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Development of recombinant B subunit of Shiga-like Toxin 1 as a probe to detect carbohydrate ligands in immunochemical and flowcytometric application

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Measurement of carbohydrate binding activity of *Escherichia coli* Shiga-like toxin in a simple and quantitative way is an important step for evaluation of antibodies with therapeutic value and of effectiveness of vaccine treatment. We constructed a plasmid vector (pVT1-B5) to express carbohydrate binding (B) subunit of Shiga-like toxin 1 without expression of toxic (A) subunit, and established a simple method to purify the recombinant B subunit, which was then labeled with digoxigenin. The binding specificity of the digoxigenin-labeled B subunit for globotriaosylceramide was established by thin-layer chromatography immunostaining. We developed an enzyme-linked immunosorbent assay using immobilized glycolipids, demonstrating high sensitivity and clear-cut specificity of the assay. The digoxigenin-labeled B subunit was also readily applicable to the detection of cell surface carbohydrate ligands by flowcytometry.

Keywords: **O157, Shiga-like toxin, globotriaosylceramide, CD77, ELISA, lectin**

Abbreviations: **BBG, bovine brain ganglioside mixture; Cer, ceramide; DIG, digoxigenin; GalCer, galactosylceramide (Galβ1-1Cer); Gb_3, globotriaosylceramide (Galα1-4Galβ1-4Glcβ1-1Cer); Gb_4, globotetraosylceramide (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer); GM_{1a}, Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc β1-1Cer; GD_{1a}, Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer; GD_{1b}, Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glcβ1-1Cer; GT_{1b}, Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glcβ1-1Cer; LacCer, lactosylceramide, (Galβ1-4Glcβ1-1Cer); SLT, Shiga-like toxin, STX, Shigella toxin.**

Introduction

Shiga-like toxins (SLTs or verotoxins) are exotoxins, and are virulence factors in gastrointestinal infections with enterohemorrhagic *Escherichia coli* (EHEC) such as serotype O157 : H7. Infection of SLT-producing *E. coli* could result in life-threatening complications such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) [1]. The SLTs are further divided into SLT-1, which have an identical amino acid sequence with *Shigella dysenteriae* toxin (STX) [2,3], and SLT-2, which is about 60% homologous with SLT-1 at the amino acid level [4]. The SLTs consist of toxin component (A subunit) and cell-binding component (B subunit). The B subunits form a pentamer and recognize specific carbohydrate ligands, globotriaosylceramide (Gb_3, Galα1-4Galβ1-4Glcβ1-1ceramide) [5,6].

Induction of protective antibodies against SLTs in animal models [7] and development of vaccines [8] has been tried but with limited success. More challenging studies may be development of therapeutic antibodies against SLTs. To this purpose, it is important to produce antibodies that are capable of neutralizing toxin activity, such as those directed against the carbohydrate recognition sites of the B subunits. It is also necessary to produce fair amount of safe materials that lack toxin activity for immunization and for assay. This study aimed at development of a simple and reliable assay that detect carbohydrate binding activity of SLT-1 B subunit to apply in the production of neutralizing antibody as well as in the assessment of effectiveness of vaccine treatment.

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Materials and methods

Construction of expression vector

A plasmid pUC118 containing an insert of 2.1-kb *Bam*HI-*Bgl*II fragment [9] that encode entire coding sequence for *slt*-1 gene (GenBank accession number: L04539) was kindly provided by Dr. Shinji Yamazaki and Dr. Yoshifumi Takeda (Research Institute International Medical Center of Japan). DNA fragments corresponding to the base position of 4083 to 4491 were prepared by polymerase chain reaction (PCR) using the pUC118 plasmid containing *slt*-1 gene as a template and TaKaRa LA *Taq* polymerase (Takara Shuzo Co., Ltd, Kyoto, Japan). PCR primers were CATGCCATGGATTCATC-CACTCTGGGGGC (sense primer) and GCTTGCGGATC-CGGCAACAACTGACTG (anti-sense primer). The fragments were subcloned into an expression vector p*Trc* 99 A (Amersham Pharmacia Biotech, Tokyo, Japan) between *Nco* I/*Bam*HI cloning sites (termed pVT1-B5) considering a possible translational coupling of *slt*-1B gene with the upstream *slt*-1A gene. Thus the construct has an insert DNA corresponding to a 3′ portion of coding sequence of the A subunit (carboxy-terminal 16 amino acids) in the same reading frame as the ATG start codon from p*Trc* 99 A, followed by Shine-Dalgarno sequence, entire coding sequence of the B subunit (*slt*-1B) and additional downstream DNA (78 bp). The DNA sequence of the coding region of the B subunit was verified by dye terminator cycle sequence analyses using ABI PRISM DNA sequence kit (PE Applied Biosystems, Warrington, UK) using an ABI 373A DNA sequencer (PE Biosystems, Foster City, CA). The plasmid pVT1-B5 was expressed in *E. coli* JM105. An *E. coli* strain JM105 (pSBC32) was kindly provided by Dr. S.B. Calderwood (Department of Microbiology and Molecular Genetics, Harvard Medical School).

Expression and purification of SLT-1 B subunit proteins

The *E. coli* JM105 (pVT1-B5) cells (1/10,000 dilution) were cultured in 600 ml of Luria-Bertani (LB) medium containing ampicillin (100 μg/ml) for 2 h at 37°C. Isopropyl *β*-D-thiogalactoside (IPTG) was added to growing cells ($A_{600} = 0.4$–0.6) at a final concentration of 1 mM, and the bacteria were cultured for additional 4 h at 27°C. The bacteria (A_{600} = approximately 1.0) were harvested by centrifugation at $8,000 \times g$ for 1 min at 4°C, and then washed once with ice cold phosphate-buffered saline (PBS) and recovered by centrifugation. Periplasmic extract was prepared by incubation of the washed bacteria in 18 ml of PBS containing 0.1 mg/ml of polymyxin B sulfate (Sigma) for 30 min at 0°C. The bacterial suspension was centrifuged at $10,000 \times g$ for 10 min, and supernatant was recovered. Ammonium sulfate was added at 4°C to the supernatant to 80% saturation, and proteins were allowed to precipitate for 14 h at 4°C. The precipitate was recovered by centrifugation at $15,000 \times g$ for 30 min at 4°C, dialyzed against 50 mM-Tris/HCl (pH 7.7) using Spectra/Por 3 membrane (MW 3,500 exclusion; Spectrum Laboratories, Inc., Rancho Dominguez, CA) and then applied onto an anion exchange column (16×100 mm; HiPrep 16/10 DEAE, Amersham Pharmacia Biotech, Tokyo, Japan) using FPLC system (Pharmacia). Proteins were eluted with a linear NaCl gradient (0 to 0.5 M) in 50 mM-Tris/HCl (pH 7.7). Pooled fractions that contain SLT-1 B proteins were concentrated using Centriprep 3 concentrators (Amicon, Inc., Beverly, MA), followed by dialysis against 25 mM-imidazole/HCl (pH 7.4). The dialyzed pool was applied to a column (1×20 cm) of Polybuffer exchanger 94 (Pharmacia) equilibrated with the imidazole buffer. Elution was performed with a diluted solution (1 : 8 with water and adjusted to pH 4.0 with HCl) of Polybuffer 74 (Pharmacia) that had been degassed. Fractions (2.2 ml) were collected, and the SLT-1 B-positive fractions were subjected to ammonium sulfate precipitation (80% saturation) to remove polybuffer. The precipitate was dialyzed against PBS. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL).

Electrophoresis

Purity of SLT-1 B proteins was examined by Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) according to the published method [10]. Total acrylamide monomer concentrations were specified in each experiment while the percentage concentration of the cross-linker relative to the total concentration was 6% throughout experiments. Proteins were stained using Coomassie Brilliant blue R250 (0.1% w/v) dissolved in 10% acetic acid. Molecular weight standards were purchased from Pharmacia.

Protein labeling with digoxigenin

DIG-antibody labeling kit was purchased from Boehringer Mannheim (Tokyo, Japan). Purified SLT-1 B proteins were labeled with digoxigenin-3-O-succinyl-*ε*-aminocaproic acid N-hydroxysuccinimide ester (DIG-NHS) according to the supplier's instructions. The molar ratio between monomer of SLT-1 B (MW 7,700) and DIG-NHS in the reaction was 1 : 2.

Thin-layer chromatography (TLC) immunostaining using DIG-SLT-1 B proteins

Glycolipids were obtained from the following materials: Gb_3 (Gal*α*1-4Gal*β*1-4Glc*β*1-1Cer), Gb_4 (GalNAc*β*1-3Gal*α*1-4Gal*β*1-4Glc*β*1-1Cer) and LacCer (Gal*β*1-4Glc*β*1-1Cer) from porcine erythrocytes and GalCer (Gal*β*1-1Cer) from bovine brain as reported previously [11]. Bovine brain ganglioside mixture (BBG) contains equal amount of GM_{1a}, GD_{1a}, GD_{1b} and GT_{1b} that were prepared from bovine brain by anion-exchange chromatography according to the published procedure [12]. TLC immunostaining was performed according to the methods previously described [11]. In brief, glycolipids were separated on a thin-layer plate (Polygram, Sil G, Macherey-Nagel, Germany) in chloroform : methanol : water (60 : 35 : 8, by vol.). The plate was dried, and then incubated in

PBS containing 1% (w/v) bovine serum albumin (BSA Fraction V, Sigma) for 16 h at 4°C to block nonspecific binding sites. Digoxigenin-labeled SLT-1 B proteins (DIG-SLT-1 B) diluted at 1.5 μg/ml in 100 mM Tris-HCl (pH 7.5)–0.15 M NaCl (TBS) containing 0.1% BSA (TBS-BSA) were loaded to the plate, and the plate was incubated for 2 h at 25°C. After extensive washing in TBS, the plate was incubated with peroxidase-labeled sheep anti-digoxigenin Fab fragments (Boehringer) diluted in TBS-BSA (1 : 500) for 2 h at 25°C. The binding of DIG-SLT-1 B was visualized by the reaction using a substrate solution (2 mM 4-chloro-1-naphthol, 1.2 mM N, N-diethyl-p-phenylenediamine, 0.003% w/v H_2O_2 in 0.1 M citrate buffer, pH 6.0), and the presence of glycolipids was visualized by orcinol/sulfuric acid reagent in a parallel TLC experiment.

ELISA assay using DIG-SLT-1 B proteins

Glycolipids were immobilized on an ELISA plate as previously described [11]. Solution of glycolipids dissolved in methanol (50 μl) was added to each well of an ELISA plate (PolySorp; Nunc, Denmark), and the wells were allowed to dry to immobilize glycolipids. Each well was blocked with 100 μl of 1% BSA-PBS for 16 h at 4°C. After extensive washing with PBS, 100 μl of DIG-SLT-1 B proteins in TBS-BSA was added, and the plate was incubated for 1 h at 25°C. The bound DIG-SLT-1 B proteins were detected by incubation with 100 μl of peroxidase-labeled sheep anti-digoxigenin Fab fragments (1 : 500 dilution in TBS-BSA) for 1 h at 25°C, followed by colorimetric detection (OD 490 nm) using 0.4% (w/v) o-phenylenediamine, 0.012% (w/v) H_2O_2 in 0.1 M citrate buffer (pH 5.0) as a substrate. The wells were washed five times with PBS between each incubation.

Flowcytometric detection using DIG-SLT-1 B proteins

Ability of SLT-1 B proteins to detect cell surface binding sites was assessed by flowcytometric analyses as previously described [13]. MOLT-4 (T cell line), and Burkitt's lymphoma-derived cell lines Daudi, Raji, and RAMOS were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Boehringer) and 60 μg/ml kanamycin sulfate in a humidified atmosphere of 5% CO_2, 95% air at 37°C as previously described [11]. Cells (1×10^6) were incubated with or without DIG-SLT-1 B proteins (1.5 μg/ml) in 1 ml of PBS containing 0.1% BSA and 0.1% NaN_3 (PBS-BSA) for 30 min at 0°C. After washing in PBS-BSA, the cells were incubated with FITC-labeled sheep anti-digoxigenin Fab fragments (Boehringer) in 1 ml of PBS-BSA (1 : 500 dilution) for 30 min at 0°C. The cells were analyzed on a flowcytometer (EPICS XL, Beckman Coulter, Tokyo, Japan) using gates of forward and side light scatter.

Results

Expression of SLT-1 B subunit

To express SLT-1 B subunits without expression of toxic A subunit, pVT1-B5 was constructed. A plasmid encoding entire sequence corresponding to *slt*-1 gene, which was originally derived from SLT-1-converting phage isolated from *E. coli* O157 : H7 strain 83–1386 [9], was used as a template to prepare DNA inserts by PCR. Because of polycistronic nature of *slt*-1 gene, pVT1-B5 was designed so that the carboxy-terminal 16 amino acids of the A subunit was also expressed to avoid problems due to a possible translational coupling [14]. The plasmid pVT1-B5 was introduced into *E. coli* JM105, and whole bacterial extracts were electrophoresed to detect B subunit (Fig. 1). Upon IPTG induction, a protein band of apparent molecular weight of 7,000 was detected by Tricine-

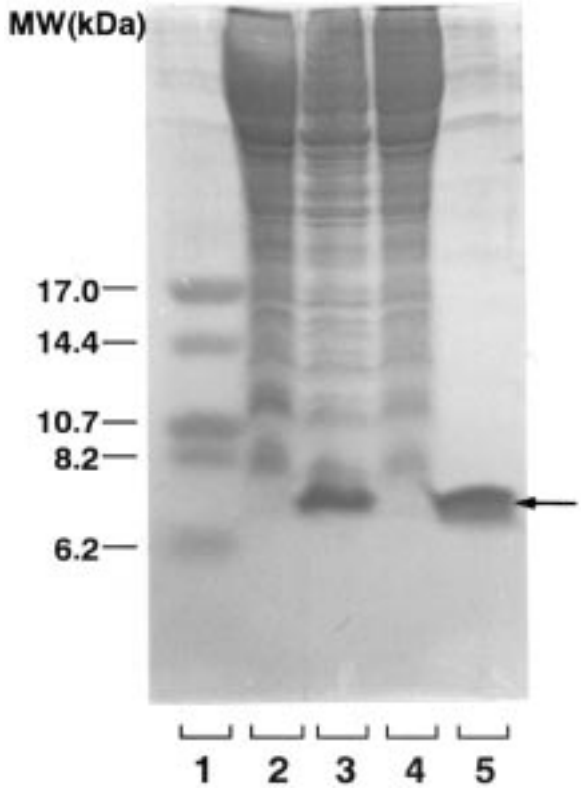

Figure 1. Synthesis of SLT-1 B subunits in *E. coli* JM105(pVT1-B5) after IPTG induction. Tricine-SDS-PAGE (12.5% gel, non-reducing conditions) analysis was carried out using whole bacterial extracts (equivalent to bacteria in 100 μl of harvest) from *E. coli* JM105 (lane 2), or from *E. coli* JM105(pVT1-B5) with (lanes 3) or without (lanes 4) IPTG induction. Purified SLT-1 B proteins (4 μg) from *E. coli* JM105(pSBC32) as a positive control (lane 5), and molecular weight standards (lane 1) were also electrophoresed. The position representing electrophoretic mobility of SLT-1 B proteins was marked by an arrow. Molecular weight standards are myoglobin (17.0 kDa), myoglobin I + II (14.4 kDa), myoglobin I + III (10.7 kDa), myoglobin I (8.2 kDa) and myoglobin II (6.2 kDa).

SDS-PAGE. This band was in the same mobility as the band of purified B subunits produced by *E. coli* JM105(pSBC32) used as a positive control. Without IPTG induction, no protein band was seen at the corresponding position. Host *E. coli* extract lacked this band.

Purification of SLT-1 B subunit proteins

The B subunits were expected to be released into periplasmic space, because signal sequence for the B subunit secretion was present in the pVT1-B5 construct. As expected, B subunit proteins were effectively recovered in the supernatant after polymyxin B treatment. Dose response experiments revealed that as low as 0.1 mg/ml of polymyxin B was sufficient (data not shown). The periplasmic extract was subjected to ammonium sulfate precipitation followed by separation on a DEAE column using FPLC system. Fractions were analyzed by Tricine-SDS-PAGE, and the B subunits were eluted with approximately 0.2 M NaCl (Fig. 2a). Although fractions 27 to 29 contained a single major protein of apparent MW of 7,000 (Fig. 2b, arrow), we further purified B subunits using chromatofocusing as reported by Ramotar *et al.* [15]. Symmetrical and base line-separated protein peak was observed at pH 5.6 (Fig. 3a, fractions 50 to 53), and Tricine-SDS-PAGE analysis of the combined fractions revealed a single band of apparent MW of 7,000 (Fig. 3b, arrow).

Specific binding to glycolipids

To prepare versatile reagent as a probe for specific carbohydrate ligands, purified SLT-1 B proteins (chromatofocusing pooled fractions 50 to 53) were labeled with digoxigenin. The binding of DIG-SLT-1 B proteins was demonstrated in the format of TLC immunostaining (Fig. 4). Gb_3, Gb_4, GalCer, LacCer and BBG were separated on a TLC plate. The presence of these glycolipids on the TLC plate was demonstrated by orcinol/sulfuric acid reaction (Fig. 4a, lane 1–5). By probing with DIG-SLT-1 B proteins, together with peroxidase-anti-DIG antibody, Gb_3 produced strong positive reaction (Fig. 4a, lane 10). In contrast, GalCer, LacCer and BBG were entirely negative. Weak cross reactivity to Gb_4 was observed. However, the signal intensity obtained for Gb_4 was much lower than that obtained for Gb_3. In addition, DIG-SLT-1 B even detected a band representing contaminated Gb_3, which is not visible by orcinol/sulfuric acid reagent, in this particular Gb_4 preparation. Dose response analyses demonstrated that as low as 31 pmol of Gb_3 can be probed in the TLC immunostaining, whereas LacCer or BBG did not produce positive reaction at 500 pmol (Fig. 4b). Faint bands represent-

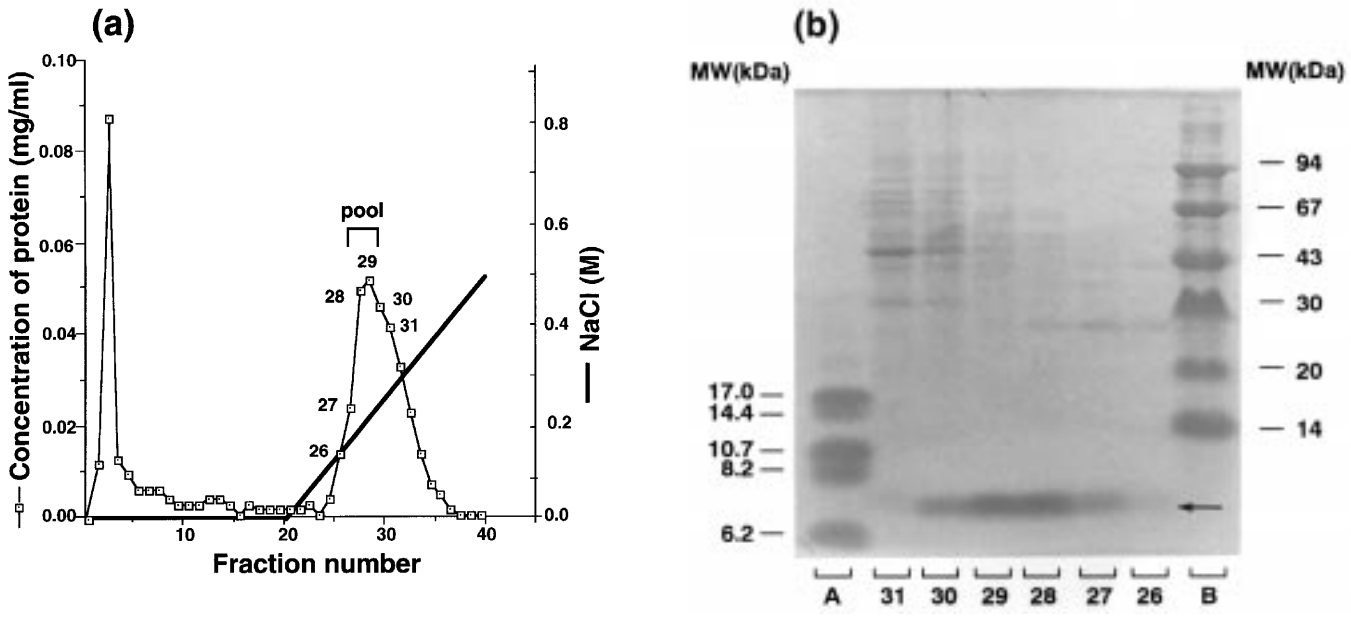

Figure 2. Purification of SLT-1 B proteins using an anion exchange chromatography. (a) Elution profile of SLT-1 B from DEAE-FPLC column. Periplasmic extract of IPTG-induced *E. coli* JM105(pVT1-B5) was subjected to ammonium sulfate precipitation and then applied on an DEAE column. Proteins were eluted with a linear NaCl gradient (right scale), and 5 ml fractions were collected. Protein concentration in each fraction was determined by BCA protein assay kit (open square, left scale). (b) Tricine-SDS-PAGE (10% gel, non-reducing conditions) analysis of fractions from the DEAE column. Fractions (26–31, indicated at the bottom) were analyzed. The protein band representing SLT-1 B was marked by an arrow. Molecular weight standards in lane A are the same as shown in Figure 1, while those in lane B are phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), lactalbumin (14 kDa). Fractions (27–29) were pooled for further processing.

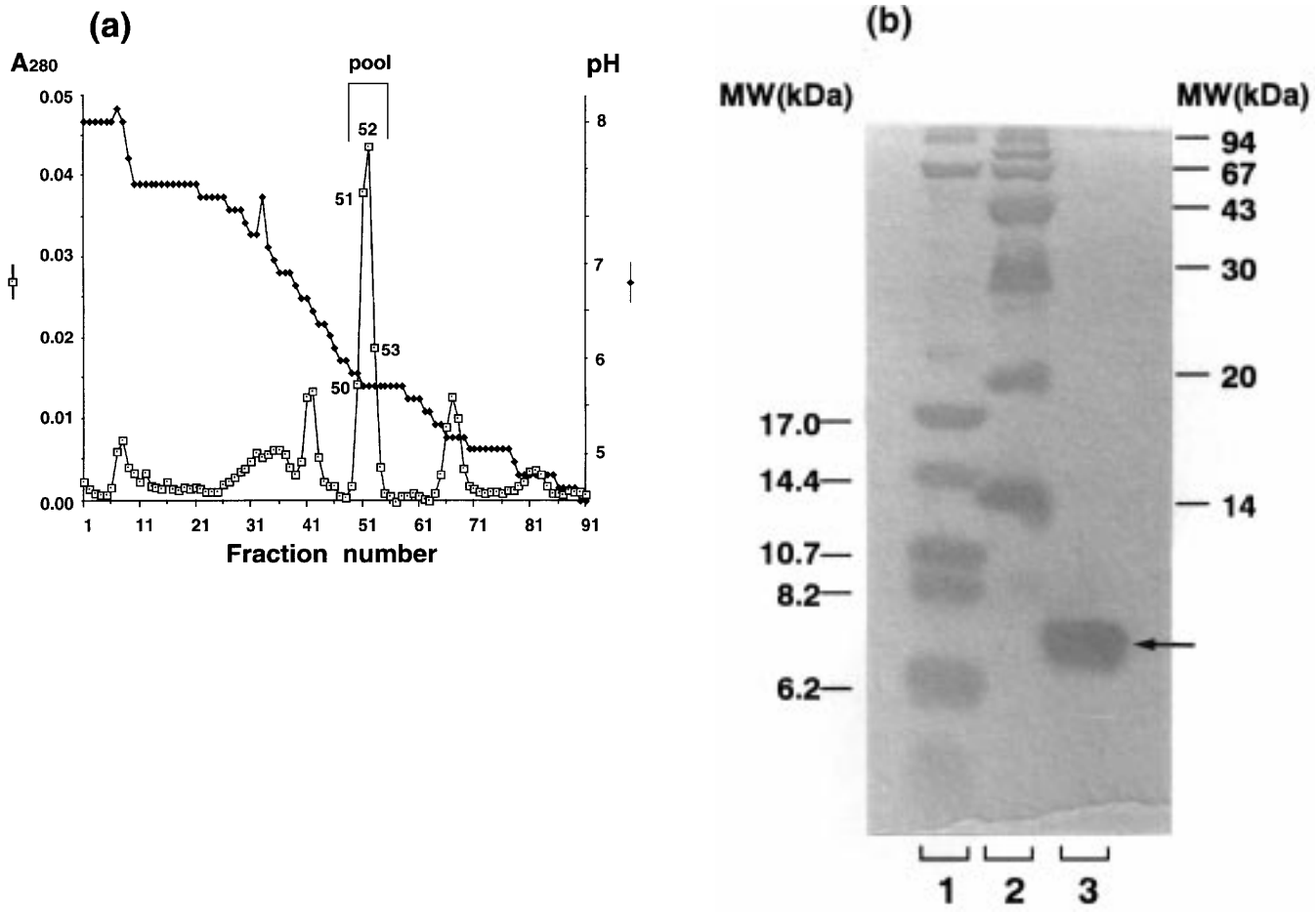

Figure 3. Purification of SLT-1 B proteins using chromatofocusing. (a) Elution profile from Polybuffer exchanger 94. Pooled fractions containing SLT-1 B (fraction 27–29 from DEAE column) were applied on a column of Polybuffer exchanger 94. Proteins were eluted with Polybuffer 74 (1 : 8, pH 4.0). A_{280} (open square, left scale) and pH (closed diamond, right scale) was measured in each fraction. Fraction 50–53 were combined and concentrated. (b) Tricine-SDS-PAGE (12.5% gel, non-reducing conditions) analysis of an aliquot (11 μg protein) of the combined fractions (lane 3). The protein band representing SLT-1 B was marked by an arrow. Molecular weight standards (lane 1 and 2) are the same as those used in Figure 2.

ing Gb_4 and particularly contamintated Gb_3 in this case were detected only at 500 pmol.

ELISA assay using glycolipids

We next tried to establish an immunoassay based on an ELISA format. Glycolipids (Gb_3, Gb_4, GalCer or LacCer) were coated on an ELISA plate, and the binding of DIG-SLT-1 B to glycolipids was detected by peroxidase-anti-DIG antibody. Dose response curve of glycolipids showed that as low as 2 pmol of Gb_3 produced a significant signal (Fig. 5a). In contrast, GalCer and LacCer did not produce positive signals up to 200 pmol. In contrast, DIG-SLT-1 B cross reacted to Gb_4. This particular preparation of Gb_4 produced significant signal at 12 pmol or more. Because small amount of Gb_3 in this Gb_4 preparation was detected by DIG-SLT-1 B in the TLC immunostaining assay (Fig. 4a), Gb_3 is likely to be responsible for at least part of the signals obtained with the Gb_4 preparation. Dose response curve of DIG-SLT-1 B proteins revealed a saturable binding of the B subunit (Fig. 5b). As low as 20 ng/ml of DIG-SLT-1 B proteins successfully detected Gb_3 on an ELISA well. Higher concentration of DIG-SLT-1 B proteins (more than 6 μg/ml) appeared to display a weak interaction with GalCer, while no cross reactivity to LacCer was observed up to 12 μg/ml. In the case of the Gb_4 preparation, significant cross reaction was observed, however, 16-fold higher concentration of DIG-SLT-1 B was required for obtaining a comparable level of signal observed with Gb_3. These results, together with TLC immunostaining, clearly demonstrate sensitivity and specificity of DIG-SLT-1 B as a probe for glycolipid ligands.

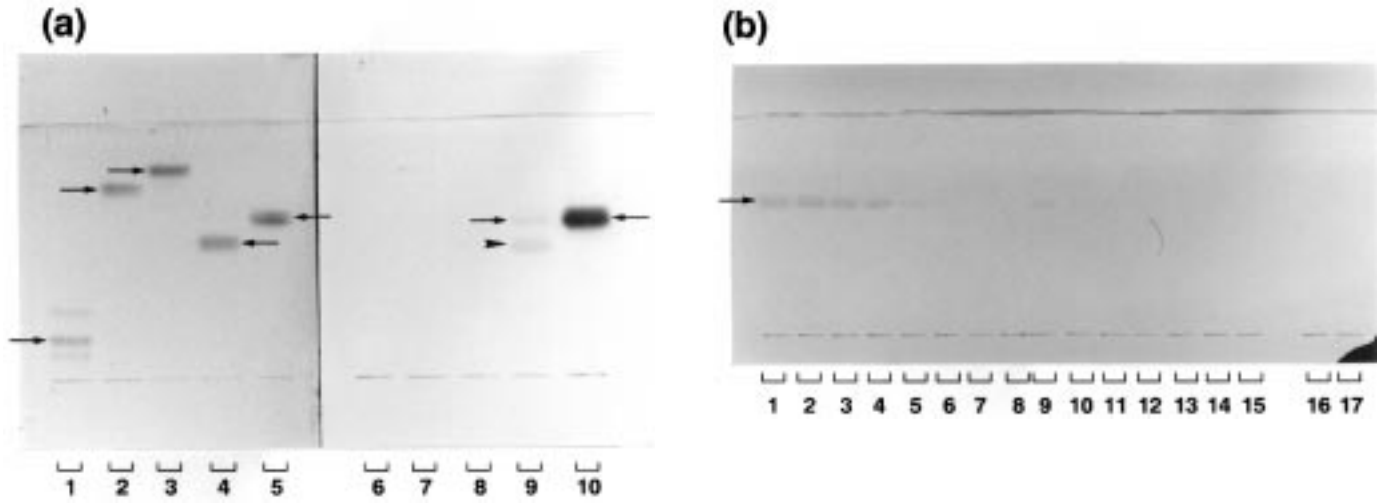

Figure 4. Specific binding of DIG-SLT-1 B to Gb_3 glycolipids. (a) BBG (lanes 1, 6), LacCer (lanes 2, 7), GalCer (lanes 3, 8), Gb_4 (lanes 4, 9) and Gb_3 (lanes 5, 10) were separated on a TLC plate. Each glycolipid was applied at 500 pmol except that BBG contained 500 pmol of each ganglioside component. Glycolipids were stained using orcinol/sulfuric acid (lanes 1–5, arrows) or probed by DIG-SLT-1 B and peroxidase-anti-DIG antibody (lanes 6–10). DIG-SLT-1 B strongly reacted to Gb_3 (lane 10, an arrow). Weak binding to Gb_4 (lane 9, an arrowhead) was observed as well as to contaminated Gb_3 in this preparation (lane 9, an arrow). (b) Dose response of TLC-immunostaining using DIG-SLT-1 B. Varying amounts of Gb_3 (lanes 1–8), Gb_4 (lanes 9–15), BBG (lane 16) or LacCer (lane 17) were separated on a TLC plate. Amounts of glycolipids were 500 pmol (lanes 1, 9, 16, 17), 250 pmol (lanes 2, 10), 125 pmol (lanes 3, 11), 63 pmol (lanes 4, 12), 31 pmol (lanes 5, 13), 16 pmol (lanes 6, 14), 8 pmol (lanes 7, 15) and 4 pmol (lane 8). BBG contained 500 pmol of each ganglioside component. Gb_3 was detected as low as 31 pmol (lane 5), whereas BBG and LacCer gave negative signal at 500 pmol. DIG-SLT-1 B weakly reacted to Gb_4 only at 500 pmol (lane 9).

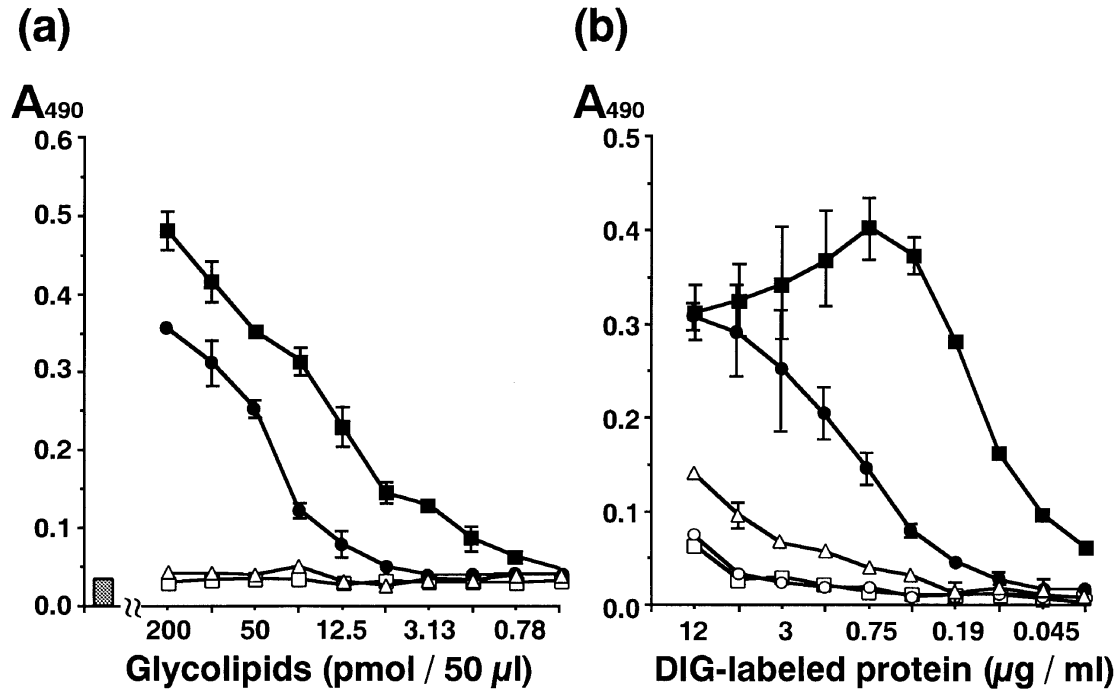

Figure 5. ELISA assay using DIG-SLT-1 B and Gb_3 glycolipids. (a) Dose response of coated-glycolipids on DIG-SLT-1 B binding. Wells were coated with varying amounts (abscissa) of Gb_3 (filled square), Gb_4 (filled circle), GalCer (open triangle). LacCer (open square), or without glycolipids (bar). Fixed amount of DIG-SLT-1 B proteins (1.5 μg/ml) was added, and their binding was detected by peroxidase-anti-DIG antibody. (b) Dose response of DIG-SLT-1 B proteins on binding to Gb_3. Wells were coated with 50 pmol of Gb_3 (filled square), Gb_4 (filled circle), GalCer (open triangle), LacCer (open square), or without glycolipids (open circle). Binding was measured in response to concentration of DIG-SLT-1 B proteins (abscissa). Values represent means of duplicate determinations, and *error bars* indicate half ranges of determination.

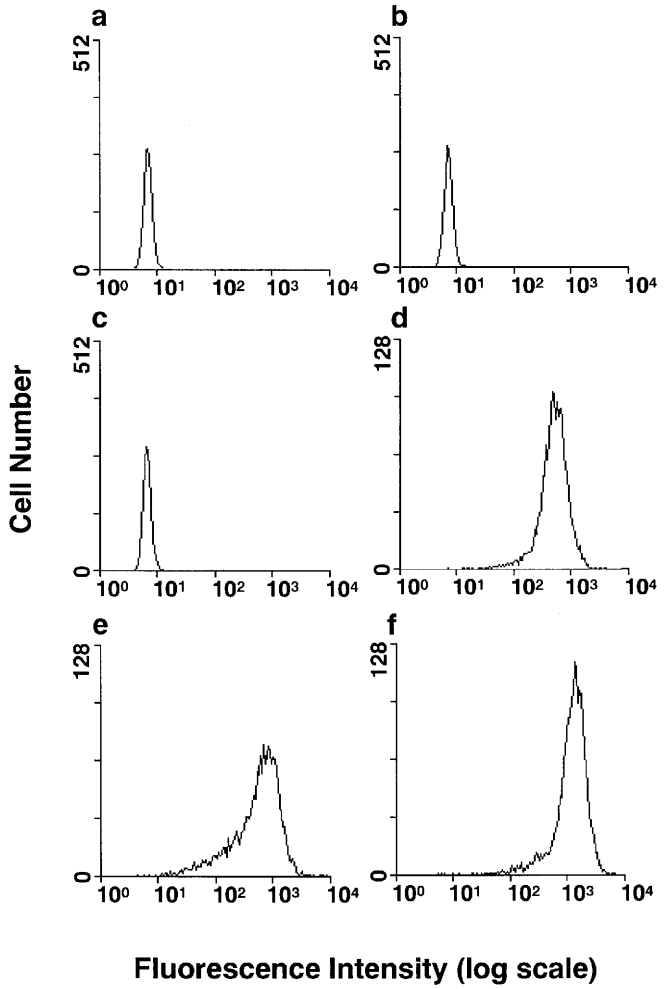

Figure 6. Flow cytometric detection of binding of DIG-SLT-1 B proteins. MOLT-4 (a, b), Daudi (c, d), Raji (e), or RAMOS (f) cells were incubated with (b, d, e, f) or without (a, c) DIG-SLT-1 B proteins. Cells were incubated with FITC-anti-DIG antibody, and then analyzed on a flowcytometer. Background levels (FITC-anti-DIG alone) for Raji and RAMOS cells were not shown, because they were same as those for Daudi (c).

Binding of SLT-1 B to live cells

We next examined whether DIG-SLT-1 B can be used as a probe for flowcytometry to detect Gb_3/CD77 cell surface antigens. Cells were incubated with DIG-SLT-1 B followed by FITC-anti DIG antibody and analyzed on a flowcytometer. CD77-positive Burkitt's lymphoma cells (Daudi, Raji and RAMOS) were strongly stained with DIG-SLT-1 B whereas MOLT-4T cells (CD77-negative) were not stained at all (Fig. 6). These results further demonstrated the specificity and sensitivity of the probe in flowcytometric application.

Discussion

To develop neutralizing antibody against SLT-1 in a systematic way, it would be helpful to establish a simple and reliable assay measuring carbohydrate binding activity of SLT-1 binding subunit (B subunit). To this end, we produced a versatile reagent, digoxigenin-labeled recombinant SLT-1 B subunit (DIG-SLT-1 B), and demonstrated its effectiveness in carbohydrate binding assay using glycolipids as well as in flowcytometric assay using CD77-positive Burkitt's lymphoma cells. DIG labeling technique has been successfully used to detect interaction between cholera toxin B subunit and DIG-labeled GM_1 ganglioside [16]. This is another way to apply DIG labeling to study interaction between enterotoxins and their ligands, though the versatility is limited.

Although recombinant SLT-1 B subunit proteins have been available, there are mainly two sources that is widely used in experiments. They are proteins expressed from pSBC32 plasmid vector [14], and those from pJLB120 and related plasmids [15,17]. Recent reports almost exclusively used recombinant proteins derived from pJLB120 and related plasmids, and interest has been limited to elucidate structural basis of protein-carbohydrate interaction by physicochemical measurements [6,18–20]. The recombinant proteins derived from pJLB120 was also used to compare immunogenicity to mouse strains [7] and induction of apoptosis in Burkitt's lymphoma cells [21].

We constructed an expression vector pVT1-B5 using plasmid DNA encoding *slt*-1 gene from an independent origin, and produced recombinant SLT-1 B proteins in an inducible manner. We purified SLT-1 B proteins into homogeneity and labeled the proteins by a simple procedure, resulting in DIG-SLT-1 B preparations. TLC immunostaining demonstrated that the binding of DIG-SLT-1 B has specificity to Gb_3 glycolipids, but with weak cross reactivity to Gb_4. The extent of cross reactivity to Gb_4 seems to be variable between experiments by unknown reasons. Although conventional staining procedure for glycolipids was not able to detect contaminated Gb_3 in the Gb_4 preparation, DIG-SLT-1 B detected such faint contaminants (Fig. 4). The cross reactivity to Gb_4 appeared to be more evident in the ELISA assay (Fig. 5). However, contaminated Gb_3 in the Gb_4 preparation may significantly contribute to the signals obtained from the wells coated with the Gb_4 preparation. Original observations using STX holotoxin demonstrated a weak binding to Gb_4 [5]. Subsequent studies using recombinant SLT-1 B proteins derived from pJLB120 and related plasmids, however, reported lack of interaction to Gb_4 [15,17]. We also expressed and purified recombinant SLT-1 B proteins using pSBC32 plasmid, and the proteins were labeled with DIG. Binding specificity to glycolipids was similar between the preparation using pSBC32 and pVT1-B5 in our hands (Miyashita *et al.*, unpublished observation). Reasons underlying the difference in the weak cross reactivity to Gb_4 are currently unknown.

Because the binding of DIG-SLT-1 B was easily detected by anti-DIG antibody, this reagent was applicable to detect binding not only to glycolipids but to cell surface carbohydrate ligands as cytochemical and presumably histochemical reagents. In flowcytometry analyses, all three Burkitt's

lymphoma lines (Daudi, RAMOS and Raji) were strongly stained with DIG-SLT-1 B with completely negative staining of control T cell line MOLT-4. To demonstrate carbohydrate binding activity of SLT-1 B, earlier studies used inhibition of the binding of radio labeled SLT-1 holotoxin to Hela cells [14] and to Gb_3 glycolipids [15], binding of radio labeled SLT-1 B itself [20], or binding detected by antiserum prepared against SLT-1 [17]. Our assay has an advantage over the previous experiments because commonly used anti-DIG Fab fragments yielded high sensitivity, low background, and versatility. The ELISA based binding assay will be ideal to screen blocking antibody directed against SLT-1 B carbohydrate recognition sites.

Although Gb_3 used in this study was prepared from porcine erythrocyte, Gb_3 has been isolated from microvillus membranes of rabbit intestinal mucosa as a target for STX [22,23]. The activity of glycosyltransferase producing Gb_3 has also been demonstrated in rabbit small intestine [23]. These results indicate pathological relevance of Gb_3 as target molecules for STX and SLT-1 during infection.

Structural and functional similarity between SLT-1 and certain plant toxins, such as ricin, has been reported [2]. Although homology in the amino acid sequence of the A subunit between ricin and SLT-1 is high, there is no significant homology between the carbohydrate binding B subunits [2]. Therefore, the mechanism for carbohydrate recognition is different. In the case of ricin, which has specificity to β-galactosides, the B subunit has been reported to be arranged into two globular domains [24]. Each domain consists of 3 subdomain units containing 40 amino acid residues. One domain consists of 1α, 1β, 1γ subdomains, while the other consists of 2α, 2β, 2γ. The subdomains 1α and 2γ have carbohydrate binding sites. In the case of 1α subdomain, for example, Trp 37 provides flat binding surface to galactose, which was hydrogen-bonded with Asn 46 and Asp 22 [24]. In contrast, SLT-1 B, which has 69 amino acid residues, has been reported to form a pentamer [6]. Initial report for crystal structure of SLT-1 B complexed with Gb_3 trisaccharides revealed 3 binding sites per each monomer. Therefore, 15 binding sites per SLT-1 molecule were proposed [6]. Subsequent studies of solution structure using NMR analysis [25] and fluorescence analysis [26] reported that only one of the three binding sites (site 2) had substantial ligand occupancy. In the case of the site 2, terminal galactose of Gb_3 trisaccharide hydrogen-bonded to Asn 32 and Arg 33 as well as to Asp 16 from neighboring B subunits of the pentamer [6]. This suggests importance of pentamer formation on the carbohydrate recognition by SLT-1. In addition, C6 hydroxyl group of the penultimate galactose interacted with Asn 55 through hydrogen bonding. This will provide a basis for Galα1-4Gal specificity of SLT-1 B. Aromatic stacking interaction was not evident in the site 2, but terminal galactose interacts with Phe 30 through hydrophobic interaction [6]. Taken together, three dimensional structures of the carbohydrate binding sites of ricin B and SLT-1 B subunits are quite different.

In conclusion, we developed a versatile system to evaluate carbohydrate binding activity of SLT-1. This system will be useful not only for screening of blocking antibody in an ELISA assay but for studies of signal transduction through CD77 antigens [21] and intracellular trafficking of SLT-1 proteins [27].

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