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Demonstration of the pH Sensitive Binding of Multivalent Carbohydrate Ligands to Immobilized Shiga-Like Toxin 1 B Subunits[1]

Yasuyuki Imai,*[2] Yasumi Matsuura,* Yousuke Ono,* Tomoyuki Ishikawa,* and Yukishige Ito†

**Department of Microbiology, University of Shizuoka School of Pharmaceutical Sciences, Shizuoka 422-8526, and †Synthetic Cellular Chemistry Laboratory, The Institute of Physical and Chemical Research (RIKEN), Saitama 351-0198*

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The cytotoxic effects of Shiga-like toxins from enterohemorrhagic *Escherichia coli* O157:H7 depend on the recognition of carbohydrate determinants by B subunits. As a specific carbohydrate ligand, globotriaosylceramide has been characterized. We developed an alternative binding assay using multivalent carbohydrate ligands. We prepared globotriose-conjugated poly-lysine, and measured their binding to immobilized recombinant B subunits by an ELISA format. The signals representing ligand binding were dependent on the amount of immobilized B subunits as well as on the concentration of the ligands. The ligand binding activity was lost in an acidic environment, in which changes in the local conformation of the B subunits have been reported. Furthermore, pH dependent dissociation of the ligands from the B subunits was observed. We also demonstrate that antiserum from mice immunized with the B subunits specifically interferes with ligand binding. This suggests further potential for an assay to screen for blocking antibodies that could inhibit toxin internalization into host cells.

Key words: blocking antibodies, carbohydrate recognition, enzyme-linked immunosorbent assay, *Escherichia coli*, Shiga toxin.

Shiga-like toxins (SLTs or verotoxins) are exotoxins that are virulence factors in gastrointestinal infections involving enterohemorrhagic *Escherichia coli* (EHEC), such as serotype O157:H7. Infection with SLT-producing *E. coli* could result in life-threatening complications such as hemolytic uremic syndrome (HUS) (*1*). SLTs consist of a toxin subunit (A subunit) and cell-binding subunits (B subunits) (*2, 3*). The B subunits form a pentamer and recognize specific carbohydrate determinants, such as those displayed on glycolipid globotriaosylceramide (Gb_3, Galα1-4Galβ1-4Glcβ1-1ceramide) (*4, 5*). After binding to cell surface receptors, Shiga-like toxins are believed to be endocytosed and reach intracellular acidic compartments by retrograde transport (*6*). The A subunit is translocated to the cytoplasm, where it inhibits protein synthesis by removing a single adenine residue from 28S rRNA (*1*).

Early studies demonstrated that the local conformation closely associated with the carbohydrate binding sites of the B subunits changes in acidic environments corresponding to an intracellular compartment. On the other hand, the binding of radiolabeled B subunits to immobilized Gb_3 is not altered under such acidic conditions (*7*). Due to this fact, the change in local conformation has been implicated in the release of the A subunits from toxin complexes and in the subsequent translocation of the A subunits to the cytoplasm.

We have previously reported the preparation of recombinant B subunits of SLT-1 (SLT-1B). We labeled SLT-1B with digoxigenin, and applied them to the detection of Gb_3 glycolipids as well as cell surface ligands using flowcytometry (*8*). Here we describe an enzyme-linked immunosorbent assay (ELISA)–based binding assay using immobilized SLT-1B and multivalent carbohydrate ligands. We asked whether the binding of multivalent ligands to the B subunits is influenced in acidic environments.

Attemps to develops vaccines against SLTs and protective antibodies against SLTs have been made but with limited success (*9, 10*). The selective induction of blocking antibodies that could interfere with carbohydrate recognition by SLTs may lead to improvements in the prevention and treatment of toxin effects. For this purpose, simple and reliable assays will facilitate the development of such antibodies. Binding assays using multivalent ligands and immobilized animal lectins have been successfully applied for L-selectin (*11, 12*) and mouse macrophage lectin MMGL (*13, 14*). This assay format has been shown to be extremely useful for differentiating between blocking antibodies, presumably against binding sites, and non-blocking antibodies (*12, 14*). Therefore, we applied this assay format to SLT-1B, which is of bacterial origin, and checked to see whether blocking antibodies could be detected.

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[2] To whom correspondence should be addressed. Tel +81-54-264-5716, Fax· +81-54-264-5715, E-mail imai@u-shizuoka-ken.ac.jp

Abbreviations: ELISA, enzyme-linked immunosorbent assay, FITC-Gb_3-PLL, fluorescein isothiocyanate–labeled globotriose-conjugated poly-L-lysine; Gal, galactose; HRP, horseradish peroxidase, OVA, ovalbumin, SLT, Shiga-like toxin.

MATERIALS AND METHODS

Synthesis of Globotriose and Preparation of Multivalent Carbohydrate Ligands—Globotriose was synthesized as previously described (*15*). Glycosylation with galactose to obtain the Gal α1-4 Gal determinant was performed under van Boom conditions (*16*), followed by complete deprotection. The purity of the globotriose was confirmed to be >95% by ^{1}H-NMR (400 MHz, D_2O). Globotriose was conjugated to poly-L-lysine (PLL; degree of polymerization of 288; Sigma P-9404, St. Louis, MO) using the ε-amino groups of lysine residues as described previously (*13*). Seventeen percent of lysine residues were connected to sugar chains as calculated on the bases of the phenol–sulfuric acid reaction. Next we introduced fluorescein isothiocyanate (FITC) groups (7 mol/mol) as a tag (*13*), and then the residual free amino groups were blocked using *N*-hydroxysuccinimidyl acetate (Sigma) to avoid excess positive charge. Carbohydrate ligands containing 48 mol globotriose, 7 mol FITC per 1 mol PLL (FITC-Gb_3-PLL), were prepared.

Preparation of Recombinant SLT-1B and Antiserum—The expression and purification of SLT-1B using *E. coli* JM105 (pVT1-B5) cells have been described previously (*8*). In brief, the plasmid allows translation of a truncated carboxy-terminal A subunit (16 amino acids) and the entire B subunit. During transportation into the periplasm, the B subunits lose their signal sequences. The natural form of the B subunits thus accumulated in the periplasm was extracted by treatment with polymyxin B, and purified by anion exchange and chromatofocusing. The purity was routinely checked by Tricine-SDS-PAGE (*17*) The purified SLT-1B gave a single band with an apparent M_r 7,000 under non-reducing conditions. BALB/c mice (SLC Japan, Shizuoka) were immunized subcutaneously with 40 μg of purified SLT-1B in complete Freund's adjuvant (DIFCO, Detroit, MI), with additional intraperitoneal injections of 30 μg of SLT-1B in saline on days 12 and 48. BALB/c mice were subcutaneously immunized with 100 μg OVA (Sigma) in complete Freund's adjuvant on day 0 and with 100 μg OVA in incomplete Freund's adjuvant (Difco) on day 14. Sera collected 1 week after the last immunization were used as anti–SLT-1B and anti–OVA antiserum, respectively. Animal care and experiments were performed in accordance with the guidelines of University of Shizuoka Ether anesthesia was used when the mice were immunized with adjuvants.

Detection of Carbohydrate Binding Activity of Immobilized SLT-1B by ELISA—An ELISA procedure developed for mouse macrophage lectin was modified (*13*). In brief, purified SLT-1B was adsorbed on the wells of an ELISA plate (Costar 9018, Corning, NY) and nonspecific binding sites were blocked using 1% BSA (Sigma) in Dulbecco's phosphate-buffered saline (PBS). FITC-Gb_3-PLL (100 μl/well) diluted in PBS containing 0.1% Tween 20 (Wako, Osaka) and 0.1% BSA (PBS-Tween-BSA) was added. After incubation for 1 h at 20°C, the bound ligands were detected by incubation with 100 μl of horseradish peroxidase (HRP)–labeled rabbit anti-FITC (Dakopatts, Denmark) diluted in PBS-Tween-BSA (1:500) for 1 h at 20°C. Between each incubation, the wells were washed three times with PBS containing 0.1% Tween 20 (PBS-Tween). As a substrate, 1 mM 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Nacalai Tesque, Kyoto) and 0.034% H_2O_2 in 0.1 M sodium citrate (pH 4.2) was added, and absorbance readings were measured using a 405 or 415 nm filter. In some experiments, the following buffers were used to dilute FITC-Gb_3-PLL: 20 mM glycine-HCl (pH 2 or 3), 20 mM sodium acetate (pH 4, 4.25, 4.5, 4.75, or 5), and 20 mM sodium phosphate (pH 6, 7, or 8). All of these buffers contained 0.15 M NaCl, 0.1% Tween 20, and 0.1% BSA. To observe dissociation, the ligands were allowed to bind to immobilized SLT-1B under the standard conditions. After the removal of unbound ligands, the wells were further incubated in buffers with different pH for 2 h at 20°C to allow ligand dissociation. The remaining ligands were detected under the standard conditions. In antibody blocking experiments, SLT-1B–coated wells were pre-incubated with 50 μl of serum at the indicated concentration in PBS–1% Tween 20–1% BSA for 1 h at 20°C FITC-Gb_3-PLL (50 μl) diluted in the same buffer was then added. After additional incubation for 1 h at 20°C, ligand binding was assessed under the standard conditions. To detect antibody binding, wells that had been pre-incubated with serum (50 μl of the indicated concentration) were washed and subsequently reacted (100 μl, 1 h each at 20°C) with rabbit anti-mouse IgG (γ-chain specific; Zymed Laboratories, South San Francisco, CA) in PBS–1% Tween 20–1% BSA (1:1,000), and then with HRP-goat-anti-rabbit $IgG_{(H+L)}$ (Zymed) in PBS–0.1% Tween 20–0.1% BSA (1:1,000). In preliminary experiments, non-specific inhibition of ligand binding was observed in the presence of serum. We also observed that ligand binding was dramatically decreased in the absence of 0.1% Tween 20 (data not shown), suggesting that Tween 20 may improve accessibility of the carbohydrate determinants to the immobilized lectin molecules. Thus, we increased the concentration of Tween 20 to 1% Furthermore, we compared the effects of Tween 20 concentration on anti–SLT-1B IgG binding. Exactly the same dose response curve was obtained regardless of the concentration of Tween 20 (data not shown). This indicates that the increase in Tween 20 concentration itself does not affect antibody binding, and that it does not induce detachment of the immobilized SLT-1B.

RESULTS

Specific Binding of FITC-Gb_3-PLL to Recombinant Shiga-Like Toxin B Subunit (SLT-1B) in an ELISA Format—To develop an ELISA-based ligand binding assay tailored for the detection of lectin activity of SLT-1B, we prepared multivalent ligand FITC-Gb_3-PLL. The binding of FITC-Gb_3-PLL to immobilized recombinant SLT-1B was detected using peroxidase-labeled anti-FITC antibodies. The signals representing the binding of FITC-Gb_3-PLL increased in response to increasing concentrations of FITC-Gb_3-PLL The signals were also dependent on the amount of SLT-1B used to coat the wells (Fig. 1).

Binding of FITC-Gb_3-PLL is Abrogated in Acidic Environments—Despite tryptophan fluorescence measurements suggest that the local conformation involved in the carbohydrate binding sites of B subunits changes below pH 5, the binding of radiolabeled B subunits to immobilized Gb_3 has been reported not to be influenced by such pH changes (*7*). We asked whether an acidic environment could

affect the binding activity of immobilized SLT-1B to FITC-Gb_3-PLL. Binding was optimal between pH 6 and 7. At pH 5 and 8, suboptimal but substantial binding was still observed. In contrast, under acidic conditions (<pH 4), no significant binding above background was seen (Fig. 2). The specific ligand binding was gradually lost as the pH decreased from 5.0. Fifty percent of maximal binding was seen around pH 4.5 (Fig. 3A). When immobilized SLT-1B was pretreated for 1 h in buffers between pH 2 and 4, and then FITC-Gb_3-PLL binding was measured at neutral pH, an approximately 75% reduction in the specific signals was observed (data not shown). Although partial renaturation may have occurred, the immobilized SLT-1B was rather sensitive to acidic environments. The binding of mouse anti–SLT-1B IgG to the immobilized SLT-1B was not affected after exposure to acidic buffers as demonstrated at several points of antiserum dilution (Fig. 3B). This result rules out the possibility of SLT-1B detachment under acidic conditions.

Release of Bound FITC-Gb_3-PLL from SLT-1B in Acidic Environments—FITC-Gb_3-PLL was first allowed to bind to immobilized SLT-1B under the standard conditions, and then the environmental pH was lowered (Fig. 3C). When the pH was lower than 5.0, significant dissociation was observed. Incubation at pH 4.5 resulted in 30% dissociation.

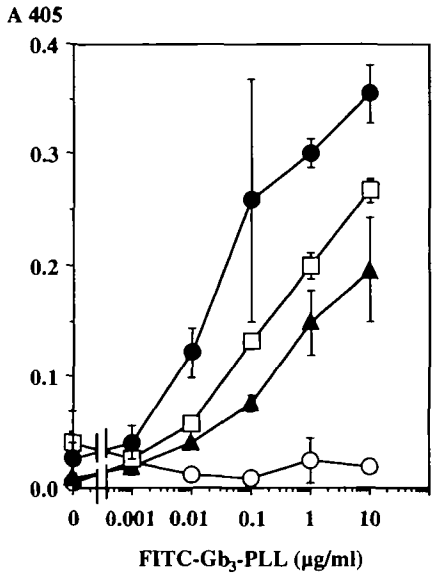

Fig 1 **Solid phase ligand binding assay for SLT-1B.** Recombinant SLT-1B proteins adsorbed on wells were incubated with various concentrations of the multivalent ligand FITC-Gb_3-PLL (abscissa, log scale) The ligand binding was colorimetrically determined using HRP-labeled anti-FITC antibody (ordinate) Proteins were immobilized at 1,000 ng/well (filled circles), 500 ng/well (open squares), 250 ng/well (filled triangles), or nil (open circles) Data are expressed as mean ± SD (n = 3)

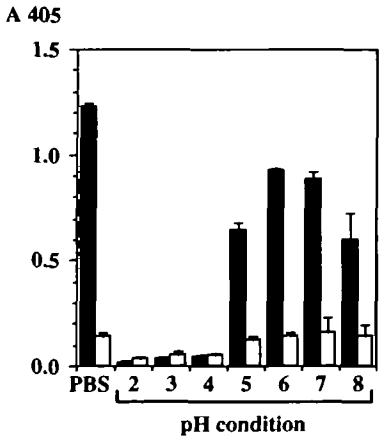

Fig 2. **Dependence of ligand binding on pH.** FITC-Gb_3-PLL (1 μg/ml) was incubated in wells coated with (filled bars) or without (open bars) SLT-1B (500 ng/well), and binding was determined (ordinate). Buffers of the indicated pH or PBS (abscissa) were used. Data are expressed as mean ± SD (n = 3)

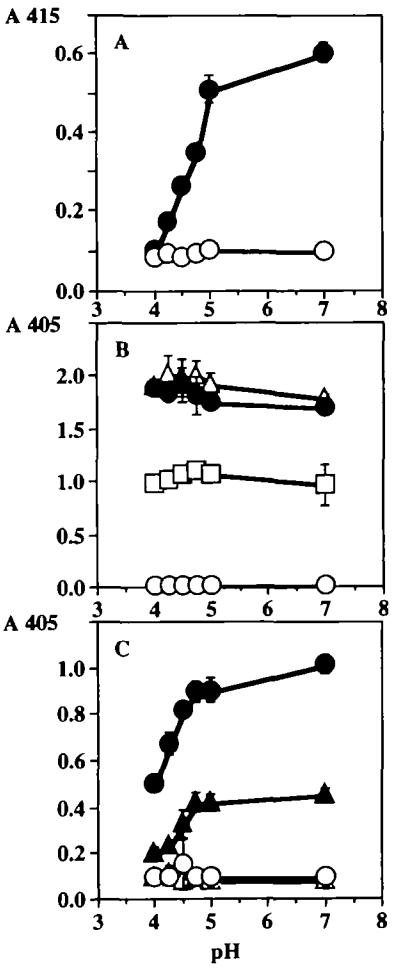

Fig. 3. **Critical pH range that affects ligand binding activity.** A: Binding of FITC-Gb_3-PLL (1 μg/ml) was tested on wells coated with (500 ng/well, filled circles) or without (open circles) SLT-1B using buffers of the indicated pH (abscissa) B Retention of SLT-1B on wells after incubation under acidic conditions. Immobilized SLT-1B was treated with buffers of the indicated pH for 1h at 20°C. The binding of antibodies was compared using anti–SLT-1B antiserum at a dilution of 1 1,000 (filled circles), 1 10,000 (open triangles), 1.100,000 (open squares) or buffer alone (open circles) under the standard buffer conditions. C· FITC-Gb_3-PLL (circles, 1 μg/ml, triangles, 0 1 μg/ml) were allowed to bind to wells coated with (filled symbols) or without (open symbols) SLT-1B under standard conditions. After 2 h incubation at indicated pH, the remaining FITC-Gb_3-PLL was measured under the standard conditions. Data are expressed as mean ± SD (n = 3).

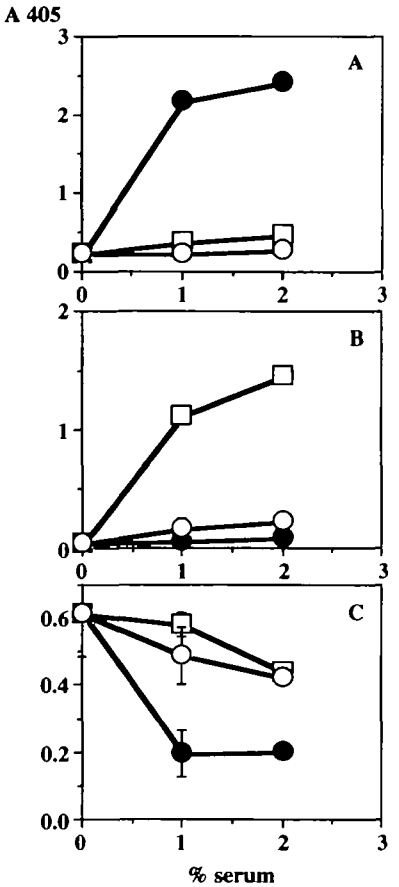

Fig 4 **Effect of anti–SLT-1B antiserum on ligand binding.** A and B Specific binding of IgG to immobilized (500 ng/well) SLT-1B (A) or OVA (B). Aliquots (50 μl) of serum from mice immunized with SLT-1B (filled circles) or OVA (open squares), or from control mice (open circles) were added at the indicated concentrations (abscissa), and the binding of IgG was determined C: Inhibition of ligand binding by specific antiserum Aliquots (50 μl) of mouse serum (as in the panel A) were added at the indicated concentrations (abscissa) to the SLT-1B–coated wells. After a pre-incubation period, 50 μl of FITC-Gb_3-PLL (1 μg/ml) was added. Ligand binding was determined after incubation for 1 h at 20°C Data are expressed as mean ± half ranges (n = 2).

When a suboptimal ligand concentration was used, dissociation was much more evident (Fig 3C, filled triangles).

Specific Antibody Blocks the Binding of FITC-Gb_3-PLL— SLT-1B–specific IgG was demonstrated in SLT-1B–immunized mouse serum but was absent from normal mouse serum or anti-OVA serum (Fig 4A). OVA-specific IgG was detected only in the anti-OVA serum (Fig. 4B). Ligand binding to immobilized SLT-1B was significantly inhibited by SLT-1B–immunized serum. Normal serum or ovalbumin (OVA)-immunized serum produced only a slight reduction at high serum concentrations (Fig. 4C). These results indicate that blocking antibodies can be specifically detected in antiserum using the present assay system.

DISCUSSION

The crystal structure of the complex between SLT-1B and globotriose derivatives initially revealed three binding sites per SLT-1B monomer (*5*). Subsequent structural analyses in solution suggested that only one of these binding sites (site 2) has substantial ligand occupancy (*18, 19*). In site 2, the terminal galactose (Gal 1) and penultimate galactose (Gal 2) were observed to form extensive hydrogen bonds with SLT-1B amino acid residues.

Earlier studies demonstrated that the fluorescence emission from the single Trp residue (Trp 34) in the B subunits decreases dramatically in acidic environments (*7*). The transition was seen at pH 4.5, which is typical as endosomal pH. Resonance energy transfer to Trp 34 from coumarin-modified Gb_3 also suggested the relevance of Trp 34 fluorescence to ligand binding (*19*). Nearby Trp 34, Asn 32 and Arg 33 are involved in hydrogen bonding to Gal 1. Furthermore, Asp 16 in an adjacent B subunit of the SLT-1B pentamer has been observed to provide hydrogen bonding to Gal 1 (*5*) It is conceivable that changes in Trp 34 fluorescence may be a reflection of an alteration in the ionization state of Asp 16 (*7*). If so, low pH conditions should produce critical changes in hydrogen bonding from Asp 16, which may affect the carbohydrate binding activity.

Despite the evidence of pH-dependent local conformational changes, the binding of radioiodinated SLT-1B to immobilized glycolipid Gb_3 has been reported to be unchanged (*7*). These results lead to a conclusion that the local conformational changes may cause the release of the A subunit from the B subunit pentamer, which may promote the subsequent translocation of the A subunit to the cytoplasm (*7*).

We developed an alternative method to detect the carbohydrate binding activity of the B subunits, and examined the effects of pH changes. We observed a disruption of ligand binding and a dissociation of the carbohydrate ligands from immobilized SLT-1B in acidic environments. Although the difference in the results may simply reflect the differing assay formats, it must be emphasized that our system lacks glycolipid. Thus, our results may only reflect the interaction between the carbohydrate binding sites of SLT-1B and carbohydrate residues without support of the lipid portion Further investigations measuring the interaction between the B subunits and carbohydrate ligands using other experimental systems would be helpful to resolve this issue These results may also influence views of the intracellular retrograde transport of SLTs (*6, 20*) The dissociation of the B subunits from carbohydrate ligands in endosomal acidic compartments may play a part in toxin transport into the cytoplasm.

To prepare multivalent carbohydrate ligands, we connected globotriose to poly-lysine by means of reductive amination. The glucose ring at the reducing end was forced open and used as a spacer between the Galα1-4Gal unit and the poly-lysine backbone. Although a hydrophobic interaction between the glucose at the third position and Asn 55 in SLT-1B has been proposed, the glucose did not appear to form a hydrogen bond with SLT-1B (*5*). Our present results are consistent with the structural analyses.

The immobilized SLT-1B seems to offer multiple binding sites for two reasons First, SLT-1B forms an oligomer (*5*) in which multiple binding sites are present. Second, the number of proteins adsorbed on the solid surface may offer binding sites for multiple carbohydrate determinants on the same ligand molecule. For these reasons, relatively weak interactions between monomeric carbohydrates and lectins would be stabilized. In this study, we prepared molecules with one of every 6 lysine residues, on average, modi-

fied with globotriose. However, the microstructure of the ligands is not known at present and the number of modifications could change depending on the reaction conditions. The use of other types of synthetic polymers with globotriose determinants with defined microstructures may produce better results. It would also be interesting to measure the interaction between a single binding site on SLT-1B and globotriose itself to know the cluster effects of multivalent ligands in the binding to SLT-1B.

Immunization with recombinant SLT-1B has been reported to induce antibody production in BALB/c mice in an H-2 restricted manner (*10*). Subsets of BALB/c mouse antibodies could detect unique determinants on native SLT-1B that are absent from the SLT-1B mutant Phe30Ala, which lacks ligand binding activity (*10, 21*). These results suggest that antibodies produced in BALB/c mice may be able to block the carbohydrate binding activity. In the present assay system, we directly demonstrated that BALB/c antiserum against SLT-1B significantly inhibits the binding of multivalent ligands. In contrast, antiserum against OVA had only a marginal effect not greater than that of normal mouse serum. This marginal effect may be caused by interference with serum proteins.

A ligand binding assay based on immobilized lectin and multivalent carbohydrate ligands has been employed to demonstrate the activity of animal lectins (*11, 22*). This type of assay has also been useful to study the effects of blocking antibodies against carbohydrate recognition domains (CRD). For example, mAb MEL-14 against mouse L-selectin CRD efficiently blocks the binding of the carbohydrate ligand phosphomannan monoester core (PPME) to immobilized L-selectin, whereas mAb anti-Ly22 (specifically detects an allotype of L-selectin within the epidermal growth factor-like domain) do not (*12*). It is also useful for screening blocking antibodies against mouse macrophage lectin (MMGL). The binding of FITC-galactose-PLL to immobilized recombinant MMGL has been successfully employed to screen for blocking mAb against CRD of MMGL (*14, 23*) One of the important features of these experiments may be that the activity of immobilized lectins is sensitive to the conformational changes associated with the carbohydrate binding sites. Thus clear cut inhibition of the activity of these calcium-type lectins is observed when the free calcium concentration is reduced (*11, 13, 14*). Furthermore, mAbs that can detect a conformational epitope closely associated with lectin activity have been successfully screened (*14, 23, 24*). In the present experiments, this type of binding assay was employed to detect activity of lectins of bacterial origin. One of the advantages of this assay format is that the lectin activity can be evaluated in a way that is sensitive to local conformational changes of the binding sites. Another advantage is that lectins are readily used without the need to label them. Experiments on other lectins also support the usefulness of the tailor-made assay described in this study as a screening method for blocking monoclonal antibodies against SLT-1B.

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