CHARACTERIZATION OF RICIN BINDING TO MULTIVALENT BOVINE SERUM ALBUMIN-BASED NEOGLYCOCONJUGATES

A Dissertation in
Biochemistry and Molecular Biology

by

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ABSTRACT

Ricin toxin is a potent type 2 ribosome inactivating protein (RIP) from the plant, *Ricinus communis*. It has the potential to be used as a bioterrorism agent due to its high toxicity and the relative ease with which it can be obtained/purified. Ricin is a heterodimeric protein consisting of 2 subunits. The A-chain (RTA) is an N-glycosidase that halts protein synthesis by catalyzing the depurination of a specific adenine residue on ribosomal RNA. RTA is linked to the B-chain (RTB) via a single disulphide bridge. RTB is a lectin, containing 2 galactose binding sites ∼70Å apart, that plays an essential role in mediating the binding and internalization of the toxin.

While the binding of ricin to galactose has been studied, characterization of the binding of ricin to more complex carbohydrate ligands (i.e., glycoproteins and glycolipids) that might be present on the surface of a cell is lacking. In contrast to the low affinity of RTB for monovalent galactose (reported to be between 0.28 and 3.5x 10^-4 M), its affinity for asialofetuin (ASF) is about 1,000 times greater than it is to monovalent galactose. This observation supports the hypothesis that ricin exhibits multivalency when it adheres to ASF.

The goal of this work was to synthesize multivalent carbohydrate ligands, use them to determine whether ricin would bind to them in a multivalent fashion, and to identify general saccharide characteristics that affect binding. To account for the distance between the two galactose binding sites on RTB, bovine serum albumin (BSA) was selected as the oligosaccharide carrier. BSA-based neoglycoconjugates were synthesized and their efficacy as ligands for the toxin monitored using surface plasmon resonance
(SPR). The results indicated that ricin did exhibit multivalency when it interacted with appropriately derivatized BSA neoglycoconjugates and that it appeared to bind more efficiently to specific longer chain oligosaccharide ligands than to disaccharides. These observations have important implications for the development of possible antidotes for the treatment of people inadvertently exposed to ricin, for the use of RTB in drug deliver, and for optimization of ricin biosensors.
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<tr>
<td>Abs&lt;sub&gt;490nm&lt;/sub&gt;</td>
<td>Absorbance read at 490nm</td>
</tr>
<tr>
<td>ASF</td>
<td>Asialofetuin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CHAPS</td>
<td>(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride</td>
</tr>
<tr>
<td>ELLSA</td>
<td>Enzyme linked lectinosorbant assay</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GalCer</td>
<td>Galactosyl ceramide</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetyl galactosamine</td>
</tr>
<tr>
<td>GD1a</td>
<td>Disialoganglioside D1a</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl glucosamine</td>
</tr>
<tr>
<td>GM1</td>
<td>Monosialoganglioside M1</td>
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<tr>
<td>GSL</td>
<td>Glycosphingolipid</td>
</tr>
<tr>
<td>GT1b</td>
<td>Trisialoganglioside T1b</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES buffered saline</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>HPTLC</td>
<td>High performance thin layer chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration of ligand that inhibits binding by 50%</td>
</tr>
<tr>
<td>Lac</td>
<td>Lactose</td>
</tr>
<tr>
<td>LacCer</td>
<td>Lactosyl ceramide</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Dose that is lethal to 50% of the subjects</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
<td>Matrix assisted laser desorption time of flight mass spectrometry</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
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| MTS      | [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2059254/)
<p>| MWCO     | Molecular weight cut-off                                                   |
| NANA     | N-acetyl neuraminic acid                                                   |
| NHS      | N-hydroxysuccinimide                                                       |
| Oligo-AM1 | Oligosaccharide portion of asialo-GM1                                       |
| PBS      | Phosphate buffered saline                                                  |
| PBS-P    | Phosphate buffered saline containing surfactant-P20                       |
| PDB      | Protein databank                                                           |
| p-ricin  | Ricin purified in this laboratory                                          |
| POPC     | 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphorylcholine                        |
| RCA&lt;sub&gt;60&lt;/sub&gt; | Ricinus Communis Agglutinin of MW 60kDa (ricin)                           |
| RCA&lt;sub&gt;120&lt;/sub&gt; | Ricinus Communis Agglutinin of MW 120kDa (agglutinin)                      |</p>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>RIP</td>
<td>Ribosome inactivating protein</td>
</tr>
<tr>
<td>RL</td>
<td>Relative ligand concentration (RUs resulting from ligand immobilization)</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RTA</td>
<td>Ricin toxin A-chain</td>
</tr>
<tr>
<td>RTB</td>
<td>Ricin toxin B-chain</td>
</tr>
<tr>
<td>RU</td>
<td>Response units</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>sRTB</td>
<td>RTB obtained from Sigma-Aldrich</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>v-rinic</td>
<td>Ricin obtained from Vector Laboratories</td>
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<tr>
<td>vRTB</td>
<td>RTB obtained from Vector Laboratories</td>
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Chapter 1

Introduction

Ricin is a toxin produced by the castor bean plant (*Ricinus communis*). The castor bean plant can be found in all temperate and subtropical regions of the world. The main product from castor beans is castor oil. It is commonly used in medicine, as a laxative, and in industry, as a lubricant. The oil can be used safely because ricin is not present in it in significant quantities. Ricin is retained in the mash, a waste byproduct from the castor oil production process.

Useful as this plant has been, it also has a history of being exploited for more sinister purposes. One of the better known examples was its use to kill a Bulgarian journalist named Georgi Markov. He was assassinated by a <2mm platinum pellet, containing ricin, that was fired into his thigh by someone using a modified umbrella. He died in a hospital three days later (Knight, 1979). More recently, in 2003, a powdered form of ricin was found in an envelope addressed to then US Senate majority leader, Bill Frist. More commonly, complications arise in animals and children who accidentally ingest seeds from the plant. Although there are no reports of child deaths due to castor bean ingestion (Challoner and McCarron, 1990), even in small doses, ricin can cause “severe haemorrhagic gastrointestinal irritation, nausea, violent vomiting, abdominal pain, profuse watery or bloody purging, thirst, dilatation of the pupils, shivering, and fever” (Knight, 1979). The estimated lethal dose in humans is 1-25µg/kg when inhaled or injected, and 20mg/kg when ingested (Franz and Jaax, 1997; Audi et al., 2005).
Although there is no known antidote to the effects of ricin exposure, work is being done to develop a ricin vaccine. Results from a pilot clinical study indicated that a recombinant vaccine (called “RiVax”) that protected mice from ricin exposure (Smallshaw et al., 2007), was successful at inducing production of anti-ricin antibodies in humans (Vitetta et al., 2006). This is a promising treatment for people, such as military personnel, who may have a substantial risk of being exposed to toxic levels of ricin. However, it would be advantageous to continue to explore ways that could be used to treat non-immunized individuals exposed to ricin.

In order to develop a potential treatment for people exposed to ricin, it is necessary to know how ricin functions. Research has provided information about the structure, activity, and binding properties of this protein. Ricin is a member of the type 2 ribosome-inactivating protein (RIP) family. Members of this family damage ribosomes by cleavage of one or more adenine residues from rRNA. Type 2 RIPs contain two polypeptides, one that is responsible for the enzymatic activity, and another that mediates binding (Barbieri et al., 1993; Lord et al., 1994; Stirpe and Battelli, 2006). Ricin is a heterodimeric protein made up of two glycoprotein subunits (Olsnes and Pihl, 1973), the A-chain (RTA) and the B-chain (RTB), linked together by a single disulphide bond (see Fig. 1.1). RTA has a molecular weight of 28 kDa and is the catalytically active subunit, while RTB is the 32 kDa lectin portion of the toxin. RTA, an N-glycosidase, catalyzes cleavage of the N-glycosidic bond of adenine residue A-4324 of 28S rRNA (Endo and Tsurugi, 1987; Endo and Tsurugi, 1988). The proposed mechanism for this enzymatic activity is shown in Fig. 1.2. The depurination prevents binding of elongation factor 2 to
the conserved sequence of the 28S rRNA loop, preventing formation of a functional ribosome. This halts protein synthesis resulting in cell death (Stirpe and Battelli, 2006).

In order for ricin to act, the toxin must bind to the surface of a target cell, be internalized, and transported to its site of action on rRNA. The need for toxin binding to the cell surface is supported by the observation that some type 1 RIPv, which characteristically lack a binding subunit, are able to be safely consumed by humans. The requirement for the binding subunit in order to have a cytotoxic effect is exemplified by the inability of the barley RIP (a type 1 RIP) to induce toxicity in mice, while a type 2 RIP, such as ricin, has an LD<sub>50</sub> in mice of ~1picomole/kg of body weight (Barbieri et al., 1993).
Fig. 1.1: The three-dimensional structure of ricin. The ricin A-chain is shown in blue and the B-chain in pink. The 2 galactose binding sites are indicated by the arrows, and the bound galactose at each site is shown in blue. (Rutenber and Robertus, 1991).
Protein carbohydrate interactions are important in a myriad of biological functions such as coagulation of blood (Ofosu, 1989), regulation of the immune system (Lee, 1988), and pituitary hormone regulation (Baenziger, 1994). Much work has been done to examine the specifics of how these interactions occur. For many lectins, binding of the protein to a single carbohydrate moiety is of relatively low affinity with dissociation constants commonly in the high µM to low mM range (Vyas, 1991; Lee and Lee, 2000). The low affinity binding can be subverted when multiple molecules containing the
saccharide ligand recognized by the protein are clustered in a manner that makes them available for binding by the multiple binding sites present on the protein (Lee, 1995; Lee and Lee, 2000). Multiple, appropriately presented, carbohydrate ligands have the ability to enhance protein to carbohydrate binding by several orders of magnitude beyond that expected based on the additive effect of binding to several individual carbohydrate binding sites. The exponential increase in protein to carbohydrate binding affinity was originally termed the “glycoside cluster effect” (Lee and Lee, 2000), and is now frequently referred to as the “multivalent” effect.

The multivalent effect has been confirmed by results obtained from studies of a number of carbohydrate binding toxins [e.g., cholera toxin, the heat labile enterotoxin of *E. coli* (Thompson and Schengrund, 1997), and shiga toxin (Kitov et al., 2000)] each of which was found to bind with high affinity when multiple carbohydrates were present on the ligand. Lectins, carbohydrate binding proteins, can bind to glycosylated proteins and lipids present on the plasma membrane of a cell. Due to either the presence of more than one glycosylation site and/or to the branched antennary nature of the oligosaccharides, many glycoproteins can be bound by lectins in a multivalent fashion.

Unlike glycoproteins, glycosphingolipids (GSLs) contain only one oligosaccharide per molecule. However, GSLs can be found in lipid rafts (Brown and London, 2000; Prinetti et al., 2000; Simons and Toomre, 2000; Vinson et al., 2003) making them accessible as “multivalent” ligands for lectins having multiple carbohydrate binding sites. Fig. 1.3 shows a diagram of some components of lipid rafts and of how GSLs might be organized within them to form potential multivalent ligands. The glycoside cluster effect, as it pertains to GSLs, is exemplified in the binding of cholera toxin. The monosialylated
ganglioside, GM1, is a lipid raft marker, and readily bound by the pentameric binding subunit of cholera toxin (Rouquette-Jazdanian et al., 2005). Cholera toxin prefers multivalent ligands over monovalent ones. This is illustrated by the ability of multivalent ligands [derivatized with the oligosaccharide portion of GM1 (oligo-GM1)] to inhibit cholera toxin binding to immobilized GM1 as well as to cell surface GM1 more effectively than monovalent oligo-GM1 (Schengrund and Ringler, 1989; Thompson and Schengrund, 1997; Thompson and Schengrund, 1998).

![Lipid raft schematic](image)

Fig. 1.3: Schematic showing the possible composition of a lipid raft (courtesy of Steven Cook).

Adherence of ricin to the cell surface is mediated by at least two defined carbohydrate binding sites present in the RTB subunit. These binding sites are ~70Å apart and can bind to terminal galactose residues present on cell surface glycolipids and glycoproteins (Rutenber and Robertus, 1991). This promiscuity in binding is thought to
contribute to ricin’s toxicity. For example, experiments using HeLa cells and $^{125}$I labeled ricin indicated that there were between $1–3 \times 10^7$ ricin binding sites per cell (Sandvig et al., 1976).

While it is understood that ricin binds to terminal galactose moieties on the cell surface, much work remains to be done to characterize the ricin carbohydrate interaction. A better understanding of ricin binding properties could be beneficial for many areas of research. For example, identifying optimum carbohydrate presentations for ricin binding might provide the basis for development of a ricin antidote that could block its entry into cells thereby preventing damage as a result of inadvertent exposure to the toxin. Another important area of research is the development of bioweapons sensors. Many biological detection tools use antibodies to identify specific agents. While antibodies are highly specific and useful in many situations, they are subject to degradation under harsh conditions (such as a hot, humid environment). While promising results were obtained when glycosphingolipids were tested as ligands on a biosensor for ricin (Stine et al., 2004; Stine et al., 2005) the sensitivity was ~156nM. Use of an optimized ligand might further enhance sensitivity.

Ricin is similar to other lectins in the sense that the individual binding affinities of its two carbohydrate binding sites are in the mid micromolar range. More specifically, the affinities of RTB’s individual binding sites for lactose were reported to be $3.5 \times 10^{-4}$ and $0.28 \times 10^{-4}$M (Zentz et al., 1978). However, ricin binds to asialofetuin (ASF) ~1,000 times better than it does to monovalent galactose (Baenziger and Fiete, 1979). ASF contains 6 oligosaccharides per molecule, three disaccharides (also known as the T antigen, which is found on human carcinoma cells, Fig. 1.4, A), and three complex
triantennary oligosaccharides (Fig. 1.4, B). Each of the three triantennary oligosaccharides, as well as each of the three disaccharides, contains a terminal galactose residue, which means that there are 12 terminal galactose residues per molecule of ASF (Spiro and Bhoyroo, 1974; Dill and Olson, 1995; Dawson et al., 1999). The increase in binding affinity of ricin to ASF supports the hypothesis that the binding is multivalent, but the specific reason for the enhanced affinity is unknown. Rutenber and Robertus (1991) hypothesized that the distance between the terminal sugars of a branched biantennary oligosaccharide was too small to span the distance between the two RTB binding sites. Therefore, increased binding may be the result of 1) simultaneous binding of terminal galactose residues present on the triantennary saccharides, 2) concomitant binding of terminal galactosyl residues present on two different glycosylation sites, 3) secondary stabilizing interactions with other sugars in the oligosaccharide chain, or 4) a combination of these possibilities.

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**Fig. 1.4:** Structure of the two types of oligosaccharides present on ASF, A) structure of the three disaccharides, aka T-antigen, and B) structure of the three triantennary oligosaccharides present on ASF.
The hypothesis that ricin might bind to multivalent ligands more efficiently than to monovalent ones was investigated previously by Dawson, et al., (2006) using carbohydrate derivatized dendrimers. Dendrimers are polymeric molecules, composed of multiple branched monomers that emanate radially from a central core and terminate with specific functional end groups (de Brabander-van den Berg and Meijer, 1993; Lee et al., 2005). Various types of molecules, including carbohydrates, can be linked to the end groups to form multivalent structures (Turnbull et al., 2002; Rosa Borges and Schengrund, 2005). The study of the ability of dendrimers, derivatized with various carbohydrate ligands, to inhibit ricin binding to ASF indicated that the lactose-derivatized dendrimers (when corrected for the number of lactose moieties present per mole of derivatized dendrimer) were bound no more efficiently by ricin than monovalent lactose (Dawson et al., 2006). This observation may reflect the fact that the diameter of the dendrimer used was only ~36Å (Hiraiwa et al., 2006), while the distance between the two galactose binding sites of ricin is ~70Å (Rutenber and Robertus, 1991). The difference in spacing supports the hypothesis that the lactose-derivatized dendrimers were ineffective because the short distance between the carbohydrate residues effectively made them monovalent ligands.

Lee and Lee (2000), suggested that serum albumin-based neoglycoconjugates might be good ligands for determining whether carbohydrate binding proteins having multiple binding sites that are more than 54Å apart, exhibit multivalency. Their suggestion takes into consideration the size of serum albumin, a helical protein with approximate dimensions of 80x80x30Å (Sugio et al., 1999). In regard to ricin, the size of serum albumin should, theoretically, be more than sufficient to span the two carbohydrate
binding sites that are approximately 70Å apart in RTB. An additional feature of using serum albumin, is the degree to which it can be derivatized with carbohydrate. For bovine serum albumin (BSA), there are 59 lysine residues present (see Fig. 1.5) that can be used in linking carbohydrates to the protein. One can control the number of carbohydrates added to BSA by adjusting pH, temperature, and incubation time (Roy et al., 1984).

![Spacefilling model of human serum albumin with lysine residues shown in blue. The distance between Lys4 and Lys574 is ~85Å, and between Lys174 and Lys313 is ~74Å. Model adapted from PDB structure 1GNJ (Petitpas et al., 2001).](image)

Based on the foregoing, neoglycoconjugates were prepared using BSA as the saccharide carrier and used to test the hypothesis that ricin would prefer multivalent over monovalent ligands. Results indicate that when the distance between galactose binding sites on RTB are taken into consideration when designing the potential ligand, ricin will exhibit “multivalency” as seen by a significant increase in binding affinity.
Chapter 2

Materials and Methods

2.1 Materials

Ricin, fluorescein labeled ricin, and antibodies were purchased from Vector Laboratories (Burlingame, CA) while RTB was obtained from both Sigma Aldrich (St. Louis, MO) and Vector Laboratories. RTB from Sigma Aldrich will be referred to as “sRTB” while that from Vector Laboratories will be referred to as “vRTB.” Castor beans were obtained from Main Street Seed and Supply (Bay City, MI), Immunolon 1 Removawell strips from Dynatech Labs Inc. (Chantilly, VA), and high performance thin layer chromatography (HPTLC) plates from VWR International (West Chester, PA). Ganglioside standards, lactosyl ceramide (LacCer), galactosyl ceramide (GalCer), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphorylcholine (POPC) were purchased from Matreya (Pleasant Gap, PA). Gangliosides used for enzyme linked lectinosorbant assays (ELLSAs) as well as for isolation of oligosaccharides were isolated from the gray matter of bovine brains (Folch et al., 1957). Bio-Gel P-2 (fine) and protein standards were obtained from BioRad (Hercules, CA). Centiplus ultrafiltration devices were obtained from Fisher Scientific (Pittsburgh, PA). Surface plasmon resonance (SPR) was performed using a Biacore 3000 [Biacore, part of GE Healthcare (Uppsala, Sweden)]. HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3mM EDTA, and 0.005% surfactant P20, pH 7.4) was purchased from Biacore, or freshly prepared and degassed just prior to use. All other reagents used during the immobilization procedure as well as surfactant P20 were obtained from Biacore. PBS-P (10mM phosphate, 2.7mM KCl, 137mM NaCl,
and 0.005% surfactant P20, pH 7.4) was freshly prepared, degassed, and used as both running and sample buffer for all toxin binding experiments. HeLa cells were kindly provided by Dr. M. J. Tevethia (Penn State University, College of Medicine). Cell Titer 96® AQueous One Solution Cell Proliferation Assay (MTS), and sequencing grade modified trypsin were obtained from Promega Corporation (Madison, WI). Alexa Fluor® 594-Transferrin and Hoechst Dye 33342 were purchased from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA), lactose, ASF, Dowex-50W (H+, dry mesh 200-400, 4% cross-linked) and all other reagents and materials were purchased from either Sigma Aldrich or VWR International.

2.2 Enzyme linked lectinosorbant assays (ELLSAs)

Procedures for monitoring toxin binding to various lipids were performed with some modifications to the method used in this lab previously (Schengrund and Ringler, 1989). In brief, assays, done in quadruplicate, were carried out as follows: 1µg of lipid in 100µL methanol was added to each of four microtitre wells, and 100µL of methanol alone was added to each of four adjacent wells that would serve as controls. Methanol was allowed to evaporate overnight and the wells washed with PBS to remove unabsorbed lipid. All incubations were done at room temperature. After a 1 hr incubation with blocking buffer (0.1% BSA in PBS), 1µg of toxin in 100µL blocking buffer was added to each well, and the toxin allowed to bind for 1 hr. Toxin was then removed, the wells washed with PBS, and a 1:500 dilution of rabbit anti-ricin antibody in blocking buffer was added to each well. After a 1 hr incubation, wells were washed with PBS prior to exposure to a 1:1,000 dilution of secondary antibody (HRP-conjugated goat-
anti-rabbit antibody) in blocking buffer. Wells were washed with PBS, and 100µL of substrate buffer (10mg of o-phenylenediamine dissolved in 10mL of 100mM sodium citrate, pH 4.5, to which 4µL of 30% H₂O₂ was added immediately prior to use) was added to the wells. After 2 min in the dark, the reaction was stopped by addition of 10µL of 2N H₂SO₄, and binding visualized by monitoring absorbance at 490 nm.

2.3 Isolation of the oligosaccharide portion of asialo-GM1

Gangliosides were isolated from bovine brain gray matter as described by Folch et al. (1957). In brief, lipids were extracted using chloroform:methanol (2:1, v/v), polar lipids allowed to partition into water, and the aqueous phase lyophilized. After base treatment to degrade phospholipids, neutral lipids were separated from sialylated gangliosides by column chromatography on DEAE-sephadex A-25. Saccharide portions were isolated from mixed gangliosides using 2,3-dichloro-5,6-dicyanobenzoquinone to oxidize the C(3) hydroxyl of sphingosine to a ketone, followed by base-catalyzed β-elimination of the oligosaccharide as previously described (Yowler et al., 2001). Isolated oligosaccharides were desialylated using Dowex-50W in the protonated form (Schengrund and Kovac, 1999). Again, neutral saccharides were separated from free sialic acid and residual sialylated oligosaccharides by ion-exchange chromatography on DEAE Sephadex A-25. Neutral oligosaccharides were eluted with water and their recovery followed by HPTLC. Acetonitrile:isopropanol:50mM KCl (2:13.4:4.6, by vol) (Yowler et al., 2001) was used to develop the plates while bands were visualized by charring after exposure to 5% sulfuric acid in ethanol. Samples containing neutral oligosaccharides were combined and dried under vacuum from the frozen state. The
oligosaccharide portion of asialo-GM1 (oligo-AM1) was purified from other neutral oligosaccharide contaminants by size exclusion chromatography on Bio-Gel P-2 with 100mM pyridine acetate, pH 5.0 as the eluant. Purity of oligo-AM1 was confirmed by HPTLC. Charged compounds were eluted from the DEAE-Sephadex column using 1M sodium acetate. After dialysis against water to remove salt, samples containing sialylated oligosaccharides were identified by HPTLC. Plates were developed as described for neutral oligosaccharides and the bands visualized using resorcinol spray (Svennerholm, 1957). Recovered sialylated oligosaccharides could be treated with Dowex-50W a second time to enhance the yield of recovered oligo-AM1.

2.4 Preparation of BSA-saccharide conjugates

Reductive amination (see Fig. 2.1) was used to covalently link saccharides to BSA using the optimized reaction conditions described by Roy et al., (1984). Briefly, saccharide (292 µmol), BSA (1 µmol), and sodium cyanoborohydride (1.59 mmol) were dissolved in 5 mL of 0.2M sodium borate buffer (pH 9.0) and incubated, with stirring, at 50°C for 0 to 48 hrs. The reaction was stopped by reducing the pH to 3.5-4.0 with 80% acetic acid. Samples were then dialyzed against water for 48 hrs prior to being dried under vacuum in the frozen state. A visual estimation of the mass of the products was obtained using SDS-PAGE under reducing conditions (Laemmli, 1970). The average number of saccharides conjugated per molecule of BSA was determined from masses obtained by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) done as described previously (Kensinger et al., 2004), using a Perseptive Biosystems Voyager DE-PRO spectrometer. A 1:10 dilution of sample
(1mg/mL) to matrix was used. The matrix was slightly modified from that described by Lewis et al. (1993), and consisted of 600µL dH₂O, 300µL acetonitrile, and 100µL of 3% trifluoroacetic acid in water saturated with 3,5 dimethoxy-4-hydroxycinnamic acid.

Fig. 2.1: Mechanism for reductive amination. For production of BSA-based neoglycoconjugates, carbohydrates were covalently linked to the ε-amino groups of lysine residues present on the BSA.

2.5 Isolation of ricin from castor beans

Ricin was purified from castor beans using the protocol of Nicolson and Blaustein (1972) with some minor modifications. One hundred grams of castor beans were blended in 1L of PBS for 5 minutes, incubated with stirring for 3 hrs at 4°C, and then filtered through cheese cloth to remove insoluble debris. For ease of handling, the mixture was separated into four fractions and each handled separately, but identically throughout the rest of the procedure. The solution was centrifuged at 10,000xg for 30 min and the supernatant removed, taking care not to disturb the lipid layer on top of the aqueous layer. Lipid and the pellet were discarded and the centrifugation step repeated with the clarified supernatant a total of 3 times. The clear supernatant was adjusted to 0.6 saturation with ammonium sulfate and incubated overnight at 4°C with stirring. The ammonium sulfate precipitated pellet (enriched in ricin) was recovered by centrifugation at 10,000xg for 30 min. The pellet was taken up in PBS, and dialyzed against PBS, in the
cold for 48 hrs. The solution was then centrifuged at 10,000xg for 30 min and the supernatant collected. Ricin and ricin agglutinin were isolated by chromatography on a column containing 400mL of 10% non-crosslinked agarose beads. Non-adherent proteins were removed by washing the column with PBS until absorbance at 280nm was less than 0.05. Ricin (RCA$_{60}$) and ricin agglutinin (RCA$_{120}$) were then eluted with 200mM D-galactose in PBS. Galactose-binding fractions were combined and RCA$_{120}$ separated from ricin using a Centriplus ultrafiltration device having a molecular weight cut-off of 100kDa. When retentate volume was reduced to 5mL or less, filtrate (containing RCA$_{60}$) was collected and concentrated using a Centriplus ultrafiltration device with a molecular weight cut-off of 50kDa. Purity of the recovered protein was ascertained by its ability to bind to agarose, and by comparing its molecular weight to ricin standards using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under both reducing and non-reducing conditions.

2.6 Immobilization of ligands on the surface of L1 and CM5 sensor chips

All SPR experiments were carried out using a Biacore 3000. A L1 sensor chip was used for SPR experiments to monitor binding of toxin to GSLs. The surface of the L1 sensor chip is dextran coated and modified with alkyl chains to increase lipophilicity (Cooper et al., 2000). Liposomes were prepared as described previously (Yowler and Schengrund, 2004) with minor modifications. Briefly, a solution of POPC (95 mole%) and asialo-GM1 (5 mole%) in chloroform:methanol (1:1 by volume) was placed into a glass test tube and dried under nitrogen to form a thin film. The lipid film was hydrated with HBS for 20 min at 50°C. The hydrated lipids were sonicated for 2 minutes, prior to
being extruded through a 100nm filter 21 times. For use as a negative control, liposomes consisting of POPC alone were also prepared. Liposome preparations were used immediately after preparation.

Throughout GSL binding experiments, at least 2 flow cells were used. Flow cell 1 was coated with POPC and used as a negative control, while flow cell 2 was coated with 5 mole percent asialo-GM1 in 95 mole percent POPC and used as the toxin binding surface. A sample sensorogram from the immobilization of 5 mole percent asialo-GM1 onto an L1 sensor chip is shown in Fig. 2.2. Prior to coating the flow cells, contaminants were removed from the chip by washing the surface with a 5 min injection of 20mM CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate) at a flow rate of 5µL/min. The appropriate liposome mixture was then injected across the flow cell for 30 min at a flow rate of 2µL/min. To remove any loosely associated liposomes and create a stable monolayer, the flow rate was increased to 100µL/min and a 12 sec pulse of 50mM NaOH was injected over the surface. Non-specific protein binding sites were then blocked by a 5 min injection of BSA (0.1mg/mL in PBS) at a flow rate of 10µL/min. This immobilization procedure resulted in adherence of approximately 8,500 RUs of liposomes to the surface of a flow cell.
Fig. 2.2: Immobilization of asialo-GM1 onto an L1 sensor chip. Arrows indicate injection start and injection stop points of: A) 20mM CHAPS, B) 5 mole percent asialo-GM1, C) 50mM NaOH, D) 0.1% BSA.

When using SPR to monitor the binding of toxin to protein ligands, a CM5 sensor chip was used. The surface of the CM5 chip is coated with dextran containing carboxyl groups. Functional groups on the ligand to be immobilized (i.e., -NH₂) are coupled to the surface in a covalent manner. Ligands were covalently immobilized directly to the surface of the CM5 sensor chip using the amine coupling method provided with the control software. An example sensorgram from the immobilization of ASF onto a CM5 sensor chip is shown in Fig. 2.3. Throughout the immobilization procedure, buffer flow
rate was maintained at 5µL/min. The sensor chip surface was activated with a 7 min injection of a 1:1 (by volume) mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 11.5 mg/ml) and N-hydroxysuccinimide (NHS, 75 mg/ml). For surface binding kinetics, a 10mM acetate buffer, pH 5.5, containing either 50nM ASF or 25nM BSA was used to coat the appropriate flow cell surface. For general kinetic measurements, total RUs due to analyte binding to ligand should be between 100-500RUs (Karlsson et al., 1991). The amount of immobilized ligand needed to yield maximum analyte binding (RU\textsubscript{max}) of ~100 RUs was calculated using the following equation:

\[ RU_{\text{max}} = \left( \frac{MW_{\text{analyte}}}{MW_{\text{ligand}}} \right) \times RL_{\text{ligand}} \times V_{\text{ligand}} \]

where \( RL_{\text{ligand}} \) is the number of RUs of ligand to be immobilized and \( V_{\text{ligand}} \) is the stoichiometry of the ligand (assumed to be 1 for these experiments). The relative ligand concentration (RL) shown to give a maximum binding capacity of 100 RUs to ASF was 75 RUs. The immobilization of the appropriate RL concentration was achieved using a targeted immobilized level “wizard” built into the Biacore control software. This wizard analyzes the rate at which each ligand becomes immobilized to the sensor chip surface and monitors immobilization in real time. When the specified RL level is reached, the procedure stops, and remaining amino groups are blocked with a 35µL injection of 1M ethanolamine. For inhibition and solution affinity experiments, the sensor chip surface was saturated with ASF or BSA by following the steps described for immobilizing specific amounts of ASF, however, a 1µM solution of ASF or BSA, in 10mM sodium acetate buffer, pH 4.5, was used and injected over the activated surface for 45 min. This
procedure resulted in an increase in baseline response of between ~5,000 and ~8,000 RUs, depending on the ligand used.

![Graph showing immobilization of ASF onto a CM5 sensor chip. Arrows indicate injection start and injection stop points of: A) EDC:NHS, B) ASF, C) 1M ethanolamine.](image-url)

Fig. 2.3: Immobilization of ASF onto a CM5 sensor chip. Arrows indicate injection start and injection stop points of: A) EDC:NHS, B) ASF, C) 1M ethanolamine.

2.7 SPR analysis of toxin binding

SPR was used to monitor binding because the amount of material needed to obtain binding data is small, binding is directly monitored (analyte and ligand don’t need to be labeled), and binding is monitored in real-time. To observe toxin binding to ASF, SPR experiments were conducted using flow cells on a CM5 sensor chip prepared as described above. Ricin and RTBs were prepared and tested identically and will be
referred to as “toxin” when describing general aspects of each experiment. To test the ability of toxin to bind each ligand, serial dilutions (1:2) of each ligand were prepared. The toxin concentration used was 40 nM unless indicated otherwise. Toxin or toxin plus ligand was injected over the flow cells, in a random order, at a flow rate of 10µL/min for 2 min. Maximum toxin binding was recorded for each sample with that for toxin alone taken as 100%. Toxin was allowed to dissociate by exposure to running buffer at a flow rate of 10µL/min for 3 min, after which any remaining surface bound toxin was removed by a 1 min injection of PBS-P containing 200mM Lactose and 1M NaCl at a flow rate of 30µl/min. This prepared the surface for another binding assay. SPR data analyses were performed using BiaEvaluation 4.1 software. Non-specific binding to ASF was accounted for by subtracting sensorgrams obtained for binding to BSA alone from the corresponding sensorgrams obtained for binding to ASF. Percent of maximum binding was calculated for each concentration (see results section) of ligand and plotted on a graph. The IC$_{50}$ for each ligand is defined as the concentration that allowed only 50% of the binding response seen when toxin alone was injected. Experiments were repeated between three and seven times and the mean maximum binding and the standard error of the mean (SEM) calculated for each concentration of ligand.

For SPR experiments done to monitor inhibition of RTB binding to asialo-GM1, a L1 sensor chip coated with POPC-asialo-GM1 liposomes, as described above, was used. Experiments were done using PBS (no surfactant P20) as the running buffer as well as the sample buffer, and performed in a manner similar to those done with protein ligands on the CM5 chip. To control for non-specific RTB binding, sensorgrams from flow cells containing POPC alone were subtracted from those of POPC-asialo-GM1 containing flow
cells. To ensure that results were not skewed due to saturation of the flow cell surface, maximum binding obtained for several concentrations of sRTB were obtained and plotted on a graph (Fig. 2.4). The 500nM sRTB concentration is not in the range at which the flow cell surface is saturated, and therefore was used for subsequent asialo-GM1 binding experiments. To monitor binding of RTB alone, a 500nM solution of each RTB (obtained from either Sigma Aldrich or Vector Laboratories) was injected across the liposome surface at a flow rate of 20µL/min. The maximum RUs of binding were observed as well as the overall shape of each of the binding curves. For inhibition experiments, serial dilutions (1:2 by volume) of various ligands were prepared and added to 1,000nM sRTB (1:1 by volume) prior to injection over the liposome surface. The final concentration of protein ligands ranged from 0-1µM, while the final concentration of saccharide (lactose or oligo-AM1) ligand ranged from 0-100mM. Samples of sRTB plus ligand were injected across the liposomes at a flow rate of 20µL/min for 2 min. Bound sRTB was allowed to dissociate for 2.5 min in running buffer. Remaining sRTB was removed with a 1 min injection of regeneration solution (200mM lactose, 1M NaCl, in PBS) at a flow rate of 30µL/min, thus preparing the surface for another round of binding. Maximum RUs due to sRTB binding were recorded for each sample. Experiments for each sample were repeated between three and eight times and the mean and SEM calculated. Percent inhibition was calculated for each ligand in the same manner as described above. Percent inhibition was plotted against ligand concentration for each sample and the IC\textsubscript{50}s for each sample were determined.
Fig. 2.4: Binding of various concentrations of sRTB to asialo-GM1 immobilized on a L1 sensor chip surface. These results confirm that 500nM sRTB falls on the linear part of the curve and, therefore, was used throughout SPR lipid binding experiments.

2.8 Determination of toxin affinity for each ligand

The kinetics of toxin binding to immobilized ASF and BSA-Lac₃₄ were examined using SPR. Ligands were immobilized to the surface of a CM5 sensor chip as described above. For these experiments, 75 RU of ASF and 250 RU of BSA-Lac₃₄ were immobilized. Serial dilutions of toxin (from 0-500nM) were prepared and each was injected over a given protein ligand for 2 min at a flow rate of 75µL/min. Dissociation was monitored for 3 min before the surface was regenerated. Sensorgrams from the control flow cell were subtracted from those of ligand coated flow cells. The sensorgrams were evaluated using BiaEvaluation 4.1 software. Injections of 500nM
toxin at flow rates of 5, 15, 30, and 75µL/min were performed to investigate contribution due to mass transfer. No differences in RUs were seen at flow rates above 15µL/min, indicating that, since experiments were performed at 75µL/min, mass transfer effects were negligible, and therefore not included in the kinetic fit models used (see discussion for more detailed explanation of mass transfer). Sensorgrams were zeroed on the y-axis based on the average response seen just prior to sample injection and aligned to zero on the x-axis at the sample injection start point. A sensorgram from the injection of running buffer alone was subtracted from all other sensorgrams to remove any buffer effects. The data was fit to the “bivalent analyte” binding model provided with BiaEvaluation 4.1 software.

Experiments to determine solution affinity of the toxin for each of the different ligands were performed in the same manner as those used to determine IC\textsubscript{50}s for ligand inhibition of toxin binding to ASF as described above. Different concentrations of each toxin (from 5-50nM) were injected over the flow cells for 2 min at 10µl/min. Maximum binding was recorded for each concentration. This was repeated four times for each concentration tested. A standard curve was generated by plotting maximum binding response against toxin concentration using BiaEvaluation 4.1 software (see Fig. 2.5). By conducting all experiments under conditions used to generate the standard curve, free toxin concentration could be calculated based on response units (RUs) obtained when maximum binding by 40nM toxin was measured in the presence of various concentrations (0.5nM-100mM) of ligand. BiaEvaluation 4.1 software was used to calculate the concentration of free toxin in each sample based on the standard curve
generated. Free toxin was plotted against ligand concentration, data fit to the solution affinity model, and affinity of the toxin for the ligand calculated.

![Graph showing maximum binding of different concentrations of v-ricin to ASF.](image)

Fig. 2.5: Maximum binding of different concentrations of v-ricin to ASF. Maximum binding by each concentration was measured 5 times and the data obtained used to determine the concentration of free v-ricin in solution affinity experiments.

### 2.9 Amino acid analysis of RTB obtained from two different sources

RTB from both Sigma-Aldrich and Vector Laboratories was treated identically. To reduce disulphide bonds, 4µg RTB were dissolved in 10µL of a 2.5mM dithiothreitol (DTT) solution and incubated at 95°C for 20 min. Free cysteine residues were alkylated (to prevent reformation of disulphide bonds) by bringing the mixture to a volume of 20µL containing a final concentration of 50mM NH₄HCO₃ and 10mM iodoacetamide.
This reaction was allowed to proceed for 30 min at 37°C in the dark. For trypsin digestion, 70µL of 50mM NH₄HCO₃, 10µL acetonitrile, and 0.1µg sequencing grade methylated trypsin were added to the alkylated sample and incubated at 37°C overnight. The sample was dried by evaporation in a vacuum centrifuge. In order to completely remove NH₄HCO₃ and acetonitrile, the pellet was resuspended in 200µL dH₂O and dried. This step was done three times. On the last cycle, the sample volume was reduced to approximately 10µL and trifluoroacetic acid (TFA) was added to give a final concentration of TFA of 0.1% (v/v). Personnel in the Macromolecular Core Facility, at the Penn State Milton S. Hershey Medical Center (Hershey, PA), then analyzed the peptide composition of the digested protein samples using MALDI-TOF-TOF MS.

2.10 Cell culture and ricin cytotoxicity

HeLa cells were cultured in HEPES buffered Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% newborn bovine serum and 1% L-glutamine in closed flasks at 37°C. For cytotoxicity experiments, cells were grown in sterile 96-well microtitre plates. HeLa cells were seeded at a density of 10,000 cells per microtitre well and incubated at 37°C for 24 hrs. Culture media was aspirated and replaced with 100µL of media containing ricin at a concentration of 300pM and various concentrations of either ASF or BSA-Lac₃₄. After a 5 min incubation, ricin containing media was aspirated and replaced with plain HEPES buffered DMEM and the cells incubated at 37°C for an additional 24 hrs. Media was removed and replaced with 100µL of fresh HEPES buffered DMEM and 20µL of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent was
added to each well. The MTS solution contained phenazine ethosulfate (PES), a stable electron coupling reagent. When MTS is reduced by NADPH or NADH present in living cells, it forms a colored formazan product which is soluble in cell culture media. After a 4 hr incubation at 37°C, \( \text{Abs}_{490\text{nm}} \) was measured using a 96-well plate reader. \( \text{Abs}_{490} \) was plotted against ligand concentration to observe inhibition of cytotoxicity. Results from time course experiments indicated that a 5 min exposure of cells to ricin at 37°C was sufficient to induce cell death 24 hrs after removal of ricin from the media.

### 2.11 Visualization of ricin binding using fluorescence microscopy

To observe ricin binding to cells, fluorescein labeled ricin was used. HeLa cells were seeded at a density of 400,000 cells onto glass cover slips in 60mm tissue culture dishes and incubated at 37°C for 24 hrs. Cells were then exposed to labeled ricin for varying lengths of time prior to fixation using 4% para-formaldehyde in PBS. The cover slips were then inverted and mounted on slides for microscopy. Slides were visualized at room temperature. Images were acquired (objective 100x/1.4) on a Nikon Optiphot-2 epifluorescence microscope equipped with a Retiga Exi Q Imaging camera, using the acquisition software QED InVivoTM.
Chapter 3

Results

3.1 Use of ELLSA to observe toxin binding

Binding of vRTB to various GSLs, the structures of which are shown in Fig. 3.1, and ASF was examined using ELLSA. It can be seen in Fig. 3.2 that vRTB bound to ASF more readily than to GSLs. Out of the GSLs tested, vRTB bound most efficiently to asialo-GM1. While less than that to asialo-GM1, significant binding to LacCer was also observed. Interestingly, binding to GM1 was only ~30% of that seen to asialo-GM1. No significant binding to GalCer, GD1a, or GT1b was noted. Variability in ricin binding properties has been noted previously (Houston and Dooley, 1982), therefore, the binding of sRTB, vRTB, and v-ricin to asialo-GM1 were monitored using the ELLSA procedure. All three bound well to asialo-GM1, however, sRTB had a higher Abs$_{490}$ than vRTB and v-ricin at the same concentration (see Fig. 3.3). The reason for this is not clear, however, sRTB was included in several subsequent experiments to further characterize these differences. Based on these results, the saccharide portions from both asialo-GM1 and LacCer (oligo-AM1 and Lactose, respectively) were used to synthesize neoglycoconjugates tested as possible ligands for ricin.
Fig. 3.1: Structures of GSLs studied. Numbers indicate the carbohydrate residues present in the structure.

<table>
<thead>
<tr>
<th>GSL</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>LacCer</td>
<td>1,2</td>
</tr>
<tr>
<td>Asialo GM₁</td>
<td>1,2,3,4</td>
</tr>
<tr>
<td>GM₁</td>
<td>1,2,3,4,5</td>
</tr>
<tr>
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<tr>
<td>G₁₁</td>
<td>1,2,3,4,5,6,7</td>
</tr>
</tbody>
</table>
Fig. 3.2: Binding, as determined by ELLSAs, of vRTB to ASF and glycosphingolipids. Each assay was done in quadruplicate and error bars indicate the SEM.
3.2 Isolation of the saccharide portion of asialo-GM1

The products obtained at various points during the isolation of the oligosaccharide portion of asialo-GM1 are shown in Fig. 3.4. It can be seen that oxidation followed by base-catalyzed elimination resulted in release of a mixture of sialylated oligosaccharides from the gangliosides isolated from bovine brains (Fig. 3.4, lane A). After treating the sialylated oligosaccharides with Dowex-50W, ion exchange chromatography effectively separated neutral oligosaccharides from residual sialylated ones (Fig. 3.4, lane B), while their chromatography on Bio-Gel P2 resulted in the isolation of apparently pure oligo-
AM1 (Fig. 3.4, lanes C and D). Lane D was deliberately overloaded to confirm purity. Using the methods described, milligram quantities of pure oligo-AM1 were isolated and used for ligand synthesis.

Fig. 3.4: HPTLC analysis of fractions recovered during isolation and purification of oligo-AM1. The plate was developed in acetonitrile: isopropanol: 50mM KCl (2:13.4:4.6, by vol), and oligosaccharides visualized using resorcinol first and then by charring after exposure of the plate to 5% sulfuric acid in ethanol. Lane A, resorcinol positive oligosaccharides isolated from mixed bovine brain gangliosides: the bottom band corresponds to a trisialylated-, the middle to a disialylated-, and the top to a monosialylated-gangliotetraose oligosaccharide. Lanes B-D were resorcinol negative and were visualized using 5% sulfuric acid in ethanol. Lane B, neutral carbohydrates recovered after removal of sialic acid residues from the sialylated oligosaccharides using Dowex-50W followed by their isolation by ion exchange chromatography. Lanes C and D, oligo-AM1 obtained after size exclusion chromatography of the neutral oligosaccharides on a Bio-Gel P2 column. Lane D was deliberately overloaded in order to confirm the absence of other neutral saccharides.

3.3 Preparation of BSA-saccharide conjugates

Use of reductive amination to add saccharide moieties to BSA induced a change in mass of the BSA, which was detected as a change in its mobility upon analysis by
SDS-PAGE (see Fig. 3.5). The apparent increase in mass provided an estimate of the number of carbohydrate residues linked to the BSA. A more precise estimation of the average mass of each BSA-neoglycoconjugate was obtained by MALDI-TOF MS analysis (see Fig. 3.6). The number of saccharide residues was calculated by dividing the difference in mass between derivatized BSA and native BSA by the mass of the saccharide added, minus 16 (mass of the oxygen lost during reductive amination (see Fig. 2.1). The average number of carbohydrate moieties added to BSA could be manipulated by controlling how long the reaction was incubated (Fig. 3.7). Results indicated that an average of 34 lactosyl residues were added in a 48 hr reaction time (BSA-Lac$_{34}$), 18 in 4 hrs (BSA-Lac$_{18}$), 15 in 2 hrs (BSA-Lac$_{15}$), 9 in 1 hr (BSA-Lac$_{9}$), 4 in 30 mins (BSA-Lac$_{4}$), and 2 in 15 mins (BSA-Lac$_{2}$). Interestingly, it took 24 hrs to add an average of 15 oligo-AM1 residues (BSA-oligo-AM1$_{15}$). The change in molecular weight due to carbohydrate derivatization is summarized in Table 3.1. The longer time required for the addition of oligo-AM1 residues may reflect the larger size of the oligo-AM1 compared to lactose.
Fig. 3.5: SDS-PAGE analysis of BSA neoglycoconjugates. A) molecular weight standards of 100, 75, and 50 kDa, B) native BSA, C) BSA-Lac_{34}, D) BSA-Lac_{15}, and E) BSA-oligo-AM_{15}. Proteins were separated on a 5% stacking and 7.5% resolving gel and bands visualized using GelCode Blue.
Fig. 3.6: MALDI-TOF MS analysis of BSA-based neoglycoconjugates: A) BSA, B) BSA-Lac\textsubscript{34}, and C) BSA-oligo-AM\textsubscript{115}. 
Fig. 3.7: Graph depicting the relationship between incubation time and the average number of lactose moieties added to BSA.
Table 3.1: Molecular weights of BSA-neoglycoconjugates obtained from MALDI-TOF MS analysis. Average number of carbohydrates per molecule of BSA was calculated by dividing the mass due to carbohydrate by the molecular weight of the carbohydrate, minus 16 (to account for the loss of 1 oxygen during reductive amination).

<table>
<thead>
<tr>
<th></th>
<th>Average Molecular Wt.</th>
<th>Mass due to carbohydrate</th>
<th>Avg. # of carbohydrates per molecule of BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>66,861</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BSA-Lac₂</td>
<td>67,583</td>
<td>722</td>
<td>~2</td>
</tr>
<tr>
<td>BSA-Lac₄</td>
<td>68,268</td>
<td>1,407</td>
<td>~4</td>
</tr>
<tr>
<td>BSA-Lac₉</td>
<td>69,923</td>
<td>3,062</td>
<td>~9</td>
</tr>
<tr>
<td>BSA-Lac₁₅</td>
<td>72,131</td>
<td>5,270</td>
<td>~15</td>
</tr>
<tr>
<td>BSA-Lac₁₈</td>
<td>73,151</td>
<td>6,290</td>
<td>~18</td>
</tr>
<tr>
<td>BSA-Lac₃₄</td>
<td>78,468</td>
<td>11,607</td>
<td>~34</td>
</tr>
<tr>
<td>BSA-oligo-AM₁₅</td>
<td>77,546</td>
<td>10,685</td>
<td>~15</td>
</tr>
</tbody>
</table>

3.4 Isolation of ricin from castor beans

Results from GSL binding ELLSAs indicated some variability in the binding profile of sRTB with respect to vRTB and v-ricin. Therefore, ricin was isolated from whole castor beans and used in several experiments to see whether it behaved in a manner more akin to toxins from Vector Laboratories or Sigma Aldrich. Ricin was purified from castor beans as described in the Methods section 2.5. Purified ricin (p-ricin) was used to compare how toxin from different sources bound to various ligands. Both RCA₆₀ (ricin) and RCA₁₂₀ (ricin agglutinin) bound to the agarose column and were eluted with PBS containing galactose (see Fig 3.8, lane C). The use of ultrafiltration to
separate RCA_{60} from RCA_{120} eliminated the need for ion exchange chromatography. Using a Centriplus ultrafiltration device with a molecular weight cut off (MWCO) of 100kDa, RCA_{60} passed through the filter while larger contaminants remained in the retentate. The filtrate volume, containing purified RCA_{60}, was reduced using a Centriplus device with a MWCO of 30kDa (see Fig. 3.8, lane D). The slower moving component seen on SDS-PAGE analysis (Fig. 3.8, lane D) was similar to that found in commercially obtained v-ricin (for comparison, see Fig. 3.8, lanes B and D) and was not identified.

---

**Fig. 3.8:** SDS-PAGE analysis of proteins recovered during purification of ricin. A) molecular weight standards, B) v-ricin, C) p-ricin and slower contaminant present after affinity chromatography using agarose media, and D) p-ricin after ultrafiltration. Proteins were separated using a 5% stacking and 10% resolving gel under non-reducing conditions and bands visualized using GelCode Blue.
3.5 Kinetics of toxin binding to ASF and BSA-Lac$_{34}$

All toxins bound both ASF and BSA-Lac$_{34}$ immobilized on a CM5 sensor chip. However, binding to immobilized BSA-Lac$_{34}$ was much less (see Fig. 3.9). Kinetic data for the binding of all toxins to the ASF surface was obtained and used to calculate dissociation constants. Binding to BSA-Lac$_{34}$ was so low that it was not used to obtain kinetic data. Due to the fact that there are two galactose binding sites present on RTB, the data obtained was fit to the bivalent analyte model, shown below,

$$
\begin{array}{c}
A + L \rightleftharpoons \frac{k_{a1}}{k_{d1}} AL \\
\end{array}
$$

where A is the analyte (toxin) and L is the immobilized ligand (ASF). To confirm that this was the appropriate model choice, data was also fit to the 1:1 binding model,

$$
\begin{array}{c}
A + L \rightleftharpoons \frac{k_{a}}{k_{d}} AL \\
\end{array}
$$

and inspected visually (Fig. 3.10) and Scatchard analysis was performed. It is apparent in Fig. 3.10, panel A, that significant deviations between raw data and the calculated fits are present, most notably in the early association phase and the early dissociation phase. This, coupled with the fact that the Chi$^2$ value obtained for this fit was $>$10% of the calculated R$_{max}$ indicated that the data did not fit the 1:1 binding model. Scatchard analysis was performed using equilibrium data obtained when vRTB was injected across the sensor chip surface until maximum binding (R$_{eq}$) was reached (see Fig. 3.11). The maximum response from the 5 highest concentrations (C) was used to create a Scatchard plot of R$_{eq}$/C vs. R$_{eq}$, where R$_{eq}$ = “Bound” and C = “Free”. The Scatchard plot from this
data is shown in Fig. 3.12. Visual inspection of the Scatchard plot indicates that the data is not linear, confirming the inappropriateness of the 1:1 binding model. An example of a kinetic fit obtained from these experiments using the bivalent analyte model is shown for vRTB (Fig. 3.11), sRTB (Fig. 3.13), v-ricin (Fig. 3.14), and p-ricin (Fig. 3.15). Table 3.2 summarizes kinetic data obtained for toxin binding to ASF. $K_D$s in the low µM range were obtained for binding of each toxin to ASF.

![Graph](image)

Fig. 3.9: V-ricin binding to ASF and BSA-Lac$_{34}$. The dashed line shows binding of v-ricin binding to 282 RU of ASF immobilized to a CM5 sensor chip, while the solid line shows v-ricin binding to 300 RU of BSA-Lac$_{34}$ immobilized on a CM5 sensor chip.
Fig. 3.10: Kinetic data for vRTB binding to immobilized ASF fit to the 1:1 (Langmuir) binding model. Panel A shows binding data obtained during a kinetic experiment. Solid lines represent the raw data obtained for vRTB binding, while the dashed lines indicated the calculated fit using the 1:1 (Langmuir) binding model. Panel B is a plot of the residuals obtained when the binding data was fit to the calculated kinetic model.
Fig. 3.11: Kinetic data for vRTB binding to immobilized ASF fit to the bivalent analyte model. For this experiment, vRTB was injected for 2 minutes to allow the higher concentrations of vRTB to reach maximum binding. This data was also used for Scatchard analysis to confirm kinetic model choice. Panel A shows binding data obtained during a kinetic experiment. Solid lines represent the raw data obtained for vRTB binding, while the dashed lines indicated the calculated fit using the bivalent analyte model. Panel B is a plot of the residuals obtained when the binding data was fit to the calculated kinetic model.
Fig. 3.12: Scatchard plot of kinetic data obtained from vRTB binding to ASF immobilized on a CM5 sensor chip.
Fig. 3.13: Kinetics of sRTB binding to immobilized ASF. Panel A shows binding data obtained during a kinetic experiment. Solid lines represent the raw data obtained for sRTB binding to ASF, while the dashed lines indicated the calculated fit using the bivalent analyte model. Panel B is a plot of the residuals obtained when the binding data was fit to the calculated model.
Fig. 3.14: Kinetics of v-ricin binding to immobilized ASF. Panel A shows binding data obtained during a kinetic experiment. Solid lines represent the raw data obtained for vRCA\textsubscript{60} binding, while the dashed lines indicated the calculated fit using the bivalent analyte model. Panel B is a plot of the residuals obtained when the binding data was fit to the bivalent analyte kinetic model.
Fig. 3.15: Kinetics of p-ricin binding to immobilized ASF. Panel A shows binding data obtained during a kinetic experiment. Solid lines represent the raw data obtained for pRCA$_{60}$ binding, while the dashed lines indicated the calculated fit using the bivalent analyte model. Panel B is a plot of the residuals obtained when the binding data was fit to the bivalent analyte kinetic model.
Table 3.2: Summary of the kinetic values obtained for toxin binding to ASF immobilized on a CM5 sensor chip.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Calculated Rmax (RUa)</th>
<th>Chl²</th>
<th>Kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>vRTB</td>
<td>76.4</td>
<td>3.33</td>
<td>6.7x10^{-6}M</td>
</tr>
<tr>
<td>aRTB</td>
<td>171</td>
<td>0.61</td>
<td>2.9x10^{-6}M</td>
</tr>
<tr>
<td>v-ricin</td>
<td>272</td>
<td>5.35</td>
<td>3.2x10^{-6}M</td>
</tr>
<tr>
<td>p-ricin</td>
<td>182</td>
<td>0.47</td>
<td>2.3x10^{-6}M</td>
</tr>
</tbody>
</table>

3.6 Effectiveness of ASF and BSA-neoglycoconjugates as inhibitors of toxin binding to ASF

The ability of various ligands to inhibit binding of toxin to ASF was tested using SPR. An example of sensorgrams obtained during a ricin inhibition experiment is shown in Fig. 3.16. Percent inhibition for each ligand concentration was calculated using the following equation

\[ \%\text{Inhibition} = \left(1 - \left(\frac{TL}{T}\right)\right) \times 100 \]

where “TL” is equal to the maximum RUs obtained when a given sample of toxin plus ligand was injected over the surface, and “T” is equal to the maximum RUs obtained when 40nM toxin alone was used. Values were calculated for each ligand concentration, standard error of the mean (SEM) computed, and percent inhibition plotted (see Fig. 3.17). Experiments done on different days or with a different flow cell coated with ASF
gave similar results, confirming reproducibility of the method. The fact that the error bars obtained for replicate experiments with lactose are not as tight as those for the other ligands may reflect the need to use mM concentrations of lactose to inhibit toxin binding. This high concentration of lactose caused large changes in refractive index, making it more difficult to obtain accurate data. All toxins bound similarly to each ligand tested, except for sRTB to BSA-Lac$_{34}$. While IC$_{50}$s for ASF were in the nanomolar range, those for each of the BSA based multivalent ligands tested were in the low micromolar range. All of the BSA based ligands had IC$_{50}$s > 100 times less than those obtained for lactose. These results indicate that, even after correcting for the number of lactosyl moieties per BSA (Table 3.3), ricin bound the multivalent ligands more effectively than free lactose.
Fig. 3.16: Sensorgrams obtained for v-ricin binding to immobilized ASF in the presence of various concentrations of ASF. Samples were injected over flow cells on a CM5 chip coated with either BSA (control) or ASF (experimental) at a flow rate of 10µl/min for 2 min. Sensorgram curves were overlaid, injection start points aligned to 0 seconds on the x axis, and zeroed on the y axis to the average baseline seen just prior to the injection start. Concentrations of ASF incubated with 40nM ricin were, from top to bottom: 0, 3.9, 7.8, 11.25, 15.625, 22.5, 31.25, 45, 62.5, 90, 125, 180, 250, 360, and 500nM.
Fig. 3.17: Inhibition of toxin binding to immobilized ASF by different ligands. Various concentrations of each ligand were incubated with 40nM toxin and injected over flow cells coated with either immobilized ASF (experimental) or BSA (control to account for nonspecific binding). Percent inhibition was calculated for each sample and plotted on the graph. The shapes of the symbols represent the ligand used in solution: ■ASF; ●BSA-Lac34; ▼BSA-Lac15; ▲BSA-oligo-AM1; ♦Lactose. Black symbols indicate ricin, gray, vRTB; and white, sRTB. Inhibition of v-ricin by native BSA was used to confirm inhibition was due solely to the presence of carbohydrate and is noted by the X symbol.
<table>
<thead>
<tr>
<th>Ligand</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>n</th>
<th>Avg. # of terminal galactosyl residues per molecule of ligand</th>
<th>Adjusted IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>1.61x10&lt;sup&gt;6&lt;/sup&gt; ± 8.00x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
<td>1</td>
<td>1,610</td>
</tr>
<tr>
<td></td>
<td>1.79x10&lt;sup&gt;6&lt;/sup&gt; ± 3.37x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3</td>
<td>1</td>
<td>1,790</td>
</tr>
<tr>
<td></td>
<td>1.88x10&lt;sup&gt;6&lt;/sup&gt; ± 2.91x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>6</td>
<td>1</td>
<td>1,880</td>
</tr>
<tr>
<td>BSA-Lac&lt;sub&gt;14&lt;/sub&gt;</td>
<td>5,880 ± 225</td>
<td>5</td>
<td>15</td>
<td>88.2</td>
</tr>
<tr>
<td></td>
<td>1,375 = 43</td>
<td>5</td>
<td>15</td>
<td>20.6</td>
</tr>
<tr>
<td>BSA-oligo-AM1&lt;sub&gt;15&lt;/sub&gt;</td>
<td>2,638 = 94</td>
<td>6</td>
<td>34</td>
<td>90.4</td>
</tr>
<tr>
<td></td>
<td>1,178 = 40</td>
<td>7</td>
<td>34</td>
<td>40.1</td>
</tr>
<tr>
<td></td>
<td>169 ± 21</td>
<td>5</td>
<td>34</td>
<td>5.7</td>
</tr>
<tr>
<td>Asialofetuin</td>
<td>80 ± 4</td>
<td>5</td>
<td>12</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>29 ± 1</td>
<td>5</td>
<td>12</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>49 ± 0.4</td>
<td>6</td>
<td>12</td>
<td>0.59</td>
</tr>
</tbody>
</table>

Table 3.3: IC<sub>50</sub>s for each ligand tested. Values given are the average of 3 to 7 assays ± the standard error of the mean. The column on the right shows IC<sub>50</sub> data adjusted to account for the number of terminal galactosyl residues present on each molecule.

3.7 Solution affinities of the toxins for ligands

Solution affinity data was obtained by analyzing the concentration of free toxin present in solutions containing different concentrations of ligand using the equation,

$$B_{free} = \frac{B - A - K_D}{2} \pm \sqrt{\left(\frac{A + B + K_D}{4}\right)^2 - A \cdot B}$$

where, A is the concentration of ligand incubated with toxin, B is the concentration of toxin incubated with ligand, and B<sub>free</sub> is the calculated concentration of free toxin in the solution.
solution. To obtain accurate solution affinity data, solutions of toxin plus ligand must be at equilibrium. To determine effect of exposure time of the toxin with ligand on binding data, measurements were made on samples that were incubated for 24 hrs at 25°C, as well as those that were freshly mixed (see Fig. 3.18). Because no difference in binding was noted between samples incubated for 24 hrs and those freshly prepared, all experiments were done using toxin freshly mixed with ligand. Ricin, vRTB, and sRTB bound to ASF in solution with $K_D$ values around $10^{-8}$M (Table 3.4). As expected, ricin and vRTB had similar affinities for BSA-Lac$_{34}$, with $K_D$ values of 1.96 and $1.70 \times 10^{-6}$M, respectively. In contrast, that for sRTB was $5.25 \times 10^{-8}$M. While $K_D$s for binding of ricin to BSA-oligo-AM$_{15}$ and BSA-Lac$_{15}$ differed, 1.68 and $5.95 \times 10^{-6}$M, respectively, they were still in the $10^{-6}$M range. A summary of $K_D$ values obtained for each toxin–ligand combination tested is presented in Table 3.4. The high concentrations of lactose needed to inhibit toxin binding precluded obtaining acceptable fits using the solution affinity program provided in BiaEvaluation 4.1.
Fig. 3.18: Sensorgrams of v-ricin binding to ASF immobilized on a CM5 sensor chip. Samples of v-ricin (40nM) and ASF (300nM) were prepared and injected over the sensor chip surface to monitor binding. The curve in black shows binding of v-ricin after a 24 hr incubation with ASF, while the curve in red shows binding of v-ricin that had been freshly mixed with ASF. These results indicated that extended incubation times were not needed for equilibrium to be reached.
<table>
<thead>
<tr>
<th></th>
<th>ASF</th>
<th>BSA-Lac₃₄</th>
<th>BSA-Lac₁₅</th>
<th>BSA-oligo-AM₁₁₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>v-ricin</td>
<td>Kₒ=4.74x10⁻⁴M</td>
<td>Kₒ=1.96x10⁻⁴M</td>
<td>Kₒ=5.95x10⁻⁴M</td>
<td>Kₒ=1.68x10⁻⁴M</td>
</tr>
<tr>
<td>vRTB</td>
<td>Kₒ=1.73x10⁻⁸M</td>
<td>Kₒ=1.70x10⁻⁹M</td>
<td>N.D.⁵</td>
<td>N.D.⁵</td>
</tr>
<tr>
<td>sRTB</td>
<td>Kₒ=1.05x10⁻⁸M</td>
<td>Kₒ=5.25x10⁻⁸M</td>
<td>N.D.⁵</td>
<td>N.D.⁵</td>
</tr>
</tbody>
</table>

Table 3.4: Solution affinities of ricin for different glycoconjugates.  
⁵N.D. = Not assayed

3.8 Use of SPR to monitor toxin binding to asialo-GM1

Results from ELLSA experiments (Figs. 3.2 and 3.3) indicated that asialo-GM1 was bound well by all the toxins tested. Although sRTB appeared to bind better to asialo-GM1 in ELLSA experiments (Fig. 3.3), this difference was more pronounced when SPR was used to monitor binding of sRTB and vRTB to asialo-GM1. When both sRTB and vRTB were tested for their ability to bind immobilized asialo-GM1, the resulting sensorgrams were quite different. Fig. 3.19 shows binding sensorgrams for both sRTB and vRTB to asialo-GM1 immobilized on an L1 sensor chip. It can be seen that sensorgrams obtained for the binding of sRTB to immobilized asialo-GM1 were reproducible and that the surface was easily regenerated. In contrast, vRTB did not bind as well to asialo-GM1, and its binding curve was not reproducible. Furthermore, regeneration of the lipid surface after binding of vRTB was incomplete as evidenced by failure of the baseline to return to the level it was prior to injection of vRTB. The binding of v-racin to immobilized asialo-GM1 was also evaluated using SPR (Fig. 3.20). It can be seen that sRTB readily binds to immobilized asialo-GM1 (323 RUs), while v-
ricin does not (4 RUs). Due to poor binding of vRTB and v-ricin to immobilized asialo-
GM1, subsequent experiments using SPR to monitor lipid binding were conducted using
only sRTB.

Fig. 3.19: Sensorgrams of sRTB and vRTB binding to asialo-GM1 immobilized on an L1
sensor chip.
Fig. 3.20: Sensorgram of sRTB and v-ricin binding to asialo-GM1 immobilized on an L1 sensor chip. Arrows indicate injection start and stop points. Injection of 500nM sRTB resulted in 323 RU of binding, while injection of 500nM v-ricin resulted in only 4 RU of binding.
3.9 Inhibition of sRTB binding to asialo-GM1

The ability of various ligands to inhibit sRTB binding to asialo-GM1 was examined. Results were compiled and percent inhibition was plotted against ligand concentration (Fig. 3.21). It can be seen that as the number of carbohydrate residues per BSA molecule increased, the amount of lactosylated BSA needed to inhibit binding of ricin to asialo-GM1 decreased. To answer the question of whether the BSA-Lac\textsubscript{34} inhibited at a lower concentration than monovalent lactose because of the greater number of lactosyl residues present per mole of ligand, IC\textsubscript{50}s were adjusted to account for the number of galactose moieties per molecule of ligand. For example, lactose would have 1 galactose present per molecule of ligand while BSA-Lac\textsubscript{34} would have 34 galactose moieties present per molecule of ligand. The IC\textsubscript{50}s obtained from curves in Fig. 3.21 as well as the adjusted IC\textsubscript{50}s are presented in Table 3.5. Based on the adjusted IC\textsubscript{50}s, BSA-Lac\textsubscript{34} was a better ligand than ASF. Interestingly, BSA-oligo-AM1\textsubscript{15} was a better ligand than BSA-Lac\textsubscript{15}. This observation supports the hypothesis that the longer oligosaccharide chain of oligo-AM1 may have some stabilizing secondary interactions with sRTB that are lacking in lactose.
Fig. 3.21: Inhibition of sRTB binding to asialo-GM1 by various glycoconjugates. Error bars indicate the SEM.
Table 3.5: IC₅₀ values for various ligands used to inhibit binding of sRTB to asialo-GM1 immobilized on an L1 sensor chip.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC₅₀ (nM)</th>
<th>n</th>
<th>Avg. # of Carbohydrates per molecule</th>
<th>Adjusted IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>1.75x10⁴ ± 577</td>
<td>4</td>
<td>1</td>
<td>175.0</td>
</tr>
<tr>
<td>Oligo-AM1</td>
<td>9.5x10⁴ ± 3.04x10⁴</td>
<td>3</td>
<td>1</td>
<td>95.0</td>
</tr>
<tr>
<td>BSA-Lac₃</td>
<td>1.0x10⁴</td>
<td>5</td>
<td>2</td>
<td>20.0</td>
</tr>
<tr>
<td>BSA-Lac₄</td>
<td>1.2x10⁵</td>
<td>5</td>
<td>4</td>
<td>5.0</td>
</tr>
<tr>
<td>Asialofetuin</td>
<td>288 ± 7.5</td>
<td>3</td>
<td>12</td>
<td>3.5</td>
</tr>
<tr>
<td>BSA-Lac₉</td>
<td>225 ± 59</td>
<td>3</td>
<td>9</td>
<td>2.0</td>
</tr>
<tr>
<td>BSA-Lac₁₅</td>
<td>68.5 ± 22</td>
<td>4</td>
<td>15</td>
<td>1.03</td>
</tr>
<tr>
<td>BSA-Lac₁₈</td>
<td>28.5 ± 11</td>
<td>3</td>
<td>18</td>
<td>0.51</td>
</tr>
<tr>
<td>BSA-AM1₁₅</td>
<td>25 ± 4.5</td>
<td>4</td>
<td>15</td>
<td>0.38</td>
</tr>
<tr>
<td>BSA-Lac₃₄</td>
<td>11.5 ± 2.5</td>
<td>4</td>
<td>34</td>
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3.10 Primary sequence analysis of tryptic peptides from RTB

To investigate whether the different characteristics of sRTB and vRTB were due to the proteins being two different isoforms of ricin, a tryptic peptide analysis was performed. Analysis of samples using MALDI-TOF-TOF MS gave sequence data for several peptides. Several of the peptide sequences were for regions in which the amino acid sequence of the isoforms was homologous, and therefore were uninformative. However, several peptides were obtained from both sRTB and vRTB that fell in regions that were useful for differentiating between the B-chains of the variants, ricin D, ricin E, and ricin agglutinin. Fig. 3.22 highlights the peptides sequenced to >99% confidence, that fall into regions that can be used to identify the variants. It can be seen that the highlighted regions between amino acid residues 28 and 40, and residues 41 and 53, are
homologous to the sequences of ricin D and ricin E, but not to ricin agglutinin. Furthermore, the highlighted regions between amino acid residues 188 and 198, and residues 220 and 236 are homologous to the sequence or ricin D only. These results indicate that both RTB samples contained peptides that are homologous to those of ricin D, and are presumably the same protein.

Fig. 3.22: Primary sequence of ricin B-chain isotypes (Araki and Funatsu, 1987). The highlighted areas indicate peptides obtained from sRTB (yellow), vRTB (blue), or both sRTB and vRTB (green).
4.1 RTB binding to GSLs

Results from the ELLSA experiments in which binding of vRTB to various GSLs was measured (Fig. 3.2) indicate that vRTB preferred asialo-GM1 over LacCer and GalCer. This finding agrees with observations made by Ganguly and Mukhopadhyay (2007) and may be due to stabilizing interactions between vRTB and residues in the oligosaccharide other than the terminal galactose moiety. Another possibility is that only the interaction of RTB with the terminal galactose moiety is of significance in the binding of vRTB. If this is true, then the increased RTB binding seen with asialo-GM1 may be due to the distance from the microtitre well afforded by longer chain oligosaccharides. The effect of oligosaccharide chain length on ricin binding to liposomes has been noted previously (Surolia and Bachhawat, 1978; Gustafson, 2003). The reason for increased binding could also be a combination of these two possibilities. The IC$_{50}$ data, obtained in SPR experiments, indicating that oligo-AM1 was a more effective ligand than lactose when conjugated to BSA agrees with the ELLSA data and adds support to the hypothesis that oligosaccharide chain length affects RTB binding. If oligosaccharide chain length was the only contributing factor in regard to vRTB binding, one would hypothesize that the gangliotetraose ganglioside GM1 would be bound as efficiently as oligo-AM1 and more efficiently than LacCer. However, that was not observed with vRTB in ELLSAs. Instead, it appears that charge may also play a role in vRTB binding. ELLSA results indicate that binding of vRTB to gangliosides decreased
with the presence of one or more sialic acid residues. In the case of both GD1a and GT1b, the lack of binding probably reflects the presence of a sialic acid residue at the C3 position of the terminal galactose (see Fig. 3.1). Interestingly, it was hypothesized that binding of RTB is largely mediated by hydrogen bonding interactions between the protein and the C3 and C4 hydroxyls of galactose residues (Lord et al., 1994; Wu et al., 2006). The position of the sialic acid residue on C3 of the terminal galactose moiety is probably responsible for the substantial decrease in vRTB binding to GD1a and GT1b. However, the sialic acid moiety on GM1 is found on the internal, not the terminal galactose presumably bound by RTB, so in this case, the reduced binding seen by vRTB relative to its binding to asialo-GM1 may reflect the presence of the negatively charged sialic acid.

The ability of the toxins to bind asialo-GM1 and LacCer in these ELLSA experiments justified: 1) the use of asialo-GM1 as the test ligand for SPR lipid binding experiments and 2) the use of the oligosaccharide portions of asialo-GM1 and LacCer to make multivalent BSA-neoglycoconjugates. Furthermore, it was possible that use of the two oligosaccharide structures might provide information about the effect of oligosaccharide chain length on ricin binding.

4.2 Synthesis of BSA-neoglycoconjugates

Considering the distance between galactose binding sites of ricin (~70Å), BSA-based neoglycoconjugates were used as the oligosaccharide carrier molecule for the reasons discussed in the introduction to this work. The number of carbohydrate residues conjugated to BSA could be easily manipulated by adjusting the reaction time. The fact
that only 15 oligo-AM1 moieties were added to BSA in 24 hrs compared to the addition of 34 lactose moieties in the same amount of time might reflect the size of the saccharide being linked to the BSA. It is possible that the longer carbohydrate chain of oligo-AM1 reduced the rate of its conjugation to BSA by sterically hindering addition of other oligo-AM1 moieties to nearby lysine residues.

4.3 Isolation of ricin from castor beans

During the course of the ELLSA experiments, it was noted that equal concentrations of sRTB and vRTB bound to asialo-GM1 with different intensities (Fig. 3.3). To aid in determining which toxin source was the “outlier” in its binding preferences, another source of ricin was needed. This need was met by purifying ricin from castor beans. The procedure used for the purification of ricin was modified to separate ricin from the agglutinin using ultrafiltration. Although the slower moving contaminant seen on SDS-PAGE was originally presumed to be ricin agglutinin, RCA120 (Nicolson and Blaustein, 1972), it did not migrate on SDS-PAGE in a manner consistent with a protein of that size. Rather, the contaminant migrated with an apparent mass that was about 25% greater than that of ricin agglutinin (Fig. 3.8). This may have been due to oligomerization of some of the ricin. Confirming purity, MALDI-TOF-TOF MS sequence analysis of vRTB (obtained from v-ricin, which contained the same apparent contaminant as p-ricin) showed no evidence of the presence of ricin agglutinin (see section 4.8 for further discussion).
4.4 Kinetics of toxin binding to ASF

To obtain kinetic data for toxin binding to ASF immobilized on a flow cell of a CM5 sensor chip, various concentrations of each toxin were injected across the flow cell surface. Binding of analyte to the flow cell surface is a 2 step reaction. First analyte moves towards the sensor chip surface via convection and diffusion, and second, the analyte binds to the ligand. Each step has its own rate constant. If mass transfer is limiting the rate at which ligand binding occurs, injection of one concentration of analyte over the flow cell at different flow rates will result in different RUs of binding. If no change in RU is observed between a slower and a faster flow rate, the effects of mass transfer can be ignored when fitting kinetic data (Glaser, 1993). To determine whether it was necessary to consider mass transfer when fitting our kinetic data, toxin was injected over a surface immobilized with ASF for 2 min at flow rates of 5, 15, and 75µL/min. Sensorgrams from these experiments are shown in Fig. 4.1. Due to the fact that no significant change in response was seen at flow rates of 5 and 15µL/min, mass transfer was insignificant, and therefore was ignored when fitting kinetic data.
Binding of toxin was monitored and fit to the Bivalent Analyte model supplied with BiaEvaluation 4.1 software. The use of this model is supported by structural data available in the literature and by binding data obtained in our experiments. As discussed in the Introduction, ricin structural data indicates that there are two galactose binding sites present on the RTB. If those binding sites were to bind to the ligand independently of each other, the 1:1 Langmuir binding model would be appropriate. To test this model, data from a kinetic binding experiment in which vRTB binding to ASF was monitored, was fit to the 1:1 binding model (see Fig. 3.10) as well as to the bivalent analyte model (see Fig. 3.11). The fit of the data using the bivalent analyte model appeared to be more...
appropriate than the use of the 1:1 binding model for several reasons. First, visual inspection of the vRTB binding data fit to the 1:1 binding model showed marked deviations in the raw data with respect to the calculated fit data, most notably in the early association and early dissociation phases. These deviations resulted in a Chi² value that was >10% of the calculated R_{max}, which is an indication of a poor fit and/or inappropriate model choice. In the bivalent analyte model, the raw data was more appropriately comparable to the calculated fit data (Fig. 3.11) and gave a Chi² value that was <5% of the calculated R_{max} (see Table 3.2), indicating a more acceptable fit of the data. Finally, Scatchard analysis of the binding data obtained for vRTB (Fig. 3.12) indicated that the data obtained did not correlate with a 1:1 binding interaction. If vRTB bound in a 1:1 fashion, the data points plotted in Fig. 3.12 would have given a straight line. The fact that the data points do not form a straight line confirms the hypothesis that vRTB does not bind in a simple 1:1 manner. These observations supported the use of the bivalent analyte model for analyzing data in subsequent kinetic experiments.

Toxin binding to ASF was monitored using SPR. For the reasons discussed above, the bivalent analyte model was used to fit data obtained for each toxin tested. The dissociation constants for p-ricin and v-ricin binding to immobilized ASF were similar, 2.3µM and 3.2µM, respectively. These values differ from previously published dissociation constants that were in the nM range—with the actual K_{DS} reported being 36nM (Dill and Olson, 1995) and 1.9nM (Dawson et al., 1999). The reason for these discrepancies is probably due to the use of different experimental methods to obtain the kinetic data. Both published values were obtained using ELLSA type experiments in which the binding of ricin (incubated with varying concentrations of ASF) to ASF
immobilized on microtitre wells was monitored. These experiments measured the concentration of free ricin in solution, while the $K_D$s obtained here were calculated from the rates at which vRTB bound to and dissociated from immobilized ASF. [However, in solution affinity experiments, in which the equilibrium binding of ricin to ASF in solution was measured, kinetic constants similar to the published ones were obtained (see section 4.6 for further discussion).] Limitations in comparing Biacore surface binding kinetic data with other methods were noted previously (Nieba et al., 1996). However, these authors had difficulties in their experiments with analyte rebinding to the ligand surface during the dissociation phase. This may have been caused by the slow flow rate (5µL/min) used, as rebinding to the ligand surface can be minimized by increasing the flow rate. The 75µL/min flow rate used in this work should have been sufficient to prevent rebinding of analyte due to its being quickly cleared from the flow cell upon its dissociation. Despite these differences, the reproducibility of the results obtained in the kinetic experiments performed here provided relative dissociation constants that allowed comparisons to be made between the ricin samples studied. The dissociation constant for vRTB was 6.7µM and that for sRTB was 2.9µM. Even though the $K_D$ for sRTB was similar to those obtained for the intact ricins, the sRTB data is suspect. For reasons that are not clear, the sensorgrams obtained for sRTB binding to ASF did not look like those obtained for the binding of intact ricins and vRTB to ASF. The most notable difference was the apparent decrease in binding that occurred just prior to the end of injection of the toxin. Therefore, despite the similarity of the $K_D$s to those obtained for the other samples tested, the shape of the experimental sensorgrams reduced confidence in the value obtained.
4.5 Inhibition of toxin binding to ASF

To supplement the kinetic data obtained for toxin binding to ASF, the ability of several multivalent ligands to inhibit toxin binding to immobilized ASF was assessed. Toxin was injected with various concentrations of ligand and the change in maximum binding monitored. An example of the sensorgrams obtained from these experiments is shown in Fig. 3.16. The responses obtained with 40nM v-ricin in these experiments were around 500 RUs. This was due to the fact that ~8,000 RUs of ASF were immobilized for inhibition and solution affinity experiments, compared to the 75 RUs that were immobilized for surface binding kinetic experiments. Biacore recommends ~100 RUs of binding for the highest analyte concentration when performing surface binding kinetic experiments so that small changes in the binding curves can be monitored effectively. However, for inhibition and solution affinity experiments, it is recommended that the flow cell surface be immobilized with enough ligand to just about saturate the surface. This ensures that a maximum amount of ligand is available on the flow cell surface to bind relatively large amounts of the free analyte in solution. This is necessary in order to accurately measure free analyte concentrations. To monitor reproducibility, each sample injection was repeated between 3 and 7 times. Comparable results were obtained for different preparations of toxin as well as when different preparations of ligand were used. The small SEMs obtained in most experiments attest to the reproducibility of the data.

Data presented in Fig. 3.17 shows percent inhibition plotted against ligand concentration for all samples tested. It can be seen that ASF was the best inhibitor of binding for all toxins, and was approximately 5 orders of magnitude better than
monovalent lactose. BSA-Lac$_{34}$ was the next best inhibitor and was approximately 3 orders of magnitude better than monovalent lactose. BSA showed no significant inhibition of binding by any of the toxins, confirming that any inhibition seen with BSA-Lac$_{34}$ was due to the presence of lactose conjugated to the BSA, and not to the BSA itself. With the exception of BSA-Lac$_{34}$, all toxins tested were inhibited to a similar degree by each ligand. However, BSA-Lac$_{34}$ more effectively inhibited the binding of sRTB than it did vRTB or v-ricin. While the reason for sRTB’s enhanced affinity for BSA-Lac$_{34}$ was not clear, it was not entirely surprising since the sRTB behaved somewhat differently in experiments designed to obtain binding affinities. Most importantly, the graph shown in Fig. 3.17 indicates that ricin might bind more effectively to a multivalent ligand than to a monovalent one.

As mentioned previously, Dawson, et al., (2006), saw enhanced inhibition of toxin binding by multivalent carbohydrate derivatized dendrimers. When the IC$_{50}$s obtained were adjusted to account for the number of carbohydrates present on each dendrimer, it was found that the enhanced binding was due to an additive effect caused by the increased number of lactose moieties on each dendrimer. In other words, the adjusted IC$_{50}$s were comparable to that obtained for monovalent lactose. To determine whether the enhanced inhibition by multivalent ligands seen in Fig. 3.17 was truly a multivalent effect, all IC$_{50}$s were adjusted to account for the number of terminal galactosyl moieties present per molecule of ligand. It can be seen (Table 3.3) that, in contrast to the findings of Dawson, et al.(2006), the adjusted IC$_{50}$s indicate that the increased binding was not due to just an additive effect, but was a true multivalent one.
This result probably reflects the ability of the sugars on the BSA to span the 70Å distance between the two galactose binding sites on RTB.

Although the ability of ricin to bind to ligands in a multivalent fashion is the most striking data obtained from these experiments, more subtle observations can also be made. First, it appears that BSA-Lac$_{34}$ was no more efficient at inhibiting v-ricin binding than BSA-Lac$_{15}$. This suggests that there is a point at which further derivatization of BSA with lactose no longer has an effect on ricin binding. This may be due to the presence of only a finite number of lactose residues that are appropriately spaced on the BSA to elicit a multivalent ricin binding effect. Analysis of the spacing between lysine residues present on BSA (see Fig. 1.5) suggests that relatively few pairs would give the desired spacing. This observation coupled with the finding that BSA-oligo-AM$_{15}$ inhibited v-ricin binding better than both of the BSA-Lactose conjugates, supports the hypothesis that spacing and oligosaccharide chain length can affect the ability of ricin to bind to a multivalent carbohydrate ligand.

### 4.6 Solution affinities of toxins for various multivalent ligands

Solution affinities are useful measurements to investigate protein-ligand binding. In this method, the ligand is not affected by possible conformational changes induced by its covalent linkage to the flow cell surface, and the possibility that the portion of the ligand normally bound is “hidden” from the analyte by facing the surface of the flow cell instead of being exposed to the analyte in solution is eliminated. These complications, as well as those mentioned for surface kinetic analysis are largely avoided by using solution affinity (sometimes called “functional affinity”) and tend to give values analogous to
those obtained using different experimental means such as equilibrium dialysis and ELLSA (Nieba et al., 1996).

These experiments were performed in the same manner as the inhibition experiments described in the previous section. To generate solution affinity data, it is necessary to be able to determine the concentration of analyte in the solution. This was made possible by obtaining the change in RUs seen when different concentrations of toxin were injected across the ligand on the surface of the flow cells and using these values to generate a standard curve (Fig. 2.5). The relative response seen during an SPR experiment could then be related to a specific analyte concentration. Once the standard curve was generated, data obtained from inhibition experiments was used to determine the concentration of free toxin in samples containing various concentrations of ligand. Using BiaEvaluation 4.1 software, the concentration of free toxin was plotted against ligand concentration and the data fit to the solution affinity model. The solution $K_D$ obtained for ricin binding to ASF was $4.74 \times 10^{-8}$M (Table 3.4), which is similar to the $K_D$ of $3.6 \times 10^{-8}$M obtained by Dill and Olson (1995) using the ELLSA method. Similarly, affinities obtained for binding of vRTB and sRTB to ASF were 1.73 and 1.05 $\times 10^{-8}$M, respectively. Just as in the inhibition studies described above, ricin had lower affinities ($K_D$s were in the $10^{-6}$M range) for the BSA-neoglycoconjugates than for ASF. The only exception to this was the $K_D$ ($5.25 \times 10^{-8}$M) obtained for the binding of sRTB to BSA-Lac$_{34}$. As was seen in the inhibition experiments, sRTB bound to BSA-Lac$_{34}$ in a manner that was more similar to the way ricin bound to ASF than to the BSA-neoglycoconjugates.
4.7 Toxin binding to asialo-GM1 using SPR

Based on the results from the ELLSA experiments, and the fact that BSA-oligo-
AM1,15 was bound well by ricin, data obtained for toxin binding to immobilized ASF was
compared to data obtained for sRTB to asialo-GM1 immobilized to a flow cell on a L1
sensor chip. The difference in concentration of sRTB used in the experiments with
immobilized asialo-GM1 (500nM) compared to that used in experiments with ASF
(40nM) reflects the difference in toxin affinity for immobilized asialo-GM1 relative to
ASF. Support for this conclusion was provided by ELLSA data that indicated
significantly more vRTB adhered to immobilized ASF than to immobilized asialo-GM1
(Fig. 3.2). Use of 500nM sRTB gave ~500 RU's of binding to asialo-GM1, which was
similar to the amount of toxin binding obtained in the studies of the effectiveness of
BSA-glycoconjugates as inhibitors of toxin binding to ASF.

Interestingly, BSA-Lac34 inhibited sRTB binding to asialo-GM1 (Table 3.5), with
an IC₅₀ approximately 10 fold less than that for ASF. This was in distinct contrast to
results obtained when ASF was the immobilized ligand, and ASF was found to be a
somewhat more effective inhibitor of sRTB binding to ASF than BSA-Lac34 (Table 3.3).
While the reason for this difference is not clear, it could reflect the effectiveness of a
potential inhibitor at blocking the interaction of the toxin with the specific carbohydrate
residues on the immobilized ligand. An explanation that could also account for the fact
that BSA-oligo-AM1,15 was as effective, on a per saccharide basis, as BSA-Lac34 at
inhibiting toxin binding to asialo-GM1. Inhibition experiments indicated that even
though BSA-Lac2 was the poorest of the multivalent ligands, it still appeared to inhibit
sRTB binding more effectively, on a per lactose basis, than monovalent lactose. This
result suggests that 1 molecule of sRTB is binding to 1 molecule of BSA-Lac₂. If the
galactose binding sites of sRTB were binding to terminal galactose moieties from two
different BSA glycoconjugates, one would expect the binding to be no better than would
be expected with monovalent lactose. In other words, one would expect the IC₅₀ of BSA-
Lac₂ to be 2 times better than monovalent lactose. The fact that the adjusted IC₅₀ for
BSA-Lac₂ is >8 times better than that of monovalent lactose supports the idea that sRTB
is able to interact with only 1 molecule of BSA-Lac₂ in a multivalent fashion. As the
number of lactose residues per BSA increased, the IC₅₀s decreased, with the greatest
change occurring when the average number of lactose moieties increased from 2 to 4. It
can be seen (Table 3.5) that when the number of lactosyl moieties added to BSA
increased from 18 to 34, there was less than a 2-fold change in adjusted IC₅₀. These
results indicate that it should be possible to make an effective multivalent ligand with
relatively few oligosaccharides, provided they are appropriately spaced.

The adjusted IC₅₀s calculated using the IC₅₀s obtained for each ligand tested
indicate that sRTB bound more effectively to multivalent ligands than to monovalent
ones. The results also indicate the importance of the oligosaccharide moiety. For
example, the adjusted IC₅₀ for oligo-AM1 (95µM) is about half of that for lactose
(175µM), and that for BSA-AM1₁₅ (0.38µM) is approximately equal to that for BSA-
Lac₃₄ (0.39µM). The adjusted IC₅₀s for ASF and BSA-Lac₉ are also similar (3.5µM and
2.0µM, respectively). These results underscore the idea that sRTB binding is affected not
only by the number of sugar residues, but also by their size and spacing on the carrier
molecule.
Because sRTB appeared to bind ASF differently than vRTB (Figs. 3.11 and 3.13), it was decided that the efficacy of the ligands at inhibiting adherence of vRTB and of v-ricin to asialo-GM1 should be ascertained. However, in contrast to sRTB, which bound readily to asialo-GM1 immobilized on the L1 sensor chip, vRTB bound poorly and, upon repeated analyses, did not give reproducible results (Fig. 3.19). When v-ricin was injected over the asialo-GM1 surface, no binding was obtained (Fig. 3.20). Given these results, the question of whether all of the toxins were, in fact, ricin or its B-chain was addressed.

4.8 Sequence analysis of tryptic peptides from sRTB and vRTB

To answer the question of whether the toxins used in these experiments was, in fact, that which is conventionally accepted as “ricin D” (the most toxic form of the protein), peptide analyses were done. Several peptides were characterized to >99% confidence from both sRTB and vRTB. All of the peptides obtained were homologous to the published sequence for the b-chain of ricin D (Fig. 3.22). It is possible that the differences noted between sRTB and vRTB were the result of the toxins being isolated from castor beans grown in different places having slightly different characteristics. Discrepancies in binding between ricin from two different sources has been previously mentioned (Houston and Dooley, 1982). The only identifiable difference between sRTB and vRTB was the buffer in which each was supplied. Sigma included 10mM galactose in their buffer while Vector did not. It is possible that this difference affected stability and/or binding preference of the RTB, or the differences noted could have been due to the methods used to purify the toxin.
The findings presented in this work indicate that ricin does exhibit the multivalent effect when allowed to bind to appropriately spaced carbohydrate ligands. It also indicates that its binding is affected by composition of the carbohydrate ligand (e.g., oligo-AM1 appears to be a more effective ligand than lactose) and possibly by the presence of a negatively charged moiety on a carbohydrate near the terminal galactose (binding of vRTB to asialo-GM1 compared to GM1). The observation that addition of increasing numbers of lactosyl moieties to BSA eventually gave lesser increases in its efficacy as a ligand indicates that with careful analysis of spacing, it should be possible to develop a very effective ricin ligand using a relatively low number of carbohydrate moieties. In addition to providing information about what is needed to obtain efficient ricin binding, kinetic studies indicate that the binding does not follow a 1:1 binding model but appears to bind according to the bivalent analyte model. While the story is not complete (see Future Directions) the data obtained should help provide the base for development of therapeutic agents as well as more efficient biosensors.
Chapter 5

Future Directions

The results presented highlight the need for more extensive characterization of ricin’s multivalent binding. As a first step, the exact spacing requirements of galactose residues needed for optimum binding by ricin should be determined. As discussed in section 4.7, multivalent binding of ricin was obtained when as little as two lactosyl moieties were added to BSA. The fact that multivalency was seen with such a low level of derivatization, indicates that certain lysine residues (presumably ~70Å apart) on BSA may have been preferentially derivatized during the reductive amination process. Peptide analysis (similar to the type described in section 2.9) could be used to identify which lysine residues were derivatized on each of the neoglycoconjugates used. This information would permit determination of the actual distance between the first few derivatized lysines, and provide insight about the distances between saccharides needed to give multivalent binding. This type of analysis could also be done for BSA-AM1,t and might provide insight about the differences in binding of ricin to the oligo-AM1 and lactose glycoconjugates.

Although the lysines are probably derivatized in a way that is not completely random, the fact that there is some heterogeneity in the glycosylation of BSA prevents an accurate determination of spacing requirements. However, spacing requirements could be determined using a more defined carrier molecule. The protein, Spermidine/spermine N1-acetyltransferase (SSAT), is a suitable choice for several reasons: 1) it has lysine residues that are >70Å apart, 2) its crystal structure has been determined (Bewley et al.,
the number of lysines present can be controlled through site directed mutagenesis, 4) it is water soluble, and 5) it forms dimers. All of the lysines have been conservatively replaced with arginine residues (which are not subject to derivatization under the conditions used for reductive amination). Site directed mutagenesis has been used to add each of the lysine residues back to their native positions, one at a time (M. Bewley, personal communication). Since the protein exists in the form of a dimer, each lysine that is reintroduced would yield a protein dimer containing two lysine residues. The positions of the residues are known and the distance between each pair of lysines can be ascertained using structural data available for both the wild-type and mutant forms of SSAT. Because the number and location of the lysine residues is well defined, derivatization of the mutant forms of SSAT with saccharide would give a more homogeneous solution of glycoconjugates. Ambiguity due to the high number of potential derivatization sites on BSA would be avoided, allowing for more precise conclusions to be made about spacing requirements needed for multivalent ricin binding.

In regard to the data presented here, the stoichiometry of the interaction of ricin with both ASF and the BSA neoglycoconjugates tested needs to be pursued further. While evidence of a multivalent interaction with ricin was seen when BSA was derivatized with as few as 2 lactose moieties, it is not clear why further derivatization caused a decrease in IC$_{50}$ values. One hypothesis is that the first two saccharides added to BSA are moderately effective at inducing a multivalent interaction, while further derivatization provides more potential binding sites for ricin, which may happen to be more appropriately spaced than the first two thereby allowing for a tighter binding event. On the other hand, it is possible that the first two lactose residues added yield an ideal
arrangement for multivalent ricin binding, but that further derivatization allows for multiple ricin molecules to bind to a single molecule of derivatized BSA. Of course, it is entirely possible that the increase in efficacy seen with higher levels of derivatization may be the result of a combination of these two hypotheses working in tandem. Techniques such as analytical ultracentrifugation, native PAGE, or MALDI-TOF MS might be useful for determining how the level of derivatization is actually affecting the binding of ricin to the multivalent ligands.

Although some work has been published concerning ricin’s ability to bind to oligosaccharides of various compositions and complexities (Baenziger and Fiete, 1979; Gustafson, 2003; Wu et al., 2006; Ganguly and Mukhopadhyay, 2007), there is still much to be learned about what makes ricin preferentially bind to one oligosaccharide over another. Examining the binding of ricin to a wide array of carbohydrates using a carbohydrate microarray could be quite informative. Liang et al, (2007), described a high-throughput technique in which not only protein carbohydrate binding was observed, but also the strength of the interaction was characterized. This technique allows for surface dissociation constants to be obtained for protein binding to 200 different carbohydrate structures at a time. More importantly, information was simultaneously obtained for multivalent carbohydrate interactions as well. This type of information with respect to ricin’s binding preferences could have profound implications for the design of efficient biosensors. This knowledge could also allow for the use of ricin to identify aberrant glycosylation patterns on the cell surface and for exploiting those patterns in delivery of drugs to cells whose glycosylation patterns are indicative of a specific disease state.
An important downstream goal of this work was to test the ligands discussed for their ability to inhibit ricin cytotoxicity in live cells. Very preliminary studies were carried out to determine whether the BSA-Lac$_{34}$ or ASF could effectively block ricin killing of HeLa cells. HeLa cells were exposed to 300pM ricin in the presence of various concentrations of ASF or BSA-Lac$_{34}$. The ability of these ligands to inhibit cell death was monitored using MTS as described in section 2.10. Results from these experiments showed no appreciable inhibition of cytotoxicity. While disappointing, the result was not unexpected if, for no other reason than both ligands had multiple terminal galactosyl moieties that if not bound by ricin may have been available for binding by cell surface galectins (galactose binding lectins). In addition to the large number of ricin binding sites present on the cell surface [1–3 x 10$^7$ for HeLa cells (Sandvig et al., 1976)], it is possible that galectins could have bound free galactosyl residues on the BSA-Lac$_{34}$ or ASF and facilitated internalization of the glycoconjugate-ricin complex. This hypothesis could be tested in two ways. First, fluorescence microscopy could be used to monitor binding and uptake of ricin bound to fluorescently labeled ASF or BSA-Lac$_{34}$, a method that is being developed (section 2.11). If lectin binding to the ricin-glycoconjugate complex enhances its internalization, it is conceivable that the ligands tested could enhance rather than reduce ricin toxicity. Second, inhibition experiments (using SPR) similar to the ones described here, could be used to evaluate the ligands for their ability to inhibit ricin binding to immobilized plasma membrane isolated from various cell types. Furthermore, using SPR to obtain the $K_{D}$s for ricin binding to membranes from different cells could aide in the identification of cell membrane constituents that might enhance the binding of ricin to cells based on which glycoproteins and glycolipids might be more
abundant in different cell lines. The possibilities discussed here, all underscore the need for a more complete analysis of the binding requirements of ricin. A clear understanding of the subtleties that play a part in the binding of ricin to its ligands would be of benefit not only to those studying ricin, but also to those who are studying other lectins, such as mistletoe lectin, the hemolytic CEL-III lectin, and abrin, that contain galactose binding sites that have a β trefoil structure similar to that of RTB.

Although the reasons are not clear, distinct differences in the binding of sRTB and vRTB were noted throughout this work. It would be beneficial to examine this finding more closely by purifying and analyzing the binding of ricin from castor beans grown in different parts of the world. Small differences caused by genetics, environment, or purification method might reveal even greater differences in binding preferences than were noted here. This would not only be of interest from a purely biochemical standpoint, but could have significant ramifications in the realm of developing effective inhibitors of ricin binding and also in the development of more effective biosensors. Sensors developed using one source of ricin might be less sensitive for detection of ricin from different sources, which would obviously be detrimental given our inability to predict the source of a ricin contaminant.

A better understanding of ricin’s ability to bind multivalent ligands could also contribute to the identification of certain glycosylation patterns of the native cell surface proteins and glycolipids to which it preferentially binds. This knowledge could lead to the use of ricin to identify specific glycoproteins that express the ricin-specific oligosaccharide as well as to its use for identifying when those proteins are inappropriately glycosylated. The later could be caused by factors such as infection, an
inborn error in metabolism, or cancer. It would also permit use of RTB to target drugs to specific cells that express the oligosaccharides that are adhered to with the greatest affinity. All of these possibilities emphasize the need to learn more about the binding characteristics of this interesting lectin.
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Appendix

Use of surface plasmon resonance to characterize binding of botulinum type A toxin-haemagglutinin complex to gangliosides

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Abstract

Botulinum type A toxin-haemagglutinin complex, marketed as Dysport®, is used for a variety of licensed clinical applications. Currently, the accepted method for comparing potency of different preparations is the mouse lethality assay that only measures toxicity. The assay does not indicate why one preparation may be different from another. To identify cause of any differences, both endoprotease activity plus the ability of the toxin complex to bind to cell surface receptors, and to be transported to the site of action must be monitored. Inhibition of any of those steps would result in loss of lethality in vivo. As a first step in such assessments, surface plasmon resonance was used to monitor the affinity of 9 different preparations of botulinum toxin type A complex for ganglioside GT1b. The similarities in binding affinities are reflected across different preparations of the Type A complex from different sources.
Introduction

Despite the possibility that botulinum neurotoxin, the deadliest toxin known to man (1), might be used as a biowarfare agent, a quick perusal of the literature will show that the last twenty years have seen a continuous expansion in its clinical use. Clinically, the toxin used successfully during the last 15+ years has been the toxin complex produced by Clostridium botulinum (2) rather than the isolated neurotoxin. The complex, known as progenitor toxin, can exist in three different forms (3). The 12S progenitor toxin consists of one molecule of neurotoxin, one molecule of a non toxic non-haemagglutinating protein; 16S consists of the two proteins found in 12S plus 2 molecules of haemagglutinin; and 19S is a dimer of the 16S (3). The haemagglutinin is thought to facilitate binding of the progenitor complex to, and transport of the neurotoxin moiety across, the intestinal epithelium (4). The associated proteins are also believed to protect orally ingested BoNT from degradation as the complex passes down the digestive tract. Upon absorption from the upper portion of the small intestine, the progenitor toxin enters the lymphatic system and then into the blood (5). The increase in pH encountered upon leaving the digestive tract is believed to cause dissociation of the complex since free BoNT is found in both the lymphatic circulation and blood (6,7). Similar increases in pH to approximately pH 7.4 occur when the toxin complex is administered clinically, leading to very rapid dissociation of the toxin complex to free the neurotoxin molecule (8,9). The toxin complex therefore plays no role in relation to migration or spread of the toxin from the injection site.
Commercialization of botulinum type A toxin (BoNT/A) as a therapeutic has resulted in companies such as Ipsen Ltd. and Allergan developing good manufacturing practices for production of BoNT/A complex acceptable for use in the clinic. In order to make a useful drug, each preparation of a specific product must have the same potency, which for BoNT/A is measured using the mouse lethality assay (1). While results of this assay will indicate whether different preparations of toxin are equally lethal, they do not indicate whether any changes in toxicity are due to loss of BoNT/A endoprotease activity, binding, or cellular uptake and transport to the site of action. To address part of this, we have developed what is now an assay to monitor affinity of concentrated preparations of Dysport® Bulk Active Substance (BAS – bulk toxin), used in the manufacture of Dysport® finished product, to ganglioside GT1b, one of two receptors needed for optimal binding and uptake of BoNT/A by peripheral neurons (10-13). Confirmation of the need for gangliosides for optimum activity of BoNT/A was obtained when GM2/GD2-synthase knockout mice were found to be less susceptible to BoNT/A than controls (14,15).

In previous studies, surface plasmon resonance (SPR) was used to observe real-time binding of purified BoNT/A to a ganglioside-containing phospholipid monolayer (16). Results indicated that the binding of BoNT/A to GT1b was dependent upon ionic strength. It was also observed that the kinetics of binding did not follow a simple 1:1 binding model. Subsequent studies indicated that the toxin underwent a change in conformation upon binding to GT1b. Similar SPR studies of the binding of the BoNT/A complex to gangliosides have not been reported. However, Inoue et al (17) using TLC immunostaining techniques found that BoNT/A complex bound to non-sialylated
glycolipids (asialo-GM1 and paragloboside) and the glycoprotein asialofetuin. Monosialogangliosides GM1, GM2, GM3, and GM4 were not bound nor were the disialogangliosides GD1a and GD1b. Because the neurotoxin complex is the form released by Clostridium botulinum and has been extensively used clinically for many years, it was of interest to us to restudy the question of whether the BoNT/A complex bound to polysialylated gangliosides, a required component for optimum activity by BoNT/A. Using SPR, we observed that BoNT/A complex bound GT1b under conditions similar to those found to be optimum for isolated BoNT/A, and that SPR could be used to monitor reproducibility between commercial preparations of Dysport® BAS.
Materials

DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and asialofetuin (ASF) were purchased from Sigma (St. Louis, MO), gangliosides asialo-GM1, GM1, GD1a, GT1b, and GQ1b were obtained from Matreya, Inc. (State College, PA). Purified BoNT/A neurotoxin and TANC (BoNT/A complex, a Hall strain) were from BBTech, Inc. (North Dartmouth, MA). Samples of BoNT/A haemagglutinin complex (BAS, also a Hall strain) and a sample of BoNT/A produced by Metabiologics Inc. (Madison, WI) were provided by Ipsen (Wrexham, UK). An Avanti mini-extruder was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). SPR experiments were performed using a Biacore 3000 instrument (Biacore, Inc., a division of GE Healthcare, Piscataway, NJ) and CM5 and HPA chips were also from Biacore. Buffers used were prepared when needed and degassed prior to use.
Methods

Immobilization of asialofetuin on a CM5 chip

The "amine-coupling wizard" protocol provided in the Biacore 3000 control software was used to immobilize about 9000 Response Units (RUs) of ASF on the flow cell of a CM5 chip. Carboxyl groups on the surface of the CM5 chip were activated by injecting a mixture of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide [NHS (1:1)] across the chip for 7 min. ASF in 10mM sodium acetate, pH 4.0 (50 µg/ml) was injected until a level of ~9000 RUs was obtained. Any unreacted carboxyl groups were blocked by a 7 min injection of 1 M ethanolamine, pH 8.5, across the flow cell. The flow cell used as a negative control for binding experiments was activated with EDC/NHS and subsequently blocked with ethanolamine (no ASF).

Binding of BoNT/A complexes to immobilized asialofetuin

BoNT/A complex in HEPES-buffered saline, pH 7.4 (10 mM HEPES, 50 mM NaCl) containing 3mM EDTA and 0.005 percent surfactant P20 (HBS-EP) was injected simultaneously at a flow rate of 30 µl/min for 1 min over the two flow cells containing either no protein or ASF. After a one min dissociation in buffer, residual protein still bound to the chip was removed by a two min injection of 1 M NaCl (30 µl/min). This regeneration step prepared the surface for another cycle of binding. Sensorgrams of non-specific binding to the flow cell lacking ASF were subtracted from those of binding to
ASF. Purified BoNT/A was also injected over the same flow cells using the same procedure.

*Preparation of ganglioside/phospholipid liposomes*

Liposomes were prepared as follows: 5 mole percent of ganglioside was mixed with DMPC in chloroform:methanol (2:1 by vol) and dried under nitrogen. HEPES buffered saline (200 µl, 10 mM HEPES, 150 mM NaCl, pH 7.4) was then added to the lipid film and the mixture incubated at 37˚C for 15 min with occasional vortexing. After sonicating for 3 min, the lipids were extruded 21 times using the Avanti Mini-extruder fitted with a 100 nm filter. Liposomes prepared in the same way from DMPC alone were used as a negative control.

*Immobilization of liposomes on a HPA chip*

Prior to use, the surface of the HPA chip was washed using a 4 min injection of 40 mM octylglucoside at a flow rate of 5 µl/min and the needle rinsed (30 percent ethanol and water). The flow rate was then reduced to 2 µl/min and the liposomes injected over the desired flow cell(s) for 30 min. Lipids adhere to the surface by hydrophobic interactions between their hydrocarbon tails and the long-chain alkanethiol molecules that form a flat hydrophobic layer on the surface of the chip. This results in an increase in baseline of 1000–2000 RUs. To create a monolayer, 20 µl of 50 mM sodium hydroxide was injected across the cell(s) at a flow rate of 100 µl/min. Non specific binding sites were blocked by a 5 min injection of bovine serum albumin (BSA - 0.1 mg/ml HEPES buffered saline, pH 7.4) across the flow cells for 5 min at a flow rate of 10 µl/min.
Liposomes containing just DMPC were used to coat the control flow cell while test flow cells were coated with liposomes containing 95 mole percent DMPC – 5 mole percent ganglioside.

*Analysis of binding to ganglioside-DMPC lipid monolayers*

Optimum conditions for the binding of BoNT/A complex to gangliosides were determined by analyzing the effects of ionic strength, pH, concentration of ganglioside, and heat on adherence of the complex to GT1b. The effects of ionic strength and pH were determined by monitoring binding of the complex when Tris (10mM) buffer containing different concentrations of sodium chloride (20, 50, 100, or 150 mM) at various pH values (6, 7, or 8) was used as both the sample and running buffer. To determine the effects of heating, the complex was pre-incubated for 2 hr at either 4°C or 37°C prior to injection. The appropriate ganglioside content of the lipid surface was determined by measuring adherence to a surface containing either 2, 5, or 10 mole percent GT1b plus 98, 95, or 90 mole percent DMPC. In each experiment 100 nM of BoNT/A complex was injected at 20 µl/min for one min over flow cells with immobilized lipid monolayers containing DMPC alone or a mixture of ganglioside and DMPC. Non-specific binding to DMPC alone was subtracted from binding to ganglioside/DMPC. Also, for each set of conditions, purified BoNT/A (100 nM) was injected over all flow cells to serve as a positive control, while BSA was used as a negative control. In each case, BoNT/A bound to immobilized gangliosides as expected from previous observations (16), while the BSA did not bind.
Affinity and binding constants were initially determined by measuring adherence of a number of different concentrations of each complex (100 nM–0.78 nM) in 10 mM Tris, 50 mM NaCl, pH 7.4, to the ganglioside-DMPC coated flow cells. Samples were injected in a random order at a flow rate of 30 µl/min for 1 min over flow cells coated with 95 mole percent DMPC plus either 5 mole percent GT1b, 5 mole percent asialo-GM1, or 5 mole percent GQ1b. All runs were performed in duplicate. Following each sample injection, dissociation was monitored for 10 min with any remaining complex removed by a 2 min injection of 1 M NaCl. Non-specific binding to DMPC alone was subtracted from binding to ganglioside–DMPC coated flow cells. In addition, an injection of buffer was subtracted from each of the resultant sensorgrams to remove any signal attributable to buffer. Kinetic rate constants were fit to the two-state conformational change model using BiaEvaluation 4.1 software. Because binding was best to GT1b, subsequent kinetic studies of different preparations of BoNT/A complex were performed using a DMPC coated flow cell to account for nonspecific binding and also one coated with 5 mole percent GT1b, 95 mole percent DMPC to measure adherence to GT1b. Serial dilutions of BoNT/A complex from 100 nM – 3.12 nM were used. To reduce the probability of air spikes sample injection flow rate was increased to 100µl/min for one min. Dissociation time was reduced to 5 min (with no effect on kinetic analyses) followed by a 2 min injection of 1 M NaCl (30 µl/min) which gave a stable base line.
Results

Both BoNT/A complexes tested adhered to ASF while purified BoNT/A neurotoxin did not (Fig. 1). The fact that the results obtained for both BoNT/A complexes (TANC and BAS) agreed with observations reported by Inoue et al (17), led us to conclude that SPR could be used to monitor binding of BoNT/A complex to ligands of interest. The finding that purified BoNT/A neurotoxin did not adhere to ASF indicated that the binding by both BoNT/A complexes to ASF was probably mediated by one of the other proteins present in the complexes. This was not pursued further in this study.

Fig. 1: Sensorgrams showing the binding of Batch 1 (solid line) and purified BoNT/A neurotoxin (dashed line) to asialofetuin immobilized on a CM5 chip.
Ten mM Tris containing 50 mM NaCl, pH 7, resulted in more BoNT/A complex (TANC) binding to a flow cell coated with 2 mole percent GT1b–98 mole percent DMPC than was observed using 10mM Tris buffer containing either 20, 100, or 150 mM NaCl or having a pH of 6 or 8 (Table 1). Preincubation of TANC at 4 or 37˚C did not have a significant effect on its adherence to GT1b when the assay was performed using 10 mM Tris containing 50 mM or greater concentrations of NaCl (data not shown). Therefore, all subsequent assays were performed at room temperature. Use of 2 or 5 mole percent GT1b in lipid samples used to coat the flow cells had essentially no effect on TANC binding as measured using total RUs. However, total RUs for TANC binding decreased by about 50% when the concentration of GT1b was raised to 10 mole percent. Using these results as a guide, binding of BoNT/A complex to GT1b was studied using a mixture of 5 mole percent GT1b–95 mole percent DMPC. The highest level of binding by BoNT/A complex was seen at pH 7, with binding at pH 6 and 8 being 85 percent and 79 percent, respectively, of that at pH 7.0. This observation led us to use the "physiological" pH of 7.4 for kinetic analyses of BoNT/A complex (BAS) binding. RUs obtained for the binding of BAS to a flow cell coated with 5 mole percent GT1–95 mole percent DMPC indicated that it bound most efficiently when the buffer used was 10 mM Tris – 50 mM NaCl, pH 7.4. The actual number of RUs decreased from 364 when the buffer contained 50 mM NaCl, to 136 at 75 mM NaCl, down to 5 at 150 mM NaCl.
polysialylated ganglioside GT1b and to a somewhat lesser extent to GQ1b and GD1a.

BoNT/A complexes in Table 2.

Fig. 3), allowing calculation of the association/dissociation constants shown for both
sensorgrams fitted well with the calculated curves using this model (see sample data in
BoNT/A neurotoxin underwent a conformational change upon binding to GT1b (16), the
two-state conformational change model was used to fit the data. The experimental
sensorograms fitted well with the calculated curves using this model (see sample data in
Fig. 3), allowing calculation of the association/dissociation constants shown for both
BoNT/A complexes in Table 2.

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<tr>
<th>[NaCl]</th>
<th>20mM</th>
<th>50mM</th>
<th>100mM</th>
<th>150mM</th>
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<td>Not detected</td>
<td>Not detected</td>
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<tr>
<td>pH 7</td>
<td>35 ± 10</td>
<td>116 ± 3</td>
<td>19.1 ± 3</td>
<td>6.5 ± 2</td>
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<td>pH 8</td>
<td>59 ± 9</td>
<td>74 ± 10</td>
<td>11 ± 2</td>
<td>2.7 ± 2</td>
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Table 1: Binding of BoNT/A complex (TANC) (100nM) to 2 mole% GT1b–98 mole%
DMPC coated flow cell s. Values are given as RU s (Mean ± standard deviation, n=4)

Using 10 mM Tris – 50 mM NaCl, pH 7.4, and flow cells coated with 5 mole
percent ganglioside–95 mole percent DMPC, the BoNT/A complexes bound well to the
polysialylated ganglioside GT1b and to a somewhat lesser extent to GQ1b and GD1a
(Fig.2). Binding to the monosialoganglioside GM1 or to asialo-GM1 was not observed.
Kinetic analyses of the data obtained for the binding of different concentrations of the
BoNT/A complexes to either GT1b or GQ1b indicated the data did not fit well to a
traditional 1:1 binding model. Therefore, based on our previous observation that purified
BoNT/A neurotoxin underwent a conformational change upon binding to GT1b (16), the

Fig. 2: Binding of BoNT/A complex (TANC) to different gangliosides. Sensorgrams show binding of 100 mM TANC in 10 mM Tris containing 50 mM NaCl, pH 7.4 to a flow cell coated with 5 mole percent of the ganglioside indicated and 95 mole percent DMPC.
Fig. 3: Graph showing fit of sensorgrams obtained for the binding of different concentrations of BoNT/A complex Batch 8 to a flow cell coated with 5 mole percent GT1b–95 mole percent DMPC to the conformational change model. Dashed lines show the fits, solid lines, actual binding data. Concentrations of BoNT/A complex Batch 8 shown are 3.125, 12.5, 18.75, 25, 37.5, 50, and 75 nM going from bottom to top.
Table 2: Results of the Kinetic analysis of BoNT/A complexes binding to 5%GT1b and 5%GQ1b. Data were fit by the two-state conformational change model.

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<th>Sample</th>
<th>Lipid</th>
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<th>$k_{off}$ (1/s)</th>
<th>$K_{D}$ (M)</th>
<th>$R_{max}$ (RU)</th>
<th>$K_{D}$ (M)</th>
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<td>BAS</td>
<td>GT1b</td>
<td>$1.67 \times 10^6$</td>
<td>$5.12 \times 10^{-2}$</td>
<td>$3.24 \times 10^{-3}$</td>
<td>$5.62 \times 10^{-3}$</td>
<td>$1.95 \times 10^{-4}$</td>
<td>$32.8$</td>
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<tr>
<td>BAS</td>
<td>GQ1b</td>
<td>$1.94 \times 10^6$</td>
<td>$5.17 \times 10^{-2}$</td>
<td>$3.64 \times 10^{-3}$</td>
<td>$5.58 \times 10^{-3}$</td>
<td>$1.61 \times 10^{-4}$</td>
<td>$38.2$</td>
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<td>TANC</td>
<td>GT1b</td>
<td>$1.17 \times 10^6$</td>
<td>$4.09 \times 10^{-2}$</td>
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<td>TANC</td>
<td>GQ1b</td>
<td>$7.87 \times 10^5$</td>
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<td>$77.3$</td>
<td>$2.85 \times 10^{-3}$</td>
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Based on these results subsequent kinetic analyses of the binding of BoNT/A complexes to GT1b were performed using 10mM Tris containing 50 mM NaCl, pH 7.4, and liposomes prepared using 5 mole percent GT1b–95 mole percent DMPC to coat the flow cell. Results of kinetic analyses of the binding of nine different preparations of BoNT/A complex provided over a two year time period indicate that there was little difference in the kinetic constants obtained for the different batches (Table 3). Upon reanalysis of Batch 2 five months after the analysis for which data is shown, a $K_D$ of $2.6 \times 10^{-8}$ was obtained. A second sample of Batch 2, analyzed almost a year later, gave a $K_D$ of $1.8 \times 10^{-8}$. These data indicate that the analyses are reproducible even when different flow cells and lipid preparations are used.
<table>
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<th>Sample</th>
<th>$K_1$(M)</th>
<th>$K_D$(M)</th>
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<td>5.67x10^7</td>
<td>1.8x10^-8</td>
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<tr>
<td>Batch 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.84x10^7</td>
<td>2.6x10^-8</td>
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<tr>
<td>Batch 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.42x10^7</td>
<td>1.8x10^-8</td>
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<td>1.7x10^-9</td>
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<tr>
<td>Batch 9</td>
<td>4.83x10^7</td>
<td>2.1x10^-9</td>
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<td>Purified Type A neurotoxin</td>
<td>4.24x10^7</td>
<td>2.4x10^-9</td>
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Table 3: Kinetic constants obtained for nine different preparations of BoNT/A complex and one preparation of BoNT/A.

<sup>a</sup> Assayed 5 months after the first data point listed for Batch 2
<sup>b</sup> Assayed 11 months after the first data point listed for Batch 2
Discussion

The interaction of BoNT/A with gangliosides has been well documented. In contrast to the numerous studies of BoNT/A binding to gangliosides, there has been limited study of the interaction of BoNT/A complex with potential cell surface receptors. Since the BoNT/A toxin complex is extensively used clinically, this knowledge is critical to understanding its mechanism of action. In one of the few studies performed with a BoNT/A toxin complex, Inoue et al (17) showed by TLC overlay that the complex, through the haemagglutinin components, bound to the oligosaccharides on ASF. To justify the use of SPR as a valid technique, we used the method to study the binding of BoNT/A complex (TANC) to ASF. TANC was found to bind to ASF at physiological pH and ionic strength with a $K_D$ of 78 nM. Under the same conditions sample 1 of BoNT/A complex (BAS) was also found to adhere to ASF while purified BoNT/A neurotoxin did not.

Having previously reported that SPR was a valid technique for characterizing the binding of purified BoNT/A to immobilized GSLs (16), the same approach was used to study binding of BoNT/A complexes to GSLs. While the complex may dissociate at higher temperatures the observation that binding of both complexes to immobilized GT1b decreased at high ionic strengths is in agreement with previous observations, as is the finding that neither complex adhered to GM1 or asialo-GM1. While the observation that the BoNT/A complexes adhered to polysialylated gangliosides differs from results reported by Inoue et al (17), it should be noted that the TLC immunostaining experiments were performed at pH 6.0 while the SPR studies reported here were performed at pH 7.4.
In addition, the GSL binding patterns observed in the present work agree well with those observed for purified BoNT/A neurotoxin (18).

The finding that data obtained for the binding of different concentrations of the BoNT/A complexes to GT1b did not fit well to the 1:1 binding model, but did fit well to the two-state conformational change model, also agrees with observations made with BoNT/A (16). The two-state conformational change model can be described by the equation shown.

\[
\begin{align*}
\text{A} + \text{B} & \xrightleftharpoons[k_{d1}]{k_{a1}} \text{AB} \xrightleftharpoons[k_{d2}]{k_{a2}} \text{AB}^* \\
\end{align*}
\]

The similarities between results obtained in studies of the binding of BoNT/A and BoNT/A complex to GT1b support the hypothesis that adherence of the complex is mediated by the neurotoxin and not by other protein components.

\(K_D\) values obtained for samples of BoNT/A complex indicate that there is less than a 10-fold difference in values obtained for the nine different preparations studied and for seven of them \(K_D\)s ranged from 1.5 - 2.8x10\(^8\), indicating that affinities of the different preparations for GT1b were comparable. Values obtained for repeated analyses performed over the course of 11 months, showed less than a 1.5 difference in the \(K_D\) values obtained for binding to GT1b by BAS Batch 2. The consistency in results indicates that similar results were obtained even though different HPA chips and different lipid preparations were used in each of the three separate assays. The reproducibility indicates that \(K_D\)s obtained by SPR for the binding of different preparations of BoNT/A complex to GT1b can be compared to each other as long as assay conditions are maintained and consistent.
The similarity in results obtained here using BoNT/A complexes from two different sources (TANC and BAS) are important. The two BoNT/A complexes were not isolated from the same Hall strain nor by the same method. Therefore the overall composition of the BoNT/A complexes, while generally the same with analogous protein components, may differ, perhaps significantly. The similarity in results indicate that the component responsible for ganglioside binding is maintained across different Type A neurotoxin-producing strains.

Reproducibility of the results has led to use of SPR as one measure of the reproducibility of BoNT/A complex preparations. Based on experience gained when analyzing data, care must be taken to use concentrations of BoNT/A complex that result in less than 500 RUs of total binding. No analyses should be performed using curves obtained for fewer than five different concentrations of complex and data fits should have $\chi^2$ values that are 5 percent or less of the total calculated RUs. The one problem encountered using this method was the occurrence of air spikes. They are problematic because they can alter the surface of a lipid coated flow cell resulting in unusable data. When that occurs lipids should be removed using 40 mM octylglucoside, a new lipid surface laid as described above, and the experiment repeated. The benefit of this approach is that the assays are performed using unmodified complex and the data is for real time binding to and dissociation of the BoNT/A complex from GT1b.

Acknowledgements

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References


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EDUCATION

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PUBLICATIONS

   “Use of surface plasmon resonance to characterize binding of botulinum neurotoxin A haemagglutinin complex to gangliosides.” The Botulinum Journal, in press.


PRESENTATIONS
