Recombinant antibodies against *Clostridium difficile* Toxin A

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Author's declaration

This thesis is the original work of the author except where otherwise stated

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Table of Contents

Chapter 1 1

General Introduction 1

1 General Introduction 2

1.1 Pathogenicity 3

1.2 Toxin structure 8

1.3 Toxin mode of action 9

1.3.1 Receptor and receptor binding 9

1.3.2 Toxin A entry to the cell 11

1.3.3 Toxin function 12

1.4 Humoral and cellular immune response against *Clostridium difficile* Toxin A 15

1.5 Methods for control 16

1.5.1 Antibiotics 16

1.5.2 Immunotherapy 18

1.6 Toxinotypes and strains of importance 22

1.7 Antibodies and CDR conformation 26

1.8 Engineered antibodies 27

1.8.1 Humanization of antibodies 28

1.8.1.1 Chimeric antibodies 28

1.8.1.2 CDR- grafted antibodies 29

1.8.1.3 Transgenic Antibodies 29

1.9 Recombinant Antibodies and phage display 30

1.9.1 Creation of antibody libraries 33

1.9.1.1 Immune libraries 33

1.9.1.2 Non-immune libraries 33

1.9.1.3 Synthetic libraries 34

1.9.2 Formats for antibodies in phage display 35

1.10 Phage display 36

1.10.1 Selection of Phage antibody 41

1.10.2 Screening of phage antibodies 42

1.10.3 Antibody Expression 43

1.10.4 Affinity maturation 44

1.11 Aims 45

Chapter 2 46

Materials and methods 46

2 Materials and methods 47

2.1 Bacterial strain 47

2.2 Confirmation of insert encoding Toxin A C-terminal region 47

2.3 Growth of bacteria and expression of the Toxin A carboxy-terminal region 48

2.4 Purification of the protein by affinity chromatography 48

2.5 Protein assay 49

2.6 SDS-PAGE analysis 49

2.7 Electroblotting 50

2.8 Recognition in ELISA 50

2.9 Binding of native and recombinant Toxin A to cellular receptors 51

2.10 Haemagglutination activity of recombinant Toxin A 52

2.11 Sensitivity of F9 and Vero cell lines to native Toxin A 52

2.12 Kinetics of action of native Toxin A on F9 and Vero cell 53

2.13 Inhibition of native Toxin A with polyclonal anti-Toxin A and recombinant Toxin A *in vitro* 53

2.14 Inhibition of native Toxin A with polyclonal Fab fragments 54
2.14.1 Digestion of polyclonal anti-Toxin A antibody with papain 54
2.14.2 SDS-PAGE gel electrophoresis 54
2.14.3 Electroblotting 54
2.14.4 Detection of native Toxin A by Fab fragment in ELISA 55
2.14.5 Inhibition of native Toxin A with polyclonal Fab fragments *in vitro* 55

2.15 Tomlinson scFv libraries 57
2.15.1 KM13 helper phage 61
2.15.2 Objectives 63
2.15.3 Tomlinson Library I 63
2.15.3.1 Propagation of KM13 helper phage 63
2.15.3.2 Titration of KM13 helper phage stock 64
2.15.3.3 Growing the library 65
2.15.3.4 Titration of phage libraries 65
2.15.3.5 Growing TG1 and HB2151 cells 66
2.15.4 Experiments before selection 66
2.15.4.1 Growing positive controls 66
2.15.4.2 Production of soluble positive control 66
2.15.4.3 Detection of anti-BSA and anti-ubiquitin scFvs by Western blot 67

2.16 Antibody selection 67
2.16.1 Panning library I against purified recombinant C- terminal domain of Toxin A 67
2.16.1.1 First round of selection 67
Infecting TG1 cell with eluted phage antibodies 68
Rescue of selected phage antibodies 69
Titration of rescued phage 69
2.16.1.2 Second round of selection 70
2.16.1.3 Third round of selection 70
2.16.2 Screening phage by ELISA 70
2.16.2.1 Screening phage particles by ELISA (polyclonal ELISA) 70
2.16.2.2 Screening phage particles by ELISA (monoclonal ELISA) 71
Monoclonal Phage ELISA 71

2.17 Production of soluble antibody fragments 72

2.18 Genetic characterization of selected scFvs 74
2.18.1 Plasmid digestion 74
2.18.2 Sequence of selected clones 74

2.19 SDS-PAGE and Western blot analysis of selected clones 75

2.20 High level expression and purification of soluble scFv 75
2.20.1 Purification of the scFvs by affinity chromatography 76

2.21 Biological activity and immunochemical characterization of purified scFv 77
2.21.1 Recognition of native Toxin A by selected scFv 77
2.21.2 Haemagglutination inhibition assay 77
2.21.2.1 Preparation of Rabbit erythrocytes 78
2.21.2.2 Haemagglutination inhibition 78
2.21.3 Rabbit erythrocyte haemolysis assay 78
2.21.4 Indirect immunofluorescent antibody test with fixed cells 79
2.21.5 Indirect immunofluorescent antibody test with cells in suspension 80
2.21.6 SDS-polyacrylamide gel electrophoresis and Western blotting 81
2.21.7 Expression levels of selected scFvs 82

2.22 Epitope analysis 82
2.22.1 Epitope saturation curve 83
2.22.2 Epitope saturation experiment using two different scFvs 84
2.22.3 Blocking of monoclonal phage antibody epitopes by scFv of the same specificity 86
2.22.3.1 Preparation of phage antibodies 86
2.22.3.2 Optimization of epitope blocking by ELISA 87
2.22.3.3 Blocking of phage antibody by scFv 88

2.23 Assay for toxin neutralizing activity 88
2.23.1 Flow cytometric analysis of toxin binding 88
2.23.2 Inhibition of Toxin A cytotoxicity *in vitro* 89
2.23.2.1 Preparation of F9 cell line 89
2.23.2.2 Addition of Toxin A - antibody mixtures 89

2.24 Epitope mapping 90
2.24.1 Materials and methods 90
2.24.1.1 Bacteria and plasmids 90
2.24.1.2 Preparation of pET767 90
2.24.2 Polymerase chain reaction 91
2.24.2.1 PCR primers 91
2.24.2.2 PCR reactions 94
2.24.3 Elution and purification of DNA from agarose gels 95
2.24.4 Restriction enzyme digestion and ligation 95
2.24.5 DNA sequencing 96
2.24.6 Cloning 98
2.24.6.1 Cloning into pCR 2.1-TOPO vector 98
2.24.6.2 Cloning into pCG806 98
2.24.6.3 Ligation 98
2.24.6.4 Transformation 99
2.24.6.5 Preparation of synthetic DNA duplex 99
2.24.6.6 Phosphorylation of annealed oligonucleotides 99

2.25 Protein expression 100
2.25.1 SDS-PAGE and electrobloting 100

Chapter 3 *102*

Results *102*

3 Results *103*

3.1 Confirmation of sequences encoding Toxin A C-terminal binding domain 103

3.2 Purification of the protein by affinity chromatography 105

3.3 SDS-electrophoresis 105

3.4 Electroblotting analysis 107

3.5 Recognition in ELISA 107

3.6 Binding of native and recombinant Toxin A to cellular receptors 110

3.7 Haemagglutination activity of recombinant Toxin A 110

3.8 Sensitivity of F9 and Vero cell lines to native Toxin A 113

3.9 Kinetics of action of native Toxin A on F9 and Vero cell lines 113

3.10 Inhibition of the action of native Toxin A 114

3.11 Inhibition of native Toxin A with polyclonal Fab fragments 119
3.11.1 Digestion of polyclonal anti-Toxin A antibody with papain 119
3.11.1.1 SDS-PAGE gel electrophoresis 119
3.11.2 Electroblotting 122
3.11.3 Detection of native Toxin A by Fab fragment in ELISA 122
3.11.4 Inhibition of native Toxin A with polyclonal Fab fragments *in vitro* 125

