissociation constants for Stxs were

calculated from the changes in fluorescence as 3.6 x 10^{-7} and, 5.2 x 10^{-7} (M). Although the binding affinities by fluorescence were a little weaker than that by SPR, fluorescence analysis also showed that the Gb_3 mimic peptide bound strongly to Stxs.

Unfortunately, we found that the Gb_3 mimic peptide also bound to other lectins. The dissociation constants of the peptide with *B. simplicifolia* (α-Gal recognition lectin), RCA_{120} (β-Gal recognition lectin) and Con A (α-Glc/α-Man recognition lectin) were 4.1 x 10^{-7}, 3.3 x 10^{-7}, 2.4 x 10^{-7} (M) in SPR and 5.6 x 10^{-5}, 3.8 x 10^{-5} and 3.1 x 10^{-5} (M) by fluorescence measurement.

3.5. *In vitro* neutralization of Stxs with the Gb_3 mimic peptide

The Gb_3 mimic peptide was evaluated for its activity neutralizing Stxs (Fig. 3). Cell survival rates against Stxs were increased by addition of the peptide. The addition of 10^3 μg/ml peptide conferred sufficient protection on HeLa cells in the presence of Stxs. The protective effect on Stx-1 and Stx-2 was similar. The dilute peptide solution (10^{-2} – 1 μg/ml) showed a stronger protective effect on Stx-2 than Stx-1, and the concentrated solution (1-10^3 μg/ml) showed almost identical protection of Stx-1 and Stx-2. The protective effects of the peptide on HeLa cells were modest in spite of a small K_D value. The neutralization activity of the peptide in serum free medium was also investigated,

which exhibited better protective effects than that in the presence of serum.

3.6. Computer simulation of Stx-1 binding mode

Binding between Stx-1 and the peptide was simulated using the first penta-peptide (**WHWTW**) of the Gb_3 mimic peptide to analyze binding behavior and binding sites (Fig. 4). Binding was calculated using random modeling; we obtained 16 binding modes with low potential energies from 50 binding modes. The potential energy of the 16 binding modes was almost the same, within 12 kcal/mol, and the majority of the peptides (13 out of 16, ca 80 %) bound to site 2.

4. Discussion

We have been successful in the selection of a Gb_3-mimic peptide (**WHWTWLSEY**) based on biopanning for anti-Gb_3 antibody. As shown in Table 1, the selected consensus sequence was **WXWTWLS** (**X**=**H** or **Q**) (**Gb3-1**, **Gb3-3** and **Gb3-4**). Although peptides with the consensus sequence bound well to the anti-Gb_3 antibody, only those with a second His residue (**Gb3-1** and **Gb3-3**) were inhibited by Gb_3Cer, indicative of Gb_3 mimicry. Therefore the second His was considered to be essential. Besides, clones **Gb3-1** (**WHWTWLSEY**) and **Gb3-3** (**WHWTWLSDY**) had the same sequence except for the eighth residue, but **Gb3-1** bound more to Stxs than **Gb3-3**. The difference of only one

residue affected Gb_3 mimicry and Stxs binding. The Gb_3 mimic peptide (**WHWTWLSEY**) was the best ligand for Stxs, which was also confirmed by biopanning using the subtraction procedure. On the other hand, clones with the hydrophobic residues **Gb3-5** and **Gb3-7** also bound strongly to Stxs, suggesting the contribution of a hydrophobic interaction. Since these clones did not have the consensus sequence and were not selected by the subtraction procedure, **Gb3-1** was more suitable for Gb_3 mimicry. **Gb3-2** and **Gb3-6** were selected by biopanning in spite of having a weak interaction with the anti-Gb_3 antibody and Stxs. These clones were selected due to the bias of the M13 phases [34].

