A STRUCTURE-FUNCTION ANALYSIS OF SHIGA-LIKE TOXIN TYPE II OF ENTEROHEMORRHAGIC ESCHERICHIA COLI

1990

PERERA

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F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

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TEACHING HOSPITALS
WALTER REED ARMY MEDICAL CENTER
NAVAL HOSPITAL, BETHESDA
MALCOLM, GROW AIR FORCE MEDICAL CENTER
WILFORD HALL AIR FORCE MEDICAL CENTER

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Name of Candidate: Mr. Liyanage Perera
Doctor of Philosophy Degree
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Thesis and Abstract Approved:

Randall K. Holmes Committee Chairperson	7/2/90 Date
Susan K. Hoseth Committee Member	5/7/90 Date
C. D. Ross Committee Member	6/14/90 Date
Eleanor S. Metcalf Committee Member	7/2/90 Date
Alison D. O'Brien Committee Member	7/2/90 Date
Gregory P. Mueller Committee Member	7/5/90 Date

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Liyanage Perera
Department of Microbiology
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Dissertation: A Structure-Function Analysis of Shiga-Like Toxin Type II of Enterohemorrhagic *Escherichia coli*

Liyanage Parakrama Perera; Candidate, Doctor of Philosophy, 1990

Dissertation directed by: Alison D. O'Brien, Ph.D., Professor, Department of Microbiology

Shiga toxin of *Shigella dysenteriae* I and Shiga-like toxins I and II (SLT-I and SLT-II) of enterohemorrhagic *Escherichia coli* are functionally similar, protein cytotoxins. These toxin molecules have a bipartite molecular structure which consists of an enzymatically active A subunit that inhibits protein synthesis in eukaryotic cells and an oligomeric B subunit that binds to globotriosylceramide glycolipid receptors on eukaryotic cells.

The first objective of the project was to generate monoclonal antibodies against SLT-II and to investigate the molecular basis for antigenicity and toxin neutralization. Monoclonal antibodies were generated against a crude preparation of SLT-II produced by an *E. coli* K-12 strain lysogenized with the 933W toxin-converting phage of enterohemorrhagic *E. coli* 933 strain. Five monoclonal antibodies were isolated that were capable of neutralizing the cytotoxicity of SLT-II, but not SLT-I, Shiga toxin, or a variant of SLT-II produced by *E. coli* that causes edema disease of swine. All five monoclonal antibodies immunoprecipitated the isolated A subunit of SLT-II but not the B subunit. Of these five neutralizing monoclonal antibodies, four were of the immunoglobulin M class and one belonged to the immunoglobulin G_1 subclass. All five monoclonal antibodies had κ light chains.

To identify the epitopes of SLT-II reactive with the monoclonal antibodies, a

sublibrary of the *slt*-II gene was created in λ ZAP expression vector, and the recombinant phage library was screened with individual SLT-II neutralizing monoclonal antibodies. The sequences of the DNA inserts that expressed the immunoreactive epitopes contained *slt*-II gene segments larger than 1 kb in size. Therefore, sequence analysis of a larger pool of immunoreactive clones may be needed to identify the minimal coding region of each epitope.

The second objective of the project was to identify amino acid residues in the B subunit of Shiga toxin and SLT-II that are critical for either the structural or functional integrity of the holotoxin molecule. Regionally-directed, sodium bisulfite mutagenesis of the B subunit of SLT-II was used to isolate non-cytotoxic mutants. Three non-cytotoxic mutants were isolated and the responsible mutations mapped. The substitution of arginine for cysteine at codon 32, alanine for threonine at codon 42, and glycine for aspartic acid at codon 59 of the 70 amino acid mature SLT-IIB polypeptide resulted in complete abolition of cytotoxicity. Because the homologous arginine, alanine, and glycine residues are conserved at codons 33, 43, and 60 of the 69 amino acid mature B polypeptide of Shiga toxin, comparable mutations were induced in the B subunit gene of Shiga toxin by oligonucleotide-directed, site-specific mutagenesis. These mutations resulted in drastically decreased cytotoxicity (10^3 to 10^6 fold) compared to wild type Shiga toxin. The mutant SLT-II and Shiga toxin B subunits were characterized for stability, receptor binding, immunoreactivity, and the ability to be assembled into holotoxin.

The third objective of the project was to map the minimal contiguous coding segment of the *slt*-II gene required for holotoxin activity of the SLT-II molecule. The C-terminal (3' end) and N-terminal (5' end) functional boundaries of the SLT-II were mapped by creating specific deletions in the operon. Removal of six bases from the 3' end of *slt*-II did not affect the cytotoxic activity of SLT-II, whereas removal of twelve bases from the 3' end of *slt*-II resulted in complete ablation of cytotoxic activity. An "in-frame" deletion mutant which lacked the coding region between the 3rd and 18th

codons of the mature A polypeptide was devoid of any enzymatic activity. This finding indicates that the N-terminal amino acids in the SLT-IIA subunit are required for enzymatic activity or for the proper folding of the SLT-IIA subunit into an enzymatically active polypeptide.

A STRUCTURE-FUNCTION ANALYSIS OF SHIGA-LIKE TOXIN TYPE II OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI*

by

Liyanage Parakrama Perera

Dissertation submitted to the Faculty of the Department of Microbiology
Graduate program of the Uniformed Services University of the
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requirements for the degree of
Doctor of Philosophy 1990

Dedication

This thesis is dedicated to my wife Pin Yu whose love, faith and encouragement have been the mainstay of my existence.

Acknowledgement

It is with great pleasure I thank Dr. **Alison D. O'Brien** for her supervision of this work. I owe an unending debt of gratitude to my thesis advisor, Dr. Alison D. O'Brien for guidance and kindness both regarding work and otherwise. I also wish to express my appreciation and thanks to my thesis advisory committee, Drs. **Susan Hoiseth, Randall Holmes, Lee Metcalf, Greg Mueller** and **Paul Rick** for their advice and continued interest throughout this study. I am deeply indebted to Dr. **Stefanie Vogel** for her support and kindness both regarding work and otherwise. I am thankful to Dr. **Kay Holmes** for initiating me into the mysteries of electron microscopy.

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I cannot express the warmth of my gratitude to my wife **Pin Yu** with any adequacy for enduring my seeming lack of direction and almost crazy hours of work and for providing a cheerful understanding atmosphere, the environment in which this task was completed.

TABLE OF CONTENTS

Section Titles and Subtitles	Page
INTRODUCTION	1
General overview of Shiga toxin of *Shigella dysenteriae* I	1
The structure, genetics and regulation of Shiga toxin expression	4
Mode of action of Shiga toxin	5
Role of Shiga toxin in disease pathogenesis	13
General overview of Shiga-like toxins (Verocytotoxins) of *E. coli*	14
Structure, genetics and regulation of Shiga-like toxins of *E. coli*	16
Mode of action of Shiga-like toxins of *E. coli*	20
Role of Shiga-like toxins in disease	20
Structure-function relationships of Shiga and Shiga-like toxins	21
OBJECTIVES OF THE PROJECT AND STUDY PLAN	22
Objectives	22
Study plan	22
MATERIALS AND METHODS	23
Media, Enzymes, and radionuclides	23
Preparation of crude SLT-II	23
Preparation of SLT-II toxoids	24
Production of monoclonal antibodies (MAbs) to SLT-II	24
Hybridoma growth in mice	25
ELISA for screening SLT-II specific monoclonal antibodies	25
Radioimmunoprecipitation	26
Western blot (immunoblot) analysis	27

Section Titles and Subtitles	Page
Separation of A and B subunits of SLT-II	28
Cytotoxicity and neutralization assays	28
Epitope mapping	29
Receptor-analogue ELISA	30
Preparation and transformation of competent bacteria	31
Plasmid constructions	32
Construction of gapped duplex DNA for mutagenesis	34
Sodium bisulfite mutagenesis of gapped duplex DNA	40
DNA sequencing	40
Preparation of single strand DNA templates for *in vitro* mutagenesis	41
Oligonucleotide-directed, site-specific *in vitro* mutagenesis	42
Dot blot ELISA	44
Thin layer chromatogram overlay assay	45
Immunofluorescence assay	46
Pulse-chase analysis of toxin mutants	47
Inoculation of mice and rabbits	47
Bal-31 exonuclease deletion of *slt*-II gene	48
Colony screening by oligonucleotide probe hybridization	49
In vitro translation assay	50
RESULTS	52
I. Generation and characterization of SLT-II specific monoclonal antibodies	52
A. Production of MAb to SLT-II	52
B. Characterization of monoclonal antibodies	52
C. The mapping of epitopes reactive with SLT-II specific monoclonalantibodies	53

Section Titles and Subtitles	Page
II. Mutational analyses of the B subunits of SLT-II and Shiga toxin	54
A. Sodium bisulfite mutagenesis of gapped duplex DNA to isolate noncytotoxic mutants	54
B. Mapping of mutations by sequence analysis	66
C. Mutational effects on the conformation of toxin molecules as determined by dot blot ELISA	67
D. Mutational effects on receptor-analogue binding	73
E. Mutational effects on natural receptor recognition as determined by thin layer chromatogram overlay assay	74
F. Competitive inhibition of cytotoxicity by soluble receptor-analogue to determine the receptor-analogue binding ability of Shiga A43T mutant	74
G. Mutational effects on binding of toxins to Vero cells as determined by immunofluorescence assay	85
H. Intracellular degradation of Shiga toxin mutants	88
I. Animal inoculations to determine the immunogenicity of the SLT-II mutants	88
III. Deletion analysis of SLT-II to determine the minimal contiguous gene segment required for cytotoxic activity of the holotoxin	96
A. Coarse mapping of the carboxy-terminal functional boundary of SLT-II	96
B. Fine mapping of the carboxy-terminal functional boundary	97
C. Mapping of the functional boundary from the N-terminus of SLT-II	98
DISCUSSION	106
Generation and characterization of monoclonal antibodies to SLT-II	106
Mutational analyses of the B subunit of SLT-II and Shiga toxin	108

Section Titles and Subtitles	Page
Deletion mapping of SLT-II	112
SUMMARY	116
BIBLIOGRAPHY	118

LIST OF TABLES

Table		Page
1.	Sensitivity of different cell lines to Shiga toxin	2
2.	Characteristics of Shiga toxin and Shiga-like toxins	17
3.	Genotypes of the *E. coli* host strains used	33
4.	Description of primary plasmid constructs used in the study	39
5.	Characterization of SLT-II specific MAbs	55
6.	Vero cell cytotoxicity and immunoreactivity of the SLT-II and Shiga toxin mutants	68
7.	Binding of SLT-II mutants to receptor analogue, G_{b3} glycolipid and Vero cells	91
8.	Competitive inhibition of HeLa cell cytotoxicity of Shiga toxin and Shiga A43T mutant by galabiose-derivatized Sepharose beads	92
9.	Immunogenicity and lethality of SLT-II mutants	95
10.	Characteristics of Bal 31 deleted SLT-II mutants	99
11.	Cytotoxicity and immunoreactivity of SLT-II deletion mutants	103

LIST OF FIGURES

Figure		Page
1.	Model for the receptor-mediated endocytic entry of Shiga toxin and the processing of Shiga toxin in a mammalian cell	10
2.	Specific nucleotides of 28S rRNA attacked by Shiga toxin, SLT-I, SLT-II, SLT-IIv and plant toxins ricin and α-sarcin	12
3.	A working model of the organization and expression of *slt*-I operon	19
4.	Schematic map of plasmid pLP15	36
5.	Construction of plasmid pLPSH3	38
6.	Immunoprecipitation of ^{125}I-labelled crude SLT-II by MAbs	57
7.	Immunoprecipitation of ^{125}I-labelled crude SLT-II by MAbs after urea treatment and fractionation	59
8.	Schematic outline of epitope mapping using λ ZAP vector	61
9.	Screening of recombinant λ ZAP library with SLT-II specific MAbs	63
10.	Schematic outline of the method for generating gapped duplex DNA molecules for single-strand-specific bisulfite mutagenesis and enrichment for mutant selection	65
11.	Dot blot ELISA with SLT-II mutants	70
12.	Dot blot ELISA with Shiga toxin mutants	72
13.	Receptor-analogue ELISA for Shiga toxin	76
14.	Receptor-analogue ELISA for SLT-II	78
15.	Competition ELISA to assess the specificity of binding of Shiga toxin to the receptor analogue	80
16.	Binding of Shiga toxin mutants to receptor-analogue detected with MAb 13C4	82

Figure **page**

17. Binding of Shiga toxin mutants to receptor-analogue detected with MAb 4F7 84

18. TLC-overlay assay to detect binding of Shiga toxin mutants to G_{b3} and G_{b4} glycolipids 87

19. Binding of Shiga toxin mutants to Vero cells as detected by immunofluorescence assay using the Anchored Cell Analysis Station 470 90

20. Pulse-chase analysis of intracellular degradation of Shiga toxin mutants 94

21. TLC overlay assay to detect binding of SLT-II T66 and SLT-II T68 to glycolipids extracted from Vero and HeLa cells 101

22. Inhibition of protein synthesis in an *in vitro* translation system by SLT-II mutants 105

INTRODUCTION

General overview of Shiga toxin of *Shigella dysentriae* I

Shiga toxin is a potent cytotoxin produced by *Shigella dysenteriae* type 1 and was first described by Conradi in 1903. All wild-type strains of *Shigella dysenteriae* type I (Shiga's bacillus) tested to date have been found to elaborate high levels of Shiga toxin (O'Brien *et al.*, 1987; Prado *et al.*, 1986). High level Shiga toxin production is unique to *Shigella dysenteriae* type I, since neither the other serotypes (serotypes 2-15) of *Shigella dysenteriae* nor the other species of *Shigella* (*S.flexneri, S.boydii and S.sonnei*) produce high levels of Shiga toxin (Keusch and Jacewicz, 1977; O'Brien *et al.*, 1977).

Early experiments done by Conradi (1903) as well as Neisser and Shiga (1903) demonstrated that intravenous inoculation of autolysates of Shiga's bacillus (*Shigella dysenteriae* type I) killed rabbits following a prodromal phase of limb paralysis. Subsequent studies by Cavanagh *et al.* (1956) showed that various laboratory animal species manifest differential susceptibilities to the lethal effects of Shiga toxin, although only rabbits and mice display neurological symptoms. The neurological symptoms associated with Shiga toxin are believed to be secondary to its effects on the vascular system of the brain and spinal cord (Howard, 1955; Stulc, 1967). Although Wiley *et al.* (1985) reported that purified Shiga toxin can be axonally transported to rat vagal sensory neurons with subsequent killing of those neurons, other workers have not been able to substantiate this finding (J.E.Brown, W.H.Habig, J.G.Kenimer and M.C.Hardegree, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B109, p. 36). Hence, Shiga toxin is not currently classified as a neurotoxin. The cytotoxic activity of Shiga toxin was first reported by Lasfarques and Delaunay (1946). Presently, there are many established cell lines of human origin that exhibit some degree of sensitivity to the cytotoxic activity of Shiga toxin (see Table 1). By contrast, with the exception of Vero cells and LLC-MK_2 cells (Giugliano *et al.*, 1982), no animal cell lines have been

Table 1. **Sensitivity of different cell lines to Shiga toxin**[a]

Cell line	Tissue of origin	ID_{50}[b] ng/ml
$HeLa\ S_3$	Human cervical carcinoma	0.08
HeLa	Human cervical carcinoma	100,000
NHIK 3025	Hybrid of human cervical carcinoma and HeLa	4.0
EJ	Human bladder carcinoma	6.5
T 24	Human bladder carcinoma	2,000
B	Human melanoma	>10,000
V.N	Human melanoma	>10,000
FME	Human melanoma	>10,000
B.L.	Human melanoma	>10,000
A 375	Human melanoma	>1,000
Hep_2	Human laryngeal carcinoma	>10,000
A lab	Human mammary cancer	1,000
Detroit 562	Human pharyngeal cancer	4.5
HT 29	Human colonic cancer	10.0
A 549	Human lung epithelial cells	>10,000
En.	Human lung sarcoma	>10,000
2T	Human osteosarcoma	>10,000
Fibroblasts	Human skin	>10,000
Macrophages	Human blood	>10,000
Lymphocytes	Human blood	>10,000
Epithelium	Human skin	30.0

Table 1 (continued)

HE	Human embryo	>10,000
Vero	African green monkey kidney	0.6
L	Murine fibroblasts	>10,000
Schou	Murine fibroblasts	>10,000
MC F7	Rat mammary cancer	>10,000
MH ICI	Rat hepatoma	>10,000
Eveline	Mouse spleen	>1,000
Hepatocytes	Rat liver	>1,000
CHO	Chinese hamster ovary	>10,000
BHK	Baby hamster kidney	10,000
Lewis lung	Mouse carcinoma	>10,000

---

[a] Modified from Eiklid and Olsnes, 1980.

[b] The concentration of partially purified toxin which reduces protein synthesis in the cells to half the control value after 18 hours.

documented to be sensitive to Shiga toxin.

The enterotoxic activity of a partially purified preparation of Shiga toxin was first reported by Keusch *et al.* (1970) in ligated rabbit ileal segments. Following purification of the Shiga toxin to homogeneity by several laboratories (O'Brien *et al.*, 1980; Olsnes and Eiklid, 1980; Yutsudo *et al.*, 1986), it was indisputably proven that the paralytic-lethal, cytotoxic, and enterotoxic activities ascribed for extracts or culture filtrates of Shiga's bacillus were actually due to Shiga toxin (O'Brien and LaVeck, 1982; Brown *et al.*, 1982; Eiklid and Olsnes, 1983).

The structure, genetics and regulation of Shiga toxin expression.

Shiga toxin is a cell-associated toxin which is elaborated from the periplasmic space of the bacteria into the culture medium after cell death (Okell and Blake, 1930; McIver *et al.*, 1975). The Shiga toxin, like many other bacterial toxins, is a bipartite toxin which consists of an enzymatically active A subunit (M_r ~ 32,200) and a pentameric B subunit (M_r ~ 7691). The B subunit binds to receptors on the eukaryotic cell surface which facilitates the entry of the enzymatically active A subunit into the target cells.

The genes that encode the Shiga toxin, designated *stx*, appear to be located on the chromosome in close proximity to *pyrF* (Timmis *et al.*, 1985). Presently, the copy number of the *stx* gene in *Shigella dysenteriae* type I remains controversial (Timmis *et al.*, 1985; Kozlov *et al.*, 1988), although Strockbine *et al.* (1988) who cloned and sequenced the structural genes of Shiga toxin from total cellular DNA of *S. dysenteriae* type I strain 3818T detected only one copy of *stx* gene in *S. dysenteriae* I strain 3818T. As reported by Strockbine *et al.* (1988), the Shiga toxin genes, *stxA* and *stxB*, are tandemly arranged in an operon in which *stxA* is located 5' to *stxB*. The *stx* operon is translated in-frame with an untranslated region of 12 nucleotides separating *stxA* and *stxB*. In the model proposed by Strockbine *et al.* (1988), the individual subunits are

synthesized from a polycistronic mRNA that is regulated by a promoter 5' to *stxA*. The existence of individual ribosome-binding sites for *stxA* and *stxB* may be responsible for the differential translation of A and B subunits to form the holotoxin with a subunit ratio of 5B:1A (Donohue-Rolfe *et al.*, 1984). In contrast to the model proposed by Strockbine *et al.* (1988), Kozlov *et al.* (1988) presented evidence for two types of transcriptional products from the Shiga toxin operon: a larger mRNA transcript with the capacity to encode both A and B polypeptides and a smaller, more efficiently transcribed mRNA that encodes the B polypeptide.

The production of Shiga toxin is regulated by the iron concentration in the growth medium (van Heyningen and Gladstone, 1953; McIver *et al.*, 1975), such that considerably more toxin is made under low iron conditions. The mechanism by which this regulation occurs is not completely understood (see iron regulation of SLT-I expression). However, the production of a number of other bacterial toxins is similarly regulated by iron, including diphtheria toxin (Murphy *et al.*, 1976) and *Pseudomonas* exotoxin A (Frank and Iglewski, 1988). The growth temperature also appears to affect Shiga toxin production (Weinstein *et al.*, 1988), although the mechanisms involved in the thermoregulation of Shiga toxin expression are unclear.

Mode of action of Shiga toxin.

There is increasing evidence that protein-exotoxins play a pivotal role in disease pathogenesis in a number of bacterial infections in man and animals (Berkowitz, 1989). At present, the mode of action of many of these bacterial exotoxins is not well understood. In a recent review, Berkowitz (1989) categorized the microbial exotoxins with known mechanisms of action into 4 groups. Group I included those toxins that cause damage to cell membranes, such as phospholipases and thiol-activated cytolysins. Group II included those toxins that cause tissue damage by lysis of connective tissues. Group III included those toxins that cause cell damage by interfering with protein synthesis.

Group IV included those toxins that interfere with cellular functions.

A bacterial exotoxin reaches its target by a sequence of events, beginning with its attachment to the surface of the target cell. Surface binding of the toxin is followed by internalization, attachment to a subcellular target molecule, and, finally, exertion of a biologic effect. In general, bacterial exotoxins bind to cell surface receptors on sensitive cells in a manner analogous to interactions of protein hormones and other bioactive macromolecules with cellular receptors. It seems unlikely that mammalian cells possess "suicide receptors" for bacterial toxins and, therefore, it is thought that the receptors utilized by bacterial toxins under normal conditions are those utilized by physiologic molecules such as hormones (Middlebrook and Dorland, 1984). The majority of the bacterial toxin receptors which have been characterized are found to be carbohydrate-containing macromolecules, either glycoproteins or glycolipids. In the case of many toxins, the carbohydrate component itself is intimately involved in toxin binding [*eg.* cholera toxin, *E. coli* LT (heat labile toxin), tetanus toxin, and botulinum toxin], but in a few cases, the role of the carbohydrate moiety is less well defined [*eg.* diphtheria toxin, *Pseudomonas* exotoxin A, pertussis toxin, and *E. coli* ST (heat stable toxin) (Eidels *et al.*, 1983)]. The toxin is internalized after the receptor-binding domain interacts with the receptor. When toxin internalization occurs by the process of receptor-mediated endocytosis, as is the case for diphtheria toxin and *Pseudomonas* exotoxin A (Middlebrook and Dorland, 1984), the toxin-receptor complex is internalized as an endocytic vacuole. The mechanism by which the bioactive portion of the toxin is released from the endosome into the cytosol is unclear. There is evidence, however, to suggest that in the case of botulinum, diphtheria, and tetanus toxins, the amino terminus of the heavy chain (binding domain) induces channels in the membranes which might allow the light chain (toxic domain) to pass from the endosome into the cytosol (Hoch *et al.*, 1985). In the case of Shiga toxin, the pentameric B subunit mediates toxin binding to susceptible eukaryotic cells. Monoclonal antibodies directed

against the B subunit of Shiga toxin can effectively inhibit the binding of Shiga toxin to cells (Donohue-Rolfe *et al.*, 1984). In addition, it has been demonstrated that purified B subunits of Shiga toxin can competitively inhibit the binding of holotoxin to HeLa cells (Donohue-Rolfe *et al.*, 1989), a finding which supports the idea that the receptor-binding domain of the Shiga toxin is entirely composed of the B subunit of the holotoxin.

The biochemical characterization of the cellular receptor for Shiga toxin has been an active area of research (Keusch and Jacewicz, 1977; Eiklid and Olsnes, 1980; Keusch *et al.*, 1986; Lindberg *et al.*, 1987). Recent evidence indicates that there are two types of receptors for Shiga toxin on eukaryotic cells. One putative receptor is a glycoprotein that contains N-acetyl-D-glucosamine oligomers (Keusch and Jacewicz, 1977; Keusch *et al.*, 1986) and is sensitive to proteolytic enzymes and lysozymes. The binding of Shiga toxin to this glycoprotein receptor is competitively inhibited by N,N',N"-triacyl chitotriose and wheat germ agglutinin (Keusch and Jacewicz, 1977). The glycoprotein receptor is now believed to be a low-affinity, high-capacity type receptor for Shiga toxin (Lindberg *et al.*, 1987; Jacewicz *et al.*, 1989) and is presumed to be present on both Shiga toxin sensitive as well as resistant cell lines. The existence of the glycoprotein receptor may help explain the comparable binding of Shiga toxin to both sensitive and resistant cells (Eiklid and Olsnes, 1980). The second receptor is a glycolipid, globotriaosylceramide (Gb_3), which binds Shiga toxin with high affinity. This glycolipid receptor is considered to be the functional receptor for Shiga toxin (Lindberg *et al.*, 1987). The presence of this glycolipid receptor on cells correlates well with sensitivity to Shiga toxin (Lindberg *et al.*, 1987; J. E. Samuel and V. Tesh, personal communication). The critical determinant which directly interacts with the oligomeric B subunit of the Shiga toxin is the carbohydrate moiety Galα1-4Gal (galabiose). Although the galabiose moiety is present in both glycoproteins and glycolipids, according to Lindberg *et al.* (1987), the glycolipid offers a more optimal conformational fit in multivalent toxin-receptor interactions because of the unhindered

lateral mobility of the membrane glycolipids.

The internalization of the Shiga toxin occurs via a receptor-mediated endocytotic process (Keusch, 1981) as illustrated in Figure 1. Sandvig *et al.* (1989) recently demonstrated that endocytosis of receptor-bound Shiga toxin occurs from coated pits, and, thus, Shiga toxin appears to be the first example of a lipid-binding ligand that is endocytosed from coated pits. In that same study, these workers presented evidence that the endocytosed Shiga toxin is first transported to the Golgi apparatus, and then the A subunit is translocated to the cytosol from the trans-Golgi vesicle. The plant toxin ricin is another toxin known to be translocated via trans-Golgi vesicles, even though the initial endocytic pathways are different for Shiga toxin and ricin (Moya *et al.*, 1985). It has been reported that the A subunit of Shiga toxin is activated by a specific reductive cleavage mechanism into an A_1 fragment before translocation from the endocytic compartment into the cytosol (Reisbig *et al.*, 1981). Recent evidence indicates that reductive proteolytic cleavage or nicking may not be essential for the expression of the enzymatic activity of the A subunit, based on studies done in *in vitro* and *in vivo* translation systems (Donohue-Rolfe *et al.*, 1989; Saxena *et al.*, 1989). However, it is still possible that translocation of the A subunit into the cytosol mandates prior reductive cleavage.

