Kinetic Analysis of Binding between Shiga Toxin and Receptor Glycolipid Gb3Cer by Surface Plasmon Resonance*

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Shiga toxin (Stx) binds to the receptor glycolipid Gb3Cer on the cell surface and is responsible for hemolytic uremic syndrome. Stx has two isoforms, Stx1 and Stx2, and in clinical settings Stx2 is known to cause more severe symptoms, although the differences between the mechanisms of action of Stx1 and Stx2 are as yet unknown. In this study, the binding modes of these two isoforms to the receptor were investigated with a surface plasmon resonance analyzer to compare differences by real time receptor binding analysis. A sensor chip having a lipophilically modified dextran matrix or quasicyrystalline hydrophobic layer was used to immobilize an amphiphatic lipid layer that mimics the plasma membrane surface. Dose responsiveness was observed in both isoforms when either the toxin concentration or the Gb3Cer concentration was increased. In addition, this assay was shown to be specific, because neither Stx1 nor Stx2 bound to GM3, but both bound weakly to Gb4Cer. It was also shown that a number of fitting models can be used to analyze the sensorgrams obtained with different concentrations of the toxins, and the “bi-valent analyte” model was found to best fit the interaction between Stxs and Gb3Cer. This shows that the interaction between Stxs and Gb3Cer in the lipid bilayer has a multivalent effect. The presence of cholesterol in the lipid bilayer significantly enhanced the binding of Stxs to Gb3Cer, although kinetics were unaffected. The association and dissociation rate constants of Stx1 were larger than those of Stx2; Stx2 binds to the receptor more slowly than Stx1 but, once bound, is difficult to dissociate. The data described herein clearly demonstrate differences between the binding properties of Stx1 and Stx2 and may facilitate understanding of the differences in clinical manifestations caused by these toxins.

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The Shiga toxin (Stx) is a member of bacterial cytotoxins produced by Shigella dysenteriae type 1 and Shiga-toxin-producing Escherichia coli (STEC). The Stx family consists of two isoforms, Stx1 and Stx2, and ~56% of their nucleotide sequences are identical (1). Both Stxs have a subunit structure in which five 7.7-kDa B subunits are associated noncovalently with a globular 32-kDa A subunit through its carboxyl-terminal amino acids (2). The B subunits mediate binding of the toxin to the surface receptor, glycolipid Gb3Cer, on susceptible cells (3–5), whereas the A subunit inhibits protein synthesis by catalytic inactivation of the eukaryotic 60 S ribosomal subunit by its RNA N-glycosidase activity (6–10). The Stxs initially bind to the cell surface, and the receptor-ligand complex is then internalized by endocytosis mediated by clathrin-coated pits (11) targeted intracellularly to the Golgi apparatus and endoplasmic reticulum by a process called retrograde transport (12). After translocation to the cytosol, Stx displays its cytotoxicity.

These toxins have been shown to be responsible for hemorrhagic colitis and hemolytic uremic syndrome clinically (13, 14), and Stx2 has been found to cause more serious diseases than Stx1 (15–18). Stx1 and Stx2 have consistently been shown to differ significantly in their toxicity for mice in vivo, and Stx2 has been shown to be more toxic in mice than Stx1 (19). Similar results were also obtained when human renal microvascular endothelial cells were treated with Stx1 or Stx2, with Stx2 being found to be about 1000-fold more toxic (20). In contrast with these observations, however, Stx1 and Stx2 display indistinguishable enzymatic activity in cell-free systems (10), and thus possible differences in binding properties may be important in explaining differences between the toxicity of Stx1 and Stx2 in vivo.