The selected Gb_3 mimic peptide (**WHWTWLSEY**) has alternate hydrophobic (Trp) and hydrophilic residues (Thr, Ser, Glu and His). Trp can contribute to Stxs binding by a strong hydrophobic interaction [35] and π-π stacking. X-ray analysis showed that the α-anomeric surface of the Gal in Gb_3 was bound to Stx-1 through hydrophobic interaction with Leu29, Phe30 and Trp34 [30]. The Gb_3 mimic peptide amplifies the hydrophobic interaction due to its stronger hydrophobicity. The aromatic residues Pne30 and Trp34 in Stx-1 contribute to the π-π stacking with Trp in the Gb_3 mimic peptide, which was observed by computer simulation. Hydrophilic residues (Thr, Ser, Glu and His) of the Gb_3 mimic peptide can form hydrogen bonds with Stxs; this is

similar to the hydrogen bonding of Gal in Gb_3 with Thr21, Gly60, Asp17, Arg33 and Asp18 in Stx-1. His also stabilizes conformation for Stxs binding due to limited torsion angles [36].

The Gb_3 mimic peptide was predicted to bind to Stxs in the same way as Gb_3Cer, which was shown by binding inhibition with Gb_3Cer. It has been reported that Stx-1 has three types of Gb_3-binding sites (sites 1, 2 and 3) in a B-subunit, and that the binding affinity is in the order of site 2 > site 1>> site 3 [30, 37-39]. Therefore the Gb_3 mimic peptide was expected to bind to site 2 in Stx-1. The interaction between Stx-1 and the peptide was analyzed by computer simulation using the first penta-peptide (**WHWTW**). Most results showed that the peptide bound to site 2 with many different binding modes, though the potential energy of the binding modes was almost the same. Site 2 is advantageous for entropic cooperativity due to its binding modes [40, 41].

A striking feature of the Gb_3 mimic peptide was its strong binding to both Stx-1 and Stx-2, while Gb_3Cer bound strongly to Stx-1 but only modestly to Stx-2 [25, 31, 33, 34]. The conformation of site 2 in Stx-2 differs from that in Stx-1, resulting in the strong binding of the Gb_3 mimic peptide to Stx-2 and a weak binding for Gb_3Cer. It has been reported that Stx-2 is more toxic than Stx-1 and that Gb_3 binds modestly to Stx-2 compared with Stx-1. Therefore the strong interaction of the peptide with Stx-2 is a

useful feature of Gb_3 mimic peptides.

The Gb_3 mimic peptide bound not only to Stxs, but also to other lectins. Considering the Gb_3 structure of Gal(α1-4)Gal(β1-4)GlcβCer, the peptide binding to *B. simplicifolia* and RCA_{120} was adequate based on the property of Gal recognition of these lectins. However, the Gb_3 mimic peptide also bound to Con A in spite of the α-Glc/α-Man recognition property, suggesting indiscriminate binding. The Gb_3 mimic peptide was rich in hydrophobic residues, which induced the non-specific binding to α-mannose recognition lectin. Compared with Gb_3Cer, the Gb_3 mimic peptide exhibited similar or better neutralization activity to Stxs, but Gb_3Cer had better recognition specificity.

The protective effects of the peptide on Stxs were not strong in spite of the small K_D values; the unstable peptide conformation for Stxs binding is one possible reason for this. Since the synthetic 9-mer peptide (**WHWTWLSEY**) freely changes steric configurations in an aqueous solution, the frequency of forming a carbohydrate mimic structure seems to be very low, and so weakens binding affinity. But when peptides were immobilized on an SPR chip, the frequency of the mimicking Gb_3 structure seemed to increase; in fact, the dissociation constants obtained by fluorescence were larger than those obtained by SPR, indicating the weaker binding of the isolated peptide to Stxs. That confirms that, the microenvironment surrounding the peptide is important for the

peptide conformation for Stxs binding. Another possible reason is the non-specific binding of the peptide to other proteins. The protective effects of the peptide in serum free medium was better than that in the presence of serum, which suggests the peptide bound to other proteins in the serum non-specifically to reduce the protective effects. A multivalent compound or a peptide with a rigid conformation may overcome these defects.