The A subunit of Shiga toxin is an RNA *N*-glycosidase with a substrate specificity identical to that of the plant toxin ricin (Endo *et al.*, 1988). The A subunit of Shiga toxin catalytically inactivates the 60S subunit of mammalian ribosomes leading to the inhibition of protein synthesis and subsequent cell death. Both Shiga toxin and ricin depurinate a specific adenine residue near the 3' end of 28S rRNA. The cleavage site is within an evolutionarily highly conserved region of 14 nucleotides in which only a single base substitution is seen in ribosomes of *E. coli* to ribosomes of the mammalian rat (Figure 2). However, neither Shiga toxin nor ricin affect *E. coli* ribosomes or plant

Figure 1. Model for the receptor-mediated endocytic entry of Shiga toxin and the processing of Shiga toxin in a mammalian cell (taken from O'Brien and Holmes, 1987). The B subunit of the toxin binds to the mammalian cell receptor. The clathrin-coated pit is pinched off and the coated vesicle is formed. The fusion of lysosomes with the coated vesicle may lead to reductive proteolytic cleavage of the toxin, and the generation of enzymatically active A_1 fragment of Shiga toxin. The mechanism by which the A_1 fragment is released into the cytosol is currently unknown. The A_1 fragment within the cytosol binds to the 60S ribosome leading to the inhibition of protein synthesis and cell death.

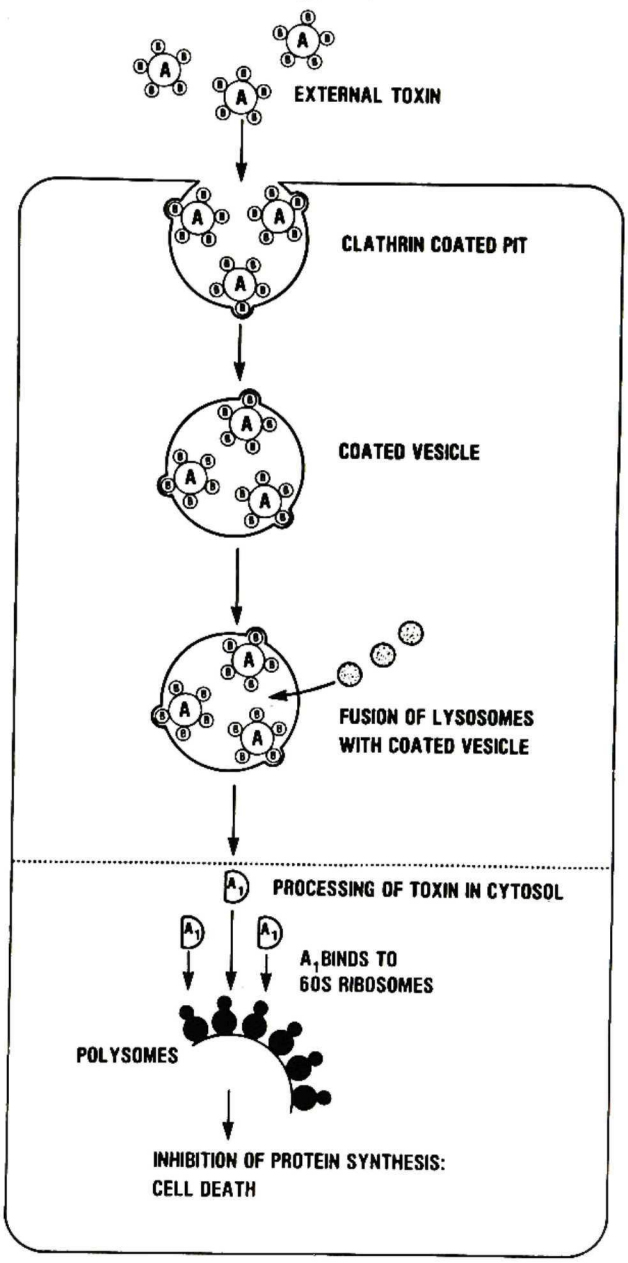
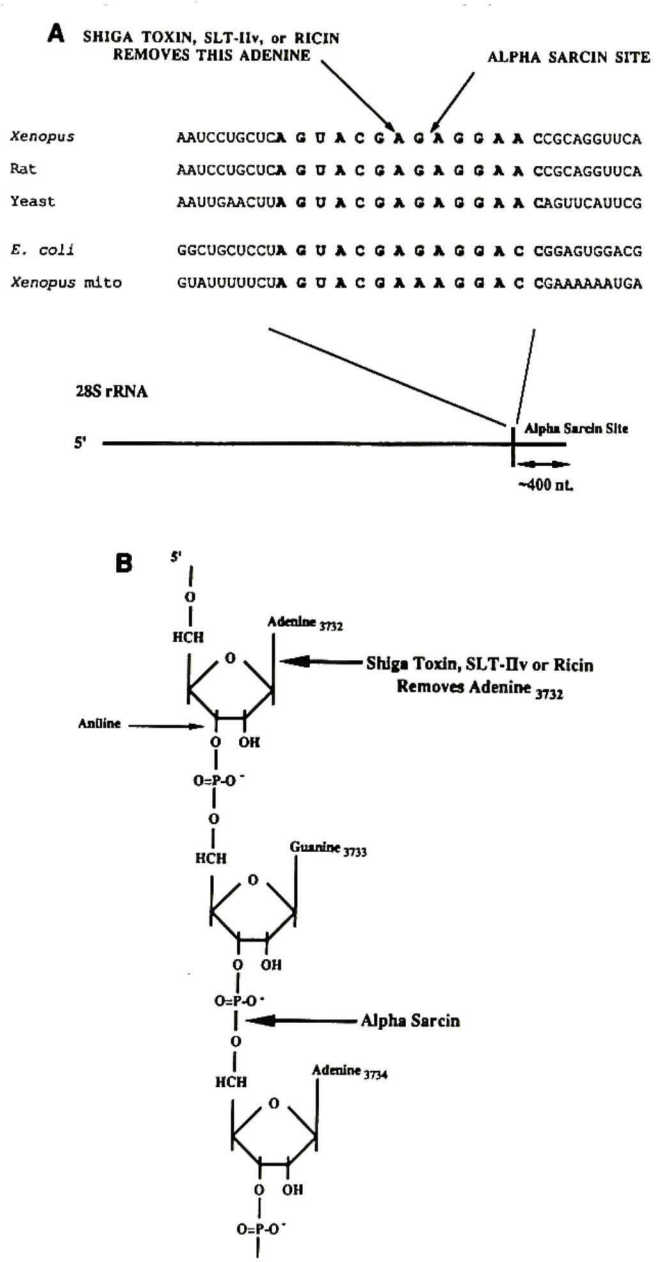

A
EXTERNAL TOXIN
CLATHRIN COATED PIT
COATED VESICLE
FUSION OF LYSOSOMES
WITH COATED VESICLE
A_1
PROCESSING OF TOXIN IN CYTOSOL
A_1 BINDS TO
60S RIBOSOMES
POLYSOMES
INHIBITION OF PROTEIN SYNTHESIS:
CELL DEATH

Figure 2. Specific nucleotides of 28S rRNA attacked by Shiga toxin, SLT-I, SLT-II, SLT-IIv and plant toxins ricin and α-sarcin (taken from Saxena *et. al.* 1989). **A.** Indicates the evolutionarily conserved 14 nucleotides comprising the α-sarcin domain in the 28S rRNA and the cleavage sites for α-sarcin, Shiga toxin, SLT-IIv and ricin. The cleavage sites of SLT-I and SLT-II are also identical to the cleavage site of ricin. (Endo *et al.* 1988). **B.** Ricin, Shiga toxin, and SLTs of *E. coli* are specific *N*-glycosidases for 28S rRNA. These toxins specifically remove the adenine adjacent to the α-sarcin cleavage site. The α-sarcin site in *Xenopus* 28S rRNA is at G3733, the ricin site is A3732 (the analogous sites in the 28S rRNA of the rat are G4223 and A4324). The removal of these bases by the toxins render the phosphodiester bond vulnerable for hydrolysis by aniline treatment.


A
SHIGA TOXIN, SLT-IIv, or RICIN
REMOVES THIS ADENINE
ALPHA SARCIN SITE
Xenopus AAUCCUGCUCA G U A C G A G A G G A A CCGCAGGUUCA
Rat AAUCCUGCUCA G U A C G A G A G G A A CCGCAGGUUCA
Yeast AAUUGAACUUA G U A C G A G A G G A A CAGUUCAUUCG
E. coli GGCUGCUCCUA G U A C G A G A G G A C CGGAGUGGACG
Xenopus mito GUAUUUUUCUA G U A C G A A A G G A C CGAAAAAAUGA
28S rRNA
5'
Alpha Sarcin Site
~400 nt.
B
5'
HCH
Adenine 3732
Shiga Toxin, SLT-IIv or Ricin
Removes Adenine 3732
Aniline
OH
O=P-O
Guanine 3733
Alpha Sarcin
Adenine 3734

ribosomes (Stirpe and Barbieri, 1986; O'Hare *et al.*, 1987). Another RNA *N*-glycosidase cytotoxin, α-sarcin from the mould *Aspergillus giganteus,* cleaves a specific guanine residue within the same conserved 14 nucleotide region and affects both prokaryotic and mammalian ribosomes (Endo and Wool, 1982).

Role of Shiga toxin in disease pathogenesis.

Since its discovery, Shiga toxin has been implicated in many of the clinical manifestations of shigellosis, and attempts have been made to generate toxoid vaccines to prevent shigellosis in humans (Farrel *et al.*, 1944). At present, no definitive proof exists to indicate that Shiga toxin functions as a major virulence factor in the pathogenesis of shigellosis. However, accumulating evidence suggests that Shiga toxin may contribute to the severity of the disease. Levine and coworkers (1973) reported that human volunteers fed an invasive but low-toxin producing, chlorate resistant mutant of *S. dysenteriae* I developed less severe disease than the volunteers fed an invasive, highly toxinogenic parent strain. The enterotoxic activity of the Shiga toxin, as determined by the ligated rabbit ileal loop model (Keusch *et al.*, 1972), has been implicated in the prodromal diarrheic phase of shigellosis. Keenan *et al.* (1986) demonstrated that purified Shiga toxin selectively destroys the mature absorptive epithelial cells of the rabbit ileum. In a recent study, Kandel *et al.* (1989) reported that Shiga toxin selectively inhibits NaCl absorption by villus cells without altering active anion secretion by crypt cells. This inhibition of absorption leads to fluid accumulation in the rabbit jejunum. The cytotoxic activity of Shiga toxin for primary human colonic epithelial cells, as demonstrated by Moyer *et al.* (1987), led to the speculation that Shiga toxin may play a role in the dysenteric phase of the disease. Fontaine *et al.* (1988) investigated the role of Shiga toxin in the pathogenesis of bacillary dysentery using a tox$^-$ isogeneic mutant of *S. dysenteriae* I in monkeys and concluded that Shiga toxin contributes to the severity of dysentery by inducing colonic

vascular damage. The observation that Shiga toxin is cytotoxic for primary endothelial cells from umbilical veins (Obrig *et al.*, 1987) supports the hypothesis that Shiga toxin has a direct effect on endothelial cells of the microvasculature of the kidneys and is responsible for the hemolytic uremic syndrome seen in children following shigellosis.

General overview of Shiga-like toxins (Verocytotoxins) of *E. coli*.

In 1977, Konowalchuk *et al.* reported the discovery of a new cytotoxin from *E. coli*, and these workers named the newly discovered toxin as verocytotoxin (VT) based on its irreversible cytopathic effect on Vero cells. Further characterization of VT revealed its enterotoxic activity (Konowalchuk *et al.*, 1978), and this prompted more extensive studies to investigate the prevalence of VT-producing *E. coli* (VTEC) in diarrheic stools of man and animals (W.G.Wade, B.T.Thom and N.Evans, Letter, Lancet ii: 1235,1979; Kashiwazaki *et al.*, 1980; Scotland *et al.*, 1980). [However, it should be noted that the enterotoxic activity of the toxin which subsequently became known as VT had earlier been demonstrated by Smith and Lingood (1971)]. The immunological cross-reactivity, as well as similar biological activities between VT of *E. coli* and Shiga toxin of *Shigella dysenteriae* I (O'Brien *et al.*, 1982), led Strockbine and coworkers (1986) to propose a new nomenclature, namely Shiga-like toxin (SLT) for the Verocytotoxin of *E. coli*. Currently, both terms, Verocytotoxin and Shiga-like toxins, are used synonymously.

In early studies, it became apparent that most of the *E. coli* strains isolated from humans that elaborated SLT belonged to classic enteropathogenic *E. coli* serogroups (EPEC), and these EPEC strains did not produce either heat labile toxin (LT) or heat stable toxin (ST_A) (Scotland *et al.*, 1980; Wilson and Bettelheim, 1980; Okerman, 1987; Smith and Scotland, 1988). Further impetus to study SLT came when it was recognized that the sudden outbreaks of bloody diarrhea and hemorrhagic colitis occurring in the U.S.A., Canada, and the U.K. were associated with a heretofore

unrecognized *E. coli* O157:H7 serotype that produced elevated levels of SLT (Johnson *et al.*, 1983; O'Brien *et al.*, 1983; and Riley *et al.*, 1983). As a consequence of intense research by many laboratories, rapid strides were made in the isolation and characterization of the Shiga-like toxins of *E. coli*. Scotland *et al.* (1985) reported the presence of an antigenically different verocytotoxin which resisted neutralization with antisera to Shiga toxin, and these workers designated this toxin VT2. Strockbine *et al.* (1986) confirmed these observations, but named the toxin SLT-II. Soon it became apparent that certain strains of *E. coli* produced both SLT-I and SLT-II while other strains produced either SLT-I or SLT-II (Strockbine *et al.*, 1986; Smith and Scotland, 1988). Although the original cytotoxicity assays for SLTs (VTs) were done on Vero cells, both SLT-I and SLT-II are cytotoxic to certain other cell lines including HeLa cells (see Table 1). According to a recent classification of diarrheagenic *E. coli* (Levine,1987) SLT-producing *E. coli* implicated in bloody diarrhea and hemorrhagic colitis are classified as enterohemorrhagic *E. coli* (EHEC). Currently, the EHEC group consists of 3 serogroups, O157, O26 and O111, although there is accumulating evidence that other serogroups can also cause bloody diarrhea and hemorrhagic colitis (Bopp *et al.*, 1987; Smith and Scotland, 1988). The *E. coli* strains that cause edema disease of swine elaborate a toxin antigenically related to SLT-II (cross-neutralized by polyclonal SLT-II antiserum). This edema disease toxin has been designated Shiga-like toxin II variant (SLT-IIv) because, unlike SLT-II, it is cytotoxic for Vero cells but not HeLa cells (Blanco *et al.*, 1983; Marques *et al.*, 1987).

Certain strains of *E. coli* and other species including *Vibrio cholerae*, *Vibrio parahemolyticus,* and *Campylobacter jejuni* have been reported to produce low levels of a cell-associated cytotoxin for HeLa cells that can be neutralized by monoclonal and polyclonal anti-Shiga toxin (A.D.O'Brien, M.E.Chen, R.K.Holmes, J.Kaper, M.M.Levine, Letter, Lancet, i:77-78, 1984; Strockbine *et al.*, 1985). However, the significance, if any, of these low-level cytotoxins in pathogenesis is not clear.

Structure, genetics and regulation of Shiga-like toxins of *E. coli.*

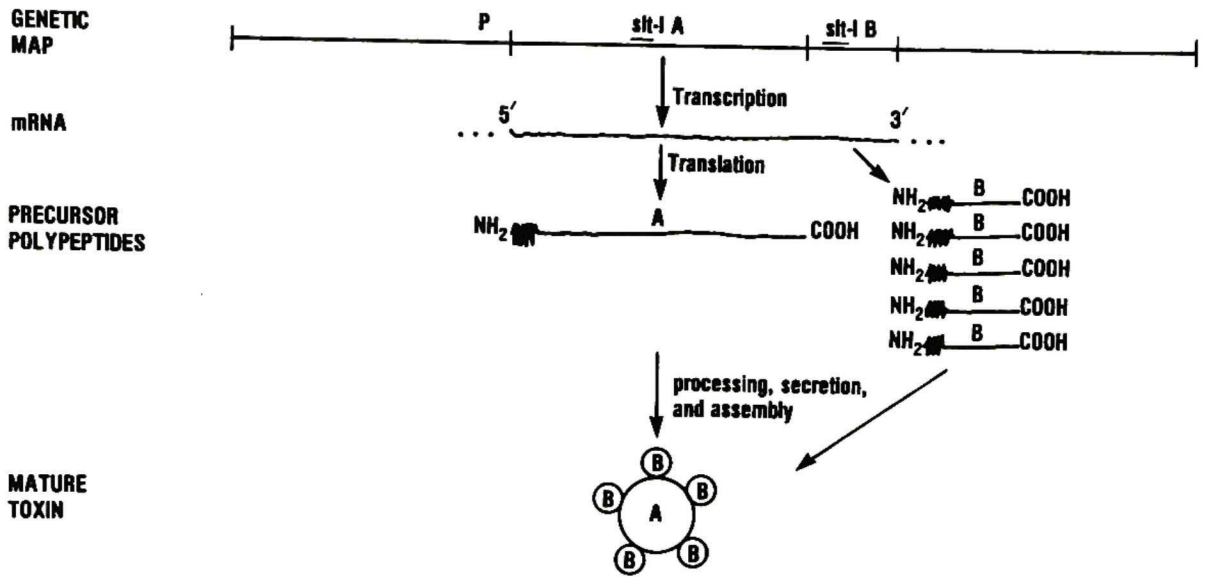
The SLT-I, SLT-II, and SLT-IIv have been purified to homogeneity by several laboratories (O'Brien and LaVeck, 1983; Downes *et al.*, 1988; Yutsudo *et al.*, 1987; Samuel *et al.*, 1990). All three toxins are bipartite toxins which consist of an enzymatically active A subunit and a receptor-binding oligomeric B subunit. The structural properties and biological activities of these Shiga-like toxins are summarized in Table 2. The genes coding for both SLT-I and SLT-II are borne on coliphage, and toxin expression by *E. coli* occurs as a consequence of phage conversion (Strockbine *et al.*, 1986). Both SLT-I and SLT-II genes have been cloned and the nucleotide sequences have been determined. There is greater than 99% homology between SLT-I and Shiga toxin genes, with a single conservative amino acid substitution in the A subunit as determined from the deduced amino acid sequences of the two toxins. Thus, SLT-I and Shiga toxin are almost identical bacterial proteins (Strockbine *et al.*, 1988). The genetic organization and expression of the SLT-I/Shiga toxin operon are shown schematically in Figure 3. As with the expression of Shiga toxin, (Van Heyningen and Gladstone, 1953), the expression of SLT-I is repressed in the presence of high iron concentration (Weinstein *et al.*, 1988a). Calderwood and Mekalanos (1987) reported that SLT-I expression is under the negative control of an iron-responsive element encoded by the *fur* locus of *E. coli*. Although a near-consensus *fur* protein-binding sequence is present in the *stx* operon (Kozlov *et al.*, 1988; Strockbine *et al.*, 1988), a functional fur-analogue has not been reported in *Shigella dysenteriae I*.

The structural genes for SLT-I and SLT-II share approximately 57% overall nucleotide-sequence homology with localized regions of much lower or much greater homology. The lowest homologies are found in the signal peptides and the carboxy end of the A and B subunits. The genetic organization and expression of SLT-II operon is believed to be similar to SLT-I (Jackson *et al.*, 1987), although SLT-II expression is not iron regulated (Weinstein *et al.*, 1988b; L.Sung, *personal communication*) .

Table 2. **Characteristics of Shiga toxin and Shiga-like toxins**

Toxin	M_r A subunit	M_r B subunit	No. of amino acid residues		Biological activity	Receptor	Mode of action
			A subunit	B subunit			
Shiga	32	7.7	293	69	Hela and Vero cell cytotoxicity, enterotoxicity in ileal loops, lethality to mice	G_{b3}	RNA *N*-glycosidase
SLT-I	32	7.7	293	69	HeLa and Vero cell cytotoxicity, enterotoxicity in ileal loops, lethality to mice	G_{b3}	RNA *N*-glycosidase
SLT-II	33	7.8	296	70	HeLa and Vero cell cytotoxicity, enterotoxicity in ileal loops, lethality to mice	G_{b3}	RNA *N*-glycosidase
SLT-IIv	33	7.6	297	68	Vero cell cytotoxicity, enterotoxicity in ileal loops, lethality to mice	G_{b4}	RNA *N*-glycosidase

Figure 3. A working model of the organization and expression of the *slt*-I operon (taken from O'Brien and Holmes, 1987). The genetic map shows the location of the promotor (P) for the *slt*-I operon and the structural genes *slt*-IA and *slt*-IB. The mRNA is presumed to be polycistronic. The primary translation products correspond to the unprocessed A and B polypeptides of SLT-I, which have amino-terminal signal sequences represented by the wavy lines. The oligomeric holotoxin is presumed to be assembled from the A and B polypeptides after they are secreted and processed to remove the signal sequences.


GENETIC MAP
P
slt-I A
slt-I B
Transcription
mRNA
5′
3′
Translation
PRECURSOR POLYPEPTIDES
NH2
A
COOH
B
processing, secretion, and assembly
MATURE TOXIN

Unlike SLT-I or SLT-II, the structural genes that encode SLT-IIv appear to be chromosomal in origin (Weinstein *et al.*, 1988 b; Gyles *et al.*, 1988). The nucleotide-sequence analysis of SLT-IIv indicates approximately 93% overall nucleotide-sequence homology with that of SLT-II (Weinstein *et al.*, 1988b; Gyles *et al.*,1988), and the genetic organization of SLT-IIv operon conforms to that of SLT-I and SLT-II.

Mode of action of Shiga-like toxins of *E. coli.*

The Shiga-like toxins of *E. coli* (SLT-I, SLT-II, and SLT-IIv) have biological activities similar to those of Shiga toxin (see Table 2). The B subunits of SLT-I and SLT-II bind to the same glycolipid receptor Gb_3 as the B subunit of Shiga toxin (Lingwood *et al.*, 1987; Waddel *et al.*, 1987). However, recent evidence indicates that the B subunit of SLT-IIv binds predominantly to the larger glycolipid Gb_4 (DeGrandis *et al.*, 1989; Samuel *et al.*, 1990). The enzymatic activities of the A subunits of all three Shiga-like toxins are identical to the activity of Shiga toxin (Endo *et al.*, 1988; Saxena *et al.*, 1989) *i.e.*, all are RNA *N*-glycosidases and depurinate the same adenine residue in 28S rRNA. This depurination leads to the inhibition of protein synthesis and cell death (see Figure 2).

Role of Shiga-like toxins in Disease.

In 1977, Konowalchuk *et al.* proposed a role for VT as a virulence factor in diarrheal disease. However, the importance of Shiga-like toxin-producing *E. coli* in human disease only became apparent in the last few years, when the epidemiological evidence clearly established the association of EHEC with hemorrhagic colitis and the hemolytic uremic syndrome. Furthermore, recent reports have indicated that SLT-producing *E. coli* may play a role in non-bloody diarrheas in young children, as well as in cases of intussusception (Lopez *et al.*, 1989a; Lopez *et al.*, 1989b). SLT-producing *E. coli* have gained importance in the field of veterinary medicine and animal husbandry

as well, since the accumulating epidemiological evidence clearly implicates hemorrhagic colitis as a zoonosis with cattle as the potential reservoir (Chapman *et al.*, 1989). In addition, SLT-producing *E. coli* have also been associated with disease in domestic animals, particularly calves and pigs (Mohammad *et al.*, 1985; Dobrescu, 1983; Smith *et al.*, 1983).

Structure-function relationships of Shiga and Shiga-like toxins.

Although considerable progress has been made in the biochemical characterization of the Shiga toxin of *Shigella dysenteriae* I and the Shiga-like toxins of EHEC and in the identification of the toxin receptors, the biophysical mechanisms involved in toxin-receptor interactions, subunit oligomerization, and holotoxin formation have not yet been defined. Furthermore, the immunodominant domains of these toxin molecules have not been defined. Thus, structure-function analyses of these toxins will increase our understanding of the role of Shiga and Shiga-like toxins in pathogenesis and may assist in the design of effective vaccines to prevent, or immunological therapeutic agents to treat, EHEC and *S. dysenteriae* I infections.

OBJECTIVES OF THE PROJECT AND STUDY PLAN

Objectives.

1. To generate monoclonal antibodies against SLT-II, and to investigate the molecular basis for antigenicity and toxin neutralization

2. To identify amino acid residues in the B subunit of Shiga toxin and SLT-II that are critical for either structural or functional integrity of the holotoxin molecule

3. To map the minimal contiguous coding segment of the *slt*-II gene required for holotoxin activity of the SLT-II molecule

Study plan.

These objectives are to be achieved by

1. generating toxin-neutralizing monoclonal antibodies to SLT-II and mapping the epitopes reactive with the monoclonal antibodies.

2. mutagenizing the cloned *slt*-II gene with sodium bisulfite and isolating and characterizing the non-cytotoxic mutants.

3. identifying the C-terminal and N-terminal functional boundaries of the SLT-II by creating deletions in the *slt*-II operon.

MATERIALS AND METHODS

Media, Enzymes, and radionuclides.

All bacterial strains, bacteriophage, and plasmids were routinely propergated in Luria broth or Luria broth agar (Maniatis *et al.*,1982). Where indicated 2YT broth (Maniatis *et al.*,1982) and M9 minimal salt agar (Maniatis *et al.*,1982) supplemented with glucose [0.2% (wt/vol)], thiamine (0.5 µg/ml), and NAD (1 µg/ml) were also used. When indicated, media were supplemented with antibiotics (Sigma Chemical Co., St. Louis, MO) at the following concentrations: ampicillin (200 µg/ml) and chloramphenicol (34 µg/ml). All restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis IN), unless otherwise indicated. Sequenase DNA sequencing kit was purchased from U. S. Biochemicals Corp., Cleveland, OH. Muta-Gene *in vitro* mutagenesis kit was purchased from Bio-Rad Laboratories (Richmond, CA). *In vitro* protein synthesis kit was purchased from Stratagene Cloning Systems (La Jolla, CA). Radionuclides were purchased from NEN Research Products (Boston, MA).

Preparation of crude SLT-II.

The *E. coli* lysogen C600(933W) which produces SLT-II (Strockbine *et al.*, 1986) was used in the preparation of high-titered, crude SLT-II lysate. A stock suspension of toxin-converting W coliphage was prepared by inducing the phage from the *E. coli* C600(933W) lysogen with mitomycin C as described previously (Marques *et al.*, 1987). An appropriate amount of the W coliphage stock was added to an exponential culture of *E. coli* 600 to obtain a M.O.I. (multiplicity of infection) of about 1. The mixture was then plated on LB agar and incubated overnight at 37° C to obtain a confluent lawn of plaques. The confluent plaque lawn was then overlaid with 5 ml of SM buffer (Maniatis *et al.*,1982) and gently rocked on a platform shaker for 3h at room

temperature to elute phage and toxin from the agar. The phage were removed from the lysate by ultracentrifugation at 50,000 x g for 3h. The clarified lysate contained 10^5-$1\,0^6$ HeLa cell 50% cytotoxic doses per ml (CD_{50}/ml) and was lethal for mice when inoculated intraperitoneally.

Preparation of SLT-II toxoids.

Crude, mouse-lethal SLT-II was converted to a toxoid by treating with either formaldehyde or gluteraldehyde. To prepare SLT-II toxoid by exposure to formaldehyde, a sample of crude SLT-II containing 100 μg of protein was treated with 1% formaldehyde in O.1 M Na_2HPO_4 (pH 8.0) for 3 days at 37° C (Donohue-Rolfe *et al.*, 1984). The residual formaldehyde was then removed by dialysis against phosphate-buffered saline (PBS) (8.0g NaCl; 0.2g KCl; 1.15g Na_2HPO_4 and 0.2g KH_2PO_4 in 1 liter of H_2O). The resultant toxoid contained no residual toxicity for HeLa cells and was not lethal for mice. To prepare SLT-II toxoid by treatment with gluteraldehyde, a crude toxin sample containing 50 μg of protein was incubated at 37° C in 0.11% gluteraldehyde in 0.1 M Na_2HPO_4 (pH 8.0) for 30 minutes (Brown *et al.*, 1982). The resultant toxoid had less than 1% of the original cytotoxicity and was not lethal for mice.

Production of monoclonal antibodies (MAbs) to SLT-II.

Female BALB/c mice (4-8 weeks of age) were immunized intraperitoneally with 25 μg of formalinized toxoid or 25 μg of gluteraldehyde-treated toxoid in complete Freund's adjuvant (CFA). The mice were boosted intraperitoneally three times at 7 day intervals with the same dose of toxoid in incomplete Freund's adjuvant (IFA). The animals were then periodically bled from the retro-orbital plexus to assess the titers of neutralizing antibodies. Four weeks after the 3rd intraperitoneal inoculation, the mice

were boosted intravenously with the same dose of toxoid in PBS via the tail vein. Three days later, mice were sacrificed by cervical dislocation and spleens were harvested aseptically. Splenocytes from two spleens were extracted and fused to Sp2/O-Ag 14 mouse myeloma cells at a ratio of 10 spleen cells to 1 myeloma cell using polyethylene glycol (Hurrell, 1982). Fused cells were distributed into 96 well microtiter plates containing syngeneic splenocytes as feeder layers. After 3 weeks in culture, hybridoma culture supernatants were assayed for toxin-specific antibodies by an enzyme-linked immunosorbent assay (ELISA) and by a cytotoxin-neutralization assay with crude SLT-II. The cultures which were positive for neutralizing antibodies were expanded and cloned twice by limiting dilution. The isotypes of the SLT-II neutralizing monoclonal antibodies were determined by immunodiffusion using murine isotype-specific goat antisera (Litton Bionetics Inc., Kensington, MD).

Hybridoma growth in mice.

To obtain high titered MAb preparations, BALB/c mice were first inoculated with 0.5 ml of Pristane (2,6,10,14-tetramethyl pentadecane) intraperitoneally. Ten days after the administration of Pristane, 10^6 - 10^7 hybridoma cells were administered intraperitoneally. The ascitic fluid was removed when the mice showed sufficient abdominal distention. The ascitic fluid was clarified by centrifugation (10,000 x g for 15 minutes) and stored at -20° C.

ELISA for screening SLT-II specific monoclonal antibodies.

An ELISA was developed to screen for SLT-II reactive monoclonal antibodies. Crude SLT-II (25 µg/ml) in coating buffer (15 mM Na_2CO_3; 34.5 mM $NaHCO_3$ and 3 mM NaN_3, pH 9.0) was dispensed into 96 well microtiter plates (200 µl/well); (Nunc-Immuno plate I type, Nunc, Roskilde, Denmark). The plates were incubated at

4° C overnight. The toxin-coated plates were then washed 6 times in high salt buffer (0.5 M NaCl; 1.5 mM KH_2PO_4; 10 mM Na_2HPO_4; 3 mM NaN_3 and 0.05% Tween 20; pH 7.4). Undiluted hybridoma culture supernatants were then added in duplicate (200 µl/well). After the plates were incubated 2 hours at 37° C, unbound antibodies were removed by washing (as described above) with high salt buffer. Next, peroxidase-conjugated goat-antimouse immunoglobulin (G + M + A; Boehringer Mannheim Biochemicals, Indianapolis, IN) was added at a dilution of 1:1000 in high salt buffer (50 µl/well). The plates were then incubated at 37° C for 2 hours and washed vigorously, as described above, to remove unbound secondary antibody. Peroxidase activity was detected colorimetrically after adding freshly made chromogen solution containing 0.04% *ortho*-phenylenediamine and 0.45% H_2O_2 in phosphate citrate buffer; pH 5.0 (33 mM citric acid; 67 mM Na_2HPO_4). *E. coli* C600 culture-supernatant coated wells were included in the assay to serve as controls so that only antibodies specific for the crude SLT-II were detected.

Radioimmunoprecipitation.

Crude SLT-II was labelled with ^{125}I using chloramine T as previously described (O'Brien and LaVeck, 1983). Immunoprecipitation of SLT-II with the MAbs was done according to published procedures (Kessler, 1976). Formalin-fixed, heat-killed *Staphylococcus aureus* cells of the Cowan I stain were purchased as a suspension (10% w/v) from Calbiochem Corp., La Jolla CA. First, the 10% suspension of bacteria was washed twice in 3 volumes of NP-40 solution (10 mM Tris pH 7.3; 0.5 M NaCl; 0.5% Nonidet P40) by centrifugation (5,000 x g for 5 minutes), and the pellet was resuspended to the original volume in NP-40 solution with 0.3% BSA. Three hundred microliters of crude ^{125}I-labelled SLT-II was precleared with 30 µl of washed

S.aureus cells by incubating the toxin and cells together at 4° C for 30 minutes with gentle agitation. After centrifugation (10,000 x g for 5 minutes), the supernatant was collected and 650 µl of NP-40 with 0.3% BSA was added to the precleared toxin sample. An aliquot of undiluted MAb culture supernatant (100 µl) was then added to the precleared ^{125}I-labelled SLT-II, and the antigen-antibody complex was incubated at room temperature for 30 minutes before adding 5 µl of rabbit anti-mouse immunoglobulin antisera (Sigma Chemical Co.). The immune-complex was further incubated at room temperature for 1 hour and then 50 µl of the *S. aureaus* cell suspension was added. This mixture was then incubated at 4° C for 1 hour. The *S. aureus* cells with the adsorbed immune-complexes were then collected by centrifugation (10,000 x g for 5 minutes) and washed three times with NP-40 solution with 0.5% SDS. The precipitated immune-complexes were resolved by SDS-PAGE on 10% or 15% polyacrylamide gels, essentially as described by Laemmli (1970). The gels were then dried and exposed to X-Omat film (Eastman Kodak Co.,Rochester N.Y.) with intensifying screens.

Western blot (immunoblot) analysis.

The subunit specificities of the SLT-II neutralizing monoclonal antibodies were assessed by Western blot analysis (Burnette, 1981). Fifty micrograms of crude SLT-II was subjected to SDS-PAGE in 15% polyacrylamide slab gels. The dissociated protein subunits were electrophoretically transferred (2 hours at 200 mA) onto a nitrocellulose membrane. The membrane was then incubated at 4° C overnight in 0.1M Tris-HCl, pH 7.4 containing 0.9% NaCl and 10% BSA to prevent nonspecific binding of protein to the membrane. The nitrocellulose membrane was immersed in undiluted hybridoma culture supernatant and incubated for 2h at room temperature with gentle agitation. The membrane was washed three times with 0.1M Tris-HCl containing 0.9%

NaCl and 0.05% Tween 20 to remove unbound MAb before incubating with peroxidase-conjugated goat antimouse immunoglobulin (G + M + A) antibody at a dilution of 1:1500 for 2h at room temperature. After washing the membrane to remove unbound second antibody as described above, the peroxidase activity was detected by adding the chromogen solution (0.05% 4-chloronaphthol and 0.015% H_2O_2 in 0.1 M Tris-HCl containing 0.9% NaCl).

Separation of A and B subunits of SLT-II.

An ^{125}I-labelled crude toxin sample (200 µg of protein per ml) was treated with 10 M urea at 37° C for 30 minutes (Olsnes *et al.*, 1981) followed by filtration with a 10 kd (kilodalton) molecular weight cut off microconcentrator (Centricon 10, Amicon Division, WR Grace and Co., Danvers, MA). The retentate and filtrate were collected separately, and, using a 3 kd molecular weight cut off dialysis tubing, the samples were dialyzed extensively against PBS.

Cytotoxicity and neutralization assays.

The assay was performed with either HeLa or Vero cells essentially as described earlier (Marques *et al.*, 1986). Freshly trypsinized cells were adjusted to a concentration to 10^5 cells/ml and dispensed into 96 well microtiter plates (100 µl of cell suspension per well). The cytotoxicity of the toxin preparation was determined by adding the toxin directly onto the cell monolayer (100 µl/well in duplicate). For certain experiments, the cytotoxicity of the toxin preparation was titrated by making 10-fold serial dilutions, and the reciprocal of the highest dilution of the toxin preparation capable of killing 50% of the cell monolayer (CD_{50}) was taken as the titer of the toxin. In the cytotoxicity neutralization assay, a standard dose (10 CD_{50}) of the toxin was mixed with an equal volume of the antibody and incubated for 1 hour at 37° C

before adding the toxin-antibody mixture onto the cell monolayer. MAb culture supernatants were used undiluted while ascitic fluid or polyclonal antisera were used at a dilution of 1:100. The plates were incubated for 48 hours before visually assessing the destruction of cell monolayers.

Epitope mapping.

To map the epitopes of SLT-II reactive with the SLT-II-specific monoclonal antibodies, a sublibrary (Mehra *et al.*, 1986) of the *slt*-II gene was created in the Lambda ZAP (λ ZAP) expression vector (Stratagene Cloning Systems, La Jolla, CA) as schematically depicted in Figure 8. The plasmid pLP15 (see Figure 4) carrying the *slt*-II gene was used to construct the *slt*-II sublibrary. Twenty micrograms of pLP15 plasmid DNA were treated with 2 ng of DNase I in 60 µl of 20 mM Tris-HCl, pH 7.5 with 1.5 mM $MnCl_2$ and 6 µg BSA at room temperature for 20 minutes to generate short, random fragments. The sample was heated for 10 minutes at 75° C to inactivate DNase I before separating the DNA by size on a 1% agarose gel. DNA fragments of approximately 500 bp (base pairs) were isolated. These fragments were end-repaired by T4 DNA polymerase in the presence of dNTP and then ligated to phosphorylated *Eco* RI linkers (Pharmacia, LKB Biotechnology Inc. Piscataway, NJ). The DNA to which the *Eco* RI linkers had been added was then digested with excess *Eco* RI enzyme for 2 hours at 37° C and then heated at 70° C for 10 minutes. Next, the DNA was fractionated on a Nensorb 20 column (NEN Research Products, Boston, MA) to remove unligated linkers (Davis *et al.*,1986). The fractions containing the DNA fragments with linkers were identified (Davis *et al.*,1986), pooled and concentrated by drying under a vacuum. The DNA fragments with *Eco* RI linkers were then ligated to phosphatase-treated λZAP arms (Stratagene Cloning Systems), and the ligated DNA was packaged into lambda phage heads using Gigapack gold packaging extract *in vitro* (Stratagene Cloning Systems). The resultant recombinant phage were amplified in *E. coli* strain BB4 (Bullock *et al.*,

1987). The library was screened with individual SLT-II specific monoclonal antibodies, and recombinant clones reactive with these MAbs were isolated as described previously (Helfman *et al.*, 1983). The nucleotide sequence of the insert DNA fragment that encoded the immunoreactive epitope was determined by sequencing accross the fusion joint using T_3 and T_7 oligonucleotide primers as depicted in Figure 8.

Receptor-analogue ELISA.

An ELISA was developed to detect the binding of Shiga toxin and SLT-II to a receptor-analogue originally described by Lindberg *et al.* (1987). The receptor-analogue, (Galα1-4Galβ-0-CETE)$_n$BSA (where n is 7 or greater), was purchased as 30-40 mole disaccharide/mole BSA from Carbohydrate International, Chicago, IL. A high-protein binding 96 well microtiter plate (Nunc-Immunoplate I type. Nunc, Roskilde, Denmark) was coated with the receptor analogue at a concentration of 2 μg/ml PBS (200 μl/well). The plate was incubated overnight at 4° C and washed once with PBS. A sonic lysate of *E. coli* expressing Shiga toxin or SLT-II was diluted 5-fold with high salt buffer containing 0.05% Tween 20 and then added to the receptor-analogue coated wells (200 μl/well). The high salt buffer with 0.05% Tween 20 was used in all the subsequent washing steps and as the diluent for antibody dilutions. The plate with the sonic lysate was then incubated at 4° C overnight and washed six times before adding toxin-specific MAb (200 μl per well). MAb culture supernatants were used undiluted while MAb ascitic fluid was used at a dilution of 1:2500 in the assay. After incubating the plates at 37° C for 2 hours, the unbound MAb was removed by washing the plates six times. Next, peroxidase-conjugated goat anti-mouse immunoglobulin (G + M + A) was added at a dilution of 1:1500 (200 μl/well). After incubating the plate at 37° C for 2 hours, unbound secondary antibody was removed by washing as described above. The bound antibody was then detected by adding 100 μl of the chromogenic substrate per well

(0.04% *ortho*-phenylenediamine and 0.45% H_2O_2 in 33 mM citric acid and 67 mM Na_2HPO_4, pH 5.0). The color development was allowed to proceed for 15 minutes in the dark at room temperature, and the reaction was terminated by adding 8N H_2SO_4 (25 µl/well). The absorbance was read at 490 nm on a microplate reader model EL308 (Bio-Tek Instruments Inc., Winooski, VT). To determine the specificity of the receptor-analogue ELISA, different dilutions (1:5, 1:25, 1:50 and 1:100) of Shiga toxin were incubated with a fixed amount of receptor-analogue (200 µg/ml) for 30 minutes at 37° C before assaying in the ELISA. BSA-coated wells served as controls to detect any non-specific binding of the toxin to the BSA moiety in the receptor analogue.

Preparation and transformation of competent bacteria.

The genotypes of the bacterial strains used in the present study are listed in Table 3. The bacterial hosts for DNA transformation were made competent according to the method described by Hanahan (1985). Briefly, a single bacterial colony was inoculated into 1 ml of SOB medium (2% tryptone with 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$) and incubated overnight at 37° C with aeration. The overnight culture was then diluted 100-fold in 2.5 ml of SOB medium and grown at 37° C with aeration until an OD_{600} of approximately 0.3 was reached. The culture was chilled on ice for 10 minutes before pelleting the cells by centrifugation at 1,000 x g for 10 minutes at 4° C. After draining the pellet thoroughly, the cells were resuspended in 800 µl of TFB solution [100 mM KCl, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 3mM $HACoCl_3$ (Hexaamine cobalt chloride) and 10 mM K-MES (morpholino ethane sulfonic acid)]. Following incubation on ice for 10 minutes, the cells were pelleted as described earlier and resuspended in 200 µl of TFB solution. Seven microliters of DnD solution (90% DMSO with 1 M DTT and 10 mM potassium acetate) was added to the cell suspension, and the mixture was incubated on ice for 10

minutes before adding a second equal aliquot of DnD. The cells were kept on ice for 20 minutes before adding the DNA in a volume less than 10 µl. After adding the DNA, the cells were incubated on ice for 30 minutes before placing them in a 42° C water bath for 90 seconds. The cells with the DNA were then immediately placed on ice for 3 minutes before adding 800 µl of SOC medium (SOB containing 20 mM of glucose) and incubated at 37° C with moderate agitation for 45 minutes. The transformants were selected by spreading the cells on agar plates that contained the appropriate antibiotics.

Plasmid constructions.

Plasmid pNN76, which carries the Shiga-like toxin II operon (*slt*-II) has been described earlier (Newland *et al.*, 1987). This plasmid was cut with *Sph* I and *Kpn* I. The resultant 2.4 kilobase (kb) fragment with the entire *slt*-II operon was subcloned into the Bluescribe phagemid expression vector pBS(-) (Stratagene Cloning Systems, La Jolla, CA), and the resultant plasmid was designated pLP15 (see Figure 4). The plasmid pNAS13 carrying the Shiga toxin operon (*stx*) has been described by Strockbine *et al.* (1988). This plasmid was cut with *Bgl* II and *Sal* I. The 5.9 kb fragment containing the entire *stx* operon was isolated by agarose gel electrophoresis. The termini of this fragment were blunted by treating with single-strand specific mung bean nuclease (Stratagene Cloning Systems). Following phenol-chloroform extraction and ethanol precipitation of DNA, the fragment was circularized by blunt-end ligation with T4 DNA ligase, and the resultant plasmid was designated pLPSH3 (see Figure 5). The 1.8 kb *Hind* III-*Eco* RI fragment of pLPSH3 was subcloned into replicative form (RF) M13mp18 vector to generate a single strand DNA template for oligonucleotide-directed, site-specific mutagenesis of *stxB* gene. The primary plasmid constructs that were used in the present study are listed in Table 4.

Table 3. **Genotypes of the *E. coli* host strains used**

Host strain	Genotype	Reference
BB4	*supF* 58 *supE* 44 *hsdR* 5 1 4 *galK* 2 *galT* 2 2	
	trpR 55 *metB* 1 tonA Δ*lac*U 169	
	F'[*proAB*$^{+}$ *lac*^{q} *lacZ* Δ M15 Tn *1 0*(*tet*^{r})]	Bullock *et al.*, 1 9 8 7
C600	*supE* 44 *hsdR thi*-1 *thr*- 1 *leuB*6	
	lacY 1 *tonA* 2 1	Huynh *et al.*, 1985
CJ236	*dut* 1 *ung* 1 *thi*-1 *rel* 1/ pCJ105 (cam^{r} F')	Kunkel *et al.*, 1987
DH5α	*supE* 44 Δ*lac*U 169 (Φ 80 *lacZ*Δ M 1 5)	
	hsdR 17 *recA* 1 *endA* 1 *gyrA* 9 6 *thi*-1 *relA* 1	Hanahan, D., 1985
MV1190	*SupE Δ(sr1-recA)306::*Tn *10 (tet*^{r}*)*	
	Δ(lac-proAB) thi	
	[F':*traD36, proAB lac* ^{q}*Z*Δ M 1 5]	Kunkel *et al.*, 1987

Construction of gapped duplex DNA for mutagenesis.

Plasmid pLP15 was transformed into *E. coli* DH5α by the Hanahan method (1985). Purified plasmid DNA of pLP15 was made from the transformants according to published procedures (Maniatis *et al.*, 1982). Plasmid pLP15 DNA was cut with *Pst* I and *Eco* RI, subjected to gel electrophoresis, and a 5 kb fragment isolated and purified by Geneclean (Bio 101 Inc., La Jolla, CA) according to the instructions provided by the manufacturer. Plasmid pLP15 was also transformed into *E. coli* CJ236 (*dut*$^-$, *ung*$^-$). *E. coli dut*$^-$ mutants lack the enzyme dUTPase and therefore contain elevated concentrations of dUTP which effectively compete with TTP for incorporation into DNA. *E. coli ung*$^-$ mutants lack the enzyme uracil *N*-glycosylase which normally removes misincorporated uracil from DNA. In the combined *dut*$^-$ *ung*$^-$ mutant, uracil is incorporated into DNA in place of thymine and is not removed (Kunkel *et al.*, 1987). Thus, only uracil-containing DNA is synthesized by *E. coli* CJ236. From these CJ236 transformants, purified pLP15 plasmid DNA with misincorporated uracil (pLP15 DNA-U) was made according to standard procedures (Maniatis *et al.*,1982). The first step in constructing a gapped duplex with a single strand region extending between *Pst* I and *Eco* RI sites of pLP15, was to linearize the pLP15 DNA-U with *Sph* I. The linearized pLP15 DNA-U (1.25 μg) was then mixed with 0.5 μg of the gel-purified 5 kb fragment (*Pst* I-*Eco* RI) of pLP15 (obtained as described above) in 40 μl of 187.5 mM KCl with 12.5 mM Tris (pH 7.5). The ratio of linearized pLP15 DNA-U to the gel purified 5 kb fragment was selected according to Kramer and Fritz, (1987). To promote gap duplex formation, the mixture was placed in boiling water for 3 minutes, incubated at 65° C for 5 minutes, and then allowed to cool to room temperature over a period of 45 minutes.

Figure 4. A shematic map of linearized pLP15. To generate pLP15, plasmid pNN76 which carries the *slt*-II operon (Newland *et al.*, 1987) was cut with *Sph* I and *Kpn* I and the resultant 2.4 kb fragment with the entire *slt*-II operon was subcloned into Bluescribe phagemid expression vector pBS(-).

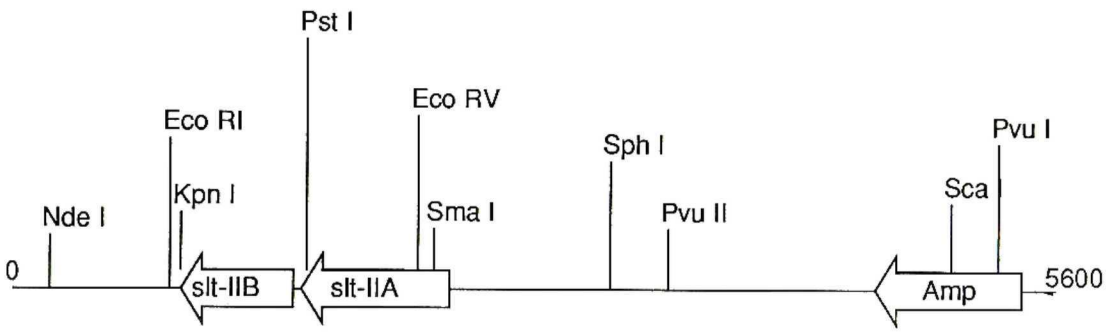

Pst I
Eco RV
Eco RI
Sph I
Pvu I
Sca I
Kpn I
Nde I
Sma I
Pvu II
0
slt-IIB
slt-IIA
Amp
5600

Figure 5. Construction of pLPSH3. The 7.6 kb pNAS13 which carries the *stx* operon was digested with *Bgl* II and *Sal* I. The 5.9 kb fragment containing the entire *stx* operon was isolated, treated with mung bean nuclease to blunt the termini, and circularized by blunt end ligation to form pLPSH3. Abbreviations for the endonuclease restriction sites: E, *Eco* RI; H, *Hind* III; B, *Bgl* II; S, *Sal* I; N, *Nru* I; NC, *Nco* I.

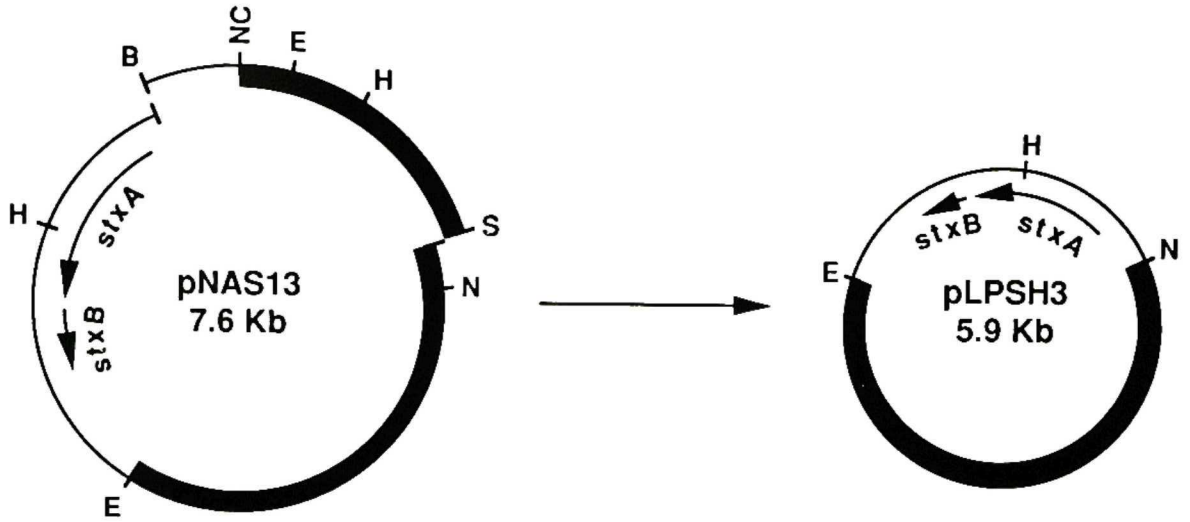

B
NC
E
H
H
stxA
stxB
pNAS13
7.6 Kb
S
N
E
H
stxB
stxA
E
N
pLPSH3
5.9 Kb

Table 4. **Description of primary plasmid constructs used in the study**

Plasmid	Description	Toxin produced	Reference
pNN76	*slt*-II in pBR329	SLT-II	Newland *et al.*, 1987.
pLP15	pNN76 *Sph* I -*Kpn* I in pBS(-)	SLT-II	This study
pNAS13	*stx* in pBR329	Shiga toxin	Strockbine *et al.*, 1988.
pLPSH3	pNAS13 *Eco* R I-*Bgl* II in pBR329	Shiga toxin	This study

Sodium bisulfite mutagenesis of gapped duplex DNA.

Fifty microliters (approximately 20 μg/ml) of gapped duplex DNA of pLP15 in 15 mM NaCl and 1.5 mM sodium citrate (pH 7.0) was mixed with 150 μl of freshly prepared 4 M sodium bisulfite solution (pH 6.0) and 2 μl of freshly prepared 50 mM hydroquinone. One hundred microliters of paraffin oil was layered above the mixture which was then incubated at 37° C for 1 hour in the dark. The mutagenesis reaction was stopped by removing the sodium bisulfite through dialysis as described by Shortle and Botstein (1983). The dialyzed sample was then extracted 3 times with ether to remove paraffin oil, and 50 μg of tRNA (Sigma chemical Co., St. Louis, MO) was added to facilitate precipitation of the DNA with ethanol. The DNA pellet was resuspended in 10 μl of 20 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$ and 50 mM NaCl. The resuspended DNA was placed on ice for 5 minutes before adding 1 μl of 10X synthesis buffer (Bio-Rad Laboratories, Richmond, CA), containing 5 mM each of dATP, dCTP, dGTP and dTTP, 10 mM ATP, 100 mM Tris pH 7.4, 50 mM $MgCl_2$, 20 mM DTT, 5 units of T4 DNA ligase and 1 unit of T4 DNA polymerase (Bio-Rad Laboratories). The sample was incubated 5 minutes on ice, 5 minutes at 25° C, and 90 minutes at 37° C to fill in the single strand gap *in vitro*. *E. coli* MV1190 (*dut*$^+$, *ung*$^+$) was transformed with 10 μl of the regionally mutagenized pLP15 DNA. Transformation of uracil-substituted DNA into an *ung*$^+$ strain results in rapid degradation of uracil-substituted DNA strands. Hence, a large proportion of the progeny from the transformants are therefore derived from replication of the uracil-free DNA strands.

DNA sequencing.

Plasmid DNA that had been purified on a CsCl density gradient was sequenced with the Sequenase DNA sequencing kit (US Biochemical Corporation, Cleveland, OH) according to the double stranded DNA sequencing protocol provided by the manufacturer

with some modifications. The purified DNA was prepared for sequencing as follows. Twelve micrograms of DNA was diluted in 54 µl of distilled H_2O to which 6 µl of 2 N NaOH was added, and the mixture was incubated for 5 minutes at room temperature to denature supercoiled DNA. Next, 24 µl of 5 M sodium acetate (pH 5.0) and 300 µl of ethanol was added to precipitate DNA in a dry ice-ethanol bath for 15 minutes. The DNA was pelleted at 12,000 x g for 10 minutes at 4° C, and the DNA pellet dissolved in 20 µl of distilled H_2O. Seven microliters of this DNA preparation (~4 µg) was used in the sequencing reaction with five nanograms of primer in a total volume of 10 µl. One microliter of undiluted Sequenase enzyme (modified T7 DNA polymerase) was used in the sequencing reactions instead of the 8-fold dilution of the enzyme recommended by the vendor.

Preparation of single strand DNA templates for *in vitro* mutagenesis.

Single strand DNA templates with misincorporated uracil were prepared from M13 recombinant phage or recombinant phagemids (see Figure 10). To generate single strand DNA templates with misincorporated uracil from recombinant M13 phage, a log culture of *E. coli* CJ236 (25 ml) grown in the presence of chloramphenicol (34 µg/ml) was infected with 1-3 x 10^6 recombinant phage (~ 0.1 MOI) and incubated overnight at 37° C with aeration. The cells were removed by centrifugation (17,000 x g at 4° C for 10 minutes). The supernatant was transferred into a sterile tube and 100 µg of RNase I was added. This mixture was incubated for 30 minutes at room temperature. The phage were precipitated from the RNase-treated supernatant by adding 1/4 volume of 3.5 M ammonium acetate containing 20% PEG 800 and incubating the sample on ice for 30 minutes. The phage were harvested by centrifugation (17,000 x g at 4° C for 15 minutes) and then resuspended in 200 µl of phage suspension buffer (300 mM NaCl, 100 mM Tris pH 8.0, 1 mM EDTA). The phage suspension was incubated on ice for 30

minutes and then subjected to centrifugation (12,000 x g for 5 minutes) to remove any insoluble debris. The efficiency of uracil misincorporation was assessed by titering the phage suspension on both *E. coli* CJ236 (*dut*$^-$, *ung*$^-$) and *E. coli* MV1190 (*dut*$^+$, *ung*$^+$). DNA was isolated from the phage suspension by extracting with phenol-chloroform three times and precipitating the DNA with sodium acetate and ethanol. The phage DNA was resuspended in 25 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA).

To prepare single strand DNA with misincorporated uracil from Bluescribe phagemid recombinants, recombinants were transformed into *E. coli* CJ236 (*dut*$^-$, *ung*$^-$) according to the Hanahan method (1985). The transformants were selected on plates containing both ampicillin (200 µg /ml) and chloramphenicol (34 µg/ml). *E. coli* CJ236 transformants were grown in 2YT containing ampicillin (200 µg/ml) and chloramphenicol (34 µg/ml) to an OD_{600} of approximately 0.3. The cells were then infected with R408 helper phage (Russel *et al.*, 1986) at an MOI of 10, and the infected cells were incubated for another 8 hours at 37^{o} C with vigorous shaking. This helper phage was used to rescue single stranded DNA from the phagemid. Single strand DNA was extracted from the rescued phagemids essentially as described for M13 phage by phenol-chloroform extraction. This single strand DNA was used as a template for oligonucleotide-directed, site-specific mutagenesis (see next section).

Oligonucleotide-directed, site-specific *in vitro* mutagenesis.

The M13 mp18 recombinant phage carrying the 1.8 kb *Hind*III-*Eco* RI fragment of the *stx* operon was used to generate single strand DNA templates to induce mutations at the 33rd, 43rd, and 60th codons of the mature B subunit of Shiga toxin. This recombinant phage yields the non-coding strand of the *stx* operon. The following synthetic mutagenic oligonucleotides that correspond to the coding strand of *stx* operon were used to introduce the above mutations (substituted bases are underlined): 5'

TTATTTACCAACTGCTGGAATCTTCAG 3' (AGA changed to TGC at codon 33); 5' CTTCTTCTCAGTACGCAAATTACGGGG 3' (GCG changed to ACG at codon 43); 5' GCCTGTCATAATGACGGGGGATTCAGC 3' (GGA changed to GAC at codon 60). To introduce *Hpa* I restriction sites in *slt*-II A and ochre terminators in *slt*-II B, single strand DNA templates rescued from recombinant phagemid pLP15 were used. The mutating oligonucleotide primer 5' CCCGGGAGTTAACGATAGAC 3' was used to introduce the first *Hpa* I site by changing the 3rd codon, TTT, of the mature A subunit of SLT-II to TTA. To introduce the second *Hpa* I site, the mutating oligonucleotide primer, 5' GTCTCTTCGTTAACTAGTATACGGAC 3' was used to change the 18th codon, AAT, of the mature A subunit of SLT-II to ACT. The ochre terminator codons were introduced at either the 67th or the 69th codons of mature SLT-II B subunit using 5' GTGCAGTTTAATTAAGACTGAGGC 3' or 5' GCTGAAGTGCAGTAAAATAATGACTGAGGC 3' mutating oligonucleotide primers, respectively. The oligonucleotide-directed, site-specific mutagenesis was done using the Muta-Gene *in vitro* mutagenesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. For mutagenesis, 0.1 pM of template DNA was mixed with 2 pM of phosphorylated mutagenic oligonucleotide primer. One microliter of 10X annealing buffer (200 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 500 mM NaCl) was added before bringing the total volume to 10 µl with distilled water. The annealing mix was heated at 70° C and allowed to cool to room temperature over a 45 minutes period. The mixture was then placed on ice, and the synthesis of the second strand was initiated by adding 1 µl of 10X synthesis buffer (5 mM dNTP, 10 mM ATP, 100 mM Tris-HCl pH 7.4, 50 mM $MgCl_2$, 20 mM DTT), 5 units of T4 DNA ligase, and 1 unit of T4 DNA polymerase. The reaction was allowed to proceed on ice for 5 minutes and then at 37° C for 90 minutes. Five microliters of the reaction mix was used to transform *E. coli* MV1190 (*dut*$^+$, *ung*$^+$).

Dot blot ELISA.

The immunoreactivities of the mutant SLT-II and Shiga toxin polypeptides with the monoclonal antibodies (MAb) against the B subunits of the SLT-II and Shiga toxin were determined by a dot blot enzyme linked immunoassay (dot blot ELISA). *E. coli* producing wild type or mutant toxin were grown overnight at 37° C in 50 ml of 2YT containing 200 ug/ml of ampicillin. The bacterial cultures were placed on ice and subjected to sonic lysis (Lab-Line ultratip labsonic system, Lab-Line Instruments, Mentrose Park, IL). To minimize proteolysis, 0.3 mM phenylmethylsulfonyl fluoride (PMSF) was added to the sonically disrupted cultures. The cellular debris was then removed by centrifugation at 10,000 x g for 15 minutes at 4° C. The supernatants of the sonic lysates were further concentrated by 60% ammonium sulfate precipitation of proteins. The precipitants were resuspended in 0.5 ml of phosphate-buffered saline (PBS) and dialized overnight in 3 liters of PBS to remove residual ammonium sulfate. A dot-blot apparatus (Schleicher & Schuell Inc. Keene, NH) connected to a vacuum was used to spot 10 µl of the concentrated sonic lysate on a nitrocellulose membrane. The nitrocellulose membrane was air dried and then immersed for 1 hour at room temperature in Tris buffered saline (200 mM Tris-HCl, pH7.5, 150 mM NaCl) with 1% bovine serum albumin (TBSB) to saturate the nonspecific protein-binding sites of the nitrocellulose membrane. The membrane was then transferred to 20 ml of a 1:5 dilution of MAb culture supernatant or a 1:2500 dilution of MAb ascitic fluid and then further incubated for one hour at room temperature. All antibody dilutions were made in TBSB. The membrane was then washed five times in 20 ml of Tris buffered saline with 0.05% Tween 20 (TBST) and incubated for 1 hour at room temperature in 20 ml of a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (G + M + A) antibody (Bethesda Research Laboratories, Gaithersburg, MD). The unbound second antibody was then removed by washing 5 times with TBST as described above. The membrane was then immersed in 10 ml of chromogen solution

containing 0.3 mg/ml of nitro blue tetrazolium (NBT) and 0.15 mg/ml of 5-bromo-4-chloro-3-indonyl phosphate (BCIP) in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$. The color development was allowed to proceed in the dark for 15 minutes after which the membrane was washed once in 20 ml of 20 mM Tris-HCl, pH 2.9 containing 1 mM EDTA. The specificities of the MAb used in the dot blot ELISA are as follows; BC5 reacts with the B subunit of SLT-II (Downes *et al.*, 1988) and 13C4, 19G8, 16E6 react with the B subunit of Shiga toxin (Strockbine *et al.*, 1985).

Thin layer chromatogram overlay assay.

The thin layer chromatogram overlay assay was done essentially as described earlier (Samuel *et al.*, 1990). Briefly, total glycolipids from HeLa and Vero cells were extracted by the methanol-chlroform extraction procedure (Magnani *et al.*,1980). The glycolipids extracted from HeLa and Vero cells as well as purified neutral and acidic glycolipids (purchased from Supelco Inc., Bellefonte, PA) serving as controls were subjected to chromatography on aluminum backed silica gel high performance TLC plates (Silica Gel 60; E Merck AG, Darmstadt, Federal Republic of Germany). The chromatograms were air dried before soaking in TBS-BSA buffer (100 mM Tris-HCl, pH 7.8 containing 150 mM NaCl and 1% BSA) for 2 hours at room temperature. The plates were then overlaid with the toxins in TBS-BSA buffer, incubated for 18 hours at 4° C, and washed five times in cold PBS. Next, the plates were overlaid for 1 hour at room temperature with MAb 4F7 (specific for the A subunit of Shiga toxin) diluted in TBS-BSA buffer. The unbound MAb was removed by washing with PBS before adding ^{125}I-labelled goat anti-mouse IgG in TBS-BSA buffer. The plates were then incubated at room temperature for 1 hour, washed four times in cold PBS, air dried, and subjected to autoradiography.

Immunofluorescence assay.

An immunofluorescence assay was developed to detect the binding of mutant toxins to Vero cells. Freshly dispersed Vero cells were suspended to a concentration of 10^5 cells/ml diluent buffer [Earles Balanced salt solution (EBSS) with 10% fetal calf serum (FCS) and 0.1% NaN_3]. Fifty microliters of the concentrated bacterial cell lysate with wild type or mutant toxin was then added to 450 µl of the Vero cell suspension. The cell suspension was incubated at 4° C for 1 hour with gentle agitation. The cells were then washed three times by centrifugation (1,000 x g for 3 minutes at 4° C) in 1 ml of washing buffer (EBSS with 1% FCS and 0.1% NaN_3) and resuspended in 495 µl of diluent buffer. Five microliters of toxin-specific MAb (ascitic fluid) was then added. The monoclonal antibody was incubated with the cell suspension for 45 minutes at 4° C with gentle agitation. Excess unbound MAb was removed by washing as described above. Next, the cells were resuspended in 495 µl of diluent buffer, and 5 µl of fluorescein-conjugated goat-anti mouse $F(ab')_2$ was added (Cappel Laboratories, Westchester, PA). The cells were incubated at 4° C for 45 minutes, washed three times with washing buffer, and then washed once with EBSS with 0.1% NaN_3 without FCS. The cells were then differentially labelled with the fluorescent cell membrane intercalating dye "di1" (1-1'dioctadecyl-3-3,3'3'-tetramethyl iodocarbocyanine perchlorate.) (Molecular Probes, Eugene, OR) as described by Hogan *et al.* (1989) for nonspecific fluorescent imaging of cells. The cells were then transferred to a flat bottom 96 well microtiter plate (100 µl cell suspension/well), and the plate was subjected to centrifugation at 1,000 x g for 3 minutes to disperse the cells into a monolayer. Fluorescent analysis of toxin-bound cells was accomplished with an Anchored Cell Analysis Station 470 (ACAS 470, Meridian Instruments Inc, Okemos, MI).

Pulse-chase analysis of toxin mutants.

Pulse-chase experiments were done (Bowie and Sauer, 1989) to compare the rate of degradation of SLT-II, Shiga toxin, and toxin mutants within the bacterial host. *E. coli* transformed with plasmid pLP15 (SLT-II), or pLPSH3 (Shiga toxin) and *E. coli* that carried plasmids with mutated toxin genes were grown overnight at 37° C in M9 medium with 1% casamino acids, 0.1 μg/ml thiamine (Sancar *et al.*, 1979) and 200 μg/ml of ampicillin. Bacterial cells were washed twice with Hershey salt solution (Sancar *et al.*, 1981) and resuspended in sulfate-free Hershey medium with 200 μg/ml of ampicillin. The cultures were diluted 50-fold in 3 ml of Hershey medium and allowed to grow at 37° C with agitation until the OD_{600} was approximately 0.5. The bacterial cultures were then pulsed with 3 μl of (^{35}S) methionine (>800 Ci/mmol,15 μCi/μl) for 5 minutes followed by the addition of excess unlabelled methionine (0.01M). Five hundred microliter aliquots of the bacterial cultures were removed at predetermined intervals and mixed immediately with 35 μl of ice-cold protease inhibitor solution (60 mM PMSF, 30 mM N ethyl maleimide and 80 mM NaN_3) on ice. The bacterial cells were pelleted by centrifugation (10,000 x g for 2 minutes) and then resuspended in 50 μl of lysis buffer (100 mM Tris-HCl, pH 8, 1mM EDTA, 10 mM 2-mercaptoethanol, 500 mM NaCl, 6 M urea, 0.1% Nonidet P-40, and 0.0125% bromophenol blue). The lysed bacterial cells were boiled for 5 minutes and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% polyacrylamide. The gel was treated with Enlightning (NEN Research Products, Boston, MA), dried, and subjected to autoradiography.

Inoculation of mice and rabbits.

To determine whether the noncytotoxic SLT-II mutants were also nonlethal to mice, concentrated sonic lysates of *E. coli* that expressed the SLT-II mutant toxins

(prepared as described under dot blot ELISA) were inoculated intraperitoneally into BALB/c mice. Each crude mutant toxin preparation was inoculated into a group of 5 mice (100 µl of sonic lysate per mouse). As a positive control for toxicity, a group of five mice was inoculated with a concentrated sonic lysate of *E. coli*(pLP15) that expressed wild type SLT-II (100 µl of sonic lysate per mouse). To assess the feasibility of using the noncytotoxic SLT-II mutants to raise neutralizing antibodies against SLT-II, three rabbits were inoculated intramuscularly (500 µl of concentrated sonic lysate) with these SLT-II mutant toxin preparations (one rabbit per mutant). The first inoculum was administered with complete Freund's adjuvant and three subsequent inocula were administered at monthly intervals with incomplete Freund's adjuvant. The three rabbits were bled and the sera from them tested for neutralizing antibody against SLT-II by the cytotoxicity neutralization assay.

Bal-31 exonuclease deletion of _slt_-II gene.

To determine the 3' (carboxy terminal) functional boundary of the *slt*-II gene, pLP15 DNA was first digested with *Eco* RI to linearize the plasmid. The unique *Eco* RI site in pLP15 is approximately 200 bp downstream from the 3' end of *slt*-II gene (see Fig. 4). The linearized plasmid was then treated with *Bal*-31 exonuclease (Bethesda Research Laboratories) according to published procedures (Maniatis *et al.*, 1982). Briefly, *Bal*-31 exonuclease was added to the linearized pLP15 DNA and then samples were obtained at 10, 12.5, 15, 17.5 and 20 minutes of incubation at 30° C. These samples were placed in eppendorf tubes that contained 3 µl of 0.5 M EDTA on dry ice. Next, the samples were pooled, and the DNA was extracted with phenol-chloroform and precipitated with ethanol. The DNA was resuspended in 50 µl of TE buffer. The ends of the deleted fragments were then filled with T4 DNA polymerase in the presence of dNTP. The fragments were then circularized by blunt-end ligation with T4 DNA ligase and transformed into *E. coli* DH5α.

Colony screening by oligonucleotide probe hybridization.

The pool of deletion mutants were first probed with an oligonucleotide probe (19mer) which spanned the 43rd and 49th codons of the 70 amino acid mature SLT-II B polypeptide to identify mutants that retained the coding sequences for at least 49 amino acid residues of the mature B polypeptide. Next, *E. coli* transformed with *Bal* 31-treated pLP15 were directly plated on nylon membrane filters [Gene Screen hybridization transfer membranes (NEN Research Products, Boston, MA)] placed over 2YT agar plates with ampicillin (200 μg/ml). The plates were incubated at 37° C until bacterial colonies reached approximately 1 mm in diameter. Replica filters were made from the original membranes, and the colonies on the replica membrane were incubated on a 2YT agar plate with ampicillin until the colonies reached approximately 1 mm in diameter. Next the replica filter was transferred onto a 2YT plate with chloramphenicol (0.15 μg/ml), and the plate was incubated at 37° C overnight (to amplify the plasmid copy number). The bacterial colonies on the nylon membrane were lysed, and the plasmid DNA was denatured according to published procedures (Davis *et al.*, 1986). The filter was baked at 80° C for 1 hour and then immersed in a prehybridization solution [6X SSC (Maniatis *et al.*, 1982), 5X Denhardt's (Maniatis *et al.*, 1982), 20 mM NaH_2PO_4, 500 μg/ml sonically disrupted salmon sperm DNA] for 2 hours at 42°C. Next, the filter was immersed in a hybridization solution (0.4% SDS, 6X SSC, 20 mM NaH_2PO_4 and 500 μg/ml sonically disrupted salmon sperm DNA) that contained the labelled oligonucleotide probe and incubated overnight at 42° C. The probe (5' CAGTTGACAGGAATGACTG 3') was used after 5' end-labelling with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P] ATP at a specific activity of 1000-3000 Ci/mM. The filter was washed three times at room temperature in 6x SSC containing 0.1% SDS. A fourth and final wash was done at 45° C in 3x SSC with 0.1%

SDS solution. The filter was exposed at -70° C, to an X-ray film with an intensifying screen. The colonies that elicited signals on the replica filter were identified. The corresponding colonies from the master filter were then picked, and the plasmid DNA isolated from them and sequenced.

In vitro translation assay.

The effect of N-terminal deletions on the SLT-IIA enzymatic activity was determined by assessing the degree of inhibition of protein synthesis in an *in vitro* translation system (Stratagene Cloning Systems). Periplasmic extracts of *E. coli* expressing the deletion mutants were prepared as described by Hovde *et al.* (1988). The mutants were grown with aeration in 300 ml of BHI medium (Difco Laboratories, Detroit, MI) with ampicillin (200 µg/ml) for 24 hours at 37° C. The cells were collected by centrifugation (5,000 x g for 10 minutes at 4° C) and washed three times in 300 ml of PBS. The washed cells were resuspended in 5 ml of PBS containing polymyxin B sulfate (2 mg/ml) and incubated at 4° C for 15 minutes. The polymyxin B-treated cells were pelleted by centrifugation (10,000 x g for 10 minutes at 4° C), and the supernatant (periplasmic extract) collected and stored at -20° C. One microliter of this periplasmic extract was added to 20 µl of nuclease-treated rabbit reticulocyte lysate provided in the *in vitro* translation kit. This mixture was incubated at 30° C for 15 minutes, and then 100 ng of Brome Mosaic virus (BMV) RNA (Promega Corporation, Madison, WI.) and 2 µl of [^{35}S] methionine (10 µCi) were added. The mixture was then incubated for 1 hour at 30° C. The translated products were analysed by autoradiography of SDS-PAGE gels as described earlier. The controls included in the assay were: a periplasmic extract of *E. coli* (pBS-) incubated with the rabbit

reticulocyte lysate mixture and BMV RNA; a periplasmic extract of *E. coli* (pBS-) incubated with the reticulocyte lysate mixture without BMV RNA; and a periplasmic extract of *E. coli* (pLP15) incubated with the reticulocyte lysate mixture and BMV RNA.

RESULTS

I. Generation and characterization of SLT-II-specific monoclonal antibodies.

A. Production of MAb to SLT-II.

Twenty microtiter plates were seeded with the mouse myeloma-spleen cell fusion mixture, and more than 900 wells showed growth after 3 to 4 weeks of incubation. Culture supernatants from these hybrids were tested in an ELISA as well as in a toxin-neutralization assay on HeLa cells to detect both toxin-neutralizing and toxin-binding antibodies. Fifty three hybrid culture supernatants were positive by ELISA for binding antibodies against crude SLT-II. Ten of these ELISA-positive supernatants were also positive by the toxin-neutralization assay. From these 10 hybrids, five stable clones were selected for further characterization (Table 5). All five of these hybrids were obtained from spleens of mice immunized with the formalinized SLT-II toxoid. Four of the five hybridoma lines, designated 11F11, 11G10, 2E1, 10E10, were of the IgM class. The fifth hybridoma, 11E10, belonged to the IgG_1 isotype. All five lines had kappa (κ) light chains.

B. Characterization of monoclonal antibodies.

The anti-SLT-II monoclonal antibodies were tested for the capacity to specifically immunoprecipitate SLT-II from an ^{125}I-labelled crude preparation of SLT-II. Four of the SLT-II neutralizing MAbs precipitated two peptides with approximate molecular weights (M_r) of 33,000 and 8,000 (Figure 6). The fifth cytotoxin-neutralizing MAb (11E10) was not included on the gel shown in Figure 5 but did immunoprecipitate two peptides of the same molecular weights on a separate gel (data not shown). The sizes of the immunoprecipitated peptides are consistent with the reported molecular weights of

the A and B subunits of SLT-II (Jackson *et al.*, 1987). These data reveal that all five SLT-II neutralizing MAbs can immunoprecipitate the SLT-II holotoxin. To define the subunit specificity of the MAbs, Western blot (immunoblot) analyses were attempted with all five of the MAbs. None of the MAbs were able to detect the denatured toxin proteins transferred onto nitrocellulose membranes after SDS-PAGE. By contrast, polyclonal antiserum to crude SLT-II was able to detect the denatured toxin (data not shown). These findings suggest that the MAbs recognize conformational, rather than sequence determined epitopes. Next, as an alternate approach to Western blot analysis, urea-treated fractions of crude SLT-II were subjected to immunoprecipitation with the individual MAbs. Olsnes *et al.* (1981) reported that urea treatment separates the A and B subunits of Shiga toxin. As shown in Figure 7, all five MAbs precipitated a protein of 33,000 daltons in the higher molecular mass urea-treated fraction (over 10,000 daltons). Immunoprecipitation performed on the lower molecular mass fraction (less than 10,000 daltons) did not precipitate any protein (Figure 7). These results indicate that the five SLT-II neutralizing MAbs react with epitopes of the A subunit of SLT-II. The cytotoxin specificities of the MAbs were assessed by a toxin-neutralization assay. None of the MAb was able to neutralize either Shiga toxin or SLT-I. The MAb 11E10 partially neutralized SLT-IIv, while the other four MAbs did not cross neutralize SLT-IIv (Table 5).

C. The mapping of epitopes reactive with SLT-II-specific monoclonal antibodies.

A sublibrary of the *slt*-II gene in pLP15, was constructed in the expression vector λ ZAP (see Figure 8). The recombinant phage library was screened with the 5 individual SLT-II specific monoclonal antibodies against the A subunit. Figure 9 shows a representative plaque lift (from a lawn containing approximately 500 plaques) screened with MAb 11E10. For each MAb tested, 15 different immunoreactive plaques

were picked. After plaque purifying the immunoreactive recombinant phages, the nucleotide sequence of the insert DNA fragment that encoded the immunoreactive epitope was determined by sequencing accross the fusion joints with T_3 and T_7 oligonucleotide primers as depicted in Figure 8. Although 15 immunoreactive plaques were isolated for each MAb, the nucleotide sequences of the insert DNA fragments were determined for only 6 of the immunoreactive recombinant clones for each of the MAb. All the immunoreactive clones that were sequenced contained gene segments larger than 800 bp. These DNA inserts were larger than expected, since in creating the sublibrary it was aimed at obtaining DNA fragments of approximately 500 bp in size by agarose gel fractionation.

II. Mutational analyses of the B subunits of SLT-II and Shiga toxin.

A. Sodium bisulfite mutagenesis of gapped duplex DNA to isolate noncytotoxic mutants.

The strategy for generation of gapped duplex DNA molecules from pLP15, mutagenesis of the gapped duplex DNA and selection for mutants in the *slt*-IIB gene is outlined in Figure 8. When *Sph* I linearized pLP15 DNA-U was mixed with pLP15 plasmid DNA from which the target *slt*-IIB region had been excised, four types of duplex molecules were, in principle, formed after renaturation (Figure 10, types I-IV). This mixture of duplex molecules was then subjected to sodium bisulfite mutagenesis. The rate of mutagenesis of the *slt*-IIB target region was controlled by varying the concentration of bisulfite used in the reaction. The type II and III molecules represented sense and antisense strands of the target region simultaneously subjected to mutagenesis. When the four types of mutagenized gapped duplex DNA were subjected to gap repair and the DNA transformed into *E. coli* MV1190 (dut^+, ung^+), a strong selection was exerted against type I molecules with misincorporated uracil in both strands. In addition the

Table 5. **Characterization of SLT-II specific MAbs**

MAb line [a]	Isotype	Cytotoxin-neutralizing activity against [b]:			
		Shiga	SLT-I	SLT-II	SLT-IIv
11F11	IgM(κ)	-	-	+	-
11G10	IgM(κ)	-	-	+	-
2E1	IgM(κ)	-	-	+	-
10E10	IgM(κ)	-	-	+	-
11E10	IgG_1(κ)	-	-	+ / -	+ / -

[a] MAbs all specific for the A subunit of SLT-II.

[b] Ability of undiluted hybridoma culture supernatants to neutralize 10 CD_{50} of cytotoxin. +, full protection from cytotoxic activity; -, no protection from cytotoxic activity; +/-, partial protection from cytotoxic activity.

Figure 6. Immunoprecipitation of ^{125}I-labelled crude SLT-II by MAbs. The immunoprecipitants were resolved by SDS-PAGE on a 10% polyacrylamide gel. Lanes A, B, C, and D contain the immunoprecipitants obtained with anti SLT-II MAbs 11F11, 11G10, 2E1, and 10E10, respectively. Lane E contains the precipitate obtained with anti-cholera toxin MAb 32D3. The numbers on the left indicate molecular masses (in kilodaltons) of the polypeptide standards. A and B to the right of lane E indicate positions of A and B subunits of SLT-II.

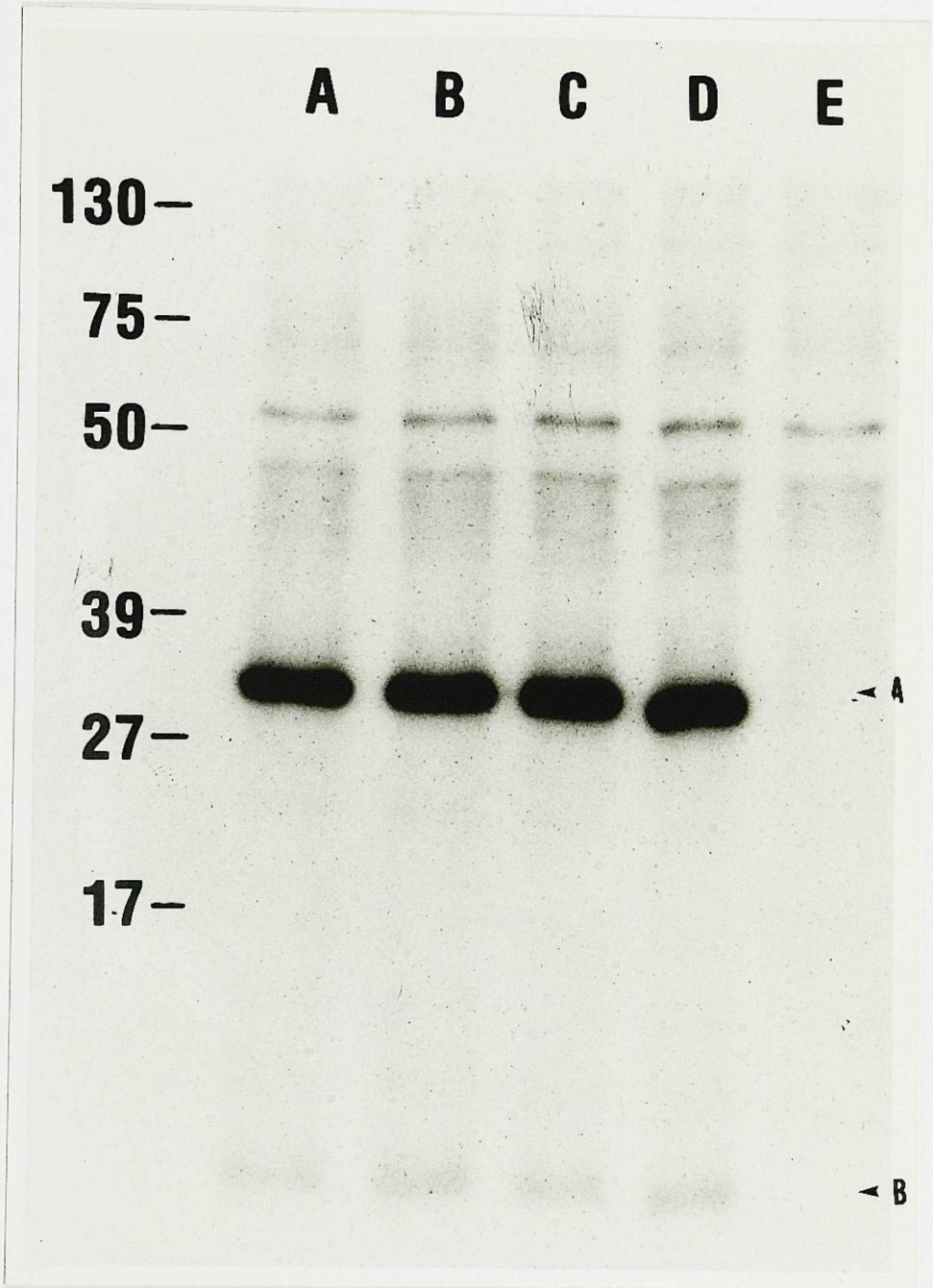

A
B
C
D
E
130
75
50
39
27
17
A
B

Figure 7. Immunoprecipitation of ^{125}I-labelled crude SLT-II by MAbs after urea treatment and fractionation. The immunoprecipitants were resolved by SDS-PAGE on 15% polyacrylamide gels. Lane A, crude SLT-II immunoprecipitated with polyclonal rabbit anti-SLT-II raised against crude SLT-II. Lane B, lower-molecular-mass fraction obtained after treatment of crude SLT-II with urea. Lane C, higher-molecular-mass fraction obtained after treatment of crude SLT-II with urea. Lane D, crude SLT-II (not urea treated or fractionated) immunoprecipitated with anti-cholera toxin MAb 32D3. Lanes E, F, G, H and I, immunoprecipitants obtained when anti-SLT-II MAbs 11F11, 11G10, 2E1, 10E10 and 11E10, respectively, were used to immunoprecipitate the higher-molecular-mass fraction obtained after treatment of crude SLT-II with urea. Lanes J, K, L, M and N, immunoprecipitants obtained when anti-SLT-II MAbs 11F11, 11G10, 2E1, 10E10 and 11E10, respectively, were used to immunoprecipitate the lower-molecular-mass fraction obtained after treatment of crude SLT-II with urea. The numbers on the left indicate molecular masses (in kilodaltons) of the polypeptide standards, A and B to the right of lane N indicate positions of A and B subunits of SLT-II.

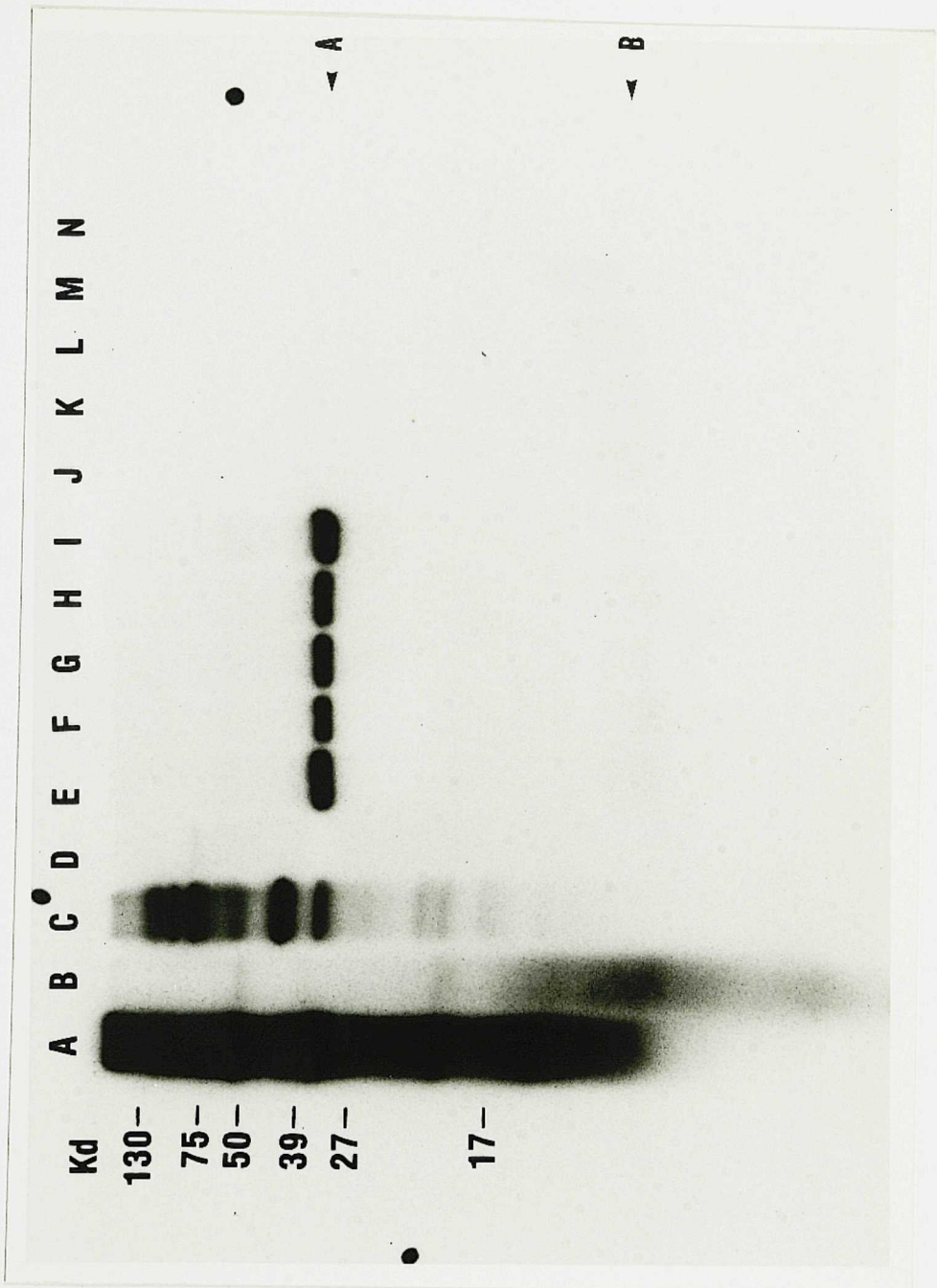

A B C D E F G H I J K L M N
Kd
130—
75—
50—
39—
27—
17—
A
B

Figure 8. Schematic outline of epitope mapping using λ ZAP vector. A sublibrary of *slt*-II gene was created in λ ZAP expression vector as described in the Materials and Methods section. The immunoreactive clones were identified and the insert DNA fragments that encoded the immunoreactive epitopes were sequenced using T_3 and T_7 oligonucleotide primers.

LAMBDA ZAP II

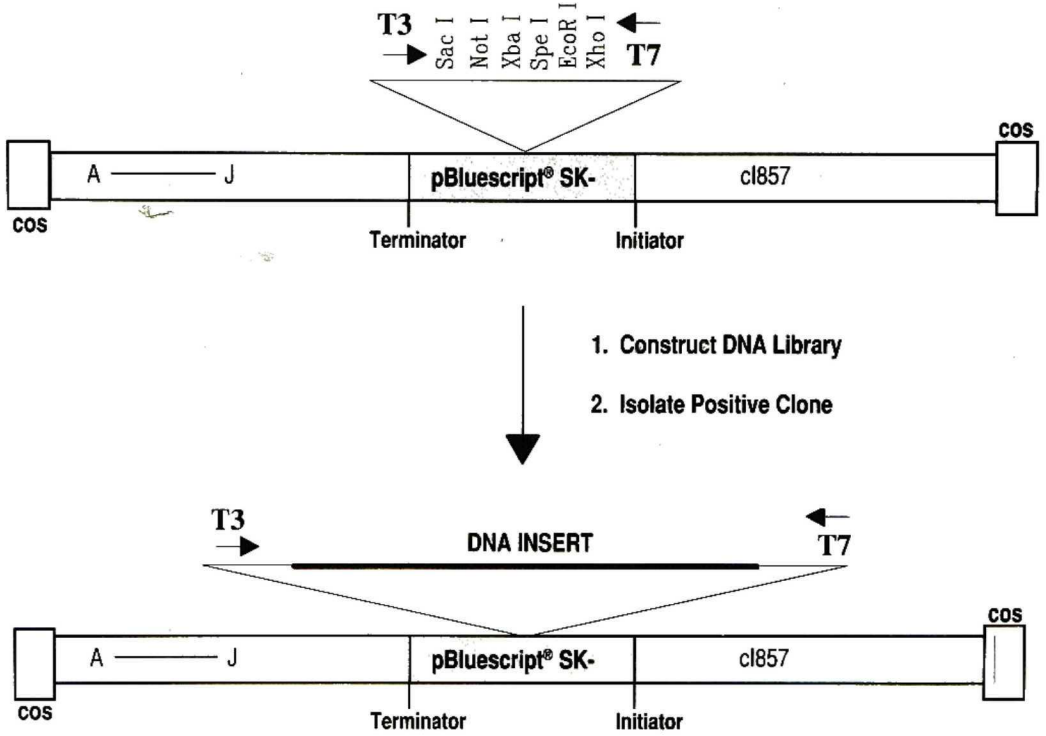


Figure 9. Screening of recombinant λ ZAP library with SLT-II specific MAbs. A representative "plaque lift" screened with MAb 11E10 is shown. An *E. coli* BB4 lawn containing approximately 500 recombinant plaques were overlaid with a nitrocellulose filter. After adsorption of antigens, the filter was removed and probed with SLT-II specific MAb. The donut-shaped blue spots represent the location of recombinant clones that expressed an immunoreactive epitope.

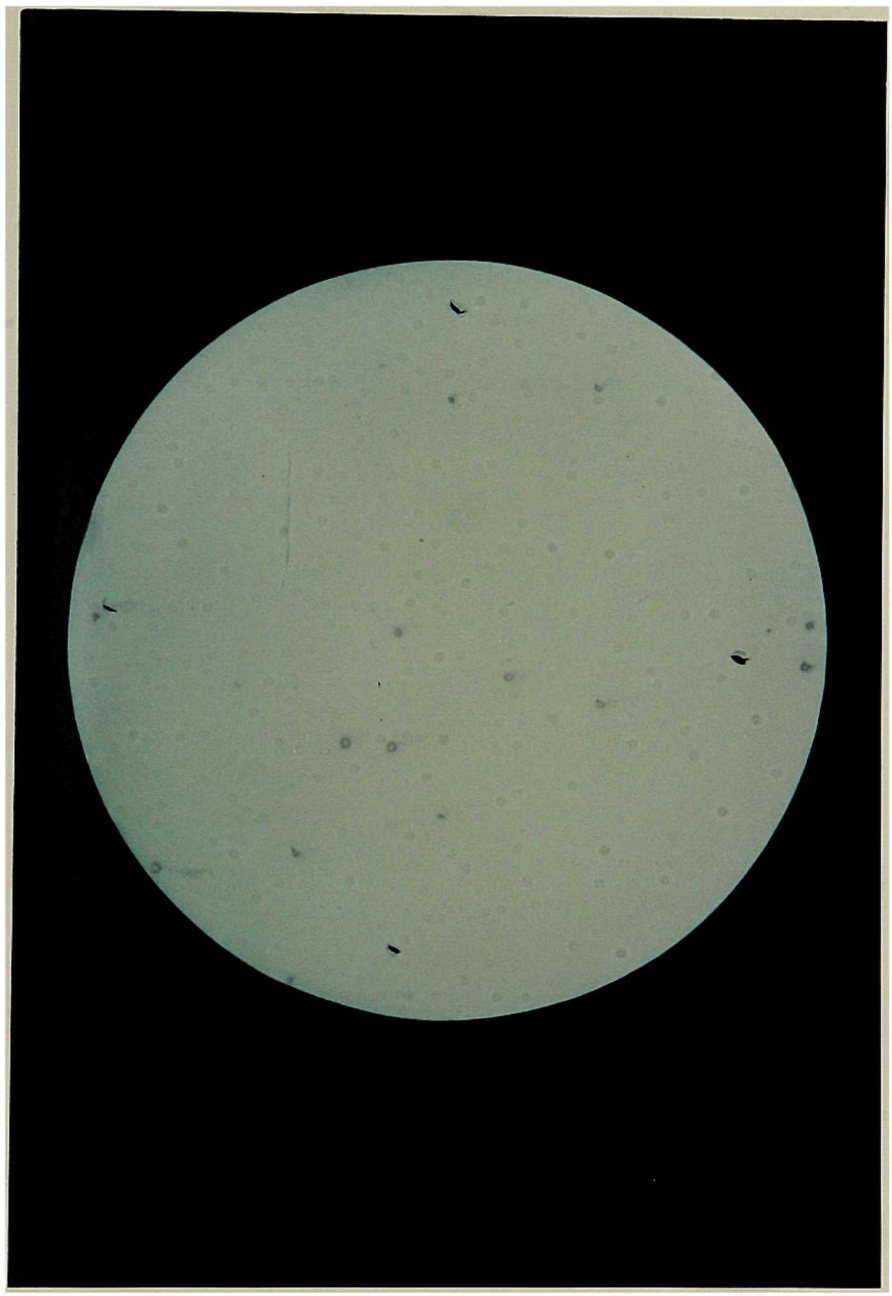
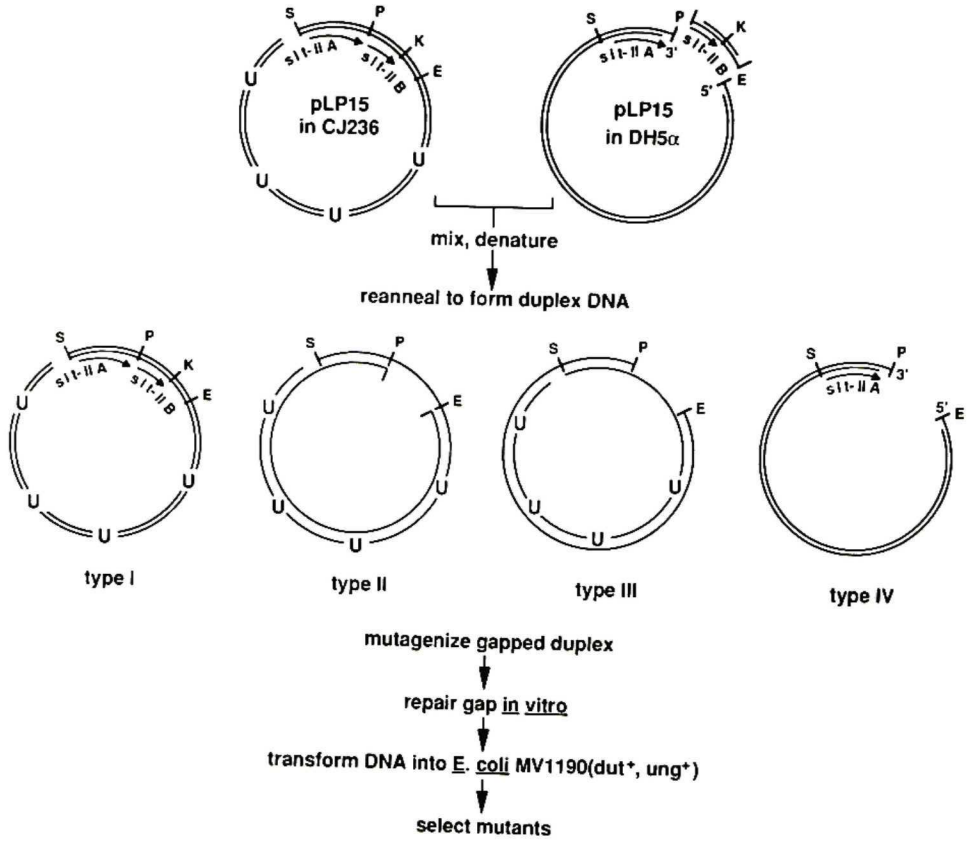


Figure 10. Schematic outline of the method for generating gapped duplex DNA molecules for single-strand-specific bisulfite mutagenesis and enrichment for mutant selection. The *Sph* I linearized uracil-containing pLP15 DNA was mixed with pLP15 from which the *slt*-IIB gene had been excised. Following denaturation and renaturation of the mixture, the gapped duplex DNA was subjected to mutagenesis. The gaps were repaired *in vitro*, and the DNA was used to transform *E. coli* MV 1190 (dut^+, ung^+). Mutants with reduced cytotoxicity were selected. This protocol favors the progeny of type II and III molecules because type I molecules contain misincorporated uracil in both strands and are rapidly degraded in *E. coli* MV1190 and type IV molecules do not form covalently closed circular molecules for efficient transformation. Abbreviations for the endonuclease restriction sites: S, *Sph* I; P, *Pst* I; K, *Kpn* I; E, *Eco* RI.


S
P
K
E
slt-II A
slt-II B
pLP15
in CJ236
U
pLP15
in DH5α
3'
5'
mix, denature
reanneal to form duplex DNA
type I
type II
type III
type IV
mutagenize gapped duplex
repair gap in vitro
transform DNA into E. coli MV1190(dut+, ung+)
select mutants

inability to form covalently closed circular molecules greatly diminished the transformation efficiency of type IV molecules. Thus, there was a strong selection for types II and III molecules. The transformants were screened for cytotoxicity. From a total of 800 transformants screened, three mutants were isolated which completely lacked any detectable cytotoxicity on both Vero and HeLa cells. The loss of any detectable cytotoxicity in these mutants represented at least a 1000-fold reduction in cytotoxicity when compared to the parental strain (Table 6). Furthermore, the sonic lysates of these *E. coli* strains which expressed the mutant toxins were not cytotoxic even after the lysates were concentrated 100-fold with ammonium sulfate.

B. Mapping of mutations by sequence analysis.

The sequence analysis of the noncytotoxic SLT-II mutants revealed that each of the three mutants contained a single point missense mutation in the *slt*-IIB gene targeted in the regionally-directed mutagenesis. In the mature B subunit of SLT-II, the three independent mutations mapped to the 32nd, 42nd, and 59th codons of the 70 amino acid residue polypeptide. The 32nd codon CGC which codes for arginine (R) was mutated to TGC which codes for cysteine (C) (mutant SLT-II R32C). The 42nd codon GCT which codes for alanine (A) was mutated to ACT which codes for threonine (T) (mutant SLT-II A42T). The 59th codon GGC which codes for glycine (G) was changed to GAC which codes for aspartic acid (D) (mutant SLT-II G59D). The mutations were designated according to Knowles, 1987. In the R32C mutation, cytosine in the sense strand had undergone deamination, whereas in the A42T and G59D mutations, cytosines in the antisense strand had undergone deamination by bisulfite. The abrogation of cytotoxicity in these mutants suggests an important role for the arginine, alanine, and glycine residues at these respective positions in mediating cytotoxic activity of the SLT-II holotoxin. Furthermore, these 3 amino acids are conserved in the 69 amino acid polypeptide of the B subunit of Shiga toxin. Because of an additional proline in the N-terminus of Shiga

toxin, the analogous codons in the Shiga toxin are the 33rd, 43rd, and 60th (Strockbine *et al.*, 1988).

To determine whether these 3 residues are critical for the cytotoxic activity of Shiga toxin, identical mutations were induced by oligonucleotide-directed, site-specific mutagenesis in M13mp18 recombinants carrying *stxB*. All three mutations caused a drastic reduction in the cytotoxic activity in comparison to the wild type Shiga toxin (Table 6). The reconstruction of each mutant *stx* operon (wild type A subunit gene with mutated B subunit gene) was facilitated by the construct pLPSH3 (see Figure 5) in which the *Hind* III and *Eco* RI sites on the vector upstream of the *stx* gene in pNAS13 were eliminated. In contrast to the corresponding SLT-II mutants, Shiga R33C, Shiga A43T, and Shiga G60D mutants still retained detectable cytotoxic activity. However, the fold reduction in cytotoxic activity between wild type toxin and the mutants was similar for Shiga toxin and SLT-II mutants.

C. Mutational effects on the conformation of toxin molecule as determined by dot blot ELISA.

To determine whether the mutations had induced conformational changes in the B subunit of Shiga toxin or SLT-II, the immunoreactivity of these mutants with B subunit specific-MAbs was analyzed using a dot blot ELISA. The immunoreactivity profiles of the mutant toxins are shown in Figures 11 and 12, and summarized in Table 6. The MAbs 13C4, 16E6, and 19G8 recognize conformational dependent epitopes on the B subunit of Shiga toxin (Strockbine *et al.*,1985). All three monoclonal antibodies reacted strongly with the mutant Shiga R33C. In contrast the A43T mutation affected the immunoreactivity of Shiga toxin drastically; the Shiga A43T mutant elicited only weak reactivity with 13C4, 16E6, and 19G8 monoclonal antibodies. The Shiga G60D mutant reacted with both 13C4 and 16E6 monoclonal antibodies but did not react with 19G8 MAb. This finding suggests that the G60D mutation affected the epitope reactive

Table 6. **Vero cell cytotoxicity and immunoreactivity of the SLT-II and Shiga toxin mutants**

Toxin	Vero cell cytotoxicity	Immunoreactivity with MAb[b]:			
	[a]CD_{50}	13C4	16E6	19G8	BC5
SLT-II	10^4-10^5	NA	NA	NA	+++
SLT-II R32C	<10	NA	NA	NA	-
SLT-II A42T	<10	NA	NA	NA	++
SLT-II G59D	<10	NA	NA	NA	++
Shiga toxin	10^9-10^{10}	+++	+++	+++	NA
Shiga R33C	10^1-10^2	+++	+++	+++	NA
Shiga A43T	10^4-10^5	+/-	+/-	+/-	NA
Shiga G60D	10^3-10^4	+++	+++	-	NA

[a] The highest 10-fold dilution of the toxin preparation that caused lysis of 50% of the cell monolayer (CD_{50}).

[b] MAbs 13C4, 16E6 and 19G8 are specific for the B subunit of Shiga toxin (Strockbine *et al.*, 1985). MAb BC5 is specific for the B subunit of SLT-II (Downes *et al.*,1988). Immunoreactivity with MAbs is indicated as follows: NA, not applicable; +++, strong reaction; ++, moderate reaction; +/-, weak reaction; -, negative reaction; determined by the dot blot ELISA (see Material and Methods for details).

Figure 11. Dot blot ELISA with SLT-II mutants. Concentrated sonic lysates of *E. coli* that expressed SLT-II R32C, SLT-II A42T, and SLT-II G59D were spotted onto a nitrocellulose membrane with a dot blot apparatus and probed with MAb BC5 (specific for the B subunit of SLT-II) to determine the effect of mutations on immunoreactivity. The controls included were a sonic lysate of *E. coli* (pLP15), a sonic lysate of *E. coli* DH5α, and a sonic lysate of *E. coli* [pBS(-)]. Immunoreactivity is indicated by purple spots. The experiment was repeated three times and similar immunoreactivity profiles were obtained in each replicate experiment.

DOT BLOT ELISA-SLT-II MUTANTS

A42T mut

R32C mut

G59D mut

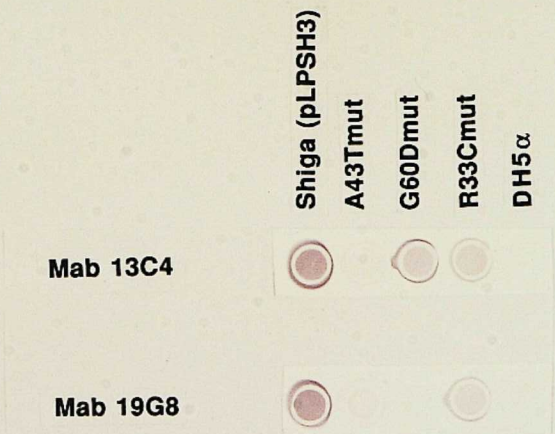
pLP15 (SLT-II)

DH5α

pBS(-)

Figure 12. Dot blot ELISA with Shiga toxin mutants. Concentrated sonic lysates of *E. coli* that expressed Shiga R33C, Shiga A43T, and Shiga G60D were spotted onto a nitrocellulose membrane with a dot blot apparatus and probed separately with MAb 13C4, MAb 19G8, and MAb 16E6 to determine the effects of mutations on immunoreactivity with each of these monoclonal antibodies. The immunoreactivity profile of the mutants with MAb 16E6 was identical to that of MAb 13C4 and is not shown in the figure. The controls included were a sonic lysate of *E. coli* (pLPSH3) and a sonic lysate of *E. coli* DH5α. Immunoreactivity is indicated by purple spots. The experiment was repeated three times and similar immunoreactivity profiles were obtained in each replicate experiment.

DOT BLOT ELISA - SHIGA MUTANTS



with 19G8 MAb. The SLT-II mutants were probed with MAb BC5 (Downes *et al.*, 1988) which is specific for the B subunit of SLT-II. The mutant SLT-II R32C did not react with MAb BC5 while SLT-II A42T and SLT-II G59D did react with that MAb (Figure 11). This observation indicates that the epitope reactive with MAb BC5 was not affected by the A42T or the G59D mutations.

D. Mutational effects on receptor analogue binding.

An ELISA that was originally developed to detect the binding of Shiga toxin and SLT-II to the receptor analogue (Galα1-4Galβ-o-CETE)n BSA was used to assess whether the mutations in the B subunit of Shiga toxin affected the receptor-binding ability of the B subunit. It should be noted that when B subunit specific MAbs are used as probes for toxin in the ELISA one can only assess whether or not the B subunit of the toxin binds to the analogue. If, however, A subunit-specific MAbs are used as probes, one can also determine whether or not the subunits have combined to form a holotoxin.

Both Shiga toxin and SLT-II bound specifically to the solid-phase, anchored receptor analogue as shown in Figures 13 and 14. Neither Shiga toxin nor SLT-II bound to BSA-coated wells. Furthermore, preincubation of toxin with the soluble receptor analogue inhibited the toxin from binding to the solid-phase anchored, receptor analogue in a dose dependent manner. This finding confirmed the specificity of the toxin-receptor binding (Figure 15).

When MAb 13C4 (B subunit specific) or 4F7 (A subunit specific) were used as probes to detect receptor analogue-bound mutant toxin Shiga R33C, no signal above background was observed. This observation suggests that the R33C mutation abrogated the receptor binding capacity of the B subunit of Shiga toxin (Figures 16 and 17). By contrast, the Shiga A43T mutant did bind to the receptor analogue very weakly (Figure 16). To exclude the possibility that this apparent weak binding was really due to less avid antigen-antibody interaction (see immunoreactivity results, Table 6), the assay

was repeated with the MAb 4F7 as a probe for receptor-analogue bound holotoxin. Marked reduction in binding of Shiga A43T mutant to the receptor analogue was still observed (Figure 17). Finally, the Shiga G60D mutant, unlike the other two mutant toxins, bound the receptor analogue well at high concentrations. However, as the Shiga G60D mutant toxin was diluted, it displayed a more precipitous reduction in receptor binding than the wild type Shiga toxin (Figures 16 and 17). When SLT-II mutants were tested in the receptor-analogue ELISA none of the three mutants elicited any signal above the background when either MAb BC5 (specific for the B subunit) or MAb 11E10 (specific for the A subunit) was used to detect the receptor-analogue bound toxin (see Table 7).

E. Mutational effects on natural receptor recognition as determined by thin layer chromatogram overlay assay.

The Shiga toxin mutants were tested in TLC-overlay assay to examine any differences in binding to the entire glycolipid moiety rather than to a receptor analogue. The Shiga R33C mutant did not bind to G_{b3}, G_{b4}, or any other glycolipid in the total glycolipid extracts of Vero or HeLa cells. The binding profile of the Shiga A43T mutant revealed a drastic reduction in binding to G_{b3} and G_{b4} but no aberrant glycolipid binding was evident. The binding profile of the Shiga G60D resembled that of the wild type Shiga toxin (Figure 18). The three SLT-II mutants did not elicit any significant binding to G_{b3} glycolipid in TLC-overlay assay (see Table 7).

F. Competitive inhibition of cytotoxicity by receptor analogue to determine the receptor-analogue binding ability of Shiga A43T mutant.

The receptor-analogue ELISA and TLC-overlay assay results indicated that the Shiga A43T mutant toxin did not bind efficiently to the receptor analogue or to the natural glycolipid receptor G_{b3} (Figures 16, 17, and 18). Preincubation of the mutant

Figure 13. Receptor-analogue ELISA for Shiga toxin. The binding of Shiga toxin to the receptor analogue (Galα1-4Galβ-0-CETE)n BSA was detected by an ELISA as described in the Materials and Methods section. Shiga toxin bound to the solid-phase, anchored, receptor analogue was detected by MAb 13C4 (specific for the B subunit of Shiga toxin). Solid-phase, anchored BSA served as a negative control to detect any nonspecific binding of Shiga toxin to the BSA moiety of the receptor analogue. The absorbance values given in the graph are the averages of three separate experiments.

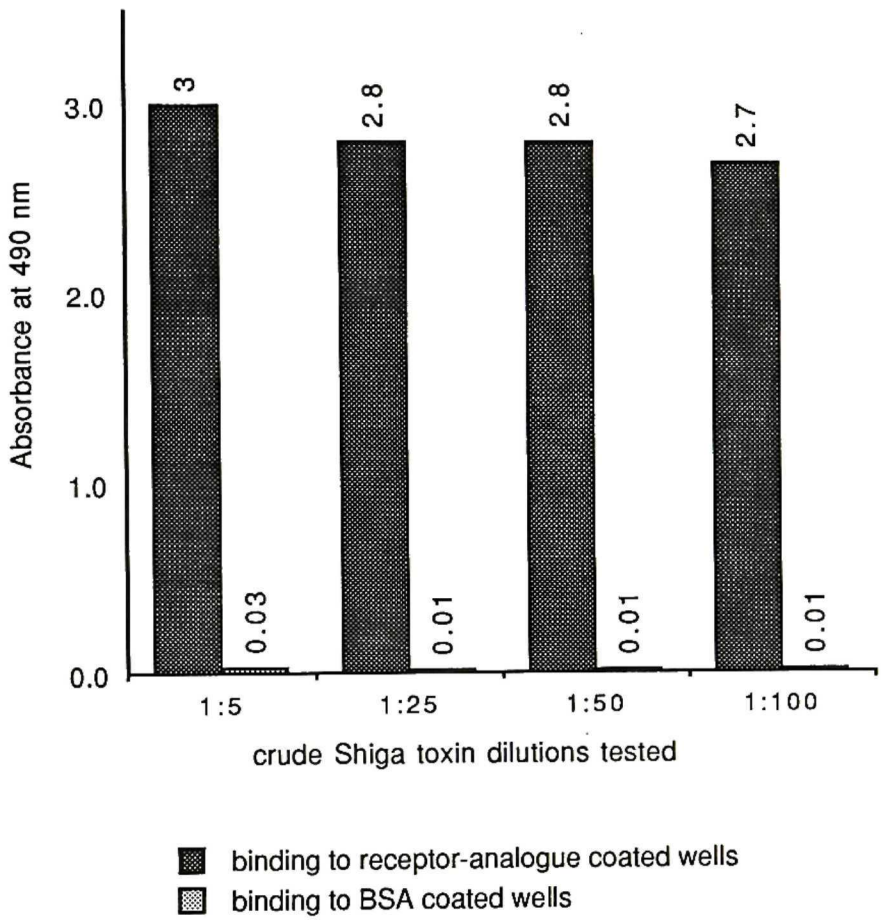

Absorbance at 490 nm
3.0
2.0
1.0
0.0
3
0.03
2.8
0.01
2.8
0.01
2.7
0.01
1:5
1:25
1:50
1:100
crude Shiga toxin dilutions tested
binding to receptor-analogue coated wells
binding to BSA coated wells

Figure 14. Receptor-analogue ELISA for SLT-II. The binding of SLT-II to the receptor analogue (Galα1-4Galβ-0-CETE)n BSA was detected by an ELISA as described in the Materials and Methods section. SLT-II bound to the solid-phase, anchored, receptor-analogue was detected by MAb 11F11 (specific for the A subunit of SLT-II). Solid-phase, anchored BSA served as a negative control to detect any nonspecific binding of SLT-II to the BSA moiety of the receptor analogue. The absorbance values given in the graph are the averages of three separate experiments.

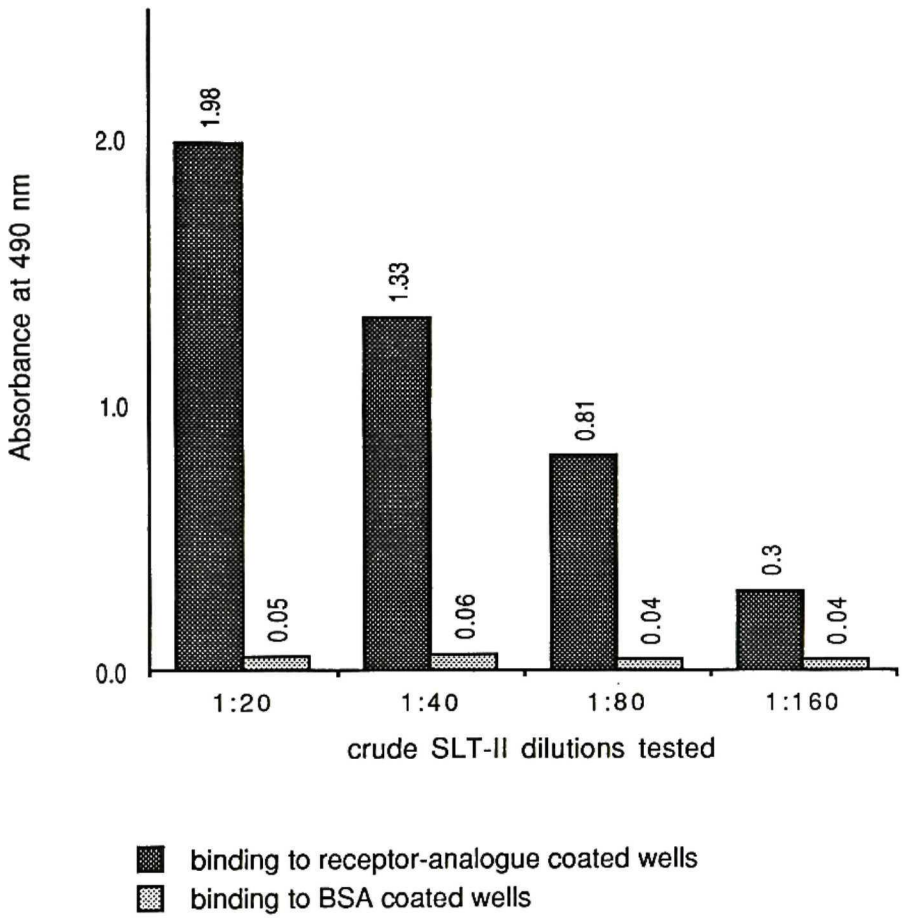

Absorbance at 490 nm
2.0
1.0
0.0
1.98
0.05
1.33
0.06
0.81
0.04
0.3
0.04
1:20
1:40
1:80
1:160
crude SLT-II dilutions tested
binding to receptor-analogue coated wells
binding to BSA coated wells

Figure 15. Competition ELISA to assess the specificity of binding of Shiga toxin to the receptor analogue. The binding of Shiga toxin to the receptor-analogue (Galα1-4Galβ-0-CETE)n BSA was determined as described in the Materials and Methods section. To determine the specificity of binding, different dilutions of crude Shiga toxin were preincubated with a fixed amount of soluble receptor-analogue for 30 minutes before assaying in the ELISA. The toxin dilutions preincubated with an equivalent quantity of BSA were included in the assay as a control. The absorbance values given in the graph are the averages of three separate experiments.

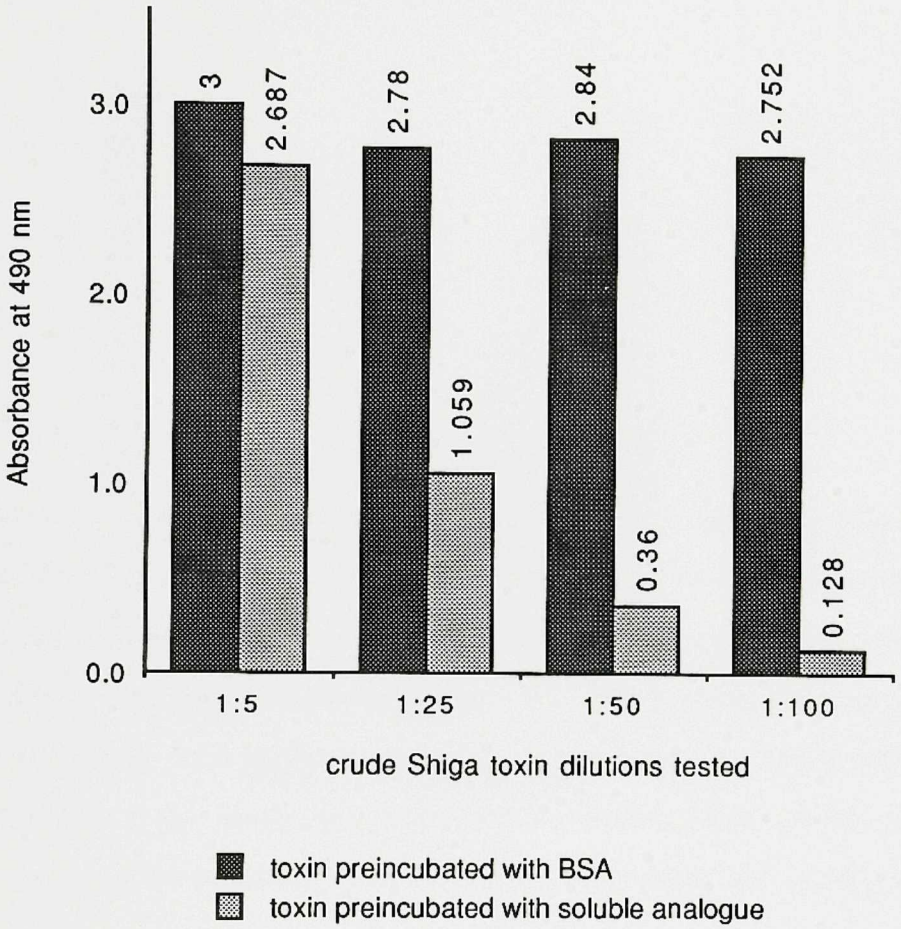

Absorbance at 490 nm
3.0
2.0
1.0
0.0
3
2.687
2.78
1.059
2.84
0.36
2.752
0.128
1:5
1:25
1:50
1:100
crude Shiga toxin dilutions tested
toxin preincubated with BSA
toxin preincubated with soluble analogue

Figure 16. Binding of Shiga toxin mutants to receptor analogue. Different dilutions of concentrated sonic lysates of *E. coli* that expressed the Shiga toxin and the Shiga toxin mutants were tested for receptor-analogue binding by a receptor-analogue ELISA as described in Materials and Methods. The receptor-analogue bound toxin was detected with MAb 13C4 which is specific for the B subunit of Shiga toxin (Strockbine *et al.*, 1985). The Shiga R33C mutant did not elicit any signal even at the highest concentration tested. The absorbance values given in the graph are the averages of three separate experiments.

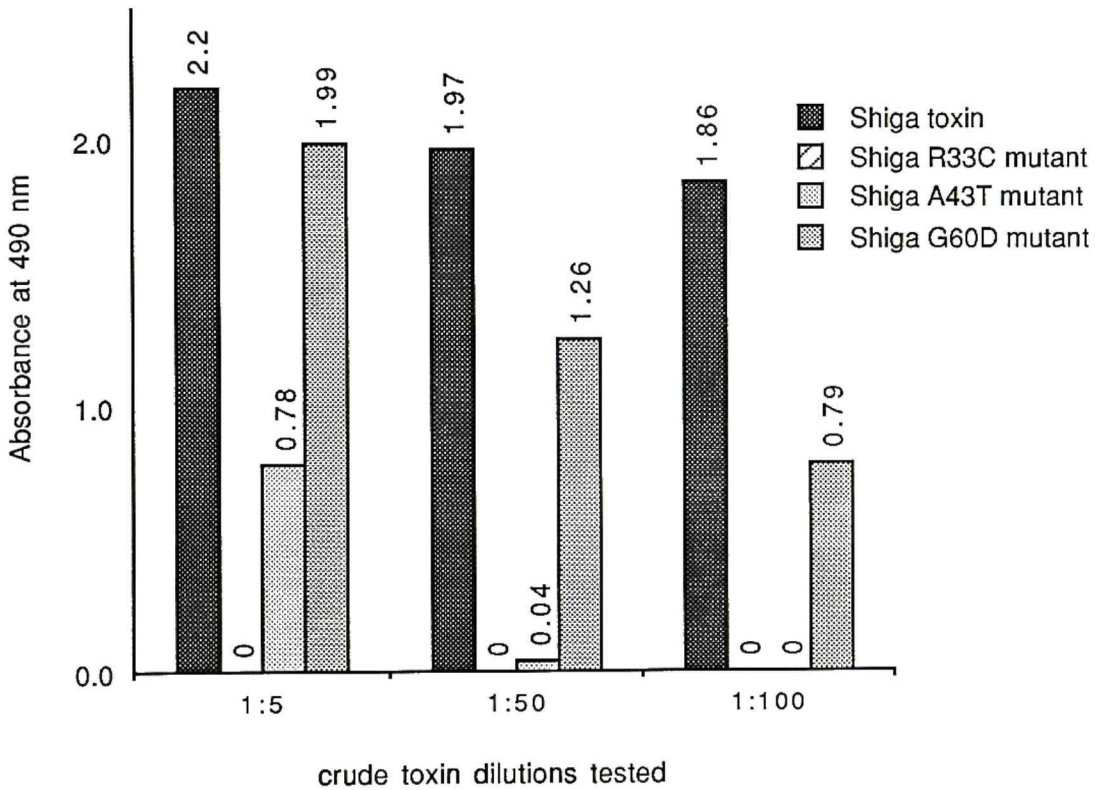
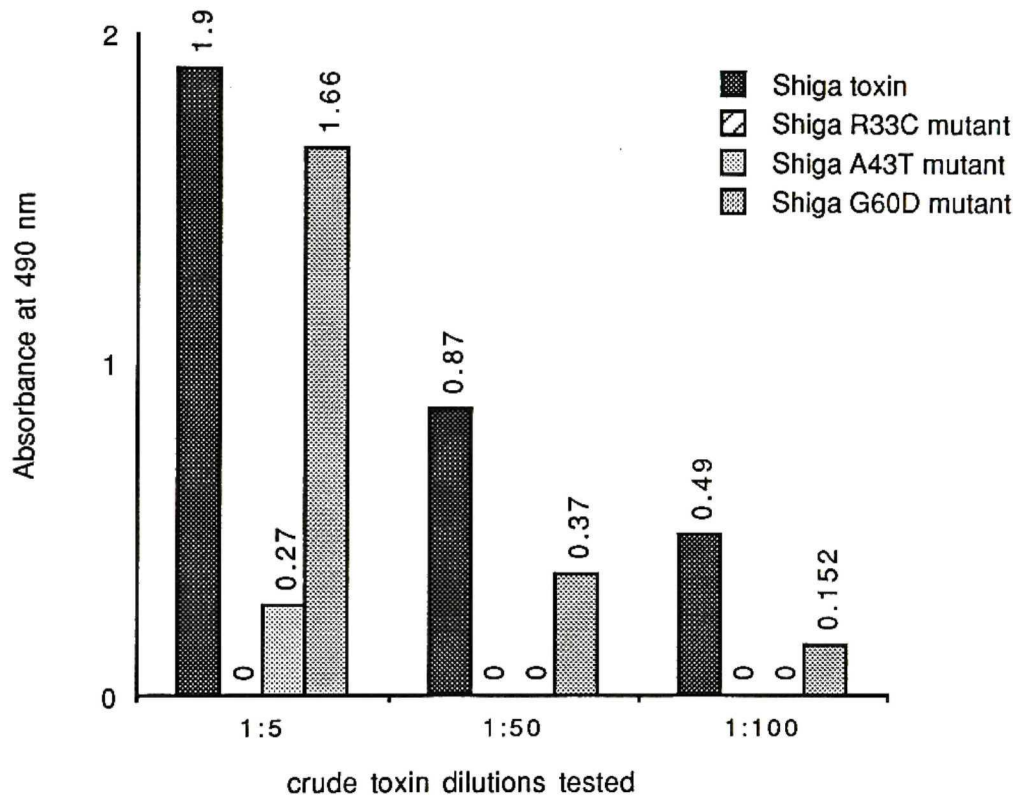

Absorbance at 490 nm
2.0
1.0
0.0
2.2
0
0.78
1.99
1.97
0
0.04
1.26
1.86
0
0
0.79
1:5
1:50
1:100
crude toxin dilutions tested
Shiga toxin
Shiga R33C mutant
Shiga A43T mutant
Shiga G60D mutant

Figure 17. Binding of Shiga toxin mutants to the receptor analogue. Different dilutions of concentrated sonic lysates of *E. coli* that expressed the Shiga toxin and the Shiga toxin mutants were tested for receptor-analogue binding by a receptor-analogue ELISA as described in the Materials and Methods section. The receptor-analogue bound toxin was detected with MAb 4F7 which is specific for the A subunit of Shiga toxin. The Shiga R33C mutant did not elicit any signal even at the highest concentration tested. The absorbance values given in the graph are the averages of three separate experiments.

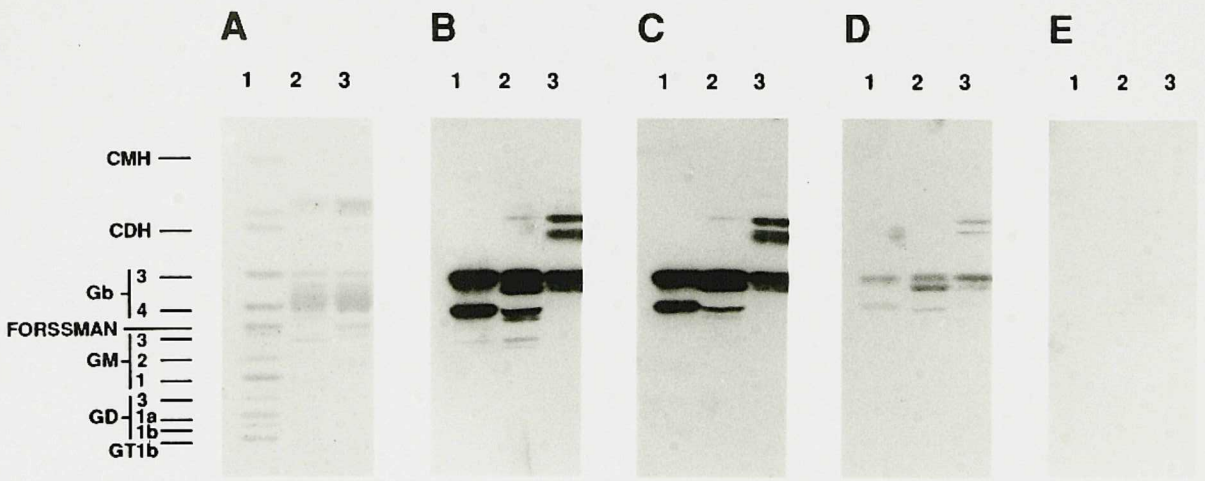

Absorbance at 490 nm
2
1
0
1.9
0
0.27
1.66
0.87
0
0
0.37
0.49
0
0
0.152
1:5
1:50
1:100
crude toxin dilutions tested
Shiga toxin
Shiga R33C mutant
Shiga A43T mutant
Shiga G60D mutant

toxin Shiga A43T with galabiose-derivatized Sepharose beads (galabiose is Galα1-4Gal, the carbohydrate moeity in G_{b3}) for 1 hour at 4°C resulted in a 10-fold reduction in HeLa cell cytotoxicity. By contrast, when wild type Shiga toxin was similarly treated, there was approximately a 10,000-fold reduction in cytotoxicity (see Table 8). This differential binding of Shiga A43T and wild type Shiga toxin to galabiose is consistent with the results of the receptor-analogue ELISA and the TLC-overlay assay and further substantiates the conclusion that Shiga A43T does not bind strongly to the natural toxin receptor.

G. Mutational effects on binding of toxins to Vero cells as determined by immunofluorescence assay.

Shiga toxin mutants were tested in an indirect immunofluorescence assay to determine the effects of the mutations, on binding of toxin to Vero cells and on subunit assembly. Both binding of B subunits to Vero cells as well as the assembly of A and B subunits into holotoxin were required to elicit fluorescence since the A subunit of Shiga toxin is incapable of binding to Vero cells directly (Donohue-Rolfe *et al.*, 1989). During the entire assay, cells were maintained in buffer with sodium azide to prevent any internalization of bound toxin. The results of the immunofluorescence assay are presented in Figure 19. The fluorescence of differentially labelled cells in the scanned field by the membrane intercalating fluorescent dye "di1", is depicted in the detector 2 column of Figure 19. The toxin-specific fluorescence elicited by wild type Shiga toxin (pLPSH3) is shown in Figure 19, panel E. It should be noted that only a subset of Vero cells bound the Shiga toxin (compare detector 1 and 2 columns of panel E in Figure 19) thus indicating possible heterogeneity of the cell population with respect to receptor distribution. The Shiga R33C mutant did not elicit significant toxin-specific fluorescence (Figure 19, panel B), whereas Shiga G60D mutant showed significant toxin-specific fluorescence (Figure 19, panel D). By contrast, Shiga A43T mutant

Figure 18. TLC-overlay assay to detect binding of Shiga toxin mutants to G_{b3} and G_{b4} glycolipids. Total glycolipids from Vero and HeLa cells were subjected to chromatography on TLC plates in lanes 2 and 3, respectively of each panel. Purified neutral and acidic glycolipids were included as controls in lane 1 of each panel. The binding of toxin mutants to separated glycolipids was detected as described in the Materials and Methods. The glycolipid-bound toxin was detected with MAb 4F7 specific for the A subunit of Shiga toxin. In panel A, glycolipids on the TLC plate visualized with orcinol reagent; panel B, wild type Shiga toxin; panel C, Shiga G60D mutant; panel D, Shiga A43T mutant and panel E, Shiga R33C mutant. The positions of the glycolipid standards are indicated to the left of panel A. The experiment was repeated twice and similar binding profiles were obtained in each replicate experiment.


A
B
C
D
E
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
CMH
CDH
Gb
3
4
FORSSMAN
GM
3
2
1
GD
3
1a
1b
GT1b

elicited strong toxin-specific fluorescence which was almost comparable in intensity to the wild type Shiga toxin (compare panel C and E of Figure 19). No significant toxin-specific fluorescence was detected for any of the three SLT-II mutants when a monoclonal antibody specific for the A subunit (11E10) or the B subunit (BC5) was used as a probe to detect cell-bound toxin (see Table 7).

H. Intracellular degradation of Shiga toxin mutants.

Pulse-chase experiments were done to eliminate the possibility that the ablated or reduced activities of the toxin mutants were due to enhanced susceptibility to intracellular proteolytic degradation. The B subunit of the Shiga toxin migrated to a position on the gel that was easily identified and distant from other bands (Figure 20). The Shiga toxin and the three mutants had apparent intracellular half lives greater than 60 minutes (compare panels, A, B, C, and D of Figure 20). Therefore, these mutants appeared to be as stable as wild type Shiga toxin, a finding which favors the hypothesis that reduced cytotoxicity of these mutants is due to attenuation of activity rather than instability of the toxin. The stability of SLT-II mutants could not be determined because the B subunit of SLT-II co-migrated with host cell proteins in the pulse-chase experiment. This observation is consistent with the slower mobility of SLT-II B subunit (M_r 7.8) in SDS-PAGE analysis when compared to Shiga toxin B subunit (M_r 7.7) as described by other investigators (Oku *et al.*, 1989).

I. Animal inoculations to determine the immunogenicity of the SLT-II mutants.

To determine whether the ablation of Vero cell cytotoxicity of the SLT-II mutants correlated with decreased toxicity for animals, BALB/c mice and rabbits were inoculated with concentrated sonic lysates of *E. coli* expressing the SLT-II mutant toxins. Mice inoculated with lysates that contained SLT-II mutant toxins survived,

Figure 19. Binding of Shiga toxin mutants to Vero cells as detected by immunofluorescence assay using the Anchored Cell Analysis Station 470. The concentrated sonic lysates of the *E. coli* that expressed Shiga toxin or the Shiga toxin mutants were incubated with freshly trypsinized Vero cells at 4^{o} C in the presence of sodium azide. A sonic lysate of *E. coli* DH5α served as the negative control. The surface-bound toxins on Vero cells were detected by MAb 4F7 which is specific for the A subunit of Shiga toxin. The detector 1 column shows cells labelled for bound toxins, and the detector 2 column shows the same cells labelled differentially with the membrane lipid probe "di1". In panel A, *E. coli* DH5α lysate; panel B, Shiga R33C mutant; panel C, Shiga A43T mutant; panel D, Shiga G60D; panel E, wild type Shiga toxin. Each panel shows a single, representative field. The experiment was repeated three times and similar binding profiles were obtained in each replicate experiment.

A
B
C
D
E
Color Values
4095
3839
3583
3327
3071
2815
2559
2303
2047
1791
1535
1279
1023
767
511
255
0
Detector 1 Data
Detector 2 Data

Table 7. **Binding of SLT-II mutants to receptor analogue, G_{b3} glycolipid and Vero cells**

Toxin	Binding of SLT-II mutants to:		
	receptor analogue [a]	Gb3 glycolipid [b]	Vero cells [c]
SLT-II	+++	+++	+++
SLT-II R32C	-	+/-	+/-
SLT-II A42T	-	-	-
SLT-II G59D	-	+/-	+/-

[a] Binding to the receptor analogue was determined by receptor-analogue ELISA.

[b] Binding to the G_{b3} glycolipid was determined by TLC-overlay assay.

[c] Binding to Vero cells was determined by immunofluorescence assay using ACAS 470

+++, strong binding; ++, moderate binding; +, weak binding; +/-, marginal binding; -, no detectable binding.

Table 8. **Competitive inhibition of HeLa cell-cytotoxicity of Shiga toxin and Shiga A43T mutant by galabiose-derivatized Sepharose beads**

Toxin	Cytotoxicity titer (CD_{50})[a]	
	before preincubation with galabiose	after preincubation with galabiose
Shiga toxin	10^9	10^5
Shiga A43T mutant	10^5	10^4

[a] The highest 10-fold dilution of the toxin preparation that caused lysis of 50% of the cell monolayer (CD_{50}).

Figure 20. Pulse-chase analysis of intracellular degradation of Shiga toxin mutants. *E. coli* expressing the wild type Shiga toxin or Shiga toxin mutants were pulse-labelled with (^{35}S) methionine for 5 minutes as described in the Materials and Methods section. The labelled bacterial cells were harvested at 5 minutes (lane 1), 15 minutes (lane 2), 30 minutes (lane 3) and 60 minutes (lane 4) after the cold methionine chase. The cells were lysed by boiling and then subjected to SDS-PAGE electrophoresis as described in the Materials and Methods section. Lysates containing the wild type Shiga toxin are in panel A. Lysates of Shiga R33C mutant are in panel B. Lysates of Shiga A43T mutant are in panel C. Lysates of Shiga G60D mutant are in panel D. Lysates of *E. coli* DH5α labelled for 5 minutes and *E. coli* DH5α carrying the pB5 (pLPSH3 from which *stx* has been deleted) labelled for 5 minutes are included as controls. The position of the B subunit of Shiga toxin is indicated by the arrows. The experiment was repeated three times and similar results were obtained in each replicate experiment.

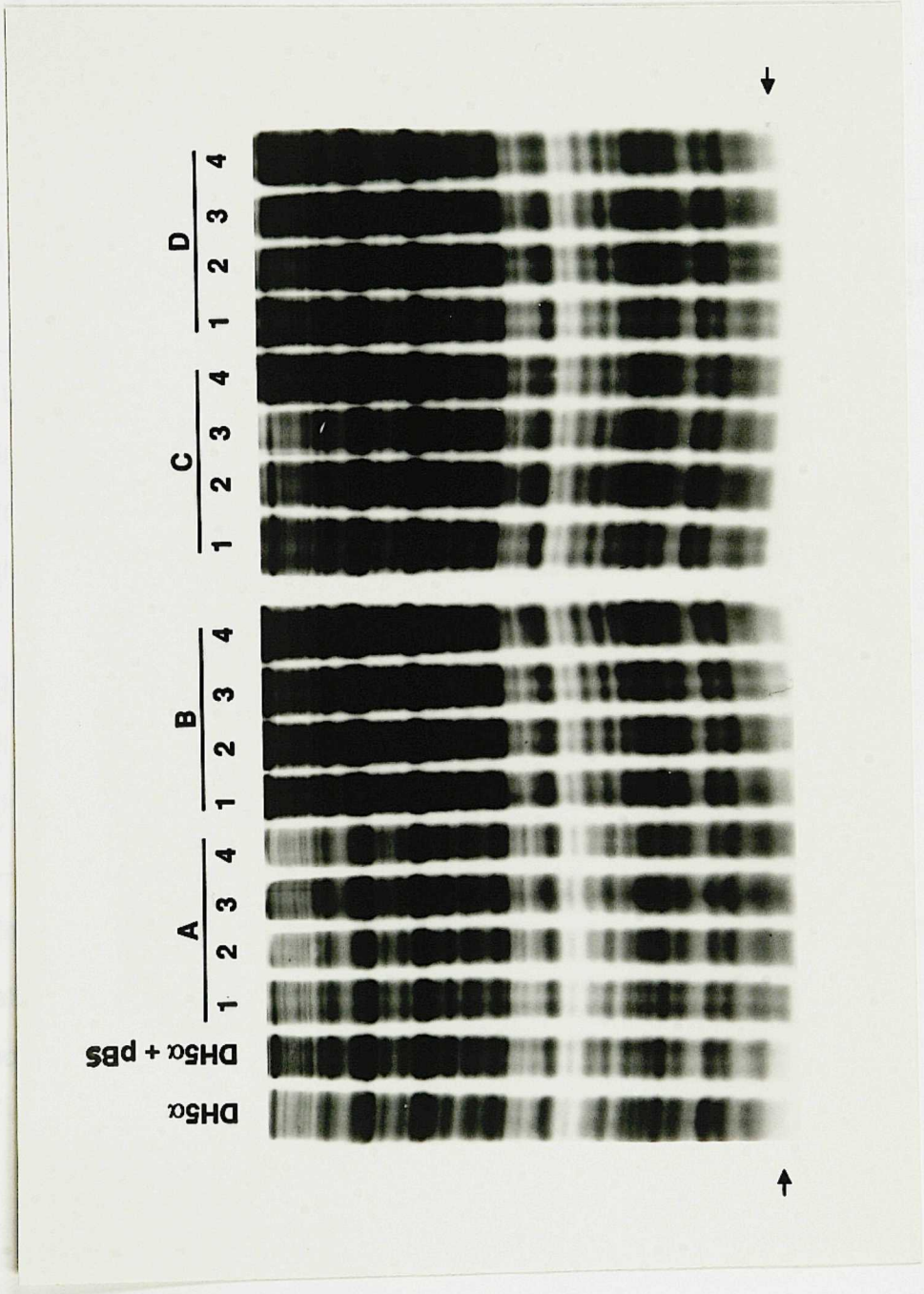

DH5α
DH5α + pBS
A
1 2 3 4
B
1 2 3 4
C
1 2 3 4
D
1 2 3 4

Table 9. **Immunogenicity and lethality of SLT-II mutants**

Toxin	Species inoculated	Number survived / Number inoculated	Post-inoculation [a] antibody response
SLT-II	mice	0 / 5	NA
SLT-II R32C	mice	5 / 5	NA
SLT-II A42T	mice	5 / 5	NA
SLT-II G59D	mice	5 / 5	NA
SLT-II R32C	rabbit	1 / 1	+
SLT-II A42T	rabbit	1 / 1	+
SLT-II G59D	rabbit	1 / 1	+

[a] The presence of toxin neutralizing antibodies were detected by cytotoxicity neutralization assay after diluting the sera 100-fold in PBS.

NA, not assayed; +, toxin neutralizing antibodies detected at a titer above 100.

whereas mice inoculated with sonic lysates of *E. coli*(pLP15) died 4-5 days post inoculation. Rabbits inoculated with the SLT-II mutant toxins also survived, and, after 3 inoculations, the sera from these rabbits contained neutralizing antibodies against SLT-II (see Table 9). The Shiga toxin mutants were not evaluated for lethality to mice or rabbits since all three mutants retained cytotoxic levels over and above the reported lethal dose for these animals (O'Brien and LaVeck,1982).

III. Deletion analysis of SLT-II to determine the minimal contiguous gene segment required for cytotoxic activity of the holotoxin.

A. Coarse mapping of the carboxy-terminal functional boundary of SLT-II.

The first step in mapping the functional boundaries of SLT-II was to coarsely map the carboxy-terminal functional boundary. To achieve this goal, progressive deletions of *slt*-II in linearized pLP15 were made with *Bal* 31 exonuclease under controlled conditions. The single disulfide linkage between the two cysteine residues encoded by codon 3 and 56 of the mature SLT-II B polypeptide has been shown to be critical for the holotoxin activity (Jackson *et al.*, 1990). Thus, any deletion extending beyond the 56th codon will result in ablation of holotoxin activity due to disruption of the disulfide linkage. Hence in the present experiment, the pool of deletion mutants were first probed with an oligonucleotide probe (19 mer) which spanned the 43rd and 49th codons of the 70 amino acid mature SLT-II B polypeptide to identify mutants that retained at least 49 amino acid residues of the mature B polypeptide. Twenty five mutants which hybridized with the oligonucleotide probe were selected for further analysis. Nine of these 25 probe-positive mutants retained cytotoxic activity on HeLa cells. These nine cytotoxic mutants contained the entire coding region of the *slt*-II gene. The 3 mutants with least extensive C-terminal deletions from the remaining 16 probe-positive,

noncytotoxic, mutants are listed in Table 10. The mutant pLP22 in which the last 2 amino acids from the C-terminus has been deleted retained cytotoxic activity although at a reduced level when compared to wild type SLT-II. The mutant pLP24 in which the last 5 amino acids from the C-terminus have been removed was completely devoid of any cytotoxic activity (see Table 10)

B. Fine mapping of the carboxy terminal functional boundary.

The results of the coarse mapping indicated that the deletion of the last 5 amino acids from the C-terminus of SLT-II resulted in complete ablation of cytotoxic activity of SLT-II. To determine the precise carboxy terminal functional boundary for holotoxin activity of SLT-II, an ochre terminator codon (TAA) was introduced by oligonucleotide-directed, site-specific mutagenesis at the 67th codon of the SLT-IIB in which TTT that codes for phenylalanine was changed to TAA (SLT-II T66). The cytotoxic activity of SLT-II was completely abolished by this mutation. When an ochre terminator was introduced at the 69th codon of the SLT-IIB [AAT that codes for asparagine was changed to TAA (SLT-II T68)], the mutant retained cytotoxic activity but at a reduced level compared to wild type SLT-II (Table 11).

The concentrated sonic lysates of *E. coli* that expressed SLT-II T66 and SLT-II T68 were probed with the MAb BC5 (specific for the B subunit of SLT-II) in a dot blot ELISA to determine whether premature truncation of the B polypeptide had affected the immunoreactivity with MAb BC5. Neither sonic lysate reacted with MAb BC5 (Table 11). Hence, the deletion of the last two amino acids in the B subunit of SLT-II leads to the abolition of the epitope reactive with MAb BC5.

The TLC-overlay assay was then done to assess whether the premature truncation of the B polypeptide of SLT-II had affected the receptor recognition function of the SLT-IIB subunit. A monoclonal antibody specific for the A subunit of SLT-II (11E10) was used to detect receptor-bound toxin. As shown in Figure 21, SLT-II T66 did not elicit

any detectable signal. This observation could be due to abolition of receptor-binding activity of the mutant B subunit or an inability to form holotoxin. By contrast, SLT-II T68 bound to the receptor but at a reduced level compared to wild type SLT-II (Figure 21, panels B and C). Thus, it appears that the last two amino acid residues of SLT-IIB polypeptide are not essential for receptor-binding activity and/or holotoxin formation of SLT-II, but they may affect the avidity of the interaction between toxin and receptor.

C. Mapping of the functional boundary from the N-terminus of SLT-II.

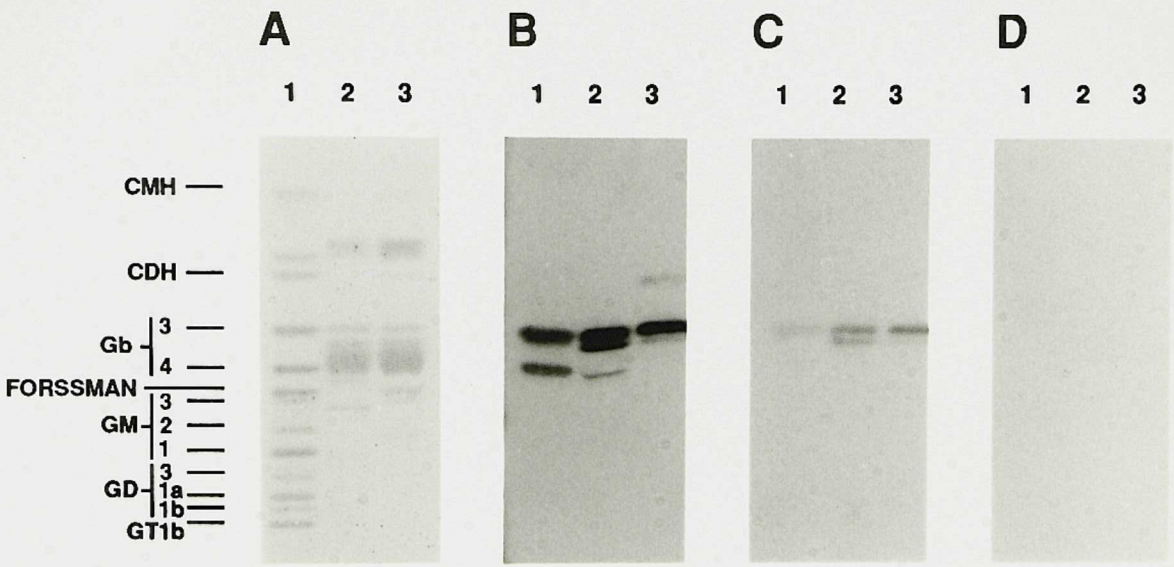
To map the functional boundary from the N-terminus of SLT-II, a unique *Hpa* I site was created by oligonucleotide-directed, site-specific mutagenesis. The 3rd codon of the mature A polypeptide is, TTT, which codes for phenylalanine. This codon was changed to TTA which codes for leucine to create the first *Hpa* I site (pLP15Hpl). Construction of this site did not affect the cytotoxicity of SLT-II (Table 11). However, an "in frame" deletion that was created (pLP15ΔHE) by removing the *Hpa* I -*Eco* RV fragment of pLP15Hpl was completely devoid of any cytotoxic activity. This deletion removed the coding region between the 3rd and the 25th codons of the mature A polypeptide of SLT-II. To create a less extensive N-terminal deletion, a second *Hpa* I site was created in pLP15Hpl by oligonucleotide-directed, site-specific mutagenesis. The 18th codon AAT which codes for asparagine was changed to ACT which codes for threonine to create the second *Hpa* I site (pLP15Hp2). The introduction of the second *Hpa* I site did not affect the cytotoxic activity of SLT-II as shown in Table 11. When the *Hpa* I -*Hpa* I fragment was removed from pLPHp2, to create an in-frame deletion, the resultant pLP15ΔHH lacked any detectable cytotoxic activity (Table 11). In the plasmid pLP15ΔHH, the coding region between the 3rd and 18th codons of the mature A polypeptide was excised to create an "in frame" deletion. Despite the lack of any detectable cytotoxic activity, sonic lysates of *E. coli* expressing pLP15ΔHE and pLP15ΔHH reacted with MAb 11E10

Table 10. **Characteristics of *Bal* 31 deleted SLT-II mutants**

Mutant	Number of amino acids deleted from the C-terminus	Amino acids added from [a] the vector sequences	Cytotoxicity for HeLa cells
pLP22	2	Ala Leu Asn Phe Cys	+ / -
pLP24	5	Arg Ala Val Asn Gly Glu Trp Lys Leu	-
pLP25	9	Asn Cys Lys Arg	-

[a] Following *Bal* 31 exonuclease treatment and blunt-end ligation, the natural terminator codon at the 3' end of *slt*-II gene was eliminated. The 3' end of the deletion mutants were derived from a terminator codon in the vector sequences.

Figure 21. TLC-overlay assay to detect binding of SLT-II T66 and SLT-II T68 to glycolipids extracted from Vero and HeLa cells. Total glycolipids from Vero and HeLa cells were subjected to chromatography on TLC plates in lanes 2 and 3, respectively of each panel. Purified neutral and acidic glycolipids were included as controls in lane 1 of each panel. The binding of toxin mutants to separated glycolipids was detected as described in the Materials and Methods section. In panel A, glycolipids on the TLC plate visualized with orcinol reagent. In panel B, binding profile of wild type SLT-II; in panel C, binding profile of SLT-II T68; in panel D, binding profile of SLT-II T66. The glycolipid-bound toxin was detected with MAb 11E10 specific for the A subunit of SLT-II. The positions of the glycolipid standards are indicated to the left of panel A. The experiment was repeated twice and similar results were obtained in each replicate experiment.


A
B
C
D
1 2 3
1 2 3
1 2 3
1 2 3
CMH
CDH
Gb
3
4
FORSSMAN
GM
3
2
1
GD
3
1a
1b
GT1b

(specific for the A subunit of SLT-II) in a dot blot ELISA (see Table 11). Thus, the first 18 N-terminal amino acids of the SLT-IIA do not appear to contribute to the formation of the epitope recognized by MAb 11E10 (SLT-IIA specific), although these amino acids are critical for holotoxin activity of SLT-II.

To determine whether the N-terminal deletions of SLT-II had affected the enzymatic activity of the A subunit of SLT-II, an *in vitro* translation assay was done in which an exogenous mRNA (Brome Mosaic Virus RNA) was translated into proteins in a cell-free rabbit reticulocyte lysate. Prior incubation of rabbit reticulocyte lysate with wild type SLT-II [periplasmic extract of *E. coli*(pLP15)] resulted in inactivation of the reticulocyte lysate as demonstrated by the finding that no proteins were synthesized from the exogenous mRNA added. When the reticulocyte lysate was preincubated with a periplasmic extract of *E. coli*(pLP15ΔHH), there was no inactivation of the reticulocyte lysate since the added mRNA was translated (Figure 22, lane A). A reticulocyte lysate preincubated with a periplasmic extract of *E. coli* DH5α carrying pBS (-) translated the added mRNA efficiently and served as the negative control (Figure 22, lane C). Thus, the first 18 N-terminal amino acids of the SLT-IIA polypeptide are critical for RNA *N*-glycosidase enzymatic activity of the A subunit of SLT-II or for the folding of the A subunit.

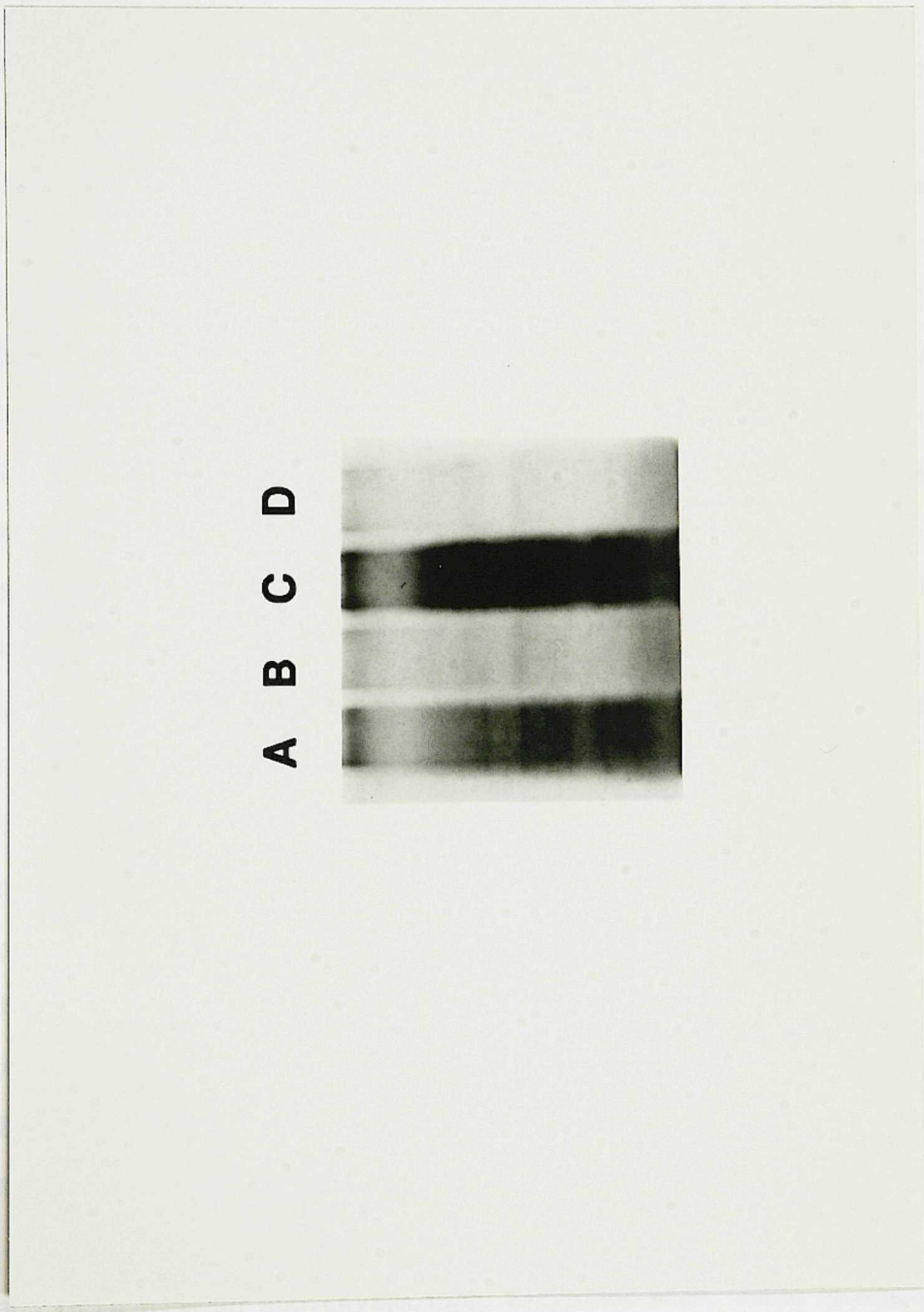
Table 11. **Cytotoxicity and immunoreactivity of SLT-II deletion mutants**

Toxin	HeLa cell cytotoxicity	Immunoreactivity with MAbs[b]:	
	CD_{50} [a]	11E10	BC5
E. coli(pLP15)	10^5	+++	+++
E. coli(SLT-II T68)	10^4	+++	-
E. coli(SLT-II T66)	<10	+++	-
E. coli(pLP15Hp1)	10^5	+++	ND
E. coli(pLP15ΔHE)	<10	++	ND
E. coli(pLP15Hp2)	10^5	++	ND
E. coli(pLP15ΔHH)	<10	++	ND

[a] The highest 10-fold dilution of the toxin preparation that caused lysis of 50% of the cell monolayer (CD_{50}).

[b] MAb 11E10 is specific for the A subunit of SLT-II (see Table 5). MAb BC5 is specific for the B subunit of SLT-II (Downes *et al.*, 1988). Immunoreactivity with MAbs is indicated as follows: ND, not done; +++, strong reaction; ++, moderate reaction; +/-, weak reaction; -, negative reaction; determined by the dot blot ELISA (see Materials and Methods).

Figure 22. Inhibition of protein synthesis in an *in vitro* translation system by SLT-II mutants. The enzymatic activity of *E. coli*(pLP15ΔHH) was evaluated in an *in vitro* rabbit reticulocyte lysate translation system as described in the Materials and Methods section. The translated products were analysed on a 10% polyacrylamide gel. Lane A, reticulocyte lysate containing the BMV mRNA that had been incubated with a periplasmic extract of *E. coli*(pLP15ΔHH); lane B, reticulocyte lysate containing the BMV mRNA that had been incubated with a periplasmic extract of *E. coli*(pLP15); lane C, reticulocyte lysate containing the BMV mRNA incubated with a periplasmic extract of *E. coli*(pBS); lane D, reticulocyte lysate without BMV mRNA incubated with a periplasmic extract of *E. coli*(pBS). This experiment was repeated twice and similar results were obtained in each replicate experment.


A B C D

DISCUSSION

Generation and characterization of monoclonal antibodies to SLT-II.

All the neutralizing MAbs generated in the present study recognized the A subunit of SLT-II, which suggests that the A subunit of the SLT-II toxoid was more immunodominant than the B subunit. In previous studies involving the production of MAbs to Shiga and Shiga-like toxins (Donohue-Rolfe *et al.*, 1984; Strockbine *et al.*, 1985), most of the neutralizing MAbs were directed against the B subunit of the toxin. One possible explanation for these differences in the immunodominant subunits among the Shiga-like toxin family is that the tertiary structure of Shiga toxin and SLT-I may differ from that of SLT-II. Alternatively, the process of inactivating SLT-II with formaldehyde or gluteradehyde could have altered the conformation of the native SLT-II molecule such that either the A subunit epitopes which are normally masked become exposed or, conversely, the B subunit epitopes which are normally exposed become masked. Furthermore, it is of interest to note that most of the monoclonal antibodies generated in the present study belonged to the IgM class of antibodies. This may reflect the relatively short immunization schedule followed in immunizing the mice with SLT-II toxoid. It should be noted that in the present study the emphasis was not to generate an extensive battery of monoclonal antibodies reactive with all antigenic determinants of SLT-II molecule. Rather, it was to obtain one or more toxin-neutralizing MAb to use as a probe in the colony ELISA (Perera *et al.*, 1988). Therefore, non-neutralizing antibodies which were capable of binding to crude SLT-II as detected by the ELISA were not characterized, and no further attempts were made to generate monoclonal antibodies to the SLT-IIB subunit.

Immunoprecipitation of the SLT-II holotoxin revealed a more heavily iodinated A subunit than B subunit in contrast to previous reports on Shiga toxin (O'Brien *et al.*, 1980) and SLT-I (O'Brien and LaVeck,1983; Strockbine *et al.*, 1985) in which the B

subunit was more heavily iodinated than the A subunit. The weaker iodination of the SLT-II B subunit compared to Shiga toxin/SLT-I cannot be explained by differences in the number of B subunit tyrosine residues (Strockbine *et al.*, 1988; Jackson *et al.*, 1987); the SLT-I B subunit and the SLT-II B subunit have two tyrosine residues each. However, the differences in the number of tyrosine residues in the A subunit between Shiga toxin/SLT-I and SLT-II could contribute to enhanced iodination of the SLT-IIA subunit because the SLT-II A subunit contains 11 tyrosine residues, whereas the Shiga toxin/SLT-IA subunits contain only 7 tyrosine residues. Another possible explanation for the intensity of the "A" band is that unassembled A subunits in the crude toxin preparation as well as holotoxin molecules were immunoprecipitated by the SLT-IIA specific MAbs, and therefore, the A subunit concentration in the immunoprecipitant was increased.

None of the MAbs neutralized Shiga toxin/SLT-I. Furthermore, none of the monoclonal antibodies except MAb 11E10 neutralized SLT-IIv, even though the A subunits of SLT-II and SLT-IIv share approximately 93% homology at the deduced amino acid sequence level. Therefore, these MAbs must be capable of detecting subtle antigenic differences amongst the SLT-II subfamily (Perera *et al.*, 1988). Because of the extreme specificity of these monoclonal antibodies, and the fact that these MAbs react with topographic (conformationally dependent) epitopes of SLT-II, they are useful tools in structure-function analysis studies of SLT-II. Furthermore, the MAbs appear to be very specific immunological probes for the detection of SLT-II-producing EHEC (Perera *et al.*, 1988).

In an attempt to map the conformationally-dependent epitopes reactive with these SLT-II A subunit-specific monoclonal antibodies, a sublibrary of the *slt*-II gene was created in the Lambda Zap protein fusion vector. The rationale for selecting this particular protein fusion vector was that in this vector foreign DNA is inserted at the N-terminus of the β galactosidase gene which should favor formation of a hybrid protein

with only 7 N-terminal amino acids of β galactosidase. Therefore, the fusion protein should fold such that the innate conformation of the epitope encoded by the inserted foreign DNA is retained.

A number of clones that expressed the reactive epitopes for each MAb were isolated. The immunoreactive clones that were sequenced contained *slt*-II gene segments larger than 800 bp in size. Although it has been reported that topographic epitopes may require extensive linear sequences for correct conformational folding (Mehra *et al.*, 1985), the possibility exists that the imprecise size fractionation of DNA fragments may have favored large inserts. However, the study was not pursued further, and the minimal coding region of each epitope reactive with SLT-II specific MAbs remains to be determined.

Mutational analyses of the B subunits of SLT-II and Shiga toxin.

Toxin-receptor interactions of Shiga toxin and the SLTs have beeen investigated by several groups of investigators (Jacewicz *et al.*, 1986; Lindberg *et al.*, 1987; Lingwood *et al.*, 1987; Mobassaleh *et al.*, 1988). In a recent report, Jackson *et al.*, (1990) implicated two well conserved domains of the B subunit of Shiga toxin, the hydrophilic region near the amino terminus and the single disulfide bond, as important for toxin-receptor interaction. Harari *et al.* (1988) also implicated the conserved hydrophilic region of the Shiga toxin B subunit as important for binding by generating cytotoxin-neutralizing antibodies with synthetic peptides corresponding to that region. However, these investigators did not show that the neutralizing antibodies interfered with toxin-receptor interactions. In the present study, regionally-directed, chemical mutagenesis was used to identify amino acids in the B subunit of SLT-II important for cytotoxic activity of the holotoxin.

Regionally-directed, bisulfite mutagenesis has been widely used to analyze structure-function relationships of cloned gene products. This technique has been

applied most often to the study of proteins for which no three dimensional structural information is available (Shortle, 1987). The principle of the mutagenesis reaction is as follows. Cytosine (C) residues in single stranded DNA are deaminated to uracil (U) when treated with sodium bisulfite, but C residues in double stranded DNA are virtually resistant to bisulfite attack (Botstein and Shortle, 1985). The uracil residues in the mutagenized strand direct the incorporation of adenosine (A) in the complementary strand synthesized *in vitro* during the gap repair reaction which results in transitions from GC to AT. Currently, several methods are available to generate a single-stranded gap region in double-stranded DNA molecules and to enrich for mutant selection after mutagenesis (Kramer and Fritz, 1987; Kunkel *et al.*, 1987; Stanssens *et al.*, 1989). In the present study, a simple strategy for generating gapped duplex DNA was developed in which both sense and antisense strands of the target gene segment were mutagenized simultaneously (see Figure 10). This strategy is applicable to any cloned gene in which the desired target region is flanked by two unique restriction sites which yield a 5' overhang and a 3' overhang at the termini.

The molecular mechanisms by which the B subunits of Shiga toxin and SLT-II interact with the cellular G_{b3} glycolipid receptors were examined by a variety of biochemical, immunological and molecular biological techniques. The three SLT-II mutants were completely devoid of any detectable cytotoxicity, but the comparable mutations in Shiga toxin retained detectable, although reduced, cytotoxic activity. It is possible that these mutations affect both toxin molecules similarly, but, due to the wide disparity in the cytotoxicity levels of the wild type SLT-II and Shiga toxin constructs (Table 6), a mutation which reduces cytotoxicity by 10,000-fold may still elicit a detectable cytotoxic response to Shiga toxin. The same reason may be responsible for the negative results obtained for SLT-II mutants in the receptor-analogue binding assay and Vero cell binding assay (see Table 7) in which the binding of these SLT-II mutants may have been below the threshold level of sensitivity of these assays to elicit any

signal. Alternatively, the identical mutations may affect Shiga toxin and SLT-II differently.

The ability of MAb 13C4 to detect receptor-analogue bound Shiga toxin (see Figure 13) deserves comment. The MAb 13C4 recognizes an epitope in the B subunit of Shiga toxin and is capable of neutralizing the cytotoxic activity of Shiga toxin. However, based on receptor-analogue ELISA it appears that the epitope reactive with MAb 13C4 remains accessible to the MAb even after toxin-receptor interaction has taken place. Thus, toxin-neutralizing capacity of MAb 13C4 appears to be due to a mechanism other than interruption of toxin-receptor interaction. Furthermore, it should be noted that when toxin-receptor interaction occurs, some epitopes of the A subunit of Shiga toxin remain accessible to antibodies as clearly demonstrated in the immunofluorescence assay (see Figure 19).

When arginine was replaced by cysteine, the mutant Shiga R33C toxin reacted with all three MAbs. This finding indicates that R33 is not critical for maintaining a conformation with exposure of immunoreactive epitopes. However, this mutation produced the most profound reduction in cytotoxicity. It is tempting to speculate that the arginine 33 (R33) in the B subunit of Shiga toxin may be directly involved in forming the binding site for interaction with G_{b3} glycolipid. The recent findings of Surewicz *et al.* (1989) supports the idea that this region of the Shiga toxin B polypeptide is involved in toxin-receptor interactions. These investigators used fluorescent spectroscopy to demonstrate that tryptophan (W34) is in close proximity to the receptor binding domain of the B subunit of Shiga toxin. The analogous arginine in SLT-II B polypeptide (R32) may play a similar role as in Shiga toxin. However, the possibility that the R32C mutation in SLT-II is unstable was not ruled out in this study.

The Shiga A43T mutant is of special interest because a relatively conservative substitution led to a significant alteration in the tertiary structure of the toxin molecule [see immunoreactivity profiles in the dot blot ELISA (Figure 12)]. Based on the

receptor-analogue ELISA (Figures 16 and 17) and TLC-overlay results (Figure 18), it appears that the A43T mutation profoundly affected the binding of the Shiga toxin B subunit to G_{b3} glycolipid receptor. Furthermore, preincubation of Shiga A43T mutant with galabiose-derivatized Sepharose beads led to a 10-fold reduction in cytotoxicity, whereas the wild type Shiga toxin after similar treatment displayed a 10,000-fold reduction in cytotoxicity (Table 8). This finding further substantiates the hypothesis that Shiga A43T mutant does not bind strongly to the natural receptor G_{b3} on the cells. However, it should be noted that the cytotoxicity was least affected by this mutation when compared to the other two Shiga toxin mutations (Table 6). The subunit assembly and holotoxin formation as well as the ability to bind to Vero cells did not appear to be affected by this mutation based on immunofluorescence assay (Figure 19). The possibility exists that the A43T mutation induced a conformational change compatible with high avidity interactions with a non-glycolipid molecule on the cell surface, such as a glycoprotein. Such a change would still result in a strong reaction in the immunofluorescence assay. This possibility is particularly intriguing in light of the reports showing avid interactions of Shiga toxin and SLT-II with glycoproteins (Keusch *et al.*, 1986; Acheson *et al.*, 1990). The inability of Shiga A43T to bind to the functional receptor, G_{b3} , on the cell surface may be responsible for the reduced cytotoxicity of Shiga A43T mutant.

The immunoreactivity profile of Shiga G60D mutant indicates that the epitope reactive with MAb 19G8 is disrupted by the G60D mutation in the B subunit of Shiga toxin. This mutation also affected the receptor interaction. That the effect of this mutation on receptor interaction may be to reduce the avidity of receptor-ligand interaction is suggested by the dose-response curve generated in the receptor-analogue ELISA (Figures 16 and 17). In addition to the reduced avidity in receptor-ligand interaction, it is also possible that the Shiga G60D mutant may be defective in translocating across the cytoplasmic membrane, which in turn would result in reduced

cytotoxic activity.

It would be of interest to evaluate the effects of comparable mutations on cytotoxicity of SLT-IIv, because a non-cytotoxic, but strongly immunogenic mutant SLT-IIv molecule would be a prime vaccine candidate for protecting swine against edema disease. The feasibility of this approach is illustrated by the finding that rabbits inoculated with non-cytotoxic mutant SLT-II developed toxin-neutralizing antibodies without any untoward symptoms.

The conclusions drawn in this section of the study are based on the assumption that comparable quantities of mutant toxin proteins were used in the various assays. However, it could be argued that mutations may have either affected the synthesis of B polypeptides of the toxins or rendered the mutant B polypeptides of the toxins unstable. Although this issue could be resolved by direct quantitation of specific toxin proteins, no monospecific polyclonal antisera against either SLT-II or Shiga toxin were available for such quantitation. However, the total protein content of the sonic lysates was standardized. The fact that the Shiga toxin mutants were as stable as wild type toxin, as assessed by pulse-chase analysis, supports the validity of the above assumption for the Shiga toxin mutants. Nonetheless, the stability of SLT-II mutants remains to be determined because the B subunit of SLT-II comigrated with host cell proteins.

Deletion mapping of SLT-II.

The goal of this aspect of structure-function analysis was to define the limits or boundaries of the functional domains of the SLT-II molecule. The holotoxin activity of SLT-II, as determined by its cytotoxicity for HeLa cells, requires correct assembly of functional (see below) A and B subunits. A functionally active B subunit possesses the ability to bind to the glycolipid G_{b3} receptor with high avidity, and to oligomerize in proper conformation for assembly with the A subunit. A functionally active A subunit possesses the RNA *N*-glycosidase enzymatic activity as well as the ability to properly

assemble with the oligomeric B subunit by non-covalent interactions. It is conceivable that the separate functions of each polypeptide are mediated by separate domains of the polypeptide as described for other toxins (Siegall *et al.*,1989).

In a preliminary study using deletion analysis, the minimal contiguous coding segment of the *slt*-II gene required for transcription and translation into an active holotoxin was determined. The importance of the carboxy-terminal amino acids for holotoxin activity as well as for binding activity of the B subunits of Shiga toxin and SLT-II was shown earlier (see mutational analyses of the B subunits of SLT-II and Shiga toxin; Jackson *et al.*, 1990). The single disulfide bond between the two cysteine residues in the B subunit of Shiga toxin was shown by Jackson *et al.* (1990), to be critical for holotoxin activity. Thus, screening of the *Bal*-31 deletion mutants with a oligonucleotide probe which detects the sequences in close proximity to the cysteine codon in the 3' end of *slt*-II prevented unnecessary sequencing of extensively deleted mutants.

The truncated mutant SLT-II T66 lacking the last 4 amino acid residues of the wild type SLT-II was completely devoid of any cytotoxicity. By contrast, SLT-II T68 which lacks the last 2 amino acid residues of the wild type SLT-II was cytotoxic. Taken together, these observations suggest that the penultimate asparagine and the last aspartic acid residues of the 70 amino acid mature SLT-II B subunit are not essential, but amino acids, 67 (phenylalanine) and 68 (asparagine) in the mature B polypeptide of SLT-II are critical for holotoxin activity. In SLT-IIv, the mature B polypeptide consists of 68 amino acid residues and the last two residues are phenylalanine (67th) and asparagine (68th) respectively (Weinstein *et al.*, 1988). In Shiga toxin, the mature B polypeptide consists of 69 amino acid residues and the last two residues are phenylalanine (68th) and arginine (69th), an amino acid that is functionally comparable to asparagine. Thus, it is tempting to speculate that the two terminal residues in the B polypeptide of Shiga toxin/SLT-I or SLT-IIv may play a critical role

in holotoxin activity although the last two residues of the B polypeptide of SLT-II are not essential for holotoxin activity of SLT-II. However, further studies are necessary to determine the exact role of these two essential amino acids in receptor binding, oligomerization, or subunit assembly, since perturbation of any of these functions of the B polypeptides can lead to abolition of the cytotoxic activity of the holotoxin.

To identify the functional boundary for holotoxin activity from the N-terminus of SLT-II molecule, "in-frame" deletions were created at the 5' end of the *slt*-II gene. The smallest deletion evaluated in the present study was pLP15ΔHH in which the coding region between the 3rd and 18th codons of the mature A polypeptide had been excised. This deletion in SLT-II led to complete ablation of cytotoxic activity. Although many explanations are possible for ablated cytotoxicity, the fact that this deletion led to abolition of enzymatic activity of the A subunit deserves comment. That the sonic lysate of *E. coli*(pLP15ΔHH) was still capable of eliciting a reaction with MAbs 11E10 and 11F11 which are specific for the A subunit of SLT-II (Table 11) argues against the possibility that this deletion drastically affected the stability of the protein. This immunoreactivity of the deletion mutant taken with its lack of cytotoxicity suggests that this deletion may have affected the enzymatic activity of the A subunit of SLT-II. Although no conclusive data are available on the location of the active site of Shiga, SLTs or ricin toxin, a putative active-site cleft has been described for the ricin A chain based on three-dimensional structure (Montfort *et al.*, 1987). Seven amino acid residues which lie within this cleft are conserved in the A subunits of Shiga toxin and SLTs (Hovde *et al.*, 1988; Kozlov *et al.*, 1988). Hovde *et al.* (1988) reported that when one of these conserved amino acids in SLT-I, *i.e.* glutamic acid 167, was substituted with aspartic acid, the enzymatic activity of the mutant SLT-I was reduced more than 1000-fold in comparison to wild type toxin. This observation led these workers to propose that glutamic acid 167 is a critical residue in the active site of SLT-I. But the subsequent work done by Schlossman *et al.* (1989) and May *et al.* (1989) indicated that

glutamic acid 177 in ricin (which is analogous to glutamic acid 167 in SLT-I) is not essential for RNA *N*-glycosidase activity. Moreover, recent results of May and coworkers (1989) indicate that the N-terminal residues of the A polypeptide of ricin are critical for its enzymatic activity. These workers showed that when the first 9 amino acids were removed from the N-terminus of the ricin A polypeptide, the enzymatic activity remained unaltered, whereas removal of the first 13 amino acid residues completely abolished the enzymatic activity. Thus, it appears that N-terminal residues are critical for the enzymatic activity of both SLT-II and ricin. It is conceivable that N-terminal deletions may cause a deleterious conformational change in a region essential for RNA *N*-glycosidase activity either due to a direct perturbation of the catalytic site itself or a domain that interacts with a receptor on the ribosome. Further work is necessary to resolve this issue.

SUMMARY

The major conclusions from this thesis are as follows:

1. The five SLT-II neutralizing monoclonal antibodies generated were directed against epitopes in the A subunit of SLT-II. None of the monoclonal antibodies were capable of completely neutralizing the cytotoxicity of SLT-I, Shiga toxin or SLT-IIv, despite a high degree of sequence homology amongst the SLTs.

2. The molecular mechanisms by which the B subunits of Shiga toxin and SLT-II interact with the cellular G_{b3} glycolipid receptor were examined by a variety of biochemical, immunological, and molecular biological techniques. Three non-cytotoxic SLT-II mutants were isolated, and the mutations were mapped. The substitutions of arginine with cysteine at codon 32, alanine with threonine at codon 42, and glycine with aspartic acid at codon 59, in the 70 amino acid mature SLT-II B polypeptide resulted in complete abolition of cytotoxicity. Comparable mutations induced in the B subunit gene of Shiga toxin, resulted in drastically decreased cytotoxicity (10^3-to-10^6-fold) compared to wild type Shiga toxin. The effects of these mutations on receptor binding and subunit assembly were assessed.

3. The minimal contiguous coding segment of the *slt*-II gene required for transcription and translation into an active holotoxin was determined by deletion analysis. Removal of 2 codons from the carboxy terminus of SLT-II had no effect on the cytotoxic activity, whereas removal of 4 codons from the carboxy terminus completely abolished the cytotoxicity of SLT-II. Removal of the coding segment between the 3rd and 18th codons of mature SLT-IIA polypeptide resulted in abolition of cytotoxicity of SLT-II, as well as the enzymatic activity of the A polypeptide. This finding suggests that the N-terminal

amino acids of the A subunit are involved in formation of an active cleft or are necessary for the proper conformation of the enzymatically active A subunit.

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