Biosensors, such as the BIACore™, which can monitor interactions between molecules in real time on the basis of surface plasmon resonance (SPR), have recently been applied to analysis on the binding of bacterial toxins to plasma membrane component glycolipids, such as the binding of cholera toxin (CT) to ganglioside GM1 (21–23). SPR assay has a number of advantages over other methods for analyzing molecular binding. We used this recently developed technology to study the interaction kinetics of Stxs toward their receptor glycolipid Gb3Cer as a means of elucidating the mechanism responsible for the difference between the biological activities of Stx1 and Stx2. To

1 The abbreviations used are: Stx, Shiga toxin; SPR, surface plasmon resonance; RU, resonance unit; STEC, Shiga-toxin-producing Escherichia coli; POPC, 1-palmitoyl 2-oleoyl-sn-glycero-3-phosphocholine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; CT, cholera toxin; GSL, glycosphingolipid.

2 Glycosphingolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (52). Gangliosides are abbreviated according to Svennerholm (53).
Fig. 1. Immobilization of liposomes for the generation of an amphipathic lipid layer on a lipophlic dextran matrix-conjugated sensor chip surface. The liposome immobilization steps are shown in chronological order. A, injection of 50 mM CHAPS to clean the sensor chip surface (5 μl/ml for 5 min); B, injection of 30% ethanol to remove the CHAPS (5 μl/ml for 5 min); C, injection of liposomes with or without GSLs to form an amphipathic lipid layer on the sensor chip surface (1 μl/min for 400 min); D, injection of 50 mM NaOH to detach multiple lipid layers and to check the stability of the immobilized lipids to alkaline pH (5 μl/ml for 1 min); E, injection of 1 mg/ml bovine serum albumin to block the unoccupied hydrophobic surface. In the last step, alternate injections of NaOH (D) and bovine serum albumin (E) were repeated.

analyze the binding properties of Stxs toward Gb3Cer as a plasma membrane component more precisely, we used modified dextran to immobilize Gb3Cer in a lipid bilayer of glycerophospholipids on a sensor chip. In this paper, we report significant differences in the binding properties of Stxs1 and Stxs2 that may enable better understanding of the differences in clinical the manifestations produced by the toxins.

EXPERIMENTAL PROCEDURES

Materials—Sphingolipids were obtained from Matreya, Inc. (Pleasant Gap, PA) and used with further purification. 1-Palmitoyl 2-oleyl-sn-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was purchased from Sigma-Aldrich. Stxs was prepared as described previously (24).

Liposome Preparation—Mixtures of glycerol phospholipids (POPC) containing only glycosphingolipid or both of glycosphingolipid and cholesterol in chloroform were dried in glass vials under a N2 gas stream to remove residual solvent. Stxs was added to each vial. The vials were then vortexed vigorously and placed in a sonic bath for 10 min. The final concentration of the total amphipathic lipid mixture was 0.5 mM. The liposomes were immediately injected onto the sensor chip.

Immobilization of Amphipathic Lipids on an SPR Sensor Chip—SPR was measured with a Biacore 2000 system using pioneer sensor chip L1 or sensor chip HPA (Biacore, Upptalsa, Sweden). Pioneer sensor chip L1 contains a dextran matrix to which hydrophobic residues are covalently bound. The surface of sensor chip HPA is composed of long-chain alkane molecules that form a flat, quasi-crystalline hydrophobic bound. The surface of sensor chip HPA is composed of long-chain alkanethiol molecules to which hydrophobic residues are covalently bound. In the last step, alternate injections of NaOH (D) and bovine serum albumin (E) were repeated.

Affinities were calculated from analysis of equilibrium binding experiments, where the values can be calculated from rate constants in the “bivalent analyte” model. By measuring the equilibrium resonance units (R_{eq}) of several ligand concentrations in the equilibrium state, binding data can be analyzed by Scatchard plots using the BIA evaluation software function and this equation,}

$$R_{eq}/C = K_a R_{max} - K_a R_{eq}$$

where $R_{eq}$ is equilibrium resonance units, $R_{max}$ is the resonance signal at saturation, C is the concentration of free analyte, and $K_a$ is the association constant.

