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AB Toxins: Recovery from Intoxication and Relative Potencies

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AB TOXINS: RECOVERY FROM INTOXICATION AND RELATIVE POTENCIES

by

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Major Professor: Ken Teter
ABSTRACT

AB-type protein toxins have a catalytic A subunit attached to a cell-binding B subunit. Ricin, Shiga toxin (Stx), exotoxin A, and diphtheria toxin are AB toxins that act within the host cytosol and kill the host cell through pathways involving the inhibition of protein synthesis. Our overall goal is to help elucidate the cellular basis of intoxication for therapeutic development. According to the current model of intoxication, the effect of AB toxins is irreversible. To test this model, we developed a system that uses flow cytometry and a fluorescent reporter to examine the cellular potency of toxins that inhibit protein synthesis. Our data show that cells can recover from intoxication: cells with a partial loss of protein synthesis will, upon removal of the toxin, increase the level of protein production and survive the toxin exposure. This work challenges the prevailing model of intoxication by suggesting ongoing toxin delivery to the cytosol is required to maintain the inhibition of protein synthesis and ultimately cause apoptosis. We also used our system to examine the basis for the greater cellular potency of Stx1 in comparison to Stx2. We found that cells intoxicated with Stx1a behave differently than those intoxicated with Stx2: cells exposed to Stx1a exhibited a population-wide loss of protein synthesis, while cells exposed to Stx2a or Stx2c exhibited a dose-dependent bimodal response in which one subpopulation of cells was unaffected (i.e., no loss of protein synthesis). Additional experiments indicated the identity of the Stx B subunit is a major factor in determining the uniform vs. bimodal response to Stx subtypes. This work provides evidence explaining, in part, the differential toxicity between Stx1 and Stx2. Overall, our collective observations provide experimental support for the development of inhibitors and post-exposure therapeutics that restrict, but not necessarily block, toxin delivery to the host cell.
EXTENDED ABSTRACT

Shiga toxin (Stx), ricin, exotoxin A (EtxA), and diphtheria toxin (Dtx) are AB-type protein toxins that act within the host cytosol and kill the host cell through pathways involving the inhibition of protein synthesis. According to the standard model, intoxication is seen as an irreversible process because it is believed that a single molecule of cytosolic toxin is sufficient to kill the host cell. Despite the fact that there is lack of convincing evidence to support the “one molecule” model, intoxication has nonetheless been widely viewed as an irreversible process. Thus, our overall goal is to help elucidate the cellular basis of intoxication for therapeutic development. Using flow cytometry and a fluorescent reporter system to monitor protein synthesis, this study found that a single molecule of cytosolic toxin is not sufficient for complete inhibition of protein synthesis or cell death. Furthermore, contradictory to the standard model, our results show that cells can recover from intoxication: cells with a partial loss of protein synthesis will, upon removal of the toxin, increase the level of protein production and survive the toxin challenge. Thus, ongoing toxin delivery to the cytosol appears to be required for the death of cells exposed to sub-optimal toxin concentrations.

We also used our cytofluorometry assay to study the potency of Stxs. Enterohemorrhagic Shiga toxin-producing *Escherichia coli* (STEC) strains like *E. coli* O157:H7 are a major public health concern worldwide. STEC strains produce Stx as their main virulence factor. There are two major antigenic forms of Stx, Stx1 and Stx2. Both forms have minor variants, which differ in potency. It has been shown that Stx2a is more potent in animal studies, while Stx1a is more toxic to Vero cells. The factors contributing to the differential potency of Stx subtypes are poorly understood.
Our results suggest that the differential potencies between Stx subtypes are partly due to the amount of toxin that reaches the cytosol of the host cell. When a cell population was intoxicated with Stx1a, a uniform loss in protein synthesis was observed. A cell population intoxicated with Stx2a and Stx2c, on the other hand, exhibited a bimodal cell distribution in which some of cells were intoxicated and some were not. Additional cytofluorometry experiments conducted to understand the bimodal response in cells exposed to Stx2 subtypes found that cells bind more Stx1a than Stx2a. Furthermore, experiments conducted with hybrid toxins in which the subunits of the Stx subtypes were swapped suggest that the identity of the B subunit is partly responsible for the different cellular responses. Cells that were challenged with a hybrid toxin in which the A1 subunit of Stx1a was combined with the B subunit of Stx2a exhibited a bimodal response to intoxication similar to the response observed with Stx2a holotoxin, while cells challenged with a hybrid toxin in which the A1 subunit of Stx2a was combined with the B subunit of Stx1a exhibited a population-wide loss of protein synthesis similar to that observed with Stx1a holotoxin.

In summary, this work presents evidence that challenges the “one molecule” model and the long-standing paradigm suggesting that intoxication is an irreversible process. Moreover, this work provides supporting evidence explaining, in part, the differential potency between Stx1 and Stx2 subtypes. These collective observations suggest there is a need to accumulate a substantial pool of cytosolic toxin to maintain the inhibition of protein synthesis and induce cell death. Thus, these observations provide experimental support for the development of inhibitors and post-exposure therapeutics that restrict, but do not necessarily completely block, toxin delivery to the host cytosol.
I dedicate this dissertation to a dear friend and a mentor, Odely Pierre Charlot, who passed away on July 24, 2013 at the age of fifty. One of my most joyful memories with Odely is when we both first played Duck Hunt on Nintendo NES. Odely is probably the reason why I am a 34-year-old man today who enjoys playing video games with kids. Odely was as great as a mentor as he was a friend: he taught me about duty and responsibility, and most importantly, he instilled upon me most of the cultural values around which my personal character revolves. Odely was the mentor who saw my potentials when I was young and invested the time to help me nurture them. I truly wish Odely was still alive today to celebrate with me the achievement of completing this Doctor of Philosophy degree.
ACKNOWLEDGMENTS

I would like to pay my regards to the collaborative effort that led to the achievement of this dissertation. First, I would like to show my kind gratitude to the Florida Education Fund (FEF) for awarding me the McKnight Doctoral Fellowship, which included a generous financial support that defrayed the cost of my doctoral studies as well as a wide range of professional career development opportunities. Moreover, I would like to extend a warm thank to Dr. Teter for the outstanding mentorship he has provided me during the last eight years in both my academic and non-academic endeavors. To the rest of my committee members, Drs. Jewett, Naser, and Zervos, I thank you all for the additional support and guidance you have provided me throughout my doctoral training. I would also like to pay special thankfulness, warmth, and appreciation, to the following list of individuals who made my research successful and/or assisted me in reaching this milestone academic achievement: my family and friends, for providing me with moral and emotional support throughout this challenging academic endeavor, all current and former members of the Teter lab, Dr. Beatriz Quiñones from the USDA, Dr. Lucia Cilenti from the Zervos’ lab, and the UCF Biomedical Sciences program. My grateful thanks are also extended to the following list of individuals from FEF for the additional support they provided me during my doctoral training: Ms. Phyllis Reddick, Mr. Charles Jackson, Ms. Lyra Logan, and Dr. Lawrence Morehouse. Finally, I would like to extend my gratitude and appreciation to UCF for being my home and my community for nearly a decade. I love UCF not only for what it is, but also for what it stands for, its philosophy. Thus, I will forever cherish my experience at UCF and will strive to exemplify every single one of its core values wherever my career and journey may take me.
Part of this dissertation is based on the following publications:


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LIST OF ACRONYMS

- 7-AAD: 7-Aminoactinomycin D
- APC: Allophycocyanin
- AV: Annexin V
- BSA: Bovine serum albumin
- DMSO: Dimethylsulfoxide
- DAPI: 4’, 6-diamidino-2-phenylindole
- Dtx: Diphtheria toxin
- EE: Early endosomes
- eEF2: Elongation factor 2
- EHEC: Enterohemorrhagic *Escherichia coli*
- ER: Endoplasmic reticulum
- ERAD: ER-associated degradation
- EtxA: Exotoxin A
- Gb₃: Globotriaosyl ceramide
- Gb₄: Globotetraosyl ceramide
- H₂O₂: Hydrogen peroxide
- HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- HB-EGF: Heparin-binding epidermal growth factor
- IPTG: Isopropyl-β-D-1-thiogalactopyranoside
- LRP: Lipoprotein receptor-related protein
- MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium
- MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
- NAD: Nicotinamide dinucleotide
- PE: Phycoerythrin
- STEC: Shiga toxin-producing *Escherichia coli*
- Stx: Shiga toxin
- StxA: Shiga toxin A subunit
- StxA1: Shiga toxin A1 subunit
- StxA2: Shiga toxin A2 subunit
- StxB: Shiga toxin B subunit
- TGN: *Trans*-Golgi network
- UCF: University of Central Florida
- USDA: United States Department of Agriculture
AB toxins are virulence factors produced by a variety of bacterial pathogens and some plants (1). They are protein toxins that are released into the extracellular environment but attack targets within the host cytoplasm (2, 3). These toxins initially enter the cell through receptor-mediated endocytosis and reach the cytosol between 30 minutes and 2 hours after internalization from the plasma membrane (4-7). AB toxins share the same basic structural characteristic: a catalytically active A subunit and a cell-binding B subunit (Fig. 1). This study will examine four AB toxins: Shiga toxin (Stx), ricin, exotoxin A (EtxA), and diphtheria toxin (Dtx). These toxins affect the host cells by inhibiting protein synthesis. The A and B moieties for each toxin can encompass different regions of a single polypeptide chain or may represent distinct proteins in various stoichiometries (e.g., AB, AB₂, AB₅, A₃B₇). Ricin, EtxA, and Dtx are single proteins comprised of one A subunit and one B subunit that represent an AB stoichiometry. The A subunit of EtxA and Dtx contains a single region, while their B subunits are comprised of two distinct regions. Stx, on the other hand, represents an AB₅ stoichiometry made of two proteins, StxA and StxB. The StxA subunit is comprised of two distinct domains: a catalytically active StxA₁ and a StxA₂ linker. The StxB subunit is made of five identical B domains assembled as a homopentamer. Two of the AB toxins examined throughout this study, Stx and ricin, inhibit protein synthesis via inactivation of the 28S ribosomal RNA (rRNA), while the other two, EtxA and Dtx, inhibit protein synthesis through inactivation of elongation factor 2 (eEF2) (8-12).

AB-type protein toxins all have a cellular receptor on the host plasma membrane and a cytosolic target. After binding to a specific receptor, these toxins are internalized and delivered to the
endosomes. Some enter the cytosol directly from the endosomes, while others move from the endosomes to the endoplasmic reticulum (ER) before entering the cytosol (1, 13). Only a small amount of internalized toxin reaches the cytosol of the host cell (14-17). A large proportion of internalized toxin is recycled to the surface of the host cell and/or degraded by the lysosomes. Unfortunately for the host cell, the small percentage of toxin that makes it to the cytosol is very effective. Our overall goal is to understand the cellular mechanism of intoxication by AB toxins. A better understanding on how these toxins work at the cellular level is essential for the development of effective therapeutics against their associated diseases.

As summarized in Table 1, the combination of receptor, entry mechanism, translocation site, cytosolic target, and action on the target are different for each AB toxin. Depending on the type of receptor used by an AB toxin, internalization takes place by exploiting the host cell endocytic pathway in a clathrin-dependent and/or clathrin-independent fashion. Some AB toxins move into the cytosol from acidified endosomes, while others follow an inefficient transport pathway to the ER before entering the cytosol. Dtx inhibits protein synthesis and belongs to the subset of toxins that cross the endosomal membrane to reach the cytosol (18); ER-translocating toxins that inhibit protein synthesis include Stx, ricin, and EtxA (13, 19, 20) (Fig. 2). A brief overview on the mechanism of action of each of the toxin will be provided next in order to establish what is currently known about their potency.
Figure 1. Structural organization of Stx1a, Stx2a, ricin, EtxA, Dtx.

(A) Ribbon diagram of Stx1a (PDB 1DM0, (21)) and (B) Stx2a (PDB 1R4P, (22)). The A1 subunit is depicted in red; the A2 linker is depicted in yellow; and the B homopentamer is depicted in blue. StxB binds to Gb3/Gb4 on the surface of the host cell, while StxA1 is an N-glycosydase that inhibits protein synthesis by removing a specific adenine residue from the 28S rRNA. (C) Ribbon diagram of ricin (PDB 2AAI, (23)). The catalytic A subunit is depicted in red and the cell-binding B subunit is depicted in blue. The B subunit of ricin binds to glycoproteins and glycolipids with terminal galactose residues, while the A subunit of ricin inhibits protein synthesis through its N-glycosidase activity that specifically removes an adenine residue from the 28S rRNA. (D) Ribbon diagram of EtxA (PDB 1IKQ, (24)). The catalytically active A subunit is depicted in red, while the B subunit containing the translocation domain and the receptor-binding domain are depicted in light blue and blue respectively. EtxA binds to α-macroglobulin receptor/low density lipoprotein receptor-related protein on the host plasma membrane, while the catalytic domain has ADP-ribosyltransferase activity and inhibits protein synthesis by modifying eEF2. (E) Ribbon diagram of Dtx (PDB 1DDT, (25)). The B fragment is located within the C-terminal domain and it is comprised of two domains, a translocation domain (light blue) and a receptor-binding domain (blue). The catalytic domain is located within the N-terminal domain. Dtx binds to epidermal growth factor on the host plasma membrane, while the C domain has ADP-ribosyltransferase activity and inhibits protein synthesis by modifying eEF2. The above ribbon diagrams were edited using Python Molecule Viewer version 1.5.6.
The toxin binds to a receptor on the surface of the target cell. Following clathrin-dependent and/or clathrin-independent endocytosis, ER-translocating toxins Stx, ricin, and EtxA are transported to the Golgi complex and ER, while Dtx is an endosome-translocating toxin that releases its catalytic subunit to the cytosol directly from the endosomes. The catalytic A subunit of Stx, ricin, and EtxA is first separated from the rest of the toxin before moving into the cytosol. The A subunits of ricin and Stx are N-glycosidases that remove an adenine moiety from the 28S rRNA, thereby halting protein synthesis. The A subunits of EtxA and Dtx, on the other hand, act as ADP-ribosyltransferases and modify eEF2. These events inhibit protein synthesis and eventually result in apoptotic cell death. The above figure was kindly provided by Ms. Alisha Kellner.
Ricin

Ricin is a natural by-product contained in the castor bean of the *Ricinus communis* plant. Ricin is a toxin known for its malevolent use in bioterrorism and it is considered a biological weapon (26-28). Symptoms from ricin poisoning depend on the exposure route. Inhalation and ingestion are the two main routes of exposure to ricin. Inhaled ricin leads to cough, respiratory problems, fever, nausea, and tightness in the chest. Ingested ricin, on the other hand, is a less toxic route that leads to severe dehydration resulting from bloody vomiting and diarrhea. The NIAID classifies ricin as a category B biothreat due to its ease of dissemination and its morbidity and mortality rates. Death from ricin depends on the route of exposure and the intoxicated dose. There are currently no known antidotes against ricin. Symptoms are routinely treated using supportive care like oral rehydration therapy.

Ricin is a type II ribosome inactivating protein (RIP II). Type II RIPs are synthesized by certain plants like pokeweed (29, 30). RIPs possess N-glycosidase activity that the toxin uses to cleave the N-glycosidic bond of adenine (depurination) in ribosomal RNA sequences. Type I RIPs are composed of a single catalytic subunit, whereas type II RIPs have an AB organization composed of a catalytic domain and a lectin domain that binds to cell surfaces. RIPs are known to be used by plants as an antiviral mechanism (29-31), and they have abortifacient activities that are reported to be used in Chinese medicine (32).

Ricin is a single-chain AB-type protein toxin made of a catalytic A subunit linked to a lectin B subunit via a disulfide bond. Using its B subunit, ricin binds to galactose residue located on glycoproteins and glycolipids on the cell surfaces (33, 34). Reduction of the disulfide bond of ricin
takes place in ER following transport from the early endosome (EE) to the Golgi via clathrin-dependent and clathrin-independent endocytosis (35, 36). Following the reduction of its disulfide bond, the catalytic A subunit of ricin exploits the ER-associated degradation (ERAD) quality control process to enter the cytosol through the Sec61p translocon (16, 37, 38). In the cytoplasm, the A subunit of ricin acts on its target, the 28S rRNA, by removing an adenine residue from the “sarcin/ricin loop” (12, 39). Depurination of the 28S rRNA takes place in a hydrolytic manner by the cleavage of the adenine residue at position 4324 (A_{4324}) (40). Ricin-induced removal of A_{4324} interferes with the proper interaction between the ribosome and eEF2, thereby leading to inhibition of protein synthesis and, ultimately, apoptotic cell death (8, 9, 12).

**Diphtheria toxin (Dtx)**

Dtx is secreted by the Gram-positive bacterium *Corynebacterium diphtheriae* (41-43). Dtx is the main cause of the respiratory infection diphtheria that is transmitted from person to person through coughing and sneezing. While diphtheria is mainly a respiratory complication, absorption of Dtx into the bloodstream also leads to damage of nearby organs (44). Symptoms of diphtheria includes swollen gland in the neck, fever, and sore throat. Serious complications that result from diphtheria include paralysis and pneumonia. If left untreated, diphtheria infection often leads to death. Currently, the treatments for diphtheria are antitoxin to target Dtx and antibiotics to kill the bacterium.

Dtx is a single protein that belongs to the AB-type protein toxin family. The B fragment of Dtx is contained within the C-terminal region and it is comprised of two domains, a translocation (T)
domain and a receptor-binding (R) domain. The catalytic (C) domain of Dtx, on the other hand, is located within the N-terminal region of the toxin (45). Following binding of Dtx to its cell surface receptor heparin-binding epidermal growth factor (HB-EGF) using its R domain, the toxin is internalized via clathrin-dependent endocytosis (42, 46-48). Following cleavage of Dtx between the C and T domains by the host protease furin, which cycles between the cell surface, endosomes, and the TGN, the two domains remain linked via a disulfide bond (48, 49). Once Dtx reaches the early endosome, the acidic environment contained within this compartment causes the T domain to undergo a conformational change that results in the insertion of its exposed hydrophobic regions into the membrane of the endosome. This forms a pore, allowing the C domain of the toxin to be translocated to the cytosol (18, 50). Within the cytosol of the host cell, the disulfide bond linking the C and T domains of Dtx gets reduced, thereby freeing the catalytic C domain of the toxin to modify eEF2 by transferring an ADP-ribose group from nicotinamide dinucleotide (NAD) (51-53). Deactivation of eEF2 by ADP-ribosylation inhibits protein synthesis, thereby leading to apoptotic cell death (54, 55).

**Exotoxin A (EtxA)**

EtxA is a single protein secreted by the Gram-negative bacterium *Pseudomonas aeruginosa*. It is an environmental bacterium commonly linked to hospital-acquired infections such as pneumonia and sepsis syndrome. Immunocompromised patients are often vulnerable to *Pseudomonas* infections. *Pseudomonas* infections can be treated using antibiotics. However, multi-drug resistant *Pseudomonas* remains an ongoing medical concern.
EtxA belongs to the AB-type protein toxin family comprised of three main functional domains: an N-terminal receptor-binding (R) domain, a translocation (T) domain, and a catalytic (C) domain (56, 57). EtxA binds to the low-density lipoprotein receptor-related protein (LRP) and enters the host cell through clathrin-coated endocytic vesicles (58, 59). At an early stage during the EtxA cellular intoxication pathway, plasma membrane carboxypeptidases engage the REDLK peptide sequence at the C-terminus of the toxin to remove the lysine residue (60, 61). A furin protease in the endosomes and/or the TGN cleaves the C and T terminal domains of EtxA in a manner similar to the cleavage of Dtx (49, 62-64). Following cleavage of the C and T terminal domains, the catalytic C domain, still attached to the T domain, migrates to the TGN through Rab9-dependent transport (65, 66). The disulfide-linked C and T domains are subsequently reduced presumably by protein disulfide isomerase (PDI) or PDI-like enzymes (66). Once located in the Golgi, the C-terminus of the toxin binds to a KDEL receptor using the C-terminal REDL sequence. This facilitates the transport of the catalytic C domain to the ER (67). Translocation of the C domain to the cytosol of the host cell is facilitated by the attached T domain (68, 69). The catalytic action of the cytosolic C domain inactivates its eEF2 target by ADP-ribosylation (70). The resulting inactivation of eEF2 leads to protein synthesis inhibition and, ultimately, apoptotic cell death.

**Shiga toxin (Stx)**

Stx is an AB-type protein toxin secreted by the Gram-negative bacterium *Shigella dysenteriae*, the first known species of Shigella. The toxin is named after Dr. Kiyoshi Shiga, who first isolated *Shigella dysenteriae* in 1896 (71-73). Other types of bacteria such as enterohemorrhagic *Escherichia coli* (EHEC) also secrete Stx as a virulence factor. Stxs secreted by STEC strains are often referred to as Shiga-like toxins (74). There are two main forms of Stx: Stx1 and Stx2. Stx1
is more potent in cell culture than Stx2. The factors contributing to the difference in potency are not fully understood. STEC strains are a major public health concern worldwide, and STEC serotype O157:H7 is frequently associated with human gastroenteritis outbreaks in industrialized countries (75). Symptoms of STEC infections include bloody diarrhea, vomiting, and fever. STEC infections can range from mild to life-threatening conditions such as hemolytic-uremic syndrome (HUS), a type of kidney failure (75, 76). The best treatment for STEC infections is supportive therapy, like hydration, against the symptoms. The use of antibiotics as treatment against STEC infections is highly discouraged as it may increase the risk of developing HUS by facilitating the release of Stx (77, 78).

Stx has a catalytic A subunit (StxA) and a binding B subunit (StxB), which places it in the family of AB toxins (79). StxA is proteolytically nicked to generate a disulfide-linked heterodimer composed of an enzymatic A1 fragment and an A2 fragment that extends into the central pore of the ring-like StxB homopentamer. StxB binding to globotriaosyl ceramide (Gb3) and/or globotetraosyl ceramide (Gb4) on the surface of a target cell leads to endocytosis through clathrin-coated pits (80-82). The host protease furin cleaves the holotoxin-associated StxA subunit in the endosomes and/or TGN to generate the StxA1/StxA2 heterodimer (83). The toxin then moves by retrograde transport to the ER where reduction of the StxA1/StxA2 disulfide bond allows StxA1 to dissociate from the rest of the toxin before entering the cytosol (84). In the cytosol, StxA1 irreversibly inactivates the ribosome through the removal of an adenine residue from the 28S rRNA of the 60S ribosomal subunit. Depurination of the ribosome interferes with protein translation and ultimately results in apoptotic cell death (11, 85).
Table 1. Toxin comparison chart.

Receptor, entry mechanism, translocation site, cytosolic target, and action on the target are summarized for Stx, ricin, EtxA, and Dtx.

<table>
<thead>
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<th>Toxin</th>
<th>Receptor</th>
<th>Endocytosis</th>
<th>Translocation Site</th>
<th>Cytosolic Target</th>
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<td>Clathrin-dependent</td>
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<tr>
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<td>Clathrin-dependent</td>
<td>ER</td>
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</tr>
</tbody>
</table>
**Probability and proportionality models of intoxication**

Table 1 summarizes several differences in the mechanism of action between AB toxins. The factors that contribute to these differences dictate the overall potency of the toxin. Many aspects of the molecular mechanism of intoxication and overall potency of these toxins are still poorly characterized. It is widely believed that the effect of AB toxins is irreversible. According to the standard model, as few as one molecule of cytosolic toxin is sufficient to completely inhibit protein synthesis and cause cell death (6, 86-88). This “one molecule” model is a probability model implying that, at the half maximal dose of a toxin (ED\(_{50}\)), half of the cells within a population receive at least one molecule of toxin and the other half receive no toxin. An alternate model of intoxication is the proportionality model, which implies that all cells within a population receive varying amounts of toxin. Per the proportionality model of intoxication, a toxin ED\(_{50}\) would indicate that all cells receive toxin, but most receive a quantity of toxin that results in 50% inhibition of protein synthesis. Most current systems that monitor toxin activity average the results from a population of cells and therefore cannot differentiate intoxicated cells from unintoxicated cells. In order to have a better understanding about the cellular potency of toxin, their effect must be monitored in single cell systems that can distinguish between the probability and proportionality models of intoxication.
CHAPTER 2: SPECIFIC AIMS AND RATIONALE

The purpose of this study is to examine two proposed models by which AB-type protein toxins affect the host cell. The probability model will be evaluated to determine the validity of the “one molecule” model for a subset of AB toxins that inhibit protein synthesis. In this model, a toxin $ED_{50}$ implies that only half of the cells are intoxicated. The proportionality model, on the other hand, will be examined to determine whether all cells receive toxin. In this model, a toxin $ED_{50}$ implies that all cells are intoxicated but the extent of intoxication is directly related to how much toxin is in the cytosol. It is important to differentiate between the two models of intoxication to determine whether intoxication is an all or nothing event. The probability model suggests that cells cannot recover, whereas the proportionality model suggest that cells can potentially recover. Therefore, this determination is important for the development of post-exposure therapeutics.

Most of the quantitative systems that are currently available to monitor the toxin-induced inhibition of protein synthesis average results from population of cells (89, 90). One of the main limitations that these systems present is that they cannot differentiate intoxicated cells from unintoxicated cells. Thus, these two models cannot be examined and differentiated by analyzing data obtained from currently available systems.

To differentiate between the probability and proportionality models of intoxication, a novel system that can monitor toxin activity in individual cells was developed. This new system is an alternative approach in which intoxicated cells can be differentiated from unintoxicated cells. The specific aims of this study were to (i) develop a novel system to investigate the mechanism of action of AB toxins in individual cells; (ii) examine the potential cellular recovery from exposure to a subset of
AB toxins using the new system; and (iii) examine the cellular basis for the differential potency between Stx subtypes. The alternative approach used in this study to examine the activity of toxins that inhibit protein synthesis will provide new insights about the cellular potencies of these toxins that would otherwise not be possible to detect using current systems that average results from population of cells.

**Aim I: Develop a new system to examine the activity of toxins that inhibit protein synthesis**

Several methods can detect the toxin-induced inhibition of protein synthesis or resulting cell death. A common procedure measures the viability of intoxicated cells by dye exclusion, MTS/MTT assay, or similar protocols (91-96). However, most of these methods require several days of toxin exposure and often involve additional processing steps for data collection. A direct method to quantify the toxin-induced inhibition of protein synthesis measures the incorporation of radiolabeled amino acids into newly synthesized proteins (97, 98). This requires the handling of radioisotopes, which is laborious, potentially hazardous, and can only accommodate a limited number of samples. Quantitative luciferase-based assays have been described that are similar to the system reported here, but these systems require several preparatory and/or processing steps to enact the detection method (99, 100). A recently described assay that monitors the production and secretion of acetylcholinesterase likewise requires additional processing steps for data acquisition (101). The main limitation for these existing technologies is that they average results obtained from population of cells. As previously mentioned, one cannot differentiate between the probability and the proportionality models of intoxication by averaging results obtained from population of cells. Therefore, the novel cytofluorometry-based system described in the present
study is a new tool that can be used to elucidate additional aspects of AB toxins potency in cell culture.

Assays that average the results from a population of cells make it difficult to distinguish between the “probability” and “proportionality” models of intoxication. As an alternative approach to monitor the activity of toxins that inhibit protein synthesis, we will use flow cytometry in conjunction with a Vero cell line that expresses a destabilized variant of the enhanced green fluorescent protein (EGFP) that is degraded by the proteasome with a 2-hour half-life, Vero-d2EGFP cells (102). Intoxicated cells degrade d2EGFP and do not replenish the lost protein due to the toxin-induced block of protein synthesis. The fluorescence level of EGFP is thus inversely proportional to toxin activity (Fig. 3). Using this cell-based assay, a direct, proportional link between the specific loss of d2EGFP intensity and the overall loss of protein synthesis in a population of toxin-treated cells has been recorded with a plate reader (103). Subsequent studies further documented the usefulness of the Vero-d2EGFP cells for measuring the activity of AB toxins (90, 104-107). Other investigators have used similar cell-based toxicity assays with destabilized reporters (99, 108, 109) and have documented a direct correlation between the toxin-induced inhibition of total protein synthesis and the toxin-induced loss of reporter signal (100). Here, our Vero-d2EGFP system was adopted to monitor the activity of toxins against individual cells within the population of toxin-treated cells. This new protocol will help further elucidate the cellular potency of AB toxins that inhibit protein synthesis.
Figure 3. Vero-d2EGFP cell-based assay.

(A) The Vero-d2EGFP cells stably expresses a GFP reporter that has a 2-hour half-life. (B) The degraded EGFP is not replenished in the presence of toxins that inhibit protein synthesis, thereby resulting in decreased fluorescence intensity. The above figure was kindly provided by Ms. Alisha Kellner.
Aim II: Determine the intoxication model for a subset of AB toxins and the potential for cellular recovery from intoxication

The overall goal of this study is to help better understand the cellular basis of intoxication for a subset of AB toxins that inhibit protein synthesis. According to the standard model of intoxication, about one molecule of AB toxin entering the cytosol of the host cell is sufficient to inhibit protein synthesis and kill a cell (6, 86-88). Based on this model, intoxication has generally been viewed as an irreversible process. The data supporting this model are not convincing. Most of the studies supporting this model use systems that average results from population of cells (6, 86, 88, 110-113). Additionally, these studies use systems that cannot differentiate cells that are intoxicated from cells that are not intoxicated. Furthermore, the results from these studies rely on extrapolations with toxin serial dilutions and/or kinetic analyses of intoxication. With the current assays, dose response curves generated with AB toxins are thought to reflect the probability of single cell intoxication in a population of cells. By this model, which represents the current working paradigm, the ED$_{50}$ of a toxin represents an all-or-nothing event in which half the exposed cells contain no cytosolic toxin and are therefore unaffected, while the other half exhibits the full effects of intoxication. An alternative interpretation for toxin ED$_{50}$ values is based on proportionality rather than probability: at the ED$_{50}$ for protein synthesis inhibition, it is possible that all cells in the exposed population contain an amount of cytosolic toxin that only reduces protein synthesis by 50%. With this proportionality model, limiting but not eliminating the quantity of cytosolic toxin could protect a cell from the lethal outcome of intoxication.

Based on the long-standing model that a single or a few molecules of cytosolic toxin are sufficient to completely inhibit protein synthesis and kill the cell, cellular recovery from exposure to as a
little as one molecule of toxin seems unlikely. The catalytic A chains of these toxins were originally believed to be quite stable in the host cytosol due to their relatively few lysine residues that allow them to escape ubiquitin-mediated proteolysis (2, 112, 114). Direct evidence in support of this assertion, however, is lacking. A single molecule of stable cytosolic toxin could, with time, theoretically block all protein synthesis and kill the cell. This theory, if true, would imply the implausibility of recovery once the host cell is exposed to as little as one molecule of toxin. However, there have been several studies indicating that toxin A chains are not stable in the cytosol: several studies have directly or indirectly documented the proteasome-dependent degradation of cytosolic toxin (115-119). These findings suggest that the severity of AB intoxication depends on the quantity of the A chain present in the cytosol of the host cell. Thus, this study examined the potential cellular recovery from exposure to AB toxins using a different approach.

Using the current systems to study AB toxins, one cannot differentiate cells that receive toxin from cells that do not. This issue has important implications for inhibitor development, as the potentially lethal effect resulting from a single molecule of cytosolic toxin would greatly limit treatment regimens that are not 100% effective or target the cell-associated toxin after first contact. Therefore, a new approach using a different system is needed in order to test the validity of the “one molecule” model of intoxication. Our study used a cytofluorometry-based system to investigate the activity of a subset of AB toxins that inhibit protein synthesis in individual cells. Using this new system, we examined how individual cells in a population respond to AB toxins that inhibit protein synthesis. This work presented evidence with quantifiable data to help understand models of intoxication used by a subset of AB toxins that inhibit protein synthesis.
Aim III: Investigate factors that contribute to the cellular potency of Stx subtypes

Stx is an AB-type protein toxin that inhibits protein synthesis by using its N-glycosidase activity to depurinate the 28S rRNA. There are two major antigenic forms of Stx, Stx1 and Stx2 (120). Moreover, Stx1 and Stx2 are composed of a diverse and heterogeneous group of subtypes (121-124). These subtypes differ in potency and species specificity, with Stx2a and Stx2c frequently linked to human disease (13, 125). Stx2a is associated with more severe infections than the other Stx2 subtypes or Stx1 subtypes (76, 125-129). In contrast, Stx1a is more toxic to cultured Vero cells than Stx2 subtypes (130, 131). The factors contributing to these differences in potency are poorly understood. Therefore, this study also used the newly developed system to examine the differential toxicity between Stx subtypes in individual cells. The B subunits of Stx1a and Stx2a share nearly 60% sequence similarity and use the same Gb₃ globoside as their primary surface receptor (132, 133), but several studies have nonetheless highlighted the role of the B subunit in the differential toxicity of Stx subtypes (131, 134, 135). In vitro, Stx1a has a greater affinity for Gb₃ than Stx2a (130, 135-138). This has led to models suggesting the B subunit is responsible for the differential potency of Stx subtypes. Studies with hybrid toxins have provided further support for this model. The O’Brien lab has shown that a hybrid Stx comprising the A₁ subunit of Stx1a with the A₂ and B subunits of Stx2a (Stx 122) is less potent in Vero cells than a hybrid toxin comprised of the A₁ subunit of Stx2a with the A₂ and B subunits of Stx1a (Stx 211) (139). Studies investigating the difference in potency between Stx subtypes have also highlighted the role of the A₁ subunit in their differential toxicity. Basu et al. showed that the A₁ subunit of Stx2a has a higher affinity for the ribosome and higher catalytic activity than the A₁ of Stx1a. Thus, the A₁ subunit is also a factor contributing to the differential potency of Stx subtypes (140). Further studies on the cellular actions of the A and B subunits are therefore needed in order to better
understand the differential toxicity between Stx subtypes. Therefore, this study used a cytofluorometry-based approach to further understand and elucidate some of the additional factors that contribute to the cellular potency of Stx subtypes.

In this study, we will examine the basis for the different cellular potencies of Stx subtypes. Flow cytometry in conjunction with a GFP reporter system will be used to monitor the Stx-induced inhibition of protein synthesis in individual cells. With this strategy, we will examine the dose-dependent cellular response to the main Stx subtypes. It has been shown that the B-pentamer of Stx1a is more stable than that of Stx2a (141, 142). More insights about the molecular basis for this difference in holotoxin stability is provided through an examination on the structures of the B-pentamers. The B subunit of Stx2a contains a destabilizing amino acid residue, Gln40, that lies in a hydrophobic pocket, while Leu41 is the corresponding hydrophobic residue for Stx1a that potentially contribute to its more stable structure (143). Moreover, each of the 5 B subunit monomers has up to three binding sites. For Stx2a, one of the Gb3-binding sites is partially blocked by the Stx2 A2 linker (22, 144, 145). Thus, previous experimental evidence suggest that the cellular potency of a Stx subtype is linked to the stability, identity, and affinity of the B-pentamer. Therefore, this study aims to explain, in part, the molecular basis for the differential cellular toxicity between Stx1 and Stx2 subtypes and the resulting implications for the cell biology of Stxs. Hence, we will use a new experimental approach to investigate the involvement of the B subunit of Stx1a and Stx2a in the differential cellular potency of Stx subtypes.
Significance and implication

There have been numerous studies highlighting the mechanism of action of AB-type protein toxins. However, a lot remains unknown about the mechanism of action of these toxins. Furthermore, AB toxins and chimeric AB toxins involving ricin, Dtx, and EtxA are being investigated as anti-cancer agents because their mode of action ultimately kill cells (88, 146). Type II RIPs, for example, have been shown to exhibit higher toxicity in cancer cells as compared to normal cells (147). It has been suggested that the high toxicity to cancer cells may be due to the fact that these cells have a higher rate of protein synthesis than normal cells (31). Thus, studies on RIPs like ricin, and other similar toxins, are being explored as immunotoxins and conjugates that can potentially be used in cancer therapy (148-150). As of today, the use of these toxins as cancer therapy has not yet been successful. Thus, a better understanding on how AB-type protein toxins work at the cellular level will be beneficial in the development of effective immunotoxins.

In this study, we will use a new system to assess the validity of the long-standing paradigm regarding the cellular potency of AB toxins. Moreover, the new system will be used to examine and better understand the differential cellular potency between Stx subtypes. The novelty of the present work lies in the fact that no other studies have previously examined and determined the intoxication models employed by AB-type protein toxins. The data that can be gathered using this new system and the insights that can be gained from these data cannot be accomplished using existing systems that average results from population of cells. Consequently, the new findings from this study can potentially be used as a foundation in the development of new therapeutics against diseases caused by AB toxins as well as in the development of immunotoxins to target cancer cells.
CHAPTER 3: MATERIALS AND METHODS

Cell culture materials

- Parental Vero cells (ATCC #CCL-81; Manassas, VA) and a clonal population of Vero-2dEGFP cells with stable, constitutive expression of the d2EGFP reporter (90, 103).

- 100× 20 mm tissue culture dishes (Techno Plastic Products; Switzerland).

- Costar flat bottom cell culture 24 well plates (Techno Plastic Products; Switzerland).

- Black-walled 24 well plates with glass bottom (Cellvis; Mountain View, CA)

- Costar black-walled 96 well polystyrene plates with a clear, flat bottom (Corning Inc.; Kennebunk, ME).

- Costar flat bottom cell culture 96 well plates (Techno Plastic Products; Switzerland).

- Complete Dulbecco’s Modified Eagle Medium (DMEM) for carrying cells: DMEM, high glucose (4.5 g/L d-glucose) with 584 mg/L l-glutamine and 110 mg/L sodium pyruvate (GIBCO; Waltham, MA), supplemented with 10% fetal bovine serum (Atlanta Biologicals; Flowery Branch, GA), 1% antibiotic-antimycotic solution, and 1 mg/mL Geneticin (G-418) (GIBCO; Waltham, MA). Geneticin was used as the selective pressure for isolation of the Vero-d2EGFP cell line. Maintaining drug selection (1 mg/mL) in the passage medium of the Vero-d2EGFP cells ensures continued, uniform expression of the EGFP reporter.

- Intoxication medium: F-12 + GlutaMAX-I nutrient mixture (Ham’s F-12) (GIBCO; Waltham, MA).

- HyClone antibiotic-antimycotic 100× solution: 10,000 U/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin B (GE Healthcare; Logan, UT).
- Trypsin-EDTA 1× solution containing 0.25% trypsin, 0.9 mM EDTA, and phenol red (GIBCO; Waltham, MA).
- HyClone phosphate-buffered saline (PBS) 1× solution: 6.7 mM PO₄ without calcium or magnesium (GE Healthcare; Logan, UT).

**Routine protocol for plating cells**

- Maintain the parental Vero and Vero-d2EGFP cells in a DMEM-based medium and passage when the cells form a >90% confluent monolayer on a 10 cm dish.
- Working in a tissue culture hood, remove the spent medium from the tissue culture dish and wash the cells with 10 mL of sterile 1× PBS.
- Detach cells from the dish by adding 1 mL of trypsin/EDTA for about 10 minutes at room temperature.
- Resuspend detached cells in 9 mL of complete DMEM medium for a total volume of 10 mL.
- Determine the cell concentration with a hemocytometer before seeding plates with appropriate amount of cells.
- Grow cells for ~24 hours at 37°C in a 5% CO₂ humidified incubator.

**Toxins**

- Ricin agglutinin II was purchased from Vector Laboratories (Burlingame, CA).
- Stx1a was obtained from BEI Resources (Manassas, VA) or Dr. Alison O'Brien (Uniformed Services University of the Health Sciences (USUHS) (Bethesda, MD).
- Stx2a, Stx2c and the B subunits from Stx1a and Stx2a were obtained from BEI Resources (Manassas, VA).
- EtxA and Dtx were purchased from List Biologicals (Campbell, CA).

**Stx supernatant**

The plasmids encoding wild-type and hybrid Stxs (pLPSH3, pJES120, pMJS122, and pMJS211) were kindly provided by Dr. Angela Melton-Celsa, Department of Microbiology and Immunology, USUHS (Bethesda, MD) (Fig. 4). The toxin-containing cell extracts from transformed *E. coli* strain BL21(DE3)pLysS were generated following a previously established protocol (151). Briefly, plasmids encoding Stx1a, Stx2a, Stx 122 (Stx1a A1 with the A2 and B subunits from Stx2a), or Stx 211 (Stx2a A1 with the A2 B subunits from Stx1a) were transformed into BL21 *E. coli* cells via heat shock. Following transformation, the samples were placed on ampicillin-containing plates and allowed to grow overnight at 37°C. The next day, a single colony was picked from each plate and grown in 5 mL ampicillin-containing LB broth overnight at 37°C. The following day, the bacterial cultures were expanded in 50 mL ampicillin-containing LB broth and allowed to reach an optical density (OD) between 0.4 and 0.6 before a 3-hour induction with 1 mM IPTG (Sigma Aldrich; St. Louis, MO). Following induction, the cells were pelleted at 6,000 RPM at 4°C for 20 minutes. The collected cell pellets were resuspended in lysis buffer (300 mM NaCl, 20 mM Tris-HCl pH 7) supplemented with protease inhibitor cocktail (Fisher Scientific; Pittsburgh, PA). Following incubation of the cell pellets with 0.4 mg/mL lysozyme (Fisher Scientific; Pittsburgh, PA) at room temperature for 30 minutes, the samples were sonicated using a Digital Sonifier® (Branson Ultrasonics Corporation; Danbury, CT) to generate cell extracts. Stx-containing supernatants were obtained by spinning the cell extracts down at maximum speed using a Prism tabletop microcentrifuge (Labnet International; Edison, NJ).
Figure 4. Shiga holotoxin and hybrid Stx structures.

Native Stxa subunits are depicted in grey and Stxa subunits are depicted in black. The Stx 122 hybrid is comprised of the A1 subunit of Stxa with the A2B5 subunits of Stxa, while the Stx 211 hybrid is comprised of the A1 subunit of Stxa with the A2B5 subunits of Stxa.
Antibodies

Antibodies against Gb3/CD77 and Gb4 were purchased from GeneTex (Irvine, CA) and Matreya LLC (State College, PA), respectively. A goat anti-rat IgG antibody conjugated to allophycocyanin (APC) was purchased from Invitrogen (San Diego, CA), and a donkey anti-rabbit IgG antibody conjugated to phycoerythrin (PE) was purchased from Invitrogen (San Diego, CA).

Fluorescence measurements

Vero or Vero-d2EGFP cells were seeded in 500 μL volume to black-walled 24 well plates with glass bottom or flat bottom cell culture 24 well plates at a density of 100,000 cells per well. For 96 well plates, cells were seeded in 100 μL volume at a density of 20,000 cells per well. After an overnight incubation at 37°C with 5% CO2, the cells were incubated in serum-free Ham’s F-12 medium containing 20 μg/mL of cycloheximide (Sigma Aldrich; St. Louis, MO), inhibitors, or the stated toxin dilutions. Following incubation, the cells were washed with 1× PBS and then bathed in 1× PBS for EGFP measurement using a Synergy H1 Multi-Mode Microplate Reader (Biotek; Winooski, VT) with bottom optics position and 485 nm excitation / 528 nm emission filter set. For subsequent cytofluorometry analysis, the cells were detached from the 24 well plates using 1× PBS without calcium and magnesium. EGFP fluorescence was measured using an Accuri C6 Flow Cytometer (BD Biosciences; San Jose, CA) or a CytoFLEX Flow Cytometer (Beckman Coulter; Fullerton, CA). All experiments recorded 10,000 events (except for the experiment in Figure 5C, which recorded 20,000 events). For quantification of both plate reader and cytofluorometry data, background levels of autofluorescence from the parental Vero cells were subtracted from the experimental measurements. Background-subtracted data from treated samples were expressed as percentages of the control value obtained from untreated Vero-d2EGFP cells.
**[35S]-methionine incorporation**

Vero-d2EGFP cells were seeded to a 24 well plate at a density of ~100,000 cells per well and allowed to reach ~90% confluency overnight. The next day the spent medium was carefully aspirated off the wells. Then, serum-free Ham’s F-12 nutrient mix medium containing one of several toxin dilutions was added to the cells at 500 µL per well. A set of cells treated with serum-free and toxin-free Ham’s F-12 nutrient mix medium was used as the 100% control value in each experiment. After 18-24 hours, the spent medium was carefully aspirated off the plate and 500 µL of methionine (met)-free DMEM (GIBCO; Waltham, MA) was added to each well for a 15 minute 37°C incubation. Then, the met-free DMEM was aspirated off the plate and replaced with 500 µL of fresh met-free DMEM containing [35S]-met (PerkinElmer; Akron, OH) at a final concentration of 5 µCi/mL for an additional 15-minute incubation. To precipitate the proteins, 500 µL of 10% trichloroacetic acid (TCA) (Fisher Scientific; Pittsburgh, PA) in 1× PBS was added to each well and incubated at 4°C for 30 minutes. After the 30 minutes, the TCA was aspirated off the plate, and another 500 µL 10% TCA in 1× PBS was added to the plate for an additional 10 minutes at 4°C. After the 10 minutes, the TCA was aspirated off and 500 µL 0.2 N NaOH was added to each well for 15 minutes to lyse the cells. 5 mL of scintillation solution fluid (National Diagnostics; Atlanta, GA) was then added to scintillation vials. Cell lysates generated in the 0.2 N NaOH were loaded into scintillation vials, and the acid-precipitated radiolabel was quantified with an LS 6500 multipurpose scintillation counter (Beckman Coulter; Fullerton, CA).

**Detection of apoptosis**

Cells treated with toxin in parallel with the fluorescence experiments described above were washed with 1× PBS and detached from the plate with 400 µL 1× PBS lacking calcium and magnesium.
The cell suspension was then supplemented with 1× binding buffer (0.01 M HEPES/NaOH [pH 7.4], 0.14 M NaCl, 0.25 mM CaCl₂) containing PE annexin V and 7-AAD (BD Biosciences; San Jose, CA). The cell suspensions were protected from light, mixed, and incubated at room temperature for 15 minutes. Following this incubation, binding buffer was used to bring the final sample volume to 500 μL. Samples were then analyzed using an Accuri C6 Flow cytometer. Unintoxicated cells that were unstained, stained with PE annexin V (AV) alone, or stained with 7-aminoactinomycin (7-AAD) alone were used to establish the quadrant for healthy, viable cells lacking AV and 7-ADD staining. The fraction of unintoxicated Vero-d2EGFP cells in this quadrant was arbitrarily set as the 100% control value, and the fraction of viable cells after toxin or H₂O₂ (Fisher Scientific; Pittsburgh, PA) challenge were expressed as percentages of the control value.

**MTS viability assay**

To monitor cell viability through cellular metabolism, 20,000 Vero-d2EGFP cells were seeded in a 96 well plate and allowed to reach ~80% confluency overnight at 37°C under 5% CO₂. Cells were then incubated in serum-free Ham’s F-12 containing-toxins, drugs, and/or toxins with drugs. Following the indicated incubation period, 20 μL of MTS reagent (Promega; Madison, WI) was added to each well of the plate and incubated for 3 hours at 37°C. NADPH and NADH from live, metabolically active cells reduce the MTS reagent into a colored formazan product that can be detected at an absorbance of 490 nm using a Synergy H1 Multi-Mode Microplate Reader. Absorbance is directly proportional to the extent of cell viability. Background readings taken from wells without cells were subtracted from the experimental measurements. After background subtraction, the absorbance value obtained from untreated control cells was arbitrarily set at 100%. Data from treated or intoxicated samples were then expressed as percentages of the control value.
As a control to ensure substantial cell death could be detected, cells were incubated with 20% DMSO (Fisher Scientific; Pittsburgh, PA). Each experiment was run with 6 to 12 replicate samples per condition.

**Light microscopy**

Vero-d2EGFP cells were seeded to 24 well plates in 500 µL volume at a density of 100,000 cells per well. Following overnight growth at 37°C and 5% CO₂, the cells were treated with toxins diluted in serum-free Ham’s F-12 medium. Following incubations of the indicated times, phase contrast pictures were taken using a Nikon Eclipse TE200 microscope equipped with 20× objective lens and a Nikon Digital Sight camera (Nikon Instruments Inc.; Melville, NY). The intoxicated cells were not washed prior to image capture.

**Confocal microscopy**

Vero cells and Vero-d2EGFP cells were seeded to 24 well plates containing microscope cover glass (Fisher Scientific; Pittsburgh, PA) in 500 µL volume at a density of 100,000 cells per well. Following overnight growth at 37°C and 5% CO₂, the cells were washed once with 1× PBS before being fixed for 10 minutes at room temperature with 4% paraformaldehyde (Sigma Aldrich; St. Louis, MO) in 1× PBS. Following the fixation reaction, the cells were washed twice with 1× PBS. One drop of DAPI Fluoromount-G (SouthernBiotech; Birmingham, AL) was added directly to the cover glass before being mounted on a microscope slide (Fisher Scientific; Pittsburgh, PA). The slide was then allowed to air dry prior to being examined using a Leica TCS SP5 II confocal microscope equipped with 20× objective lens (Leica Microsystems Inc.; Buffalo Grove, IL).
StxB labeling

Individual B subunit monomers from Stx1a and Stx2a were conjugated to Alexa Fluor 594 using the Alexa Fluor 594 Microscale Protein Labeling kit from Invitrogen (San Diego, CA) according to the manufacturer’s instruction. Briefly, 5 μL of 1 M sodium bicarbonate was added to 50 μL of 1 mg/mL StxB subunit monomers in a reaction tube. Then, the vial of Alexa Fluor 594 succinimidyl ester (the reactive dye) was reconstituted with 10 μL of deionized water. The conjugate reaction was prepared by adding 2.4 μL of reactive dye solution to the reaction tube, mixed thoroughly by pipetting up and down several times, and incubated at room temperature for 15 minutes. The upper chamber of a spin filter column was filled with 800 μL of suspended resin gel and centrifuged at 16,000 × g for a total of 15 seconds (including run-up time). Following preparation of the conjugate reaction mixture, 50 μL was loaded onto the center of the resin bed surface and centrifuged at 16,000 × g for a total of 1 minute. The resin bed had a reddish purple color as indicative that the unreacted dye was retained in the filter. The resulting Alexa Fluor® 594 dye-labeled protein fluoresces under excitation and emission maxima similar to the Texas Red® fluorophore, 590/617 nm.

Stx binding assay

Vero or Vero-EGFP cells were seeded to a 10 cm dish about two days before staining and allowed to reach ~90% confluency. They were then lifted from the dish with trypsin/EDTA and distributed to microcentrifuge tubes at a quantity of ~500,000 cells per tube. Pelleted cells (2 minutes at 8,000 × g) were washed once with 1× PBS and incubated under moderate shaking at 4°C for 30 minutes in Ham's F-12 medium containing 2% BSA (Fisher Scientific; Pittsburgh, PA) and various concentrations of the labeled B subunit from either Stx1a or Stx2a. The cells were then washed
with 1× PBS, resuspended in 400 µL of 1× PBS, and processed using the red laser of an Accuri C6 Flow Cytometer. Cells incubated in the absence of toxin were used to establish the background level of autofluorescence. Pilot experiments found that cells exposed to an unlabeled toxin B subunit exhibited a fluorescent output that was similar to that of cells incubated without a toxin B subunit.

**Gb3 and Gb4 detection**

Vero cells were seeded to a 10 cm dish about two days before staining and allowed to reach ~90% confluency. They were then lifted from the dish with trypsin/EDTA and distributed to microcentrifuge tubes at a quantity of ~500,000 cells per tube. Pelleted cells (2 minutes at 8,000 × g) were washed once with 1× PBS and incubated under moderate shaking at 4°C for 2 hours in Ham’s F-12 medium containing 2% BSA and a 1:250 dilution of either a rat anti-Gb3 antibody or a rabbit anti-Gb4 antibody. The cells were then washed once with 1× PBS and incubated under moderate shaking at 4°C for 1 hour in Ham’s F-12 medium containing 2% BSA and a 1:250 dilution of either an APC-conjugated goat anti-rat IgG antibody or a PE-conjugated donkey anti-rabbit IgG antibody. The cells were then washed with 1× PBS, resuspended in 400 µL of 1× PBS, and processed using an Accuri C6 Flow Cytometer. When indicated, both Gb3 and Gb4 antibodies were added to the same population of Vero cells. To establish the background level of fluorescence, cells were exposed to the secondary antibodies alone.
CHAPTER 4: RESULTS

Aim I: Develop a new system to examine the activity of toxins that inhibit protein synthesis


The goal of Aim I was to optimize a previously developed system to detect the activity of AB toxins that inhibit protein synthesis in individual cells. Moreover, the system was evaluated for its capability to screen for toxin inhibitors. Lastly, the advantages of our system were compared to current systems that are used to detect the activity of AB toxins that inhibit protein synthesis.

Effect of ricin on Vero-d2EGFP cells

This study used a previously generated Vero cell line that stably expresses d2EGFP-N1, an EGFP variant that contains a C-terminal PEST sequence for rapid degradation by the ubiquitin-proteasome system (102, 103). Steady-state fluorescence in the Vero-d2EGFP cell line is easily detected by flow cytometry, microscopy (Fig. 5), or with a plate reader. When challenged with a toxin that inhibits protein synthesis, toxin-susceptible cells will degrade d2EGFP and will not produce more of the protein. Thus, toxin activity is inversely proportional to the EGFP fluorescence output. Productive intoxication accordingly results in the loss of d2EGFP fluorescence. As shown in Figure 6A, ricin reduced the Vero-d2EGFP fluorescent signal and the $[^{35}\text{S}]-\text{met}$ incorporation signal in a dose-dependent manner. The $[^{35}\text{S}]-\text{met}$ incorporation method, a common technique to monitor the activity of toxins that inhibit protein synthesis, was used as a basis of comparison to the Vero-d2EGFP system. These results documented that the two methods
were equally capable of detecting the ricin-induced inhibition of protein synthesis. Moreover, we also monitored toxin activity by an MTS cell viability assay. The loss of EGFP fluorescence was much more dramatic than the loss of cell viability after an 18-hour intoxication: a ricin ED$_{50}$ of 0.03 ng/mL was recorded by the Vero-d2EGFP assay, whereas the MTS cell viability assay reported an ED$_{50}$ of 0.7 ng/mL (Fig. 6B). Both fluorescence and viability were measured in the same cell population. The loss of viability eventually mirrored the loss of fluorescence after 42 hours of toxin exposure, with both EGFP and MTS assays documenting an ED$_{50}$ of 0.02–0.04 ng/mL (Fig. 6C). These collective results highlight several advantages of the Vero-d2EGFP system, including (i) relatively rapid detection of toxin activity, (ii) high sensitivity, (iii) minimal sample handling for data acquisition, and (iv) a noninvasive/nonterminal measurement that allows the cells to be used for other purposes such as an MTS assay.
Figure 5. Detection of fluorescence from the Vero-d2EGFP cells.

The fluorescence output from Vero cells (A) and Vero-d2EGFP cells (B) by confocal microscopy using a 20× objective lens. All cells in panels A and B were stained with DAPI and analyzed under the same exposure time. The scale bar (top left corner of panels A and B) represents 15 µm. (C) The fluorescence output of 20,000 Vero cells (grey) and Vero-d2EGFP cells (black) were detected by flow cytometry.
Figure 6. Effect of ricin on protein synthesis and cell viability.

(A) EGFP signal (filled circles) and [35S]-met incorporation signal (filled squares) of Vero-d2EGFP cells exposed to various concentrations of ricin for 18 hours. (B-C) Fluorescence (filled circles) and cell viability via MTS assay (open squares) were measured in the same population of Vero-d2EGFP cells after an 18-hour (B) or 42-hour (C) incubation with serial dilutions of ricin. Results were expressed as percentages of the maximal signal obtained from unintoxicated Vero-d2EGFP cells. The means ± standard errors of the means of at least four independent experiments with six replicate samples for each condition are shown.
**Time frame for loss of Vero-d2EGFP fluorescence**

To examine how quickly the Vero-d2EGFP assay can detect toxin activity, we monitored the time-dependent decay of EGFP fluorescence from intoxicated cells (Fig. 7, circles). Using a single concentration of ricin (1 ng/mL), we found the EGFP signal begins to decrease after about 4 hours of toxin exposure and continues to decrease until 14 hours when a minimal signal of 5–8% is achieved. With a 2-hour half-life for d2EGFP, a signal strength corresponding to 6% of the unintoxicated control value could theoretically be reached 8 hours after exposure to a toxin that inhibits protein synthesis. The longer time frame required to reach this point for ricin-treated cells reflects the temporal delay between toxin binding to the cell surface and A chain delivery to its site of action in the cytosol (20), as well as the asynchronous nature of intoxication in a population of cells. These cellular events also explain why the loss of EGFP fluorescence is slower in toxin-treated cells than in cells treated with cycloheximide, a membrane-permeant protein synthesis inhibitor (Fig. 7, squares). Nevertheless, our experiment demonstrated the cellular activity of ricin can be detected about 4 hours after toxin exposure and reaches its maximal effect on protein synthesis about 14 hours after toxin exposure.
Figure 7. Time frame for loss of Vero-d2EGFP fluorescence.

Measurements of fluorescence intensity were taken at 2-hour intervals after incubation of Vero-d2EGFP cells with 1 ng/mL of ricin (circles) or 20 μg/mL of the protein synthesis inhibitor cycloheximide (squares). Results were expressed as percentages of the maximal EGFP signal obtained from untreated Vero-d2EGFP cells. The averages ± standard deviations of three independent experiments with six replicate samples per condition are shown.
Use of the Vero-d2EGFP assay to screen for toxin inhibitors

Disruptions to the intoxication process will permit the continued synthesis of d2EGFP in toxin-treated cells. This principle was used to identify grape seed extract as a potent inhibitor of both Stx1a and Stx2a (103). The antitoxin property of grape seed extract was then confirmed with an independent toxicity assay that monitored the overall level of protein synthesis in cells exposed to Stx2 (103). In the presence of grape seed extract, the Vero-d2EGFP cells were also protected from ricin, Dtx, and EtxA (104). Use of the Vero-d2EGFP assay to screen for toxin inhibitors is shown in Figure 8. A previous screen of 26 purified polyphenolic compounds from grape extract failed to identify any individual inhibitor of Stx1a/Stx2a (104), so we next screened fractions from the grape seed extract itself for antitoxin effects. Seven polyphenolic fractions, generated by using a modified normal-phase high-performance liquid chromatography (HPLC) method (152) (Fig. 8A), were mixed with a cell-free culture supernatant containing Stx1a/Stx2a and applied to Vero-d2EGFP cells for 18 hours. The fluorescent signal from cells exposed to toxin alone was reduced to 25% of the signal from un intoxicated Vero-d2EGFP cells (Fig. 8B). A similar loss of fluorescence was recorded for Vero-d2EGFP cells challenged with toxin in the presence of fractions 1–6. However, intoxicated cells co-incubated with fraction 7 retained a stronger fluorescent signal, representing 42% of the unintoxicated control value and a statistically significant difference from the intoxicated control cells (p = 0.0217, Student’s t-test). A similar screen indicated fractions 5–7 contain compounds that block ricin activity (Fig. 8C). In this screen, known polyphenol inhibitor of ricin - epigallocatechin gallate (EgCg) (104, 153) - was used as a positive control. Further separation of the compounds in fractions 5–7, combined with additional Vero-d2EGFP assays, could potentially identify the specific polyphenols that confer resistance to Stxs and/or ricin. In summary, these results show that this new system is capable of detecting the
activity of toxins that inhibit protein synthesis, as well as screening for toxin inhibitors. The data in the next section highlight the use of this GFP reporter system with flow cytometry to determine intoxication models, test the validity of the “one molecule” model, and investigate potential cellular recovery from toxin exposure.
Figure 8. Use of the Vero-d2EGFP assay to screen for toxin inhibitors.

(A) A modified method using normal-phase HPLC separated grape seed extract into seven fractions enriched in polyphenol monomers (catechin and epicatechin), dimers, trimers, or tetramers as indicated. The HPLC fractions were kindly provided by Dr. Salem Elkahoui. (B) Vero-d2EGFP cells were incubated with grape seed extract fractions (5% final volume) and a 1:250 dilution of a cell-free culture supernatant from an *E. coli* strain that expresses both *Stx*1a and *Stx*2a. After an 18-hour incubation, EGFP fluorescence was measured with a plate reader. Results were expressed as percentages of the maximal EGFP signal obtained from a parallel set of un intoxicated Vero-d2EGFP cells. The means ± standard errors of the means of at least four independent experiments with six replicate samples per condition are shown. (C) Vero-d2EGFP cells were incubated with 0.5 ng/mL of ricin and either grape seed extract fractions (5% final volume) or 10 μg/mL of epigallocatechin gallate (EgCg). After an 18-hour incubation, EGFP fluorescence was measured with a plate reader. Results were expressed as percentages of the maximal EGFP signal obtained from a parallel set of un intoxicated Vero-d2EGFP cells. The averages ± ranges of two to four independent experiments with six replicate samples per condition are shown. For panels B and C, fraction 1 (F1) represents material eluted from 0 to 5 minutes; F2 represents material eluted from 5 to 10 minutes; etc.
Aim II: Determine the intoxication model for a subset of AB toxins and the potential for cellular recovery from intoxication


The results in Figure 5 show that the Vero-d2EGFP cells exhibited a fluorescence output that was distinguishable from the Vero cells as detected by both confocal microscopy and flow cytometry. Moreover, the flow cytometry results in Figure 5C indicated that the intensity of the fluorescent signal from the Vero-d2EGFP cells resulted in a peak that was relatively stronger than the one from the Vero cells. Thus, the ability to detect the Vero-d2EGFP fluorescence output and intensity by flow cytometry indicated that this system could be used to monitor the activity of toxin in individual cells. We predicted that the probability model of intoxication would yield results in which distinct levels of fluorescence intensity would be detected: cells with no cytosolic toxin would exhibit a fluorescence output similar to the unintoxicated control (i.e., no loss in protein synthesis as detected by the EGFP intensity), whereas cells that received at least one molecule of toxin would exhibit a peak with lower fluorescence output that matched the parental Vero cells. On the other hand, we predicted that the proportionality model would yield results showing a uniform loss in the EGFP intensity. In this intoxication model, individual cells within a population would receive varying amounts of toxin and the overall loss of EGFP intensity would be reflected as a uniform shift of the fluorescence peak.

Detection of EGFP expression in Vero-d2EGFP cells by flow cytometry

Distinct populations of Vero and Vero-d2EGFP cells were resolved by cytofluorometry when the two cell types were mixed together (Fig. 9). The individual peaks of background autofluorescence
(Fig. 9A) and EGFP fluorescence (Fig. 9B) from pure populations of Vero and Vero-d2EGFP cells, respectively, were both seen in mixed populations containing 1:1 (Fig. 9C) and 4:1 (Fig. 9D) ratios of Vero:Vero-d2EGFP cells. Although the number of cells contributing to the EGFP signal was reduced in the mixed populations, the peak fluorescent intensity from Vero-d2EGFP cells did not change. These results demonstrated it was possible to differentiate between populations of cells with or without EGFP expression.

Consistent with previous reports (109, 154) (Fig. 7), a time-dependent reduction in the fluorescent intensity from cycloheximide-treated Vero-d2EGFP cells was detected by cytofluorometry and with a plate reader (Fig. 10). The fluorescent peak from untreated Vero-d2EGFP cells shifted to uniform peaks at progressively lower intensities after both a 4-hour and 8-hour incubations with cycloheximide, a protein synthesis inhibitor (Fig. 10A). Quantification of the remaining signals with both a plate reader and cytofluorometer recorded a 50% loss of fluorescence after 4 hours of cycloheximide treatment and an ~80% loss of fluorescence after 8 hours of cycloheximide treatment (Fig. 10B). Thus, our cytofluorometry system gives the same average quantitative data as the standard plate reader method. Using an MTS assay, only a minor loss of cell viability (22%) was detected after an 8-hour exposure to cycloheximide (n=2, range=3%) (data not shown). These pilot studies demonstrated it was possible to detect and quantify the population-wide loss of EGFP fluorescence resulting from exposure to an inhibitor of protein synthesis.
Figure 9. Detection of separate cell populations with or without EGFP expression.

(A) Vero cells, (B) Vero-d2EGFP cells, (C) a 1:1 ratio of Vero:Vero-d2EGFP cells, and (D) a 4:1 ratio of Vero:Vero-d2EGFP cells were subjected to cytofluorometry. The range of background fluorescence generated by parental Vero cells and a minor population of the Vero-d2EGFP cells is in black. The distribution of higher levels of fluorescence for the Vero-d2EGFP cells is in dark green, while light green highlights the lower level of fluorescence from a subpopulation of Vero-d2EGFP cells. The peak fluorescent intensity from the population of Vero-d2EGFP cells with the highest fluorescence levels in panel B is represented by the red line in all panels.
Figure 10. Detection of population-wide loss of fluorescence from cycloheximide-treated Vero-d2EGFP cells.

(A) Untreated parental Vero cells (black), untreated Vero-d2EGFP cells (green), or Vero-d2EGFP cells treated with cycloheximide for 4 hours (blue) or 8 hours (red) were subjected to cytofluorometry. (B) Using data collected from the same cells by either cytofluorometry (grey bars) or with a plate reader (black bars), signals from the cycloheximide-treated cells were expressed as percentages of the value recorded for untreated Vero-d2EGFP cells.
Effect of short-term toxin exposure on EGFP fluorescence

We next used cytofluorometry to examine how EGFP fluorescence was affected in Vero-d2EGFP cells challenged with the AB toxins ricin (Fig. 11A), Stx1a (Fig. 11B), EtxA (Fig. 11C), or Dtx (Fig. 11D). In each case, cells were incubated with a toxin concentration that produced a roughly 50% reduction in EGFP fluorescence after 20 hours of incubation. Because the fluorescence intensity is displayed on a log rather than linear scale, the cytofluorometry profiles corresponding to a 50% inhibition of protein synthesis were not observed exactly midway between the unintoxicated Vero-d2EGFP cells with maximal EGFP expression and the parental Vero cells without EGFP expression. If a single molecule of toxin could elicit a cytotoxic effect, then a 50% loss of EGFP fluorescence would represent a bimodal cell population: half the cells would be intoxicated with no protein synthesis, while the other half would be unintoxicated and therefore producing normal levels of protein with full EGFP fluorescence. However, our cytofluorometry data from intoxicated cells did not detect two distinct fluorescent peaks representing background fluorescence and a full EGFP signal. We instead recorded a uniform, population-wide downshift in mean fluorescent intensity of intoxicated Vero-d2EGFP cells. A bimodal distribution of cells with either full EGFP expression or no EGFP expression was clearly absent from the intoxicated cells. These results were similar to the effects observed in Vero-d2EGFP cells that had been treated with cycloheximide for 4 hours and showed a population-wide downshift in their EGFP signal to 50% of the maximal value (Fig. 10). We accordingly concluded that the entire population of Vero-d2EGFP cells had been intoxicated, but the quantity of cell-associated toxin was only sufficient to reduce protein synthesis to 50% of normal levels.
A dose-dependent, population-wide loss of fluorescence was recorded for cells exposed to a range of concentrations for each of the tested AB toxins (Fig. 11). This effect was confirmed by quantifying the fluorescent signal from intoxicated cells with a plate reader before collecting the cells for cytofluorometry. Furthermore, the loss of protein synthesis detected by cytofluorometry mirrored the loss of protein synthesis detected with a plate reader. Only cells treated with Stx1a or EtxA showed an obvious phenotypic effect from a 20-hour challenge with higher toxin concentrations (Fig. 12). MTS cell proliferation assays recorded a 25% loss of viability in cells treated with the highest concentration of Stx1a and less toxicity in cells challenged with lower Stx1a concentrations or any concentration of the other three toxins (Fig. 12). For comparative purposes, the oxidative stress resulting from a 20-hour exposure to 1 mM H$_2$O$_2$ lowered cell viability to 56 ± 3% (n = 3, ± std. dev.) of the untreated control value (data not shown). These results indicated substantial cell death did not occur after a 20-hour toxin challenge despite the reduction in protein synthesis.
Figure 11. Dose-dependent, population-wide loss of fluorescence from toxin-treated Vero-d2EGFP cells.

Cells were incubated for 20 hours with various concentrations of (A) ricin, (B) Stx1a, (C) EtxA, or (D) Dtx. Left column: using data collected from the same cells by either cytofluorometry (grey bars) or with a plate reader (black bars), signals from the toxin-treated cells were expressed as percentages of the value recorded for untreated Vero-d2EGFP cells. Right column: cytofluorometry data from the same pool of intoxicated cells analyzed in the left column are shown, along with results from unintoxicated parental Vero cells (black lines) and unintoxicated Vero-d2EGFP cells (green lines). Yellow, blue, and red lines were generated from Vero-d2EGFP cells incubated with the color-coded toxin concentration.
Figure 12. Cell viability after 20-hour intoxications.

As indicated, Vero-d2EGFP cells were incubated for 20 hours with various concentrations of ricin, Stx1a, EtxA, or Dtx. Representative images were taken at 200x magnification. Viability, as assessed by MTS assay (n = 3, avg. ± std. dev.), is reported below each image along with the applied dose of toxin.
Effect of long-term toxin exposure on EGFP fluorescence

Our results demonstrated a single molecule of cytosolic toxin is not sufficient to completely inhibit protein synthesis and kill the target cell after a 20-hour incubation. However, there is a lag between the loss of protein synthesis and cell death. We therefore extended our incubation with ricin (Fig. 13A), Stx1a (Fig. 13B), EtxA (Fig. 13C), or Dtx (Fig. 13D) to 36 hours in order to examine whether extensive cell death follows the loss of protein synthesis. Some of the intoxicated cells had detached from the plate by 36 hours, but cell viability (as assessed by MTS assay) was relatively high – between 60–80% of the unintoxicated control values (Fig. 13, left column). Cytofluorometry analysis of the remaining adherent cells documented a population-wide loss of protein synthesis (Fig. 13, center column). The fluorescent signals from these cells were substantially lower than the unintoxicated control values, yet most cells were still viable as demonstrated by a lack of staining with the apoptosis markers annexin V and 7-AAD (Fig. 13, right column). Viability as determined by MTS was lower than assessments made by annexin V / 7-AAD because the MTS assay accounted for the entire cell population whereas annexin V / 7-AAD staining only considered the subpopulation of adherent cells. Additional observations were recorded for a range of toxin concentrations, with the expected dose-dependent effects on cell morphology, viability, and protein synthesis (Fig. 14). Surprisingly, however, almost none of the remaining adherent cells – even those with extremely low levels of protein synthesis – were apoptotic. A 36-hour toxin challenge thus left a subpopulation of cells with a quantity of cytosolic toxin that inhibited protein synthesis but was not lethal. These observations strongly indicated that a single molecule of cytosolic toxin will neither completely inhibit protein synthesis nor induce cell death.
Figure 13. Cell survival with a long-term, toxin-induced inhibition of protein synthesis.

Vero-d2EGFP cells were incubated for 36 hours with (A) 0.05 ng/mL of ricin, (B) 0.01 ng/mL of Stx1a, (C) 1.0 ng/mL of EtxA, or (D) 0.05 ng/mL of Dtx. Left column: representative images were taken at 200x magnification. Cell viability, as assessed by MTS assay (n = 3, avg. ± std. dev.), is indicated. Center column: red and blue lines (corresponding to the color-coded toxin concentrations in Figure 11) were generated from cytofluorometry analysis of the adherent subpopulation of toxin-treated Vero-d2EGFP cells. Unintoxicated parental Vero cells (black lines) and unintoxicated Vero-d2EGFP cells (green lines) were also processed for each condition. Right column: cell viability was recorded by cytofluorometry analysis of annexin V and 7-AAD staining (blue), while EGFP fluorescence was recorded by cytofluorometry (grey) or with a plate reader (black). Results are presented as percentages of the values obtained from unintoxicated cells. All measurements in the matched center and right columns were performed on the same population of cells.
Figure 14. Effect of long-term toxin exposure on cell viability and protein synthesis (range of toxin concentrations).

Vero-d2EGFP cells were incubated for 36 hours with various concentrations of (A) ricin, (B) Stx1a, (C) EtxA, or (D) Dtx. Left columns: representative images were taken at 200× magnification. Cell viability, as assessed by MTS assay (n = 3, avg. ± std. dev.), is indicated along with the applied dose of toxin. Center column: the subpopulation of adherent cells were subjected to cytofluorometry, along with unintoxicated parental Vero cells (black lines) and unintoxicated Vero-d2EGFP cells (green lines). Yellow, blue, and red lines were derived from Vero-d2EGFP cells incubated with the color-coded toxin concentration. Right column: cell viability was recorded by cytofluorometry analysis of annexin V and 7-AAD staining (blue), while EGFP fluorescence was recorded by cytofluorometry (grey) or with a plate reader (black). Results are presented as percentages of the values obtained from unintoxicated cells. All measurements in the matched center and right columns were performed on the same population of cells. These experiments were performed at the same time as the data presented in Figure 13 and, for comparative purposes, include the toxin concentrations used in Figure 13.
Cellular recovery from exposure to AB toxins

Our collective data suggested cells can tolerate low levels of cytosolic toxin and the partial inhibition of protein synthesis without a terminal effect. Thus, cells could potentially recover from intoxication. To further examine this possibility, we challenged Vero-d2EGFP cells with ricin (Fig. 15A), Stx1a (Fig. 15B), EtxA (Fig. 15C), or Dtx (Fig. 15D) for 20 hours. Toxins were applied at concentrations that were at or below the 20-hour ED$_{50}$ values. One set of cells for each applied toxin was processed for cytofluorometry, while another set was washed and incubated in toxin-free medium for an additional 48-hour before cytofluorometry. The population-wide loss of EGFP fluorescence recorded after 20 hours of intoxication demonstrated all cells had a cytosolic pool of toxin at this time, as indicated by the observed downshift in peak fluorescence intensity (Fig. 15, left column). Cells that were chased in toxin-free medium for 48 hours exhibited higher levels of fluorescence than were recorded after the initial 20-hour toxin challenge (Fig. 15, center column), and a substantial pool of viable, adherent cells remained at the end of the chase (Fig. 15, right column). For these experiments, the quantity of cytosolic toxin present at 20-hour of intoxication was therefore insufficient to completely inhibit protein synthesis and kill the entire population of intoxicated cells. Removal of the toxin after a 20-hour exposure consequently allowed most cells to recover from the toxin-induced inhibition of protein synthesis.

As expected, recovery from intoxication was dependent on the applied dose of toxin: cells exposed to higher toxin concentrations did not return to high levels of EGFP fluorescence by the end of the 68-hour experiment, and substantial cell death was recorded (Fig. 16). These experiments were performed at the same time as the experiments presented in Figure 15 and are shown as a separate figure to emphasize cells can recover from sub-optimal levels of toxin (Fig. 15) but not all toxin
concentrations (Fig. 16). Combined, Figures 15 and 16 present data with the three toxin concentrations used throughout this work. Bimodal fluorescent signals were detected for cells that were exposed to intermediate concentrations of Stx1a or EtxA, which demonstrated our system could detect an all-or-nothing signal distribution with intoxicated cells. It also indicated protein synthesis had been completely inhibited in one subpopulation of toxin-challenged cells, while another subpopulation had completely recovered from intoxication and produced normal levels of protein. The bimodal fluorescent profiles detected in many conditions from the 68-hour experiment would skew the mean fluorescent intensities, so the EGFP signals were not quantified. However, the population-wide loss of protein synthesis recorded at 20-hour of intoxication for all toxin concentrations confirmed every cell had at least one molecule of cytosolic toxin at this time. As such, the greater level of cell death resulting from exposure to higher toxin concentrations could not be attributed to a greater number of intoxicated cells (i.e., the probability model of intoxication). Instead, transient exposure to higher toxin concentrations apparently produced a greater quantity of cytosolic toxin that overwhelmed the cellular capacity to withstand intoxication (i.e., the proportionality model of intoxication). Productive intoxication thus requires either transient exposure to high toxin concentrations or continual exposure to sub-optimal toxin concentrations. The exact quantity of toxin required for cell death by either of these mechanisms has yet to be determined, but both scenarios involve exposure to more than one molecule of toxin.
Figure 15. Cellular recovery from intoxication.

Vero-d2EGFP cells were incubated with (A) 0.025 ng/mL of ricin, (B) 0.001 ng/mL of Stx1a, (C) 1.0 ng/mL of EtxA, or (D) 0.01 ng/mL of Dtx for 20 hours and then chased for 48 hours in the absence of toxin. Blue and yellow lines (corresponding to the color-coded toxin concentrations in Figures 11 and 14) were generated from cytofluorometry analysis of toxin-treated Vero-d2EGFP cells at the end of the 20-hour toxin incubation (left column) or 48-hour chase (center column). Unintoxicated parental Vero cells (black lines) and unintoxicated Vero-d2EGFP cells (green lines) were also processed for each condition. Percentages represent the strength of the EGFP signal from intoxicated cells in comparison to unintoxicated cells. Right column: representative images of cells at the end of the 48-hour chase were taken at 200× magnification. Cell viability, as assessed by MTS assay (n = 3, avg. ± std. dev.) or annexin V/7-AAD staining of the same cell population processed for EGFP cytofluorometry, is indicated.
Figure 16. Dose-dependent recovery from intoxication (additional toxin concentrations).

Vero-d2EGFP cells were incubated with various concentrations of (A) ricin, (B) Stx1a, (C) EtxA, or (D) Dtx for 20 hours and then chased for 48 hours in the absence of toxin. Left columns: yellow, blue, and red lines were derived from cytofluorometry analysis of toxin-treated Vero-d2EGFP cells at the end of the 20-hour toxin incubation or the end of the 48-hour chase. Unintoxicated parental Vero cells (black lines) and unintoxicated Vero-d2EGFP cells (green lines) were also processed for each condition. Right columns: at the end of the 48-hour chase, representative images of cells initially exposed to the stated toxin concentrations were taken at 200× magnification. Cell viability, as assessed by MTS assay (n = 3, avg. ± std. dev.) or annexin V/7-AAD staining of the same cell population processed for EGFP cytofluorometry, is indicated. These experiments were performed at the same time as the data presented in Figure 15.
Aim III: Investigate factors that contribute to the cellular potency of Stx subtypes

Cherubin P, Fidler D, Quiñones B, Teter K. Bimodal Response to Shiga Toxin 2 Subtypes Results from Relatively Weak Binding to the Target Cell. Infection and Immunity. Under revision.

Stx1a is more potent to Vero cells than Stx2 subtypes. The factors contributing to the difference in cellular potency are mostly unknown. Therefore, we used our cytofluorometry-based system to examine the differential potency between Stx subtypes. Since we already monitored the activity of Stx1a in Aim II using our new system, the goal of Aim III was to monitor the activity of Stx2 subtypes in individual cells and compare the results with that obtained for Stx1a. A comparison between the results obtained for these Stx subtypes will provide better insights into their differential cytotoxicity.

Stx1a is more potent against cultured cells than Stx2 subtypes

Vero-d2EGFP cells were used to establish the relative potencies of Stx1a, Stx2a, and Stx2c. Cells were seeded on a 96 well plate overnight before an 18-hour incubation with 10-fold serial dilutions of each toxin. Stx1a was very effective at inhibiting protein synthesis in the target cells, with an ED$_{50}$ of 0.005 ng/mL (Fig. 17A, triangles). Stx2a, on the other hand, was less effective than Stx1a, with a 140-fold higher ED$_{50}$ of 0.7 ng/mL (Fig. 17A, circles). An ED$_{50}$ of 10 ng/mL was recorded for Stx2c (Fig. 17A, squares), which was 2,000-fold less toxic than Stx1a. Our results were consistent with previous reports on the relative cellular activities of Stx1a, Stx2a, and Stx2c (120, 130, 131). As previously observed when examining the toxicity of Stx1a in Vero cells (Figures 11, 12), there was a greater level of protein synthesis inhibition than cell death after an 18-hour intoxication with Stx2a and Stx2c subtypes (Fig. 17A-B). Extending the toxin challenge to 36
hours resulted in greater cell death than seen at 18 hours (Fig. 17C). ED$_{50}$ values for cell viability after a 36-hour toxin exposure demonstrated Stx1a (ED$_{50}$ of 0.06 ng/mL) was 17-fold more potent than Stx2a (ED$_{50}$ of 1 ng/mL) and 667-fold more potent than Stx2c (ED$_{50}$ of 40 ng/mL). Thus, consistent with previous reports (130, 131, 155), Vero cells were much more sensitive to Stx1a than either Stx2a or Stx2c as determined by both protein synthesis and cell viability assays. Extending the toxin challenge to 72 hours did not result in greater levels of cell death (Fig. 17D).
Figure 17. Relative potencies of Stx subtypes.

Vero-d2EGFP cells were challenged with 10-fold serial dilutions of Stx1a (triangles), Stx2a (circles), or Stx2c (squares). The extent of protein synthesis after 18 hours of incubation (A) or cell viability after 18 hours (B), 36 hours (C), and 72 hours (D) of incubation was then recorded. Values for toxin-treated cells were expressed as percentages of the maximal signal obtained from unintoxicated control cells. Data represent the means ± standard errors of the means (SEMs) of at least 6 independent experiments with 6 replicate samples per experiment.
**Bimodal cellular response to Stx2 subtypes**

With our Vero-d2EGFP system, the toxin-induced loss of protein synthesis can be detected with a plate reader or by flow cytometry. Both methods recorded the same level of toxin activity on cells incubated overnight with Stx1a (Fig. 11). Previous analyses using flow cytometry further documented the population-wide loss of protein synthesis in cells exposed to Stx1a (Fig. 11), which was replicated here: increasing concentrations of Stx1a elicited an increasingly dramatic downshift in the peak fluorescent intensity of Vero-d2EGFP cells (Fig. 18A). This effect was not seen for cells challenged with Stx2a (Fig. 18B) or Stx2c (Fig. 18C). Both toxins generated a bimodal fluorescent profile from the intoxicated Vero-d2EGFP cells in which one subpopulation of cells maintained the peak fluorescent intensity observed for unintoxicated cells and the other subpopulation exhibited reduced levels of fluorescent intensity (i.e., protein synthesis). A substantial number of Vero-d2EGFP cells were therefore resistant to moderate doses of Stx2a and Stx2c, with no appreciable loss of protein synthesis. The subpopulation of Vero-d2EGFP cells that maintained the peak fluorescent intensity after challenge with Stx2a or Stx2c were not intrinsically resistant to the toxins, however: the subpopulation of resistant cells progressively decreased with increasing toxin concentrations (Fig. 18B-C). This indicated Stx2a and Stx2c can generate substantial, population-wide decreases in protein synthesis when present at relatively high toxin concentrations of 10 ng/mL or greater.
Figure 18. Distinct cellular responses to Stx subtypes.

Vero-d2EGFP cells were subjected to cytofluorometry after an 18-hour incubation with the stated concentrations of Stx1a (A), Stx2a (B), or Stx2c (C). Unintoxicated parental Vero cells (black lines) and unintoxicated Vero-d2EGFP cells (green lines) were also processed for each condition. One of three representative experiments is shown.
Uniform distribution of Gb$_3$ and Gb$_4$ in the population of Vero cells

Globoside Gb$_3$ serves as a functional surface receptor for all Stx subtypes including Stx2e, which preferably binds to the Gb$_4$ surface receptor (156-159). Gb$_4$ also has a moderate affinity for Stx1a and a weak affinity for Stx2a or Stx2c (137, 138). We accordingly predicted that the Stx2a/Stx2c-resistant subpopulation of Vero-d2EGFP cells lacked Gb$_3$ but still expressed the alternate Gb$_4$ receptor with preferential affinity for Stx1a. To test this hypothesis, we examined the distribution of Gb$_3$ and Gb$_4$ in a population of Vero cells. Analysis by flow cytometry documented a uniform distribution of Gb$_3$ (Fig. 19A) and Gb$_4$ (Fig. 19B) in cell populations exposed to Gb$_3$ or Gb$_4$ primary antibodies and fluorophore-labeled secondary antibodies, which, in agreement with previous studies (159-161), confirmed that the Vero cells employed for these experiments contained both Gb$_3$ and Gb$_4$ cell surface receptors.

Vero cells were next incubated with both Gb$_3$ and Gb$_4$ antibodies and then the corresponding fluorophore-labeled secondary antibodies. Preliminary experiments confirmed the signals from each fluorophore did not bleed into the emission wavelength of the other fluorophore (data not shown). A scatter plot of the resulting data revealed a linear relationship between the two globosides, indicating Gb$_3$ content was proportional to Gb$_4$ content (Fig. 19C). Only 3% of cells were positive for Gb$_3$ but not Gb$_4$ (lower right quadrant), and only 5% of cells were positive for Gb$_4$ but not Gb$_3$ (upper left quadrant). Therefore, contrary to our hypothesis, the collective experiments with Gb$_3$ and Gb$_4$ staining suggested that nearly the entire population of Vero cells express both Gb$_3$ and Gb$_4$. The minor population of Gb$_3^-$/Gb$_4^+$ cells (5%) could not account for the
relatively high number of cells that were completely resistant to moderate concentrations of Stx2a or Stx2c (Figs. 18B-C).
Gb$_3$ and Gb$_4$ antibodies were applied independently (A-B) or simultaneously (C) to Vero cells before analysis by cytofluorometry. (A) Staining pattern for the combination of a rat antibody against Gb$_3$ and an APC-conjugated goat anti-rat IgG antibody (red line). (B) Staining pattern for the combination of a rabbit antibody against Gb$_4$ and a PE-conjugated donkey anti-rabbit IgG antibody (red line). Cells incubated with the secondary antibody alone (black lines in panels A and B) were processed as well. (C) The combined staining patterns for Gb$_3$ and Gb$_4$ are represented on a scatter plot of 10,000 individual cells. Cells exposed to the secondary antibodies alone were used to set background gates represented by the red lines.
The B subunit of Stx1a binds to host cells with better efficiency than that of Stx2a

To further examine the dose-dependent binding of the B subunits of Stx1a or Stx2a with their surface receptors, Vero cells were incubated with the fluorophore-labeled B subunits for 30 minutes at 4°C before analysis by flow cytometry (Fig. 20). A uniform distribution of toxin binding was observed in cells exposed to the B subunits of either Stx1a (Fig. 20A) or Stx2a (Fig. 20B). As seen for the distribution of Gb3 and Gb4 (Fig. 19), there was no obvious subpopulation of cells that did not bind the monomeric B subunit. The labeling efficiency for the B subunit of Stx1a (0.093) was slightly weaker than the labeling efficiency for the B subunit of Stx2a (0.13), yet a stronger cell-binding signal was recorded for the B subunit of Stx1a than Stx2a when an equivalent amount of B subunit (2 µg) was tested (Fig. 20C). Figure 20D further shows that 0.5 µg of the Stx2a B subunit was required to generate the same cell-binding signal as 0.2 µg of the Stx1a B subunit, indicating a 2.5-fold more binding for the B subunit of Stx1a. A similar observation was seen for other pairs of toxin concentrations (data not shown). We also noted that binding of the Stx2a B subunit appeared to reach saturation at 1 µg/mL, whereas 2 µg/mL of the Stx1a B subunit produced a stronger cell-associated signal than its 1 µg/mL concentration. This observation might reflect the relatively higher affinity of Stx1a for both Gb3 and Gb4 in comparison to Stx2a: binding of the Stx2a B subunit may reach saturation when all Gb3 receptors are occupied, but the B subunit of Stx1a could bind additional Gb4 receptors in addition to the saturated Gb3 receptors. Our collective observations were consistent with reports that have documented a higher affinity for the interaction between Stx1a and cultured mammalian cell receptors when compared to Stx2a (131, 135, 137).
Competition assays further emphasized the different affinities of Stx1a and Stx2a for Vero cells. In this experiment, a fixed concentration of Stx1a (0.1 ng/mL) was mixed with one of two different concentrations of Stx2a (1 ng/mL and 10 ng/mL). Flow cytometry was then used to assess the effect on protein synthesis after 18 hours in the presence of Stx1a, Stx2a, or both. Cells exposed to Stx1a alone (red peak) exhibited a uniform drop in protein synthesis, whereas cells exposed to either concentration of Stx2a alone (orange and blue peaks) exhibited a bimodal response (Fig. 21A). These results were consistent with the data presented in Figure 18. Cells exposed to toxin mixtures consisting of a 10-fold (orange) or 100-fold (blue) excess of Stx2a when compared to Stx1a exhibited a uniform drop of protein synthesis that was similar to the fluorescent profile of cells exposed to Stx1a alone (Fig. 21B). Thus, Stx2a does not appear to effectively compete with Stx1a for binding to the target cell. Previous studies have documented the reduced potency of Stx2a in cell culture and animal models when mixed with the Stx1a B subunit (162), but our current work - which was only possible because of the different population responses to Stx1a vs. Stx2a - provides the first experimental evidence that Stx1a holotoxin can out-compete Stx2a holotoxin in a cell culture model of intoxication.
Vero cells were processed for cytofluorometry after a 30 minutes 4°C incubation with the fluorophore-labeled B subunits of Stx1a or Stx2a. The intensity of the Alexa Fluor® 594 dye indicates the extent of Stx binding. (A-B) Cells were incubated without toxin (black line) or in the presence of various quantities of either the Stx1a B subunit (A) or Stx2a B subunit (B): 0.2 μg (magenta line), 0.5 μg (yellow line), 1.0 μg (blue line), or 2.0 μg (red line), all in 500 µL volume. (C) The signals recorded for 2 μg of either the Stx1a B subunit (red line) or Stx2a subunit (blue line) were overlaid on the same plot. (D) The signals recorded for 0.2 μg of the Stx1a B subunit (red line) and 0.5 μg of the Stx2a B subunit (blue line) were overlaid on the same plot. One of three representative experiments is shown for the aggregate data.
Figure 21. Competition between Stx1a and Stx2a.

Vero-d2EGFP cells were processed for cytofluorometry after an 18-hour incubation with Stx1a and Stx2a alone (A), or as a mixture (B). The red peak in A denotes incubation of cells with 0.1 ng/mL of Stx1a, whereas the blue and orange peaks denote incubation of cells with 1 ng/mL and 10 ng/mL of Stx2a, respectively. In panel B, the orange peak represents incubation of cells with a mixture of 0.1 ng/ml Stx1a and 1 ng/ml Stx2a, whereas the blue peak represents incubation of cells with a mixture of 0.1 ng/ml Stx1a and 10 ng/mL Stx2a. Unintoxicated Vero-d2EGFP cells (green lines) were also processed for each condition.
The identity of the B pentamer is responsible, in part, for the bimodal response observed with Stx2 subtypes.

Our results suggested that the different cellular responses to Stx1a vs. Stx2a resulted from more Stx1a binding to the target cell (Fig. 20). In this model, the origin of the toxin B subunit may determine whether the cellular response to intoxication is uniform or bimodal. To test this model, we used two hybrid toxins which consisted of either the A₁ subunit from Stx2a with the A₂ and B subunits from Stx1a (Stx 211) or the A₁ subunit from Stx1a with the A₂ and B subunits from Stx2a (Stx 122) (Fig. 4). It has been shown that replacing the binding subunit of Stx2a with that of Stx1a results in an increase toxicity for Stx2a, whereas replacing the binding subunit of Stx1a with that of Stx2a results in a decrease in toxicity for Stx1a (139). The A₂ linker is matched to its cognate B subunit in these chimeric toxins because it is important for holotoxin stability (21, 22, 139). Both toxins were isolated from the cell extracts of transformed E. coli BL21(DE3)pLysS. The control experiment in Figure 22A demonstrates the cell extract from untransformed E. coli did not have an effect on protein synthesis when added to the culture medium of Vero-d2EGFP cells. Additional control experiments ensured cell extracts containing Stx1a holotoxin (Fig. 22B) or Stx2a holotoxin (Fig. 22C) produced the expected cellular responses that were previously documented using purified toxins (i.e., a uniform loss of protein synthesis in cells exposed to Stx1a and a bimodal response in cells exposed to Stx2a) (Fig. 18). Vero-d2EGFP cells challenged with the Stx 211 hybrid toxin exhibited a uniform downward shift in protein synthesis, similar to that of Stx1a holotoxin (Fig. 22D). In contrast, exposure to the Stx 122 hybrid toxin produced a bimodal response from the Vero-d2EGFP cells that was similar to the response elicited by Stx2a holotoxin (Fig. 22E). These results suggest the A₂ fragment and B subunit of a Stx contribute to the different responses between Stx1a and Stx2a as observed by flow cytometry, resulting in a uniform profile.
for toxins containing the B subunit of Stx1a and a bimodal profile for toxins containing the B subunit of Stx2a.
Figure 22. Cellular response to hybrid Stxs.

Vero-d2EGFP cells were processed for cyttofluorometry after an 18-hour incubation with 10-fold serial dilutions of cell extracts from a non-pathogenic (Stx) BL21 *E. coli* strain that was untransformed (A) or transformed with expression vectors encoding Stx1a holotoxin (B), Stx2a holotoxin (C), the 211 hybrid toxin consisting of the A1 subunit from Stx2a with the A2 and B subunits from Stx1a (D), or the 122 hybrid toxin consisting of the A1 subunit from Stx1a with the A2 and B subunits from Stx2a (E).
CHAPTER 5: DISCUSSION

AB toxins are so potent that a single molecule of cytosolic toxin is thought to be sufficient for the complete inhibition of protein synthesis and cell death. The supporting evidence for this model is based upon extrapolations from in vitro studies with toxin serial dilutions or kinetic analyses of intoxication (6, 86, 110). It would be nearly impossible to directly demonstrate that only one or a few toxin molecules are in the cytosol of a dead cell. As an alternative approach, we have shown cells with a toxin-induced inhibition of protein synthesis can, upon removal of sub-optimal toxin concentrations from the medium, survive the toxin challenge and restore normal levels of protein synthesis. Our results show that one molecule of Stx1a, ricin, EtxA, and Dtx is not sufficient to completely inhibit protein synthesis and lead to cell death. Moreover, we showed that cells can recover from intoxication. The key experiment involved a 20-hour toxin challenge and 48-hour recovery period (Fig. 15, 16). At least one molecule of toxin must have been present in the cytosol after 20 hours of intoxication in order to have an inhibitory effect on protein synthesis, yet the intoxicated cells survived, with normal levels of protein synthesis, 68 hours after the initial toxin challenge. We accordingly conclude that one or a few molecules of cytosolic toxin are not sufficient to kill the cell.

Catalytic toxin A chains were originally thought to be stable in the host cytosol (2, 112, 114, 163), although direct evidence for this assertion is lacking. Theoretically speaking, a single molecule of stable cytosolic toxin could, with time, block all protein synthesis and ultimately kill the cell. However, there are evidence suggesting that toxin A chains are not stable in the cytosol: several studies have directly or indirectly documented the proteasome-dependent degradation of cytosolic
The turnover of cytosolic toxin is apparently faster than the time required for a single toxin molecule to inactivate enough ribosomes for the cessation of protein synthesis and corresponding cell death. Hence, as demonstrated here, productive intoxication requires either ongoing toxin delivery to the cytosol or a large initial bolus of cytosolic toxin.

The proportionality model of intoxication suggests the cytosolic stability of the toxin A chain will directly impact the capacity for recovery from intoxication, which is consistent with our observations for Dtx. As shown in Figures 15 and 16, cells could only recover from a minimal initial inhibition of protein synthesis by this relatively stable toxin (115). Other reports further suggest the extent of intoxication is linked to the efficiency of toxin clearance from the cytosol. For example, cellular resistance to ricin or EtxA results from enhanced degradation of the cytosolic toxin (117-119). Conditions that impede the turnover of cytosolic toxin likewise generate cellular sensitivity to ricin or Stx1a (6, 116, 117, 119). In fact, new studies have shown a protective effect by ricin antibodies post-toxin exposure (164, 165). These collective observations thus suggest that the extent of intoxication is directly linked to how much toxin is in the cytosol, with the amount of cytosolic toxin representing a balance between toxin delivery to the cytosol and toxin removal from the cytosol.

Stx, ricin, and EtxA enter the host cell by receptor-mediated endocytosis and follow an inefficient transport to the ER before reaching their cytosolic targets. Dtx, on the other hand, enters the host cell and reaches its cytosolic target directly from the endosomes using a more efficient process. Thus, the faster delivery and stability of Dtx seem to correlate to higher amount of total cytosolic...
toxin, which in turn explain the higher cellular potency we observed for Dtx in comparison to Stx1a, ricin, and EtxA (Fig. 11, 14).

Our study does not minimize the extreme potency of AB toxins, but it does challenge the long-standing assertion that a single or few molecules of cytosolic toxin are sufficient to completely inhibit protein synthesis and kill the cell. As previously stated, this model was formulated before the proteasome-mediated clearance of cytosolic toxin was characterized. Cells treated with a proteasome inhibitor have been shown to be sensitized to Stx and other AB toxins (6, 116, 117, 166). Likewise, the accelerated degradation of cytosolic toxin produces a toxin-resistant phenotype (117, 118, 167-169). Because there is a lag between the inhibition of protein synthesis and cell death (Fig. 6, 17), the clearance of cytosolic toxin provides an opportunity to restore normal levels of protein synthesis and recover from transient exposure to sub-optimal toxin concentrations. Thus, conditions that prevent efficient and/or ongoing toxin delivery to the cytosol could allow a cell to withstand the effects of intoxication. This was demonstrated here by removing low levels of toxin from the medium. Such observations provide experimental support for the development of inhibitors and post-exposure therapeutics that restrict, but do not necessarily completely block, toxin delivery to the host cytosol. Experimental support for this model has been previously generated in cell culture using neutralizing antibodies against Stx2a or the plant toxin ricin (165, 170, 171). In these studies, cellular protection was observed under conditions that neutralize intracellular ricin as well as conditions that interfered with ricin and Stx2a transport.

Our observations also indicate the effective application of an anti-cancer immunotoxin (88, 112) will require either ongoing delivery or efficient initial delivery of the toxin A chain into the cytosol.
of a targeted cancer cell. Our results suggest that a threshold of cytosolic toxin is required to cause cell death. Furthermore, our observations indicate that the extent of intoxication represents a balance between toxin delivery to the cytosol and toxin clearance from the cytosol. Cells that were affected by at least one molecule of Stx1a, ricin, EtxA, and Dtx survived the toxin challenge and recovered from intoxication. Thus, these findings suggest that in order to elicit and maintain a desired toxic effect to kill a cancer cell, there must be enough toxin in the cytosol to overcome its constant turnover as well as compensatory host mechanisms such as the synthesis of new ribosomes. Overall, our work indicates that the development of effective immunotoxins may require repeated application and/or the use of more stable toxins.

Most quantitative assays that monitor the toxin-induced inhibition of protein synthesis average the results from a population of cells (89, 172). Using these methods, it is not possible to differentiate between intoxicated and unintoxicated cells within the population. As an alternative approach, we used flow cytometry in conjunction with a Vero-d2EGFP cell line that expresses an EGFP variant with a 2-hour half-life. This allowed us to record the toxin-induced inhibition of protein synthesis and resulting loss of EGFP fluorescence from individual cells in the population of toxin-challenged cells. Using our cytofluorometry-based system, we were able to differentiate the probability model from the proportionality model of intoxication. Our system monitored toxin activity in individual cells and allowed us to detect cells that were affected from those that were not. In our system, toxin-affected cells exhibit a lower fluorescence peak, whereas cells unaffected by toxin maintain a fluorescence peak similar to unintoxicated cells.
Our cytofluorometry-based system also allowed us to make a contribution to the understanding of the cellular basis for the different potencies of Stx1a and Stx2 subtypes. The factors contributing to the higher toxicity of Stx1 subtypes to Vero cells are poorly understood and/or unknown. All Vero-d2EGFP cells exposed to Stx1a were affected by the toxin and thus, exhibited a uniform drop in protein synthesis that was detected by the EGFP signal intensity. Even at the lowest concentration of Stx1a (0.001 ng/mL), a minor population-wide drop in fluorescent intensity was detected (Fig. 11B, 18A). In contrast, the bimodal fluorescent profile of Vero-d2EGFP cells exposed to Stx2a or Stx2c indicated a subpopulation of the toxin-challenged cells were completely resistant to these toxins and therefore maintained their full level of protein synthesis. This bimodal response to Stx2a or Stx2c has not been previously reported.

The uniform response to Stx1a and bimodal response to Stx2a could not be attributed to the lack of Gb3 receptor in a subpopulation of cells. However, the cells did bind more Stx1a than Stx2a. Stx1a could also out-compete Stx2a, as cells challenged with a combination of Stx1a and Stx2a exhibited a population-wide loss of protein synthesis even when there was a 100-fold excess of Stx2a over Stx1a. Additional studies with hybrid toxins indicated the uniform vs. bimodal response to intoxication is linked to the subtype of the B subunit, with the B subunit from Stx2a producing a bimodal response. Our collective results suggest the bimodal response to Stx2a or Stx2c involves (i) relatively weak binding between Stx2a and the host cell that reduces the total cell-associated pool of Stx2a in comparison to Stx1a, and (ii) the need to accumulate a substantial pool of cytosolic toxin to inhibit protein synthesis and induce cell death. These observations further suggest the inhibition of protein synthesis and resulting cell death from Stx2a or Stx2c requires a threshold concentration of cell-associated toxin, which is consistent with our conclusions for Aim II.
The number of Stx2-resistant cells decreased with increasing toxin concentration, which indicated there is no subpopulation of Vero-d2EGFP cells with intrinsic resistance to Stx2 subtypes. We did not observe a bimodal pattern for Gb3 content, so it is unlikely that the bimodal response to intoxication was due to a subpopulation of cells that lack Gb3. Instead, the relatively poor binding of Stx2a seemed to limit the amount of cell-associated toxin to the point where some cells were resistant to intoxication. In contrast, the relatively strong binding of Stx1a to its target cell ensured all cells in the population received an effective dose of cytosolic toxin.

Data gathered from various epidemiological studies indicate that patients carrying *E. coli* O157:H7 strains that only express Stx2a are more prone to develop HUS (125, 173-176). The factors contributing to the observed difference in potency are not fully understood. In an attempt to understand the differential potency between Stx subtypes, localization experiments have shown that Stx1a mostly accumulates in the lungs of mice, while Stx2a does not (177). It has been suggested that the lungs serve as a “sink” where Stx1a strongly binds to toxin-resistant cells, thereby preventing the toxin from reaching the more sensitive kidney cells. Moreover, Stx1a has been shown *in vivo* to protect mice intoxicated with Stx2a (178). The B subunit of Stx1a was also shown to reduce the potency of Stx2a in mice and the cytotoxicity of Stx2a to Vero cells (162). In our study, we further demonstrated that Stx2a could not effectively compete with Stx1a for intoxication of Vero-d2EGFP cells: cells exposed to a 1:100 mixture of Stx1a to Stx2a still produced the uniform loss of fluorescence characteristic of the Stx1a response. We believe that the low receptor affinity of Stx2 subtypes may be a factor that enables the toxin to escape lung cells. Moreover, it is possible that the higher catalytic activity of the Stx2a A subunit is a contributing factor to its higher potency in animal studies. The present study highlighting the role of the binding
subunit in the differential cellular toxicity of Stx subtypes provides additional evidence to better understand how Stx2a evades lung cells and how Stx1a suppresses the potency of Stx2a. Thus, these collective observations suggest that the binding subunit of Stx subtypes is an important factor that contributes to potency and disease pathogenicity.

As we have previously demonstrated with Stx1a and other AB toxins (Aim II), cell death is not an inevitable outcome of toxin binding or even toxin activity in the cytosol. Therapeutic approaches that involve toxin neutralization at a post-exposure stage could thus be effective strategies for the treatment of STEC infections. These strategies may be particularly effective against STEC strains that express a Stx2 subtype, as the dose-dependent bimodal response to Stx2a or Stx2c suggests it is possible to reduce the cell-associated pool of toxin to the point where cells are either completely resistant to intoxication or more prone to recover from a partial inhibition of protein synthesis. Toxin inactivation could involve the administration of inactive Stx1a variants that compete with Stx2a for binding to target cells. This approach has been shown to reduce the toxicity of Stx2a in mice (162, 178). Neutralizing antibodies could likewise reduce the amount of Stx2a that reaches the cytosol of host cells, either by blocking adherence to the target cell or disrupting intracellular toxin trafficking (179, 180).

Our data showing Stx1a is more potent against cultured cells than Stx2 subtypes were consistent with published work (130, 131, 155). Our observation that Stx2a binds to target cells with lower affinity than Stx1a is also consistent with the literature (130, 135, 136, 138). However, those previous studies averaged the results from an entire population of toxin-challenged cells and could not detect variable cellular responses within the population. The use of a cytofluorometry-based
intoxication system allowed us, for the first time, to document the bimodal population response to Stx2 subtypes that appears to result from the relatively low affinity of toxin binding to the cell surface. These findings provide a new basis to better understand the differential toxicity between Stx subtypes and establish a conceptual foundation for the development of post-exposure toxin therapeutics that function by lowering the amount of toxin that reaches the host cytosol.

In conclusion, we developed a new system to study the cellular potency of AB toxins that inhibit protein synthesis. Using the newly developed system, we showed that a single or a few molecules of AB toxin are not sufficient to completely inhibit protein synthesis and ultimately kill the host cell. Additionally, we showed for the first time that cells can recover from intoxication. Lastly, we showed that cells intoxicated with Stx2 subtypes exhibit a bimodal cellular response, while cells intoxicated with Stx Ia exhibit a uniform cellular response. Additional experiments on the different cellular response to Stx subtypes showed that the cellular response we observed is mainly due to the identity of the B-pentamer. Thus, this study provided a set of new evidence to help elucidate the cellular potency of a subset of AB toxins that inhibit protein synthesis.
APPENDIX A:
LIST OF PUBLICATIONS
Ph.D. publications


M.S. publication


Undergraduate publication

LIST OF REFERENCES


28. Office F. N. P. 2013. FBI Response to Reports of Suspicious Letters Received at Mail Facilities.