3.12 Tomlinson scFv library 125
3.12.1 Growing the library 125
3.12.2 Detection of anti-BSA and anti-ubiquitin scFvs by Western blot 127
3.12.3 Phage display against the C-terminal domain of Toxin A 127
3.12.4 Screening polyclonal phage by ELISA 130
3.12.5 Screening monoclonal phage by ELISA 130
3.12.6 Screening monoclonal soluble scFv antibodies by ELISA 133

3.13 Genetic characterization of selected scFvs 135

3.14 Sequence of selected clones 135

3.15 Detection of native Toxin A by selected scFv 136
3.16 Haemagglutination inhibition 143
3.17 Haemolysis assay 143
3.18 Indirect immunofluorescent antibody test with fixed cells 145
3.19 Indirect immunofluorescent antibody test with suspended cells 147
3.20 Characterization of selected scFvs 149
3.20.1 SDS-polyacrylamide gel electrophoresis and Western blotting 149
3.21 Expression levels of selected scFvs 149
3.22 Purification of the scFvs by affinity chromatography 152
3.23 Epitope saturation curve 153
3.24 Epitope saturation experiment using two different scFvs 153
3.25 Competitive ELISA with monoclonal phage antibodies and scFv 157
3.25.1 Preliminary experiments 157
3.25.2 Competitive ELISA experiments 160
3.26 Flow cytometric analysis of Toxin A-receptor interaction 162
3.27 Inhibition of Toxin A cytotoxicity by scFvs 164
3.28 Epitope mapping 167
3.28.1 Cloning of three regions of Toxin A into pCR 2.1-TOPO vector 167
3.28.1.1 Restriction analysis 167
3.28.2 Cloning Toxin A fragments into a maltose-binding protein vector 171
3.28.3 Cloning of DNA linker into pCG806 MBP vector 171
3.29 Characterisation of MBP fusions 177
Chapter 4 *1025*
4 Discussion *185*
4.1 Purification and characterization of recombinant C-terminal domain of Toxin A 185
4.2 Screening of the Tomlinson library 192
4.3 Biological activity of anti-Toxin A scFvs. 195
4.4 Epitope mapping of anti-Toxin A scFv 200
4.5 Conclusion 202
5 Appendices *204*
5.1 Luria-Bertani (LB) broth or agar 204
5.2 Preparation of antibiotic solutions 204
5.3 SOC medium 204
5.4 Citric acid phosphate buffer 205
6 Bibliography *206*

List of tables

Table 1.1: RFLP characteristics used for typing of toxin genes 24

Table 1.2: C. difficile strains with variant toxin genes 25

Table 2.1: Inhibition of native Toxin A with polyclonal Fab fragments in vitro experimental design: 56

Table 2.2 : Design of epitope saturation experiment 85

Table 2.3: Bacterial strains used for epitope mapping 92

Table 2.4: List of primers used for epitope mapping and characterization of scFv genes 93

Table 2.5: List of restriction enzymes and their target sequences 97

Table 3.1: Recoveries of phage during selection 129

Table 3.2 : List of the primer pairs and predicted size of the products used in cloning of four regions of Toxin A 168

Table 3.3: List of scFvs and the detected fragments 183

List of figures

Figure 1.1: Summary of the proposed mode of action of Toxin A ____14

Figure 1.2: The various formats of engineered antibodies ____32

Figure 2.1: Structure of phagemid pIT2 ____59

Figure 2.2: DNA sequence of pIT2 vector____60

Figure 2.3 : Principle of proteolytic selection (Goletz et al., 2002) ____62

Figure 3.2: Complete nucleic acid sequence encoding recombinant Toxin A from pET767 ____104

Figure 3.3: SDS-PAGE analysis of recombinant Toxin A by affinity chromatography ____106

Figure 3.4: Western blotting analysis of recombinant Toxin A purified by affinity chromatography ____108

Figure 3.5: Detection of recombinant Toxin A in ELISA. ____109

Figure 3.6: Immunofluorescence assay to detect the binding of recombinant and native Toxin A to receptors on F9 cells ____111

Figure 3.7: Haemagglutination activity of recombinant Toxin A ____112

Figure 3.8: Sensitivity of F9 cell lines to native Toxin A ____115

Figure 3.9: Sensitivity of F9 cell lines to native Toxin A ____116

Figure 3.10: Sensitivity of Vero cell lines to native Toxin A ____117

Figure 3.11: Effective time of exposure on the action of native Toxin A on F9 and Vero cells.____118

Figure 3.12: Inhibition of Toxin A by polyclonal anti-Toxin A and rTA ____120

Figure 3.13: Non reducing SDS-PAGE analysis of papain digested anti-Toxin A antiserum. ____121

Figure 3.14: Western blotting analysis of papain digested anti Toxin A antiserum_123

Figure 3.15: Reaction of intact IgG and Fab fragments with native Toxin A in ELISA ____124

Figure 3.16: Inhibition of Toxin A by papain digested polyclonal anti-Toxin A antiserum ____126

Figure 3.17: Polyclonal phage ELISA of Tomlinson library I during panning against recombinant Toxin A ____131

Figure 3.18: Monoclonal phage ELISA against recombinant Toxin A _____132

Figure 3.19: Monoclonal scFv ELISA against recombinant Toxin A _____134

Figure 3.20: Restriction analysis of clones from Tomlinson library I _____137

Figure 3.21: Restriction analysis of 7 anti-Toxin A Tomlinson library I_____138

Figure 3.22: Structure of phagemid pIT2_____139

Figure 3.23: complete nucleic acid sequence of 3 selected clones of anti-Toxin A from library I - Heavy chain _____140

Figure 3.24: complete nucleic acid sequence of 3 selected clones of anti-Toxin A from library I - Light chain _____141

Figure 3.25: Amino acid sequence diversity of 7 selected clones of anti-Toxin A scFvs from library I_____142

Figure 3.26: Reactivity of scFvs with native Toxin A in ELISA_____144

Figure 3.27: Haemolysis test _____146

Figure 3.28: Immunofluorescence assay binding activity of Toxin A to F9 cells.___148

Figure 3.29: Immunofluorescence assay for binding activity of Toxin A to F9 cells in suspension._____150

Figure 3.30: Recognition of recombinant Toxin A in Western blotting using scFv _151

Figure 3.31: Purification of anti-Toxin A scFv D5. _____154

Figure 3.32: Expression of the panel of scFvs. _____155

Figure 3.33: Epitope saturation with scFv _____156

Figure 3.34: Epitope saturation with two scFvs_____158

Figure 3.35: Competitive ELISA with monoclonal phage antibodies scFv preliminary experiment _____159

Figure 3.36: Toxin A competitive ELISA using phage antibodies and their soluble scFv derivatives _____161

Figure 3.37: Flow cytometry assay for the binding of Toxin A to F9 cells. _____163

Figure 3.38: Inhibition of Toxin A cytotoxicity by scFvs _____166

Figure 3.39: PCR amplification of fragments I, II and III from pET767._____169

Figure 3.40: Restriction analysis of recombinant pCR2.1 plasmids. _____170

Figure 3.41: Line digram showing locations of fragments used in epitope mapping172

Figure 3.42: DNA sequencing of IA fragment 173

Figure 3.43: DNA sequencing of IA fragment 174

Figure 3.44: DNA sequencing of IIIF fragment 175

Figure 3.45: DNA sequencing of R1 fragment 176

Figure 3.46: Recognition of maltose-binding protein in Western blotting with anti-MBP antiserum. 179

Figure 3.47: Recognition of recombinant Toxin A fragments in Western blotting with anti-Toxin A antiserum. 180

Figure 3.48: Recognition of recombinant Toxin A fragments in Western blotting with anti Toxin A scFv. 181

Figure 3.49: Recognition of recombinant Toxin A fragment repeat (generated by a linker) in Western blotting with anti Toxin A scFv D5. 182

Abstract

Clostridium difficile is a major cause of nosocomial intestinal infection. The pathogen possesses two potent toxins, Toxin A and Toxin B, both of which are responsible for diarrhea, intestinal inflammation and tissue damage. Antibiotics are effective against the disease; however, around 20 % of patients on treatment relapse after the termination of antibiotic therapy. The binding of Toxin A to a receptor on human intestinal epithelial cells initiates disease. This is considered the starting point from which the toxin elicits its effect. One feature of the carboxy-terminal domain of Toxin A is the presence of repeating units of amino acids. These repeats form a series of binding domains that create a multivalent lectin able to recognise trisaccharides and disaccharides on glycolipid and glycoprotein receptor molecules. Antibody response against the toxin can protect against *C. difficile* disease and efforts to generate vaccines have focused upon the carboxy-terminal, receptor binding domain. The aims of this project were to use phage display to isolate recombinant antibodies against part of the receptor-binding domain of Toxin A and to assess if the antibodies were capable of blocking this aspect of toxin function. Potentially, antibodies with this activity might protect against the action of Toxin A.

To assess the feasibility of these goals, Fab fragments were generated from a polyclonal antiserum against Toxin A and tested for protection against *in vitro* challenge with native toxin. This established that monovalent antibody fragments of appropriate specificity can neutralize the action of Toxin A. Recombinant scFv proteins against the receptor binding domain of Toxin A were then sought using phage display.

The carboxy-terminal region of Toxin A was expressed as a recombinant protein (rTA), purified, and shown to behave as would be expected of the receptor-binding domain. rTA was then used to isolate scFv antibodies from a phage display library using conventional bio-panning methods. Progressive enrichment of rTA-specific scFvs through 3 rounds of selection was observed. Those scFvs that showed strongest reaction with rTA

in ELISA were sequenced, expressed as soluble antibodies and purified. A panel of diverse scFvs was established that appeared to bind to non-overlapping epitopes on rTA.

To assay for protective activity, scFvs were mixed with native Toxin A, added to cells in culture, and the response monitored over a 2 hour period. The scFvs were able to consistently delay the cytopathic activity of Toxin A but were unable to match the neutralizing activity of a polyclonal serum or its Fab derivatives. The epitopes recognised by the scFvs were localized using a sub-cloning strategy. Defined parts of the rTA reading frame were recovered by PCR and fusions created with maltose binding protein. scFvs were tested for recognition of the maltose binding fusion proteins. In one instance, a scFv reacted with multiple fusion proteins suggesting recognition of a repeated peptide motif. A maltose binding protein fusion carrying an candidate sequence was successfully recognised by this scFv.

Overall, phage display enabled assembly of a panel of scFv antibodies against epitopes in the carboxy-terminal domain of Toxin A. The inability of these antibodies to block the activity of Toxin A may be due to the multivalent interaction between the toxin and its receptor. Alternatively, the rTA used in scFv isolated may lack functions that are crucial for receptor interaction and hence potential targets for antibody-mediated inhibition.

Definitions

BSA: Bovine serum albumin

C_H: constant region of heavy chain

C_L: constant region of light chain

cDNA: complementary DNA

CDR: complementary determining region

DMEM: Dulbecco's Modified Eagle Medium

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

FR: framework region

g/mg/μg/ng: grams/milligrams/micrograms/nanograms

H chain: heavy chain

HRP: horse radish peroxidase

Ig: immunoglobulin

IPTG:Isopropyl-β-D-thiogalactopyranosde

L chain: light chain

LB: Luria broth

MAb(s): monoclonal antibody (ies)

MBP: maltose binding protein

MPBS: milk in phosphate buffered saline

mRNA: messenger RNA

pAb: phage antibody

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

PCR: polymerase chain reaction

p.s.i: pounds per square inch

RNA: ribonucleic acid

rpm: revolution per minute

Recombinant Toxin A or rTA: recombinant carboxy terminal domain of Toxin A

SDS: sodium dodecyl sulphate

scFv: single chain antibody fragment

TNFα: tumor necrosis factor alpha

V: variable region

v/v: volume per volume

V_H: variable region of heavy chain

V_L: variable region of light chain

w/v: weight per volume

Chapter 1

General Introduction

1 General Introduction

Clostridium difficile, a Gram-positive micro-aerophilic (Weiner *et al.*, 1999), rod shaped, spore-forming bacterium, is a major causative of nosocomial intestinal infections (Samore, 1993). This pathogen is responsible of about one fifth of the cases of antibiotic-associated diarrhea, three quarters of antibiotic-associated colitis, and more importantly almost all the cases of life threatening pseudomembranous colitis (Bartlett, 1994; Kelly *et al.*, 1994b). These are estimated to be 3 million cases of pseudomembranous colitis in the United States alone (Bartlett, 1992; Mylonakis *et al.*, 2001) regardless of the seropositivity of the majority of adults in the USA (Weiner *et al.*, 1999). Symptoms can range from mild disease characterized by mild diarrhea, low grade fever and leukocytosis to a very serious disease characterized by fulminant course of colitis (toxic megacolon) with a high mortality rate (Wilcox, 2003). Frequent symptoms reported include fever, abdominal pain, and cramping (Vora *et al.*, 2006). In England and Wales reports of infection with *C. difficile* increased about six times in the three years starting from 1990 (Settle & Wilcox, 1996) and have risen steadily therafter. *C. difficile*-associated disease (CDAD) is estimated to cause problems to 1 % of hospitalized patients especially the immunocompromised and the elderly (Kelly *et al.*, 1994b) and causes considerable morbidity, increased financial implications on hospitals, and longer hospital stays (Riley, 1996; Silva, 1989; Wilcox *et al.*, 1996). In some instances ward closure or even hospital closure (Cartmill *et al.*, 1994) are nessessary to control infection. *C. difficile* is a very persistent nosocomial pathogen; transmission in hospital wards and nursing homes is assisted by the heat-resistance and resilience of the spores. With the increased use of broad-spectrum antibiotics such as clindamycin, cephalosporins, and amoxicillin during the last three decades, CDAD has become a leading clinical problem (Bartlett, 1981). Unlike many other bacteria, C. *difficile* infection requires antibiotic exposure in order to elicit its effect. Virtually all antibiotics, including vancomycin (Hecht & Olinger, 1989) and some other agents like cancer

chemotherapeutics can induce CDAD (Anand & Glatt, 1993). Even so, the use of antibiotics for the treatment of CDAD remains one of the few options for treatment of the disease.

1.1 Pathogenicity

Many strains of *C. difficile* are able to produce two potent toxins, Toxin A and Toxin B. Both are responsible of the virulence that is associated with the tissue damage. Toxin A is the primary mediator of intestinal tissue damage (Lyerly *et al.*, 1982; Lyerly *et al.*, 1985). Toxin A is an enterotoxin with glucosyltransferases activity and causes tissue damage (Faust *et al.*, 1998). The binding of Toxin A to a trisaccharide receptor on the human intestinal epithelial cells initiates disease (Wren *et al.*, 1991). Indeed, only Toxin A has been shown to recognize a binding domain on epithelial cell surface carbohydrates (Krivan *et al.*, 1986) and it is considered that this marks the starting point from which both toxins can elicit their effects on the epithelial cells on a later stage. There are a limited number of reports indicating that Toxin B alone can initiate an effect on the gastrointestinal cells (Riegler *et al.*, 1995; Savidge *et al.*, 2003). Unlike some enzymes like exoenzyme C3, Toxin A does not require assistance to permeabilize cells (Bartlett, 1994) which gives it the flexibility to invade damaged or intact tissues. Reports suggest that Toxin B has little effect when administered orally to animals without Toxin A, and its lethality only became evident after Toxin A had exerted an effect on the intestine (von Eichel-Streiber *et al.*, 1987).

When cells are exposed to Toxin A they tend to retract and round up. This is a direct effect of the loss of filamentous F-actin and the accumulation of G-actin (Dillon *et al.*, 1995; Pothoulakis & Lamont, 2001). This opens tight junctions, disturbing the barrier function of the epithelium, and leading to the watery diarrhea which is typical for *C. difficile* antibiotic-associated disease (Poxton *et al.*, 2001). The fluid exudates in the intestine consists of serum and cellular components that indicates a strong inflammatory

response, a feature that is not be seen in other enterotoxins (Tucker *et al.*, 1990). In contrast to some other bacterial toxins like cholera toxin and *E. coli* enterotoxins which trigger intestinal secretion without causing intestinal inflammation, when Toxin A was injected to the colonic loops in experimental animals, it induced secretion of fluid, and triggered epithelial cell destruction and neutrophil infiltration (Pothoulakis & Lamont, 2001).

Toxin A has potent biological activities that are described later. However, its receptor-binding activity may activate biological responses such as signal transduction cascades, cytokines release, and lymphocyte proliferation by clustering molecules at the surface of host cells (Castagliuolo *et al.*, 2004). These effects are likely due to the lectin-like structure of the C-terminal region of *C. difficile* Toxin A which can stimulate IL-2 and gamma interferon (IFN-ϒ) production *in vitro*. The integrity of the C-terminal domain of Toxin A is required for these effects; short recombinant peptides carrying C-terminal were not found to induce any inflammatory responses (He *et al.*, 2000).

Amongst the important effects of Toxin A on the epithelium is the disruption of the tight junctions, fluid accumulation and induction of neutrophil infiltration. The disruption of the tight junction is reported to arise from effects on cell surface projections and rearrangement of micro-villi, as studied by electron microscopy (Chang *et al.*, 1979; Fiorentini *et al.*, 1990). It was reported that in cell culture, the disruption of cell integrity takes place before the cell shrinkage (Hecht *et al.*, 1988). Some observations reported a retraction phenotype that is characterized by marginalization of the nucleus, an effect associated with the disruption of the cytoskeleton.

Neutrophil infiltration and accumulation is an important step in the development of pseudomembranous colitis. Neutrophil infiltration is thought to occur after the loss of epithelial integrity. It was reported that direct neutrophil-Toxin A interaction is essential for neutrophil infiltration (Kelly *et al.*, 1994a).

There is evidence that sensory neurons are regulated by Toxin A. This is supported by studies of substance P, an immunoreactive peptide that is linked to sensory neurons in the epithelium (Mantyh *et al.*, 1996). Substance P is thought to activate neutrophils via the activation of macrophages and the release of TNF-α. Substance P was also shown to mediate mast cell activation and degranulation. Hence, it may be also be involved in the pathogenesis of *C. difficile*-induced colonic inflammation.

Another potentially important effect is the triggering of apoptosis when intestinal cells, mast cells, and endothelial cells are exposed to Toxin A (Alcantara *et al.*, 2001; Brito *et al.*, 2002; Mahida *et al.*, 1996). This may have a significant role in the pathogenesis of pseudomembranous colitis induced by *C. difficile*. It was postulated that apoptosis could be induced by two different pathways. In the first pathway, apoptosis is initiated as a result of the transmission of a death signal after binding of TNF-α or Fas receptor to cell surface ultimately leading to activation of intracellular cysteine protease caspase 8 (Ashkenazi & Dixit, 1998). The second pathway is the activation of caspase 9 (Green & Amarante-Mendes, 1998) as a result of cellular stress and mitochondrial changes. The activation of caspase 8 or 9 leads to the activation of caspases 3, 6 and 7 that have crucial consequences on a group of important proteins and DNA fragmentation in the cell. It was reported that both pathways are induced by Toxin A (Carneiro *et al.*, 2006).

In cell culture, the detachment of epithelial cells from the basement membrane in response to Toxin A may also lead to apoptosis (Brito *et al.*, 2002; Fiorentini *et al.*, 1998).

Although apoptosis is an important natural process that corrects cell numbers by management of growth and death, it can be triggered by extracellular factors leading damage and disfunction of tissues. This damage is seen in the form of cell shrinkage, damage nucleic acid and paradoxically, impaired inflammation activity (Kroemer *et al.*, 1995).

One feature of C. *difficile* infection is the strong inflammatory response seen in the intestine. Linevsky and colleagues found that production of IL-8, IL-1β and TNF-α by macrophages can be activated as a result of Toxin A and B (Linevsky *et al.*, 1997). These factors up-regulate the expression of neutrophil and endothelial cells adhesion receptors, guide neutrophils to the vascular endothelium, promote diapedesis and tissue infiltration (Strieter *et al.*, 1994). This provides a link between macrophage activation and neutrophil infiltration in the pathogenesis of *Clostridium difficile* (Linevsky *et al.*, 1997). When cytokine synthesis was inhibited by drugs, the damaging effect of Toxin A in the intestine was minimized (Triadafilopoulos *et al.*, 1989). This finding shows the role of cytokines as important proinflammatory mediators in the pathophysiology caused by virulence factors from the bacteria. Monokines like IL-1β and IL-6 can also be released from monocytes in the presence of Toxins A and B (Flegel *et al.*, 1991). Another group (Kelly *et al.*, 1994a) showed that Toxin A can stimulate sensory neurons and trigger degranulation of mast cells causing release of inflammatory mediators that eventually lead to infiltration of neutrophils into the affected region. Castagliuolo and his colleagues reported that Toxin A induces the production of the neutrophil chemoattractant macrophage-inflammatory protein-2 (MIP-2) from the intestinal epithelial cells in the rat. The importance of MIP-2 is that it is a potent neutrophil chemoattractant and leads to neutrophil activation and attraction which is a unique phenomenon of Toxin A (Castagliuolo *et al.*, 1998a). It was also reported that Toxin A can act directly as a chemotactic factor for neutrophils (Pothoulakis *et al.*, 1993) and peripheral blood monocytes. Although these cells are unlikely to be exposed directly to significant amounts of the toxin in the peripheral circulation, the situation may be different in areas of colonic damage (Linevsky *et al.*, 1997). Interestingly, Toxin B lacks this activity (Pothoulakis *et al.*, 1988). Inflammation may also be exacerbated by down regulation of other activities. For example one study has reported that Toxin A leads to activation of adenosine deaminase, an enzyme that can down-regulate immune responses by acting as an anti-inflammatory agent (Cavalcante *et al.*, 2006).

These findings show how the pathogenesis of *C. difficile* enterocolitis is influenced by inflammatory mediators like IL-8 and the response of infiltrating monocytes and neutrophils. These responses probably underlie the massive vascular blockage, infiltration of the lamina propria with neutrophils and formation of pseudomembrane in the colon (Pothoulakis, 1996).

Surprisingly, in the last few years, it has been repeatedly reported that pseudomembranous colitis and nosocomial CDAD can be caused by *C. difficile* Toxin A-negative, Toxin B-positive stains (A^-/B^+) (Johnson *et al.*, 2001; Kato *et al.*, 1998; Limaye *et al.*, 2000). These strains were distinguished by a small to large deletions in *tcdA* (Sambol *et al.*, 2000). It seems that the deletion did not impair the toxin function as expected, but produced other forms or variants of the toxin with equivalent potential for disease initiation. Pathogenicity of A^-/B^+ strains was very similar to A^+/B^+ when measured by fluid secretion in rabbit intestinal loops (Borriello *et al.*, 1992). These variants were found to be very comparable in potency to *C. sordellii* lethal toxin (Soehn *et al.*, 1998).

The occurance of these strains appears widespread but variable. One study has reported that 39 clinical isolates out of 50 contained small deletions of 1.7 or 1.8 kb and were considered as Toxin A^-/B^+ strains. Out of those 39 A^-/B^+ isolates, 85 % were clindamycin resistant. This properly may boost the risk of *C. difficile* disease (van den Berg *et al.*, 2004). Another study by Sambol (Sambol *et al.*, 2000) reported only 7 cases of Toxin A^- strains amongst 5000 screened, suggesting that their frequency can be very low. These conflicting findings suggest that there is a very considerable difference in the occurrence of Toxin A^-/B^+ in different parts of the world. These contradictory findings raise some important questions ; in the absence of Toxin A, does Toxin B play a more active role than was thought and does Toxin A^- represent a selection event within the host (Voth & Ballard, 2005). These issues have not yet been resolved because most

experimental work carried out on the association of *C. difficile* with colitis has used of Toxin A or Toxin A producing *C. difficile*.

1.2 Toxin structure

Toxins A and B are to some extent related to each other with nearly 50 % amino acid similarity in their sequences. The highest similarity is found in the N-terminal (70 %) and in the C-terminal regions of the toxins (von Eichel-Streiber *et al.*, 1990). This finding helps to explain the common mechanism of action adopted by both toxins and the degree of similarity of substrate for both toxins. Both toxins are related to virulence factors from *C. sordelli* and *C. novyi*, based on the similarity of their sequences (von Eichel-Streiber *et al.*, 1996).

Cloning and sequencing of the *C. difficile* toxins showed that the Toxin A gene is 8.1 kb in length and encodes a 2710 amino acid polypeptide of 308 kDa (Dove *et al.*, 1990; von Eichel-Streiber *et al.*, 1992a). For many other smaller toxins that act on intracellular targets, it has been possible to identify three main regions: the receptor-binding domain, the transfer domain and the enzymatic domain. Toxin A is an extraordinarily large molecule and this property has presented a challenge to the analysis of its structure and toxicity (Faust *et al.*, 1998). Progress to unravel the mechanism of toxicity for this toxin has been relatively slow.

One of the first clues to toxin action came from analysis of the C-terminal part of the toxin, where so-called CROPS (combined repetitive oligopeptides) were identified (Florin & Thelestam, 1983) These consist of 31 short repeats (SRs) and 7 long repeats (LRs) of peptide sequence. The repeat sequences appear to be unique among extra-cellular bacterial proteins (Ho *et al.*, 2005). However, four repeat motifs show homology to features in the C-terminal region of Toxin B. Each repeat consists of a β-hairpin and a loop structure as shown by structural analysis. These features are thought to play a role in the

receptor-binding activity of the toxin. The sequence conservation in the SRs and LRs are thought to play a role in formation of kinks in structure, speculated to interfere with receptor recognition and cell entry. It was found that between 5 and 15 repeats are able to form stable secondary structures without interference with other structures. The repetition of these features may augment the binding affinity of the C-terminal domain with its receptor (von Eichel-Streiber *et al.*, 1992b; Weis & Drickamer, 1996). It has also been suggested that due to the repetitive manner of the carbohydrate binding sites of Toxin A, toxin binds to the receptor in a multivalent pattern. The binding sites present on the toxin were not thought to bind to carbohydrate receptor molecules simultaneously because they are orientated in different directions on the C-terminal domain (Greco *et al.*, 2006).

The central region of each toxin is hydrophobic and this region is thought to play a role in translocation of the N-terminal domain across the host membrane and into the cytosol (Dove *et al.*, 1990; von Eichel-Streiber *et al.*, 1992a). The third part of the toxin is the N-terminal region that carries biological activity with a hypothetical nucleotide binding site (Barroso *et al.*, 1994; von Eichel-Streiber *et al.*, 1996) that was analysed by Faust and colleages (Faust *et al.*, 1998). The following sections describe these features in more detail.

1.3 Toxin mode of action

1.3.1 Receptor and receptor binding

The repeating units of amino acids present in the C-terminal domain of Toxin A represent sites that act as multivalent lectin, recognizing the trisaccharide Galα1-3Galβ1-4GlcNAc and the disaccharide Galβ1-4GlcNac. These features are found on the I, X, and Y blood group antigens (Tucker *et al.*, 1990; Tucker & Wilkins, 1991). It has been suggested that Toxin A binds to these receptors in the intestine of animals but not humans where α-galactosyltransferase activity that is essential for the assembly of Galα1-3Galβ1-4GlcNAc does not exist. This implicates the involvement of other receptors which are not

yet identified (Krivan *et al.*, 1986; Pothoulakis & Lamont, 2001). It has been postulated that the receptor for Toxin A on mammalian cells may be a glycoprotein. On rabbit erythrocytes the receptor is a glycolipid (Pothoulakis & Lamont, 2001). A receptor for Toxin B has not yet been identified (Stubbe *et al.*, 2000).

One study (Castagliuolo *et al.*, 1998b) has pointed out the importance of the neurokinin-1 receptor in activation of sensory neurons and immune cells of the intestinal lamina propria after the injection of Toxin A into animal intestine. This study demonstrated that mice genetically deficient in the neutokinin-1 receptor were protected from the severe consequences of inflammation and secretion observed after Toxin A introduction into the intestine.

It was thought that all or some of the receptor-binding domain of Toxin A interacts with the receptor on target cells. If the structural analysis reported recently (Greco *et al.*, 2006) accurately reflects the organization of the C-terminal domain of Toxin A, it is difficult to imagine how all potential receptor-binding sites could be occupied simultaneously unless major reorganization of the domain takes place. This is because the receptor-binding pockets identified by Greco and colleages face in different directions. It is thought that cross-linking of receptors by Toxin A drives clustering, leading to endocytosis (von Eichel-Streiber *et al.*, 1996). This simple model is not consistent, however with the finding (Florin & Thelestam, 1983) that recombinant protein containing 50 % of the receptor-binding domain of Toxin A failed to bind to the cellular target receptor and to initiate endocytosis. An alternative model would be that one toxin molecule binds to one cellular receptor despite the repetitive nature of the toxin binding domain. This is supported by another study (Lyerly *et al.*, 1986) showing that blocking two epitopes in the 109 kDa receptor-binding domain with monoclonal antibody was enough to render the toxin completely inactive.

Conditions that affect the receptor binding of Toxin A have been studied by different groups. To understand the behavior of binding, it has been reported that a recombinant fragment of the binding domain of Toxin A containing 11 repetitive sequences was able to bind to the receptor of F9 cells (Sauerborn *et al.*, 1997) in high concentration of the recombinant protein which was also able to inhibit the cytotoxicity.

Genth and colleages (Genth *et al.*, 2000) reported similar findings. However, they also noted that inclusion of central, amino acids from the hydrophobic domain in a recombinant form of the binding domain enhanced inhibition of Toxin A action. The recombinant protein was also able to undergo internalization after binding to the surface of target cells *in vitro*. This shows that the hydrophobic domain has an importance in influencing the binding activity. The central domain alone, or parts of the C-terminal domain lacked these properties (Frisch *et al.*, 2003).

The importance of metals in binding activity of Toxin A to the receptor has been investigated by some groups (Cho, 2001; Dodd & Drickamer, 2001) as carbohydrate receptors often incorporate metal ions for binding. One study (Demarest *et al.*, 2005) pointed out that Toxin A-receptor interaction is Ca^{2+} dependent. The role of intracellular Ca^{2+} in cytotoxicity is still controversial (Demarest *et al.*, 2005; Fiorentini *et al.*, 1993) as some investigators report that exposure to *C. difficile* can increase the cellular Ca^{2+} concentrations (Castagliuolo *et al.*, 2004).

1.3.2 Toxin A entry to the cell

Internalization of Toxin A into the host cell is an important step for the toxin to exert full activity which ends with changes to cell morphology and tissue damage. Figure 1.1 summarises the mode of action of Toxin A.

Toxin A gains entry to the cell by endocytosis into vesicles which then undergo acidification. The low pH of the endosome is thought to play an important role in changing

the structure of the toxin. It is thought that the toxin unfolds, exposing the hydrophobic central domain of the protein (Qa'Dan *et al.*, 2000). The role of the C-terminal binding domain and the hydrophobic central domain of Toxin A after endocytosis is not well understood. One study (Pfeifer *et al.*, 2003) reported that the N-terminal domain of Toxin B is the only part of the toxin which gains access to the cytosol and binds to its target. The finding may help understanding the importance of the C-terminal and the hydrophobic domains of Toxin A.

1.3.3 Toxin function

Toxin A is an intracellular toxin with an action that is characterized by the enzymatic glucosylation of the low molecular mass GTP-binding proteins Rho, Rac, and Cdc42 (Aktories & Just, 1995; Just *et al.*, 1995). To do so, the toxin uses UDP-glucose as substrate. Glucosylation takes place at threonine 37 and threonine 35 in the target proteins in that order. This leads to the deactivation of the small GTPases (Chardin *et al.*, 1989; Paterson *et al.*, 1990). As a result, signal transduction pathways controlled by the GTPases are inhibited (Just *et al.*, 1995; Prepens *et al.*, 1996) causing destruction of the cytoskeleton, transcription of some stress-activated protein kinases, and a drop in synthesis of phosphatidylinositol 4,5 bisphosphate which could lead to the loss of cell polarity (Mackay & Hall, 1998). In the absence of the acceptor protein, Toxin A hydrolyzes UDP-glucose to UDP and glucose (Ciesla & Bobak, 1998). This is analogous to the ADP-ribosyltransferase activity of toxins like cholera toxin in that the absence of acceptor protein, hydrolysis of NAD to nicotinamide and ADP-ribose takes place.

Immediately after Toxin A has gained access to the cytosol of epithelial cells, it initiates glucosylation of Rho, Rac, and Cdc42, the actin cytoskeleton collapses and inflammation is initiated. It was reported that the induction of chemokine expression is provoked by the transcription factor NF-κB. The activation of NF-κB in the early stage of *C. difficile* pathogenesis is accompanied by release of reactive oxygen species and

activation of mitogen-activated kinases. It is thought that this pathway is responsible for the neutrophil infiltration (Pothoulakis & Lamont, 2001). It has also been reported that cellular infiltration may occur before the Rho glucosylation, suggesting an independent route of action that requires further studies (Pothoulakis & Lamont, 2001).

The small GTPases Rho, Rac and Cdc42 which share similar mechanisms of regulation, are found in eukaryotic cells and regulate the actin cytoskeleton (Hall, 1990). Rho and other small GTPases hydrolyze GTP to GDP which result conversion from the "on" to the "off" state. This state of fluctuation is controlled by guanine exchange factors that are needed for regulation of activity. When Rho is activated as part of its role in the cytoplasm, it hydrolyzes bound GTP to GDP. GDP dissociation inhibitors then keep the Rho blocked from interaction with guanine exchange factors (Zhou *et al.*, 1998). When the GDP bound form of Rho is dissociated, Rho is able to interact with guanine exchange factors, leading to binding of GTP, activation, and initiation of regulatory cascade. Hydrolysis of GTP to GDP returns Rho to the off state. Furthermore, through activation of Rho-kinase or other signal proteins, Rho participates in regulation of stress fiber formation (Fujisawa *et al.*, 1998). The later events take place after an extracellular signal. When the interaction of Rho with the signal proteins is blocked, the actin cytoskeleton is disrupted.

Although small GTPases like Rac and Cdc42 share a common mechanism with Rho, Rac and Cdc42 activity is directed more towards the formation of flamellipodia and filopodia which are important for movement of cells (Nobes & Hall, 1995).

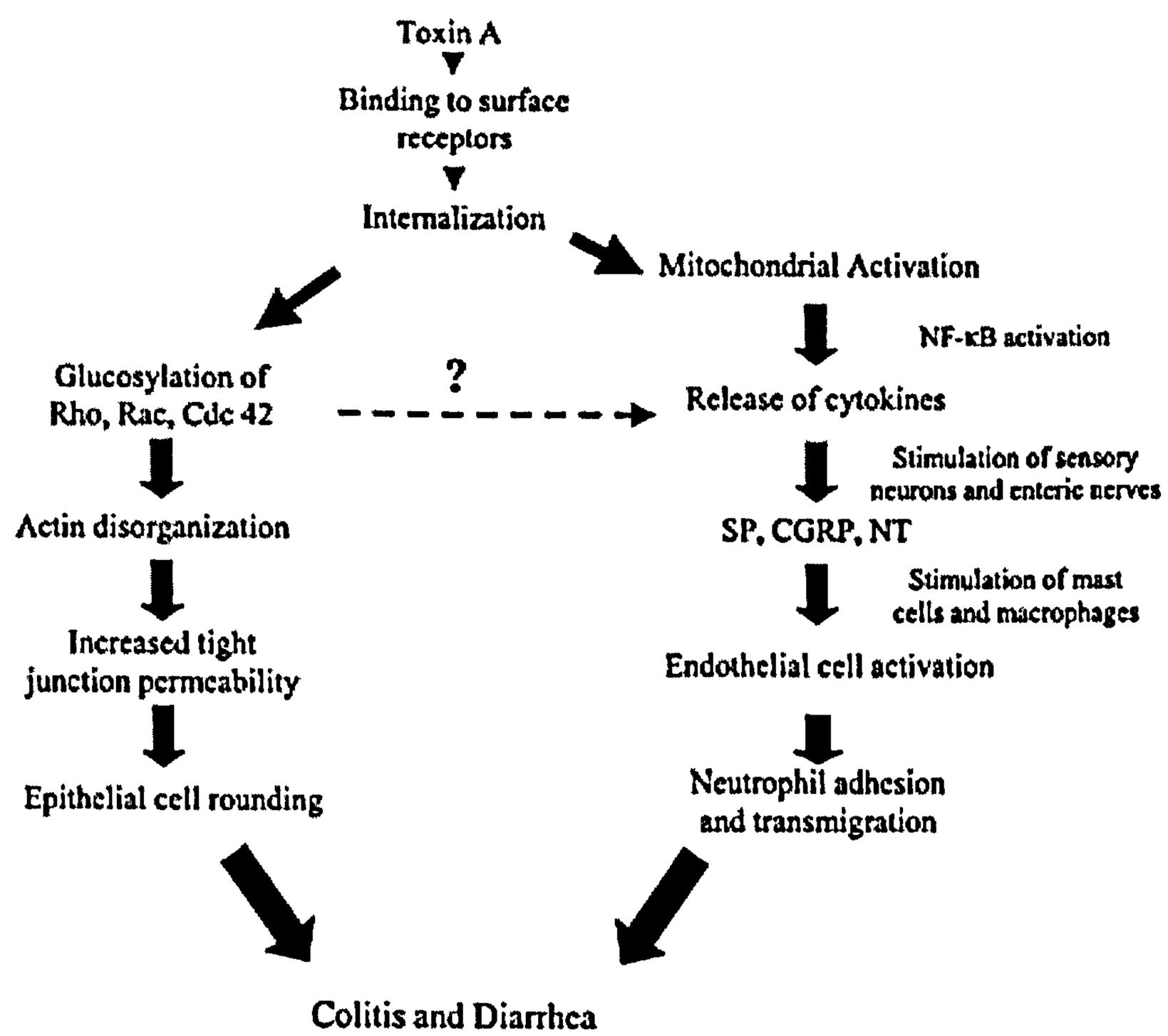

Figure 1.1: Summary of the proposed mode of action of Toxin A

(Pothoulakis & Lamont, 2001).

1.4 Humoral and cellular immune response against *Clostridium difficile* Toxin A

To date, no comprehensive studies have been done with a significant number of cases to establish the importance of immunoglobulin responses during and after infection with *C. difficile*. Some reports have investigated different aspects of the immune response in normal individuals and patients to correlate with infection, age, and sex. One study suggested that sex may have a significant role in disease prevalence as women are more prone to CDAD (Aronsson *et al.*, 1985). Age was also found to be an important factor of *C. difficile* has been found capable of colonizing the intestinal tracts of 90 % of healthy newborns and children under one year. Although toxin appears to be produced, newborns are resistant to the disease. In normal adults, two-thirds of normal subjects develop anti-Toxin A antibodies but the incidence of CDAD is much higher than in newborns. The role of different immunoglobulin classes and their levels were reported to be of significance in immune response against Toxin A. One study has linked the development of anti-Toxin A IgG in the serum of colonized patients on antibiotic therapy and the asymptomatic carriage of *C. difficile*. These patients were found to be 48 times more resistant to diarrhea than those who failed to develop IgG against Toxin A in serum (Kyne *et al.*, 2000). This finding highlights the importance of the humoral response in protection. The elevation of IgG anti-Toxin A antibody in serum was also accompanied by the elevation of IgG in the stool. This suggests a filtration mechanism from the serum to the lumen of the intestine (Warny *et al.*, 1994). Serum levels of IgM and IgG antibodies were monitored in patients who did not relapse and it was found that both immunoglobulins were markedly elevated. In contrast, patients who relapsed after the first occurrence of the disease developed much less serum IgM and IgG (Kyne *et al.*, 2001). Warny and colleages (Warny *et al.*, 1999) reported that patients who developed recurrent CDAD had lower IgA and IgG anti-Toxin A antibodies levels in the stool than those who recovered from infection. These findings clearly suggest

that elevated immunoglobulins are a good marker of the immune response against the disease and may also be critical protective factors in recurrence of CDAD.

It has also been reported (Warny *et al.*, 1994) that anti-Toxin A antibody level is an important factor in the length of CDAD. In patients with low levels of antibody, the duration of disease and the chances of relapse were both higher than in patients with high levels of anti-Toxin A antibodies.

Toxins A and B were found to have an influence on the cellular immune system *in vitro*. The T-cell response against Toxin A was studied in parallel with the monocyte response and it was found that monocyte-independent T-cell proliferation was not affected while monocyte-dependent T-cell proliferation was inhibited by the toxin. This suggested that Toxin A interacts with monocyte receptors and leads to cytopathic effects which are not seen with T-cells (Daubener *et al.*, 1988)

1.5 Methods for control

1.5.1 Antibiotics

The two most effective antibiotics for treatment of CDAD are metronidazole and vancomycin for mild or severe cases respectively. Most patients respond to these two antibiotics, but 20 % of patients relapse after the termination of antibiotic treatment (Fekety, 1997). Further treatment of CDAD with antibiotics can be successful in 70 % of patients but some may suffer many rounds of relapses (Buggy *et al.*, 1987; Jobe *et al.*, 1995). Other antibiotics like bacitracin, fusidic acid, rifampicin and teicoplanin are also reported as first line antibiotics in the treatment of CDAD. However, high relative toxicity, the cost of these antibiotics and the high rate of carriage of the pathogen in stool after treatment, leaves metronidazole and vancomycin as first and second line antibiotics of choice for CDAD (McFarland *et al.*, 2005; Young *et al.*, 1985). Many strategies have been employed to control the disease, like the normalization of microbial flora and the use of

combination of antibiotics, but success tends to be limited (Buggy *et al.*, 1987; Gorbach *et al.*, 1987). Although it has been found that some strains of *C. difficile* carry genes for antibiotic resistance (Roberts *et al.*, 1994), relapse is more often due to reinfection or failure to complete antibiotic treatment (Fekety, 1997).

Rounds of antibiotic treatment in recurrent cases can be extended to months or, in some cases, years as the percentage of recurrence increases at each round of relapse (Fekety *et al.*, 1997; McFarland *et al.*, 1999). Some studies have reported that relapse was often due to the discontinuation of antibiotic therapy (Kato *et al.*, 1996; Tang-Feldman *et al.*, 2003; Wilcox *et al.*, 1998). The authors discovered that 50 % of recurrent infections were due to relapse with the original strain that caused the first infection. New strains of *C. difficile* were blamed for the other 50 % of recurrence. Discontinuation of antibiotics may take into account factors like age, health and the severity of the disease. Moreover, the decision to cease antibiotic treatment may be made because the fecal culture has gone from positive to negative. Relapse may arise when spores germinate in the susceptible intestine. Metronidazole was found to be very effective in the majority of CDAD; however, it might not be a favorable choice for treating recurrence because it has been found that metronidazole can be associated with occurrence of multiple resistant strains. In addition, metronidazole has a relatively high toxicity (Beloosesky *et al.*, 2000; Pelaez *et al.*, 2002). Vancomycin appears to be an effective and relatively safe therapy for recurrence with very few side effects (Jarvis, 1998).

The use of broad spectrum antibiotics inhibits the normal flora in the gut causing overgrowth of resident or incoming *C. difficile* which consequently produces the toxins responsible for CDAD (Nord & Edlund, 1990). The irresponsible use of broad spectrum antibiotics is accelerating the emergence of multiple resistant bacteria. Of particular concern is the appearance of bacterial pathogens that are resistant to vancomycin (1997; Watanabe *et al.*, 1997). Moreover, metronidazole, vancomycin or other choices of

antibiotics do not allow recovery of intestinal normal flora which can inhibit enteric pathogens like *C. difficile*. The sole use of antibiotics for control of *C. difficile* infection also fails to limit the exposure to pathogens in hospital environments. Hand washing and the isolation of infected patients in parallel with antibiotic therapy are important issues (Schroeder, 2005; Valiquette *et al.*, 2004).

In mild cases of CDAD, discontinuation of the offending antibiotic or shifting to a less potent antibiotic is usually enough to reduce symptoms. Electrolyte replacements are usually administered in severe forms of diarrhea (McFarland *et al.*, 2000).

It was also reported that probiotics may be of value in recurrent cases of CDAD by competing with the pathogen for colonization and inducing the other normal organisms to establish in the colon. Micro-organisms like *Saccharomyces boulardii* and *Lactobacillus* species were introduced to the intestine. The outcome of this strategy was a significant decrease of recurrence. However, these results were achieved only when probiotics were combined with vancomycin or metronidazole (Surawicz *et al.*, 2000).

Other treatment strategies like bowel irrigation, or use of ion exchange resins were reported in some studies. In spite of some promising results, these studies were either performed on a small scale lacking placebo controls or antibiotics were still required to achieve success (Mogg *et al.*, 1982; Persky & Brandt, 2000).

1.5.2 Immunotherapy

Alternative strategies to antibiotic treatments are required to reduce the increasing incidence of *C. difficile* infection. Active vaccination against Toxins A and B has been used successfully to protect animals against *C. difficile* disease. However, vaccines appropriate for use in human patients are still under development. One study has assessed the safety and immunogenicity of Toxoids A and B vaccines in humans (Kotloff *et al.*, 2001). This study has found that the two toxoids were tolerated without serious side

effects. Minimal local or systemic reactions were observed. Antibody responses to the different doses of the toxins were strong in more than 90 % (serum IgG) and 50 % (fecal IgA) of subjects respectively. However, the importance of IgG and IgA against Toxins A and B in human infection remains incompletely defined. Stubbe and his group have demonstrated that dimeric IgA is more effective against Toxin A than either monomeric IgA or IgG immunoglobulins that share the same variable domain. The authors proposed that the neutralization efficacy of dimeric IgA and its extended duration of protection are due to the degree of polymerization (avidity) and not to the peristaltic movement of dimeric IgA as tested in the absence of mucus (Stubbe *et al.*, 2000). Salcedo *et al.* have reported the use of human Igs pooled from healthy individuals for therapy against *C. difficile*. The immunoglobulin therapy was administered intravenously to patients suffering from severe pseudomembranous colitis that was unresponsive to antibiotic treatments. Despite the success in controlling the disease, diarrhea recurred four weeks after infusion of immunoglobulins. This study suggested that passive immunotherapy using immunoglobulins from normal donors accompanied by antibiotic therapy can speed up recovery and may prevent recurrence (Salcedo *et al.*, 1997). Kyne and co-workers, also reported a successful trial in which chronic recurrent *C. difficile* colitis was treated with pooled immunoglobulins (Kyne *et al.*, 2001). Immunotherapy has been suggested as a routine treatment for recurrent CDAD where antibiotics including vancomycin have failed (Murphy *et al.*, 2006). However, the lack of a supply of immunoglobulin against the toxin for therapeutic use and the cost of the treatment imposes a limitation to the wider use in CDAD (Wilcox, 2004).

Studies on animals have shown that the protective effect is correlated to *in vitro* neutralization of the toxins and not to the quantitative level of antibodies as measured by ELISA. Moreover, active immunization with Toxoids A and B may not be an ideal prophylactic or therapeutic treatment for hospitalized patients and individuals at high risk because the immune response requires time to develop (Corrado *et al.*, 1990). The risk of

adverse immune interactions and inflammation are relevant considerations. Under these circumstances, passive immunization might be a more suitable therapeutic choice.

Animal studies have evaluated these ideas. Few have devised effective immunotherapy for CDAD after the onset of the disease (Kink & Williams, 1998). Instead, considerable effort has been invested in the study of active immunization (Corthier *et al.*, 1991; Kim *et al.*, 1987; Libby *et al.*, 1982; Lyerly *et al.*, 1991). In one study, hamsters were immunized with toxoids inactivated with formaldehyde. These were made from Toxins A or/and Toxin B. Although immunization stimulated high antibody titers, vaccination against Toxin A or Toxin B individually failed to produce antibodies that were protective against the lethal consequences of infection. However, the study was able to demonstrate the role of both toxins in the pathology of antibiotic-associated cecitis (AAC) (Libby *et al.*, 1982). A more recent study by Aboudola *et al.* (Aboudola *et al.*, 2003) has confirmed this finding and despite the high level of antibody titers, these antibodies failed to neutralize the toxin when tested by cytotoxicity assay *in vitro*. Other groups have reported different outcomes. Fernie *et al.* (Fernie *et al.*, 1983) showed that 80 % protection could be achieved when Toxoid A and B were used together to vaccinate hamsters. Interestingly, Toxin B immunization gave more protection than Toxin A alone. Kim *et al.* (Kim *et al.*, 1987) suggested that more studies are required to resolve these discrepancies. This group also suggested that Toxin B might have less importance than Toxin A in the pathogenesis of ileocecitis because immunization against Toxin A was required in order to protect hamsters against antibiotic-associated intestinal disease caused by *Clostridium difficile* (Kim *et al.*, 1987). This was supported by later studies which showed that the introduction of a high concentration of Toxin B alone to the intestinal loop did not show damaging effects. This conflicts with earlier studies suggesting equal roles for Toxins A and B in the disease in hamsters (Lyerly *et al.*, 1985). When both Toxins A and B were introduced together to the intestinal tract in low, non-lethal doses, animals developed the disease and died. Immunization of the animals against Toxin B failed to protect from

effects of Toxin A and death (Kim *et al.*, 1987). This suggests a synergistic effect in which Toxin B appears to be dependent upon Toxin A, but more aggressive in its action.

Kink and Williams (Kink & Williams, 1998) have studied the impact of antibodies against Toxins A and B upon disease in hamsters. Antibodies were raised in chickens against epitopes of Toxin A and Toxin B located in the carboxy-termini of each protein. Anti-Toxin A provided efficient protection against disease when delivered before infection with *C. difficile*, but protection against diarrhea, weight loss and death was only partial if applied after infection. This is consistent with a previous report (Kim *et al.*, 1987). When antibodies against Toxin A and B were combined, protection against infection was solid and exceeded that of vancomycin treatment. Significantly, it protected against on-going *C. difficile* disease.

One study (Lyerly *et al.*, 1991) has evaluated passive immunization of hamsters using bovine antibodies against *C. difficile* toxoids. Regardless of the failure of antibodies to treat the CDAD after the onset of the disease, the authors were able to show protection against disease, raising the prospect of a novel, high volume source for persons at high risk of *C. difficile* infection. Bovine colostrum has particularly high concentrations of IgG whereas in human colostrum, IgA is the main class. Kelly *et al.* prepared bovine immunoglobulin concentrate against *C. difficile* from the colostral milk of Holstein cows by immunizing them with the toxoids of the pathogen. The outcome was a specific IgG directed against *C. difficile* Toxin A and B which was able to neutralize the action of both toxins actions when tested *in vitro* and in animal models. The authors proposed the use of bovine immunoglobulin concentrate as an oral treatment for CDAD and colitis. It was expected that loss of antitoxin activity due to the acidic gastric secretions might take place. Therefore, the anti-toxin was combined with an anti-acid and administered to human volunteers. This showed the IgG survived passage through the full length of the intestine (Kelly *et al.*, 1996; Kelly *et al.*, 1997; Warny *et al.*, 1999). Aside from its safety, antitoxin

has the advantage of not interacting with the normal flora in the colon. This should assist re-establishment and normalization of the normal flora which may also help protect against subsequent relapses.

The use of murine monoclonal antibodies against Toxin A in passive protection has been examined by Corthier *et al.* The study investigated if MAbs that neutralize the toxic activity of Toxin A can protect mice by passive transfer. Three monoclonal antibodies were selected according to their ability to recognize the repeating units of the C- terminus of Toxin A. Each of the selected MAbs protected against *C. difficile* disease when injected intravenously to the mice (Corthier *et al.*, 1991). In human vaccination with MAbs, however, a second MAb against Toxin B might be required as Toxin A and Toxin B do not share neutralizing epitopes. A further issue of potential importance is the existence of *C. difficile* strans of different toxinotypes of Toxin A, as discussed below (Giannasca & Warny, 2004).

One distinctive study (Sauerborn *et al.*, 1997) proposed the use of recombinant fragments from the repetitive receptor-binding domain of Toxin A as passive or/and active vaccine depending on the route of introduction. The recombinant fragment recognized the toxin receptor on epithelial cells and thereby blocked the action of Toxin A. The recombinant fragment can also act as an immunogen triggering an immune response. The efficacy of the recombinant fragment to protect against Toxin A was evaluated and it was able to protect against the toxicity of Toxin A *in vitro* and *in vivo.*

1.6 Toxinotypes and strains of importance

Most of the work done on Toxin A and Toxin B has been based on one strain, VPI 10463, which may represent around 20 % of clinical isolates. However, there are 22 different serogroups of *C. difficile* that can be further classified based on restriction length polymorