Quantitative Analysis of Bacterial Toxin Affinity and Specificity for Glycolipid Receptors by Surface Plasmon Resonance*

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The primary virulence factors of many pathogenic bacteria are secreted protein toxins which bind to glycolipid receptors on host cell surfaces. The binding specificities of three such toxins for different glycolipids, mainly from the ganglioside series, were determined by surface plasmon resonance (SPR) using a liposome capture method. Unlike microtiter plate and thin layer chromatography overlay assays, the SPR/liposome methodology allows for real time analysis of toxin binding under conditions that mimic the natural cell surface venue of these interactions and without any requirement for labeling of toxin or receptor. Compared to conventional assays, the liposome technique showed more restricted oligosaccharide specificities for toxin binding. Cholera toxin demonstrated an absolute requirement for terminal galactose and internal sialic acid residues (as in G₃₁₁₁). Escherichia coli heat-labile enterotoxin bound to G₃₁ and tolerated removal or extension of the internal sialic acid residue (as in asialo-G₃₁ and G₁₁₁₁, respectively) but not substitution of the terminal galactose of G₃₁. Tetanus toxin showed a requirement for two internal sialic acid residues as in G₁₁₁₁. Extension of terminal galactose with a single sialic acid was tolerated to some extent. The SPR analyses also yielded rate and affinity constants which are not attainable by conventional assays. Complex binding profiles were observed in that the association and dissociation rate constants varied with toxin/receptor ratios. The sub-nanomolar affinities of cholera toxin and heat-labile enterotoxin for liposome-anchored gangliosides were attributable largely to very slow dissociation rate constants. The SPR/liposome technology should have general applicability in the study of glycolipid-protein interactions and in the evaluation of reagents designed to interfere with these interactions.

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The abbreviations used are: LT, E. coli heat-labile enterotoxin; CT, cholera toxin; CTB, the pentameric B-subunit of cholera toxin; DMPC, dimyristoylphosphatidylcholine; LPS, lipopolysaccharide; RU, resonance unit; SPR, surface plasmon resonance; TLC, thin layer chromatography; TT, tetanus toxin; IgG, immunoglobulin G.
venue of these interactions. We have used this technology to obtain kinetic and affinity constants to characterize the receptor specificities and affinities of CTB and LT and have shown that it can be applied also to the study of the clostridial neurotoxins, such as tetanus toxin. These toxins do not belong to the AB$_5$ toxin group but are also known to bind to gloidosaccharide receptors (17).

EXPERIMENTAL PROCEDURES

Materials—Gangloside G$_{M1}$ was obtained from BioCarb. All other glycolipids were obtained from Sigma. Cholera toxin B subunit (CTB) and TT C-fragment were obtained from Calbiochem and LT from Sigma. Dimyristoylphosphatidylcholine was purchased from Sigma. Salmonella esen serogroup B LPS was isolated by standard procedures (18). The anti-S. esen LPS monoclonal antibody Se155-4 IgG was purified from ascites fluid by affinity chromatography (19, 20).

Liposome Preparation—Mixtures containing 1 mg of DMPC and 10–100 µg of a single glycolipid in CHCl$_3$/MeOH were dried in glass vials under N$_2$. Vials were further dried under vacuum for at least 1 h, and 300 µl of phosphate-buffered saline containing 10 µg of Salmonella serogroup B LPS was then added to each vial. Vials were then vortexed vigorously and placed in a sonic bath for 20 s. Suspensions containing the liposomes were extruded (19 passes) through 50 nm polycarbonate membranes in a Liposofast apparatus (Avestin Inc., Ottawa, ON, Canada). Liposomes were separated from unincorporated material by passage through a 1-ml Sepharose CL-4B column. Liposome preparations were stored at 4 °C in 10 mM phosphate buffer, pH 7.0, containing 160 mM NaCl.

SPR Analysis—Binding kinetics were determined by SPR using a Biacore 1000$^{	ext{TM}}$ biosensor system (Biacore Inc., Piscataway, NJ) (21). Se155-4 IgG was immobilized on research grade CMS sensor chips (Biacore Inc.) at a concentration of 50 µg/ml in 10 mM sodium acetate, pH 4.5, using the amine coupling kit supplied by the manufacturer. Approximately 5000 resonance units of IgG were immobilized under these conditions, where 1 RU corresponds to an immobilized protein concentration of ~1 pg/mm$^2$ (22). Unreacted moieties on the surface were blocked with ethanolamine. All measurements were carried out in HEPES-buffered saline which contained 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA. Analyses were performed at 25 °C and at flow rates of 5 or 10 µl/min for the determination of on-rates and equilibrium binding and 50 µl/min for the determination of off-rates. Liposome preparations obtained as described above were generally diluted 1:50 in HEPES-buffered saline for capture by the IgG surfaces. In all instances, toxin concentrations were calculated using the molecular weight of the whole toxin or toxin fragment and not on a per binding site basis. Surfaces were typically regenerated with 10 µl of 1% (w/w) LPS and 1–10% of selected glycolipids in 10 mM sodium acetate buffer, pH 4.5, followed by 5 µl of 4 mM cholate in the same buffer. Milder conditions (less cholate) were used in instances of less strong-chain Fv form of Se155-4 was bound to the LPS presented on the control liposome surface (data not shown) and nonlinear analysis of the data gave association rate and dissociation rate constants that were the same as those previously reported for the binding of this scFv (mutant B5-1), as determined by SPR using bovine serum albumin-O-polysaccharide surfaces (24).

RESULTS

Liposome Surfaces—Liposome surfaces generated by a capture mechanism involving Salmonella serogroup B LPS and Se155-4 IgG, which is specific for serogroup B LPS, were used to obtain profiles for toxin binding to a panel of glycolipids (Fig. 1). Typically, 1000 RU of liposomes prepared from mixtures containing 1% (w/w) LPS and 1–10% of selected glycolipids were captured by 5000 RU of IgG surfaces. There was complete retention of the liposomes by the IgG surface, thereby providing a stable baseline for the determination of toxin binding specificity and kinetics. An analysis cycle showing G$_{M1}$ liposome capture, LT binding to the liposomes and surface regeneration is shown in Fig. 2. Liposomes containing only DMPC and serogroup B LPS were used as blank surfaces for the measurement of nonspecific binding. Liposomes containing only DMPC and LPS were used as blank surfaces for the measurement of nonspecific binding. Liposomes containing only DMPC and LPS also provided a positive control for demonstrating the accuracy of the SPR/liposome format for measuring the kinetics of protein-sugar interactions. A dimeric single-chain Fv form of Se155-4 was bound to the LPS presented on the control liposome surface (data not shown) and nonlinear analysis of the data gave association rate and dissociation rate constants that were the same as those previously reported for the binding of this scFv (mutant B5-1), as determined by SPR using bovine serum albumin-O-polysaccharide surfaces (24).

The IgG surfaces were typically stable to at least 100 regenerations using the cholate-acetate buffer regeneration protocol. One advantage to using Se155-4 as the capture antibody is that

\[ \frac{R_{R_0}}{C} = K_R R_{\text{max}} - K_K R_{\text{eq}} \]
it dissociates from the LPS antigen at pH 4.5 (20), thereby permitting relatively mild surface regeneration conditions.

**Toxin Binding Kinetics**—The amount of glycolipid in the liposomes had a marked effect on the association phase of CTB binding to GM1. At CTB concentrations of 20 nM, more rapid association kinetics were observed with 1 and 2% GM1 liposomes than with 3 and 4% GM1 liposomes (Fig. 3A). Linear transformation of the sensorgram data showed the existence of two distinct on-rates for CTB binding to GM1 (Fig. 4A), but only the faster rate was observed at GM1 concentrations of 1 and 2%. This is reflected in the derivative plot (Fig. 4A) which shows linearity for the binding of 20 nM CTB to liposomes containing 1% GM1. Liposomes containing 2% glycolipid were used for kinetic analyses since they gave good levels of binding, attributable to the faster on-rate. At this glycolipid concentration, the association rate constants derived at different toxin concentrations were in good agreement (Table I). Association kinetics were not significantly affected by changes in toxin concentration, liposome density or flow rate indicating that there wasn’t any mass transport limitation under these experimental conditions.

The dissociation rate constants for CTB binding to glycolipids were also influenced by toxin:receptor ratios. For example, at CTB concentrations of 20 nM faster off-rates were observed at liposomal GM1 concentrations of 1 and 2% than at higher concentrations. At constant receptor concentrations, higher toxin concentrations resulted in faster off-rates (Fig. 3B). Transformation of the dissociation data clearly shows these differences (Fig. 4B). Also, whereas there was some curvature to the transformed data plot at 50 nM CTB/2% GM1, the 10 nM CTB/2% GM1 plot was linear. Presumably, the slow off-rates resulted from pentavalent binding with the faster off-rates at low GM1 to toxin ratios reflecting a lower mean valency of binding. Nonlinear analysis of the rapid association data and slow dissociation data showed good fitting to a one to one interaction model (Fig. 4, C and D) even though multivalency leads to a more complex interaction than is described by such a model. The slow dissociation rates are given in Table I, and these values were used for the calculation of the KD values shown in Table I. The slow off-rates are at the lower end of the instrument range, and there is considerable error associated with their calculation because of the significant influence of even marginal baseline drift. To improve the accuracy of the off-rate calculations, analyses were performed on data for dissociation of at least 10% of the bound toxin.

**Equilibrium Binding**—For CTB, equilibrium binding studies were conducted as an alternative means of deriving affinities and determining the glycolipid concentrations that give optimal toxin binding. Scatchard plots of equilibrium data showed distinctly different binding profiles for liposomes containing 2 and 4% GM1 (Fig. 5). The equilibrium data were in good agreement with the calculated rate constants in that stronger binding was observed at the lower glycolipid concentration. The KD values derived from the Scatchard plots were 1.7 nM for 2% GM1 liposomes and 6.5 nM for 4% GM1 liposomes. The 1.7 nM value is also in reasonable agreement with the rate constant-derived KD of 0.73 nM for the binding of CTB to 2% GM1 liposomes (Table I). A rate constant-derived affinity was not calculated for 4% GM1 liposomes because of the nonlinearity of the transformed association data at higher GM1 concentrations (Fig. 4A).

**Binding Specificities and Affinities of CTB, LT, and TT**—The profiles of CTB, LT, and TT C-fragment binding to different glycolipids are shown in Fig. 6. Both CTB and LT showed highest affinity for GM1 with an approximately 10-fold lower affinity for GD1b (Table I). In both instances, the lower affinity for GD1b was attributable to a faster off-rate. CTB showed insignificant specific binding to the other gangliosides that were tested (Fig. 6A). There was, however, some evidence of adsorption of CTB to liposome surfaces as the post-injection response on all liposomes (including the blank surface liposomes containing only DMPC and LPS) was higher than on the IgG capture surface. This nonspecific binding did not significantly influence on-rate calculations because it gave little response with time relative to the response for specific binding. However, with slow off-rates, such as those observed for CTB and LT binding to GM1, this low level nonspecific binding could influence the calculations and would have the effect of giving artificially fast dissociation rate constants for the specific binding. LT differed from CT in that it showed some specificity for asialo-GM1 (Fig. 6B) with an affinity that was about 5 times lower than that observed for Gd1b and 25 times lower than for GM1 (Table I).
FIG. 4. Analysis of association and dissociation phases of CTB binding to GM1-containing liposomes. A, derivative plots of 20 nM CTB binding to liposomes containing 1% (○) and 3% (●) GM1 showing the presence of two distinct association rate constants; B, transformed data showing different off-rates for the dissociation of 10 nM (○) and 50 nM (●) CTB from liposomes containing 2% GM1; C, fitting of the association phase of 40 nM CTB binding to liposomes containing 2% GM1 to the BIAevaluation one-site model (A + B → AB); D, fitting of the dissociation phase of 40 nM CTB binding to liposomes containing 2% GM1 and 2% Gb3 to the BIAevaluation one-site model (AB → A + B).

Table I

| Toxin or toxin subunit | Ganglioside | $k_{on}$ | $k_{off}$ | $K_D$ |
|------------------------|------------|---------|----------|-------|
| CTB                    | GM1        | $6.2 \times 10^6$ (±5.5)$^a$ | $4.5 \times 10^{-4}$ (±18.3) | $7.3 \times 10^{-10}$ (±19.1) |
|                        | Gb1b       | $4.1 \times 10^6$ (±12.4) | $3.1 \times 10^{-3}$ (±17.4) | $8.0 \times 10^{-9}$ (±21.4) |
|                        | GM1        | $4.2 \times 10^6$ (±22.1) | $2.4 \times 10^{-4}$ (±27.9) | $5.7 \times 10^{-10}$ (±35.6) |
|                        | Gb1b       | $7.4 \times 10^6$ (±8.0) | $2.1 \times 10^{-3}$ (±23.8) | $3.0 \times 10^{-9}$ (±25.1) |
|                        | Asialo-GM1 | $4.7 \times 10^6$ (±17.4) | $6.9 \times 10^{-3}$ (±19.5) | $1.5 \times 10^{-8}$ (±26.1) |
|                        | Gb1b       | $2.4 \times 10^6$ (±13.3) | $3.6 \times 10^{-3}$ (±21.2) | $1.5 \times 10^{-7}$ (±13.5) |
|                        | Gb1b       | $1.8 \times 10^6$ (±35) | $3.1 \times 10^{-3}$ (±17.6) | $1.7 \times 10^{-7}$ (±39.2) |

$^a$ Numbers in brackets are the S.E., expressed as % (95% confidence limits) which for 1) on-rates were based on measurements at 5–7 different concentrations in each instance, 2) CTB and LT off-rates were based on 5 measurements at 10 nM toxin for GM1 and Gb1b and 40 nM LT for asialo-GM1, and 3) TT off-rates were based on 5 measurements at 500 nM.

$^b$ Low binding precluded accuracy of rate constant determination and calculation of affinity.

Tetanus toxin C-fragment bound most strongly to Gb1b (Fig. 6C) but with an affinity that was at least three orders lower than that of the preferred CTB and LT ligand, GM1 (Table I). Extension of the terminal galactose of Gb1b with a single sialic acid, to give Gb1b, resulted in a slight reduction in affinity, and the addition of two sialic acid residues to the galactose, to give Gb1b, completely abolished binding. Removal of one of the NeuAc residues from Gb1b to give GM1, abolished binding, but removal of the terminal galactose of Gb1b, to give Gb2, was tolerated to some extent.

**DISCUSSION**

The glycolipid binding assay described here has several attractive features. The SPR technology used to monitor binding gives kinetic data that are difficult to obtain by other means, and the kinetic data in turn can be used to obtain affinities for a wide variety of glycolipid-toxin complexes. The membrane environment of the interactions resembles physiological conditions more closely than TLC overlay and microtiter plate techniques, and this should help to eliminate the artifactual binding specificities that have been observed with the solid phase assays (25). The LPS liposome capture methodology described here gives stable liposome retention during the binding cycle and has an efficient sensor chip surface regeneration protocol. Incorporation of a small amount of LPS in the liposomes was sufficient for complete liposome retention on the sensor chip. The nature of the LPS-antibody interaction permitted surface regeneration with 10 mM acetate, pH 4.5, containing cholate. Masson et al. (26) have reported the capture of biotinylated natural lipid vesicles by streptavidin, avidin, and anti-biotin antibody sensor chip surfaces but encountered rather rapid deterioration of these surfaces during regeneration.

The CTB and LT specificities determined by the SPR/liposome method are in excellent agreement with binding site features observed in crystal structures. The structure of CTB bound to the GM1 pentasaccharide (Galβ1–3GalNAcβ1(NeuAc2–3Galβ1–4Glc) revealed that the five oligosaccharide binding sites resided primarily within the individual B-subunits (2, 3). The binding was described as a two-fingered grip with the Galβ1–3GalNAc moiety representing the “forefinger” and sialic acid representing the “thumb” (2). The strongest toxin receptor interactions involve the terminal galactose which inserts into a deep pocket in the binding site and the sialic acid which occupies a shallower depression on the toxin surface. This is consistent with the lack of binding of CTB to asialo-GM1 and GM2 (Fig. 6A). The structure of LT complexed with lactose (Galβ1–4Glc) (4) revealed the binding site of the terminal galactose in the ganglioside GM1, which is known to bind to LT with high affinity. A comparison of the CT and LT structures has provided a possible explanation of the subtle differences in specificity observed for these two toxins (1–3). Whereas the His-13 residue in CT donates a hydrogen
bond from its backbone amide to sialic acid with no side-chain contribution to binding. Arg-13, found at this position in most variants of LT, can also contribute side-chain binding to the GalNAcβ1-4Galβ1-4Glc portion of G\textsubscript{M1} and other gangliosides. These structural features are in agreement with the binding profiles observed here (Fig. 6, A and B). Ganglioside binding by CTB and LT is intolerant to extension of the terminal galactose of G\textsubscript{M1} but is somewhat tolerant to the extension of the internal sialic acid. Unlike CTB, LT does not display an absolute requirement for the internal sialic acid as shown by the weak binding of LT to asialo-G\textsubscript{M1}.

The glycolipid-binding specificities of CTB and LT reported here are generally in agreement with those previously reported using microtiter plate assays and TLC overlays (12, 27–29). However, the SPR/liposome method yielded quantitative data on rate constants and affinities which are not easily obtained using conventional approaches such as radiolabeled ligand binding and microtiter plate assays. The affinity derived for CTB binding to G\textsubscript{M1} is similar to that obtained by Cuatrecasas (30) by measuring radiolabeled CT binding to cell membranes. Moreover, the toxin-receptor specificities observed by SPR are more consistent with the binding site features seen in crystal structures (2–4) than the less restricted specificities obtained by microtiter plate assays (29). For instance, the microtiter plate assay showed very good binding of LT to G\textsubscript{M2} (29), which lacks the terminal galactose residue, and this finding is difficult to rationalize in light of the crystal structure of the CTB-G\textsubscript{M1} oligosaccharide complex (2, 3).

Both CTB and LT showed a very high affinity for liposomes containing 1 and 2% G\textsubscript{M1}, provided the toxin concentrations are sufficiently low to give the maximum valency, presumably pentavalency, of attachment. Under these conditions, the off-rates are extremely slow. These toxins also had strong affinity for G\textsubscript{D1b} although in each instance the affinity was only one-tenth that observed for G\textsubscript{M1}. The SPR technique can easily detect interactions with affinities in the low micromolar range, indicating that there is no significant binding of CTB and LT to any of the other glycolipids tested (Fig. 6, A and B).

While the CT binding data presented here are generally in good agreement with previous studies (12–14, 27–29), they are not consistent with a recent SPR study of CT affinity and specificity for gangliosides (16). Kuziemko et al. (16) observed CT binding to several gangliosides with the following order of binding strength: G\textsubscript{D1b} > G\textsubscript{G1a} > G\textsubscript{G1b} > G\textsubscript{T1b} > G\textsubscript{Q1b} > asialo-G\textsubscript{M1}. This specificity profile is difficult to rationalize on the basis of the G\textsubscript{M1} pentasaccharide-CTB crystal structure and the binding to G\textsubscript{D1a}, G\textsubscript{D3} and asialo-G\textsubscript{M1} contradicts binding specificities determined by other means (1). Most striking is the high affinity reported for G\textsubscript{D1a} binding ($K_d = 31.8$ pm); this ganglioside was used as a negative control in the analysis of ligand binding to CT by titration microcalorimetry (14) and was shown to give insignificant heat release. It is possible that the specificity differences observed with the two SPR methods may be partially related to potentially different modes of oligosaccharide presentation in the two lipid bilayer environments. The glycolipid surfaces used by Kuziemko et al. (16) were prepared by fusing 5 mol % glycolipid:95 mol % palmitoyloleoylglycero-3-phosphocholine to alkylthiol monolayer on the gold surface of the sensor chip to form planar hybrid bilayer membranes (31). There is considerable evidence that glycolipid carbohydrate can be modulated by the bilayer microenvironment (32).

The TT C-fragment specificities for gangliosides are quite different from those obtained by Holmgren et al. (27) and Ångström et al. (29), using microtiter plate formats. They reported similar specificities for G\textsubscript{D1b}, G\textsubscript{T1b}, and G\textsubscript{Q1b}, whereas the SPR-liposome results indicated a slightly higher affinity for G\textsubscript{D1b} relative to G\textsubscript{T1b} and no binding to G\textsubscript{Q1b}. The observation that the affinity of TT C-fragment for its preferred ganglioside ligand is several orders of magnitude lower than that of CTB and LT for their preferred ganglioside ligands supports the view that gangliosides are not exclusive receptors for the clos-
tridual neurotoxins (17, 33). The TT C-fragment interaction with its ganglioside receptor is presumably monovalent. Tetanus toxin is comprised of a 100-kDa heavy chain linked to a 50-kDa light chain bridged by a single interchain disulfide bridge. The light chain is responsible for the blockade of neurotransmitter release. The heavy chain is composed of two domains, the N-domain which is thought to mediate cell penetration and the C-domain which is responsible for neurospecific binding via the protein and ganglioside receptors (17).

The methodology described here should provide a powerful means of testing the effectiveness of inhibitors of protein-glycolipid interactions. The assay permits real time analysis of binding to membrane bound oligosaccharides and a means of pretesting conditions necessary for inhibition at cellular surfaces. Bacterial toxins are only one of a number of carbohydrate binding events that could be studied in this way. For example, inhibition of virus binding to cell surfaces could be monitored since it is known that virus particle binding can be followed by SPR (34). The system should also be amenable to the presentation of membrane proteins through the same LPS capture procedure. It should also be possible to simultaneously incorporate two ligands which would be useful in instances such as receptor binding by Clostridium botulinum type B neurotoxin which is thought to bind with high affinity to synaptotagmin, an integral membrane protein of synaptic vesicles, in association with the gangliosides G_{T1b} and G_{D1a} (33).

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