5. Conclusion

A Gb_3 mimic peptide (**WHWTWLSEY**), based on the **Gb3-1** clone selected from a phage display, was successfully prepared as a novel neutralizer for Stxs. A **Gb3-1** clone was found to bind well to anti-Gb_3 antibody and Shiga toxins by ELISA. The affinity of the Gb_3 mimic peptide for Stx-2 was of the same order as that for Stx-1. The peptide neutralized Stxs modestly in HeLa cells *in vitro*. Investigation of efficient Stxs neutralization by a multivalent compound and the anitibiotic peptide for *E. coli* O-157:H7 is currently under way.

Due to its great efficacy, the Gb_3 mimic peptide is a useful bioactive substance. We expect that the Gb_3 mimic peptide can efficiently express not only neutralizing activity but also other carbohydrate biological activities such as cell adhesion, cell growth and

immune response.

Supplementary Data

The result of ELISA in subtraction biopanning, the neutralization of Stxs in the serum free medium and the results of computer simulation are available.

Acknowledgement

This work was supported by the Industrial Technology Research Grant Program in '03 from New Energy and Industrial Technology Development Organization (NEDO) of Japan and the 21st Century COE Program "Nature-Guided Materials Processing".

The authors are grateful to Dr. Toshinori Kondou, Ms. Chiduru Hirose and Dr. Hirokazu Ishida at Chuden CTI Co., Ltd. for computer simulation.

References

[1] M. J. Grogan, M. R. Pratt, L. A. Marcaurelle, C. R. Bertozzi, Homogeneous glycopeptides and glycoproteins for biological investigation, Annu. Rev. Biochem. 71(2002) 593-634.

[2] M. E. Tayler, K. Drickamer, Introduction to Glycobiology, 2002, Oxford Univ Press,

London.

[3] V. -D. Le, C. –H. Wong, Synthesis of 2-substituted polyhydroxyetrahydropyrimidines (N-hydroxy cyclic guanidine-sugars): transition state mimics of enzymatic glycosidic cleavage, J. Org. Chem. 65 (2000) 2399-2409.

[4] V. Notenboom, S. J. Williams, R. Hoos, S. G. Withers, D. R. Rose, Detailed structural analysis of glycosidase/inhibitor interactions: Complexes of Cex from Cellulomonas fimi with xylobiose-derived aza-sugars, Biochemistry, 39(2000) 11553-11563.

[5] V. -D. Le, C-H. Wong, Synthesis of 2-substituted polyhydroxytetrahydropyrimidines (*N*-hydroxy cyclic guanidino-sugars): transition state mimics of enzymatic glycosidic cleavage, J. Org. Chem, 65 (2000)2399-2409.

[6] B. Mozavi-Karbassi, G. Cunto-Amesty, P. Luo, T. Keber-Emmons, Peptide mimotopes as surrogate antigens of carbohydrates in vaccine discovery, Trends. Biotech 20 (2000) 207-214.

[7]G. Cunto-Amesty, B. Monzavi-Karbassi, P. Luo, F. Jousheghany, T. Kieber-Emmons, Strategies in cancer vaccines development, Int. J. Parasitology, 33 (2003) 597-613.

[8] T. Kieber-Emmons, P. Luo, J. Qiu, T. Y. Chang, O. Insug, M. Blaszczyk-Thurin, Z. Steplewski, Vaccination with carbohydrate peptide mimotopes promotes anti-tumor responses, Nat. Biotech 17 (1999) 660-665.

[9] M. A. Johnson, B. M. Pinto, Saturation-transfer difference NMR studies for the epitope mapping of a carbohydrate-mimetic peptide recognized by an anti-carbohydrate antibody, Bioorg. Med. Chem. 12(2004) 295-300.

[10] M. A. Johnson, A. A. Eniade, B. M. Pinto, Rational Design and Synthesis of Peptide Ligands for an anti-carbohydrate antibody and their immunochemical characterization, Bioorg, Med. Chem. 11(2003)781-788.

[11] M. A. Johnson, M. Jaseja, W. Zou, H. J. Jennings, V. Copie, B. M. Pinto, S. H. Pincus, NMR studies of carbohydrate and carbohydrate-mimetic peptides recognized by an anti-group B streptococcus antibody, J. Biol. Chem. 278(2003)24740-24752.

[12] N. K. Vyas, M. N. Vyas, M. C. Chervenak, D. R. Bundle, B. M. Pinto, F. A. Quiocho, Structural basis of peptide-carbohydrate mimicry in an antibody-combining ste, Proc. Acad. Natl. Sci, USA100(2003)15023-15028. (e) M. A. Johnson, A. Rotondo, B. M. Pinto, NMR studies of the antibody-bound conformation of a carbohydrate-mimetic peptide, Biochemistry 41(2002) 2149-2153.

[13] K. R. Oldenburg, D. Loganathan, I. J. Goldstein, P. G. Schultz, M. A. Gallop, Peptide ligands for a sugar-binding protein isolated from a random peptide library, Proc. Natl. Acad. Sci. USA, 89 (1992) 5393-5397.

[14] J. K. Scott, D. Loganathan, R. B. Easley, X. Gong, I. J. Goldstein, A family of

concanavalin a-binding peptides from a hexapeptide epitope library, Proc. Natl. Acad. Sci. USA, 89 (1992) 5393-5402.

[15] S. L. Harris, L. Craig, J. S. Mehroke, M. Rashed, M. B. Zwick, K. Kenar, E. J. Toone, N. Greenspan, F-I. Auzanneau, J.-R. Marino-Albernas, M. Pinto, J. K. Scott, Exploring the basis of peptide-carbohydrate crossreactivity: Evidence for discrimination by peptides between closely related anti-carbohydrate antibodies, Proc. Natl. Acad. Sci. USA 94 (1997) 2454-2459.

[16] S. H. Pincus, M. J. Smith, H. J. Jennings, J. B. Burritt, P. M. Glee, Peptides that mimic the group B streptococcal type III capsular polysaccharide antigen, J. Immunol. 160 (1998) 293-298.

[17] S.-J. Deng, C. R. MacKenzie, J. Sadowska, J. Kichniewicz, N. M. Young, D. R. Bundle, S. A. Narang, Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display, J. Biol. Chem. 269 (1994) 9533-9538.

[18] S-J. Deng, C. R. Mackenzie, T. Hirama, R. Brousseau, T. L. Laowary, N. M. Young, D. R. Bundle, S. A. Narang, Basis for selection of improved carbohydrate-binding single-chain antibodies from synthetic gene libraries, Proc. Natl. Acad. Sci USA, 92 (1995) 4992-4996.

[19] P. Valadon, G. Nussbaum, L. F. Boyd, D. H. Margulies, M. D. Scharff, Peptide libraries define the fine specificity of anti-polysaccharide antibodies to *Cryptococcus neoformans,* J. Mol. Biol. 261(1996) 11-22.

[20] D. Ishikawa, H. Kikkawa, K. Ogino, Y. Hirabayashi, N. Oku, T. Taki, GD1α-replica peptides functionally mimic GD1α, an adhesion molecule of metastatic tumor cells, and suppress the tumor metastasis, FEBS Lett. 441(1998) 20-24.

[21] K. Sandvig, Shiga toxins, Toxicon 39 (2001)1629-1635.

[22] Y. V. Kozlov, M. M. Chernaia, M. E. Fraser, M. M. James, Purification and Crystallization of Shiga Toxin from *Shigella dysenteriae,* J. Mol. Biol. 232 (1993)704-706.

[23] C. A. Lingwood, H. Law, S. Richardson, M. Petericm J. L. Brunton, S. D. Grandis, M. Karamali, Glycolipid binding of purified and recombinant *Eschericia coli* produced verotoxin *in vitro*, J. Biol. Chem. 262 (1987) 8834-8839.

[24] H. Dohi, Y. Nishida, M. Mizuno, M. Shinkai, T. Kobayashi, T. Takeda, H. Uzawa, K. Kobayashi, Synthesis of an artificial glycoconjugate polymer carrying P^k-antigenic trisaccharide and its potent neutralization activity against Shiga-like toxin, Bioorg. Med. Chem. 7 (1999) 2053-2062.

[25] E. Harlow, D. Lane, Antibodies: A laboratory manual, 1998, Cold Spring Harbor

Laboratory Press, New York, pp613-631.

[26] N. Shinohara, T. Demura, H. Fukuda, Isolation of a vascular cell wall-specific monoclonal antibody recognizing a cell polarity by using a phage display subtraction method, Proc. Natl. Acad. Sci. USA 97(2000), 2585-2590.

[27] G. Radcliff, R. Waite, J. Lefevre, M. D. Poulik and D. M. Callewaert, Quantification of effector/target conjugation involving natural killer (NK) or lymphokine activated killer (LAK) cells by two-color flow cytometry, J. Immunol. Mothods, 139(1991) 282-292.

[28] Y. Miura, T. Ikeda, K. Kobayashi, Chemoenzymatically synthesized glycoconjugate polymers, Biomacromolecules, 4(2003) 410-415.

[29] A. M. F. Cerqueira, B. E. C. Guth, R. M. Joaquim, J. R. C. Andrade, High occurrence of Shiga toxin-producing *Escherichia coli* (STEC) in healthy cattle in Rio de Janeiro State Brazil, Vet. Micro.70(1999) 111-121.

[30] Ling, H., Boodhoo, A., Hazes, B., Cummings, M. D., Armstrong, G. D., Brunton, J. L., Read R. J., Structure of the shiga-like toxin I B-pentamer complexed with an analogue of its receptor Gb_3. Biochemistry, 37(1998) 1777-1788.

[31] M. Vacatello, G. D'Auria, L. Falcigno, M. Dettin, R. Gambaretto, C. D. Bello, L. Paolillo, Conformational analysis of heparin binding peptides, Biomaterials,

26(2005) 3207-3214.

[32] M. Mourez, R. S. Kane, J. Mogridge, S. Metallo, P. Deschateletes, B. R. Sellman, G. M. Whitesides, R. J. Collier, Designing a polyvalent inhibitor of anthrax toxin, Nat. Biotechnol. 19 (2001) 958-961.

[33] H. Nakajima, N. Kiyokawa, Y. U. Katagir, T. Taguchi, T. Suzuki, T. Sekino, K. Mimori, T. Ebata, M. Saito, H. Nakao, T. Takeda, J. Fujimoto, Kinetic analysis of binding between Shiga toxin and receptor glycolipid Gb_3Cer by surface plasmon resonance, J. Biol. Chem., 276 (2001) 42915-42922.

[34] S. Elgavish, B. Shaanan, Lectin-carbohydrate interactions: different folds, common recognition principles, Trends Bio. Sci. 22 (1997) 462-467.

[35] S. E. Gibson, N. Guillo, M. J. Tozer, Towards control of χ-space: Conformationally constrained analogues of Phe, Tyr, Trp and His, Tetrahedron 55 (1999)585-615.

[36] D. J. Rodi, A. S. Soares, L. Malowski, Quantitative assessment of peptide sequence diversity in M13 combinatorial peptide phage display libraries, J. Mol. Biol. 322 (2002) 1039-1052.

[37] J. M. Richardson, P. D. Evans, S. W. Homans, A. Donohue-Rolfe, Solution structure of the carbohydrate-binding B-subunit homopentamer of verotoxin VT-1 from *E. coli.*, Nat. Struct. Biol. 4 (1997) 190-193.

[38] H. Shimizu, R. A. Field, S. W. Homans, A. Dohohue-Rolfe, Solution structure of the complex between the B-subunit homopentamer of verotoxin VT-1 from *Escherichia coli* and the trisaccharide moiety of globotriaosylceramide, Biochemistry 37(1998) 11078-11082.

[39] P. E. Stein, A. Boodhoo, G. J. Tyrrell, J. L. Brunton, R. J. Read, Crystal-structure of the cell-binding B-oligomer of verotoxin-1 from *Escherichia coli*, Nature 335 (1992)748-750.

[40] S. C. Head, M. A. Karmali, C. A. Lingwood, Preparation of VT1 and VT2 hybrid toxins from their purified dissociated subunits. Evidence for B subunit modulation of a subunit function, J. Biol. Chem. 266(1991) 3617-3621.

[41] M. E. Fraser, M. Fujinaga, M. M. Cherney, A. R. Melton-Celsa, E. M. Twiddy, A. D. O'Brien, M. N. G. James, Structure of Shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7, J. Biol. Chem. 279(2004) 27511-27517.

Figure Legends

Fig. 1 Binding assay of selected phage clones by ELISA (a) to anti-Gb_3 antibody in the absence and presence of Gb_3Cer (0.4 μg/ml in 200 μl PBS buffer) with 1.6 x 10^9 virions of phage clones, and (b) to Stxs in the absence and presence of Gb_3Cer (20 μg/ml in 200 μl PBS buffer) with 2.4 x 10^7 virions of phage clones. Experiments were repeated at least four times.

Fig. 2 SPR trace for binding of a Gb_3 mimic peptide (**AcWHWTWLSEYK**) to Stx-1, Stx-2, fibrinogen, and trypsin at 25°C at a protein concentration of 50 μg/ml.

Fig. 3 Neutralization of Stxs with a Gb_3 mimic peptide (**WHWTWLSEY**). Survival rates of HeLa cells against Stxs were followed in the presence of various concentrations of the peptide. Results were the average of at least four different samples.

Fig. 4 Representative computer-simulated binding mode between peptide (**WHWTW**) and Stx-1 B subunit. (a) Top view and (b) side view.

Tables

Table 1 Peptide sequence of phage clones selected (a) by biopanning to the Fab fragment of anti-Gb3 antibody, and (b) by biopanning with subtraction strategy.

	Clone	Sequence	Frequency
(a)	**Gb3-1**	WHWTWLSEYPPP	3/10
	Gb3-2	GGPIPTIPRPTL	2/10
	Gb3-3	WHWTWLSDYPPP	1/10
	Gb3-4	WQWTWLCEHPPP	1/10
	Gb3-5	WHRPPWWPSSSL	1/10
	Gb3-6	HELRSQIISTTS	1/10
	Gb3-7	FQWSWYTPPRPS	1/10
(b)	**Gb3-S-1 (Gb3-1)**	WHWTWLSEYPPP	3/10
	Gb3-S-2	EQWPWSLLPIHY	2/10
	Gb3-S-3	NALTSHTASPAP	1/10
	Gb3-S-4	HPPGHRLTLIPH	1/10
	Gb3-S-5	VPSLHQTTHPTP	1/10
	Gb3-S-6	HPSCNATGIPH	1/10

Table 2 Kinetic constants of **AcWHWTWLSEYK** to Stxs estimated by SPR.

	$k_a(M^{-1}\ s^{-1})$	$k_d(s^{-1})$	K_D (M)
Stx-1	1.2×10^{4}	1.6×10^{-5}	1.4×10^{-9}
Stx-2	4.8×10^{3}	1.1×10^{-5}	2.3×10^{-9}