RESULTS

Immobilization of Amphipathic Lipid Bilayer on Sensor Chip Surface—The surface of sensor chip L1 coated with an amphipathic lipid layer containing the receptor glycolipid Gb3Cer was generated by lipophilic interaction between the hydrophobic alkyl residues of the dextran matrix and the liposomes. Since alkyl residues are covalently bound to the matrix, immobilized liposomes are known to be fused and thereby to form a lipid bilayer (25). The immobilization process of the lipid layer sensor chip is shown in Fig. 1. At the end of this process, stability at high pH was checked by alternate injection of 50 mM NaOH and 1 mg/ml bovine serum albumin. As shown in Fig. 1, the mass on the sensor chip did not change after at least four washings with 50 mM NaOH solution at a flow rate 5 μl/min for 1 min, and thus, it was concluded that a lipid bilayer stable to high pH values had formed on sensor chip L1. The sensor chip was also stable after at least 50 regenerations with 50 mM Tris (pH 9.0) (data not shown). Approximately 10,500 RU of amphipathic lipids were immobilized, with 1 RU corresponding to 1 pg/mm² of the immobilized molecule, indicating that 10.5 ng/mm² of lipids had been immobilized. Based on the average molecular mass, it was calculated that 13.6 pmol/mm² or 8.2 × 10^{12} molecule/mm² had been immobilized on the sensor chip surface. The amount of immobilized amphipathic lipids remained unchanged even when the lipid composition was changed (data not shown).
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**Fig. 2. Effect of Gb3Cer concentration on Stx binding to lipid layer immobilized onto sensor chip L1.** Overlay plot of Stx1 (A) and Stx2 (B) binding to lipid layers containing 0, 0.2, 1, and 5% Gb3Cer, as indicated. a, start of Stxs injection (0.3 μm); b, end of Stxs injection.

The surface of sensor chip HPA was also coated with an amphipathic lipid layer by the same procedure (data not shown). Since this sensor chip has fixed hydrophobic residues on one side, the amphipathic lipids are thought to be immobilized as an artificial monolayer. Approximately 3200 RU (3.2 ng/mm²) amphipathic lipids were immobilized, indicating that 4.14 pmol/mm² or 2.5 × 10⁻¹¹ molecule/mm² had been immobilized on the sensor chip surface. Immobilized lipid was unstable during the multiple regeneration steps (data not shown).

**Toxin Binding Kinetics**—The dose-response relationship was estimated to assess the effect of the Gb3Cer concentration in the lipid bilayer on sensor chip L1 (Fig. 2). As shown in Fig. 2, the sensorgrams of the 0.2% Gb3Cer immobilized flow cell showed no specific binding of Stx1 over the control flow cell (0% Gb3Cer), but the sensorgrams of 1 and 5% Gb3Cer showed dose-dependent decreases in Stx1 binding (Fig. 2A). Similar dose dependence was observed when Stx2 was used, but the responses (RU) were lower than with Stx1 (Fig. 2B). We also tested Gb3Cer in the POPC monolayer immobilized on sensor chip HPA and observed a similar dose dependence (data not shown). However, the signals obtained were very weak because of the limited amount of immobilized lipid and instability during multiple regeneration steps (data not shown).

To calculate the association and dissociation rate constants, sensorgrams were obtained with different concentrations of the toxins and 1% Gb3Cer (Fig. 3). As the concentration of Stx1 increased, RU also rose dose dependently (Fig. 3, A and C, gray lines), and a similar dose dependence was observed when 5% Gb3Cer was used (data not shown). Stx2 also showed similar dose dependence in both 1% Gb3Cer (Fig. 3, B and D, gray lines) and 5% Gb3Cer sensor chips (data not shown).

Next, the binding curves (sensorgrams) obtained from Gb3Cer immobilized flow cells with both toxins were fitted to various models as described under “Experimental Procedures.” The pseudo-first order kinetics “1:1 (Langmuir) binding” model, “heterogeneous analyte” model, “heterogeneous ligand” model, and “two-state reaction (conformation change)” model did not fit well (data not shown), and the best-fit obtained was with the bivalent analyte model represented by the equation: (first step, A + B ↔ AB; second step, AB + B ↔ AB2). Simulated curves of the bivalent analyte model were superimposed on the experimental curves (Fig. 3, solid lines). Global fitting of the data using this model yielded a χ² value of <20 (Ch² < 20), as opposed to >20 with all other models, indicating that the fitting procedure by the bivalent analyte model considerably improved and better described the kinetic data. Apparent rate constants of Stx1 and Stx2 obtained from this model are shown in Table 1. The association and dissociation rate constants of Stx1 were unaffected by the concentration of Gb3Cer in the immobilized POPC bilayer on sensor chip L1, but more rapid association kinetics (kₐ1,1) of Stx2 were observed with 1% Gb3Cer than with 5% Gb3Cer in the immobilized lipid layer. The dissociation rate constants (kₐ1,2) of both Stx1 and Stx2 were unaffected by the Gb3Cer concentration.

On the other hand, in the case of Gb3Cer in the POPC monolayer on sensor chip HPA, the signals were very weak. In addition, reasonable fitting was not obtained with any of the available models (Fig. 3). Therefore, the actual kinetics could not be calculated in this case.

Simulated curves representative of each binding step acquired from the fitting with the 1% Gb3Cer concentration in the POPC bilayer on sensor chip L1 are shown in Fig. 4. The contributions of association and dissociation appeared to be more important in the second step of Stx1 (Fig. 4A, thin solid line) than in the first step (Fig. 4A, dotted line, b). This indicates that the second binding step occurs immediately after the first binding step, in other words, that the rate-determining step is the first binding step. The first step of Stx2 binding displays faster association kinetics (Fig. 4B, thin solid line, c) than the second step (Fig. 4B, dotted line, b), indicating difficulty in achieving the second step, and the second step was therefore concluded to be the rate-determining step for Stx2. The dissociation phases of the second step of Stx1 and Stx2 were slower than the first step, indicating that the rate-determining step for both is the second step.

**Comparison of the Binding Profiles of Stx1 and Stx2**—To clarify the difference between the binding profiles of Stx1 and Stx2, the sensorgrams for 0.6 μm Stx1 and Stx2 on the 1% Gb3Cer immobilized lipid bilayer on sensor chip L1 were superimposed (Fig. 5). As shown in the figure, the sensorgram of Stx1 rose more sharply in the association phase of the sensorgram than that of Stx2, indicating that Stx1 has more rapid association kinetics than Stx2. In the dissociation phase, the sensorgram of Stx1 abruptly turned downward, whereas that of Stx2 decreased more slowly, indicating that Stx1 has more rapid dissociation kinetics than Stx2 under these conditions and suggesting that Stx1 easily binds to and detaches from Gb3Cer. In contrast, Stx2 binds slowly, and dissociation also proceeds slowly. Although other concentrations of Stxs were tested on the 5% Gb3Cer immobilized lipid layer, the results obtained were essentially the same (data not shown).

**Affinities of Stx1 and Stx2 in an Equilibrium Binding Study**—Equilibrium binding studies were carried out to calculate the dissociation constants (Kₛ, affinity) using Scatchard plots (Fig. 6). The lines and intercept of the x axis obtained by Scatchard plots shifted to the right as the Gb3Cer concentration increased, for both Stx1 and Stx2, reflecting increased numbers of receptors, again indicating dose responsiveness in
the binding of Stx1 and Stx2 to Gb3Cer. The $K_D$ values of Stx2 obtained from two plots, with 1 and 5% Gb3Cer, were almost identical, and they were parallel. However, the $K_D$ values of Stx1 obtained with 1 and 5% Gb3Cer differed by a factor of ∼2, because the slopes of the two lines were slightly different.

**Binding Specificities of Stxs to Glycosphingolipids**—We examined other glycolipids to determine whether the binding profiles obtained above were specific for the combinations of Stxs and Gb3Cer using 5% GSLs in POPC immobilized on sensor chip L1. As shown in Fig. 7, A and B, both Stx1 and Stx2 exhibited significant specific binding to Gb3Cer, in comparison to other glycolipids. No interaction between the toxins and GM3 could be detected under these conditions. Although slight interactions between Stx1 and Stx2 and Gb4Cer (Globoside) were detected, most of the toxins were quickly removed during the dissociation phase.

We also examined the binding profiles under conditions without POPC. By injecting 5 μM of each GSL solution without POPC, -1450 RU of GSLs were immobilized on the sensor chip L1. The net amount of immobilized GSLs was -2.5 times that on the 5% GSLs POPC-based immobilized bilayer. Since the POPC bilayer is not present under these conditions, GSLs are thought to be immobilized randomly on the sensor chip. As shown in Fig. 7, the specificities of Stxs binding to GSLs are conserved in a POPC-independent manner, indicating that POPC does not affect the binding specificities of Stxs. It is noteworthy, however, that the signals obtained by using the POPC lacking GSLs were significantly weaker than those obtained with POPC-based GSL layers.

**Effect of the Presence of Cholesterol in Immobilized Phospholipid Bilayer on Stx Binding to Gb3Cer**—To test the effect of cholesterol present in the phospholipid bilayer on Stx binding to Gb3Cer, we examined the binding profile of Stx to 1% Gb3Cer containing a POPC layer with or without 30% cholesterol immobilized on sensor chip L1. As shown in Fig. 8, the presence of cholesterol in the lipid bilayer led to an apparent increase in the bindings of both Stx1 and Stx2 to Gb3Cer, whereas the association and dissociation rate constants were unaffected.

**DISCUSSION**

Interactions between various bacterial toxins and their receptor glycolipids have been examined by several different methods including TLC overlay (26, 27), microplate binding (10), and SPR assays (21–23). Among these methods, the SPR assay possesses a number of advantages over the others. First, SPR assays allow very sensitive real-time monitoring of association and dissociation reactions between molecules in a continuous flow. Second, the SPR assay can provide lipid bilayer environments by using newly developed sensor chips and techniques. For example, Mackenzie et al. (22, 23) reported a lipid bilayer assay system with SPR for investigation of the interaction between the CT B-subunit and GM1 that employs a LPS/liposome capture method on anti-LPS immobilized sensor chip CM5 (dextran matrix).

In this study, we developed an SPR analysis system employing a POPC-based lipid bilayer attached to the hydrophobic dextran matrix-coated sensor chip L1 (25, 28) to examine the binding of Stxs to Gb3Cer. In a lipid bilayer composed of POPC, Gb3Cer is thought to acquire flexible mobility, as in a cell membrane, and is thus expected to provide a model of Stxs-Gb3Cer interaction that more closely mimics physiological conditions in the cell membrane than other previously described methods. Indeed, our data underscore the importance of the structure and composition of the lipid bilayer. As presented in
this study, although the net amounts of Stxs are comparable, Gb3Cer present as a constituent of the POPC-based lipid bio-
layer on sensor chip L1 can bind significantly larger amounts of Stxs than when randomly immobilized by itself on the same sensor chip. The two-dimensional mobility of the immobilized receptor glycolipid which is acquired in the lipid bilayer micro-
environment may contribute to effective toxin binding.
Several lines of evidence have recently indicated that the function of the carbohydrate moiety of glycolipids can be mod-
ulated by the lipid bilayer microenvironment (29). Considering the importance of the carbohydrate moiety in the interactions between glycolipids and bacterial toxins, binding assays not employing a lipid bilayer system may not reflect the actual interactions occurring on the cell membrane. A discrepancy between SPR studies for CT carried out independently, i.e. one performed on a lipid monolayer assembled on alkane thiol groups covalently bound to gold film (21, 30) and the other employing a lipid bilayer devised by the LPS/liposome capture method (22, 29), further supports the above notion. Although both groups used the SPR system, the specificities of CT for the ganglioside differed significantly and the latter showed good agreement with previously obtained results by other methods (31) and with information predicted by the crystal structure of the GM1-CTB-subunit complex (32, 33).
Our data further revealed that the presence of cholesterol in the lipid bilayer increases toxin bindings to Gb3Cer, although the association and dissociation rate constants are unaffected. Consistently, it has been reported that antibody binding to antigens on liposomes (34) and the interaction of hemolysin with the lipid membrane (35) are enhanced in the presence of cholesterol.
Recently, cholesterol, sphingomyelin, and glycosphingolipid have been shown to form microdomain structures in the plasma membrane, called glycolipid-enriched micro domains (GEMs) or “rafts” (36), that present functional sites for ligand-receptor binding, cell signaling, and endocytosis (36–41). We previously reported that Gb3Cer is also located in GEM/rafts on the plasma membranes of various cell types (42, 43) and that Stx ligand binding to Gb3Cer induces aggregation of GEM/rafts in the plasma membrane (43). Therefore, the Stx-Gb3Cer interaction is likely to occur in the form of a cluster in GEM/rafts.
rather than being evenly distributed on the plasma membrane. Since glycolipids have been shown to form microdomain structures in artificial phospholipid membranes (44), Gb3Cer species probably also exist in the form of microdomains on sensor chip surfaces. Cholesterol could also be enriched in GEM/rafts and should enhance the Stx-Gb3Cer interaction.

The above SPR system allowed us to obtain clear sensorgrams of Stx-Gb3Cer interactions. Among the analyte models for the BIAcore system available at present, the bivalent analyte model included in the BIAevaluation 3.1 software is the most suitable for calculating rate constants. The bivalent analyte model is based on the hypothesis that a single analyte having two independent binding sites reacts with two ligands with identical binding properties. A molecular modeling study (45) and fluorescence energy transfer measurements in a coumarin binding study (46) have provided evidence for the presence of two Gb3-binding sites per B-subunit monomer: one within a cleft between adjacent monomers (site I) and the other within a shallow depression on the surface distal to the position of the A subunit (site II) (45, 47). The presence of two binding sites in the Stx B subunit monomer and/or the formation of a pentamer structure may account for the best fit with the bivalent analyte among models included in the BIAevaluation software.

CT has a structure consisting of one A-subunit and a pentamer of B-subunits, similar to Stxs. However, the SPR sensorgram for CT binding to GM1 obtained with an LPS/liposome bilayer system fit the 1:1 (Langmuir) binding model, and thus a single set of association and dissociation rate constants could be calculated (22). This result was in clear contrast to our results for Stx-Gb3Cer interactions. One possible explanation is that the CT B subunit has one binding site for GM1 ganglioside on each B subunit (32), and one CT thus has five valences per holotoxin molecule for its receptor GM1. Both Stx1 and Stx2, on the other hand, have 10 binding sites per holotoxin, because of the presence of 2 binding sites per B subunit. Alternatively, differences in the properties of the lipids used for bilayer formation may give rise to differences. In the LPS/liposome system for CT, MacKenzie et al. (22) used 1,2-dimyr-
istoyl-en-glycero-3-phosphocholine, which does not contain unsaturated fatty acids, to prepare the liposome containing the receptor gangloside GM1. By contrast, we used POPC, which contains unsaturated fatty acids to make the liposomes for analysis of the Stxs-Gb3Cer interaction, because most of the phospholipids in the plasma membrane are known to contain unsaturated fatty acids (48, 49). This latter possibility is particularly important when recent knowledge of microdomains is taken into account. Our SPR system that mimics the plasma membrane microenvironment, including the GEM/rafts structure, provides a reaction site in which the Stx binding process from the first step to the second step occurs readily as the receptor Gb3Cers are clustered in a flexible fashion, and this may have resulted in the good fit to the bivalent analyte model. The dissociation constants of Stx1 and Stx2 at room temperature, as demonstrated using a microplate binding assay were reported to be 4.6 × 10^−8 and 3.7 × 10^−7, respectively (10). These values differ by 1 order from our data, presented in Fig. 6. This discrepancy may be due to differences in the experimental systems used and conditions such as the temperature setting. The conventional assay system employs multiple washing procedures, but washing procedures are not recommended for calculating the precise dissociation constant especially when it is weak. Therefore, the constants acquired by the SPR system should be more accurate. In addition, the conventional assay system cannot display the differences in the kinetics of the dissociation phase between Stx1 and Stx2.

As mentioned above, the B-subunit monomer has two distinct binding sites. The association and dissociation rate constants of each binding site in the Stx1 B-subunit monomer were recently investigated using a combination of 125I-labeled Stx1 and immobilized Gb3Cer on a microtiter plate (50). Their association rate constants were reported to be \( k_{a1} = 0.0075 \text{ min}^{-1} \text{ nmol}^{-1} \) and \( k_{a2} = 0.275 \text{ min}^{-1} \text{ nmol}^{-1} \), respectively. Since these data were acquired using the binding assay to Gb3Cer immobilized at very low density without phospholipids or other plasma membrane components, this method is preferable for assessing the kinetics of individual binding sites. However, the binding conditions clearly differ from those of the binding that occurs on the cell membrane. Although the kinetics of each binding site on the B-subunit of Gb3Cer that might form GEM/rafts in the POPC bilayer on the sensor chip surface were not determined, we believe the interaction made possible by our system more accurately reflects physiological conditions.

The mechanisms of action of Stx1 and Stx2 are thought to be the same and these two toxins have comparable enzymatic activities in cell-free systems (10). However, the cytotoxicity of Stx2 in human diseases and in animal models is suggested to be much stronger than that of Stx1. For example, epidemiological data have indicated that Stx2-producing strains are more frequently associated with severe symptoms, in STEC infections such as hemolytic uremic syndrome, than Stx1-producing strains (16–18, 51). Consistently, the LOD\(_{50}\) of purified Stx2 for mice is reported to be ~400 times lower than that of Stx1 (19). Although the precise mechanism accounting for the apparent difference in the toxicities of Stx1 and Stx2 in vivo is unknown at present, our data may provide a plausible explanation for the discrepancy. In this study, we showed that Stx1 has more rapid association and dissociation rate constants than Stx2. In other words, Stx2 dissociates more slowly than Stx1. The SPR system employing lipid bilayers may mimic the circulatory system, such that the data obtained would more accurately represent the physiological conditions of interactions between Stxs and target cells in patients with a STEC infection. In view of this evidence, Stx1 may bind to receptors more avidly than Stx2, and therefore be able to recruit a larger number of cells but be released from Gb3Cer before endocytosis occurs. By contrast, Stx2 may bind to the receptor with less avidity and more slowly, but stay on the cells long enough to be incorporated, and therefore produce more toxicity than Stx1 in vivo.

In conclusion, the SPR assay employing sensor chip L1, on which Gb3Cer was immobilized in a POPC-based lipid bilayer, provides a new method for analyzing the binding properties of Stx to its receptor. This system should mimic cell membrane fluidity as well as the recently described local variation in glycolipid distribution, called GEM/rafts. This assay revealed quite different binding and dissociating motifs for Stx1 and Stx2. Determination of whether this difference is related to the different clinical manifestations associated with these toxins awaits further studies, but the data described herein should enhance our understanding of this disease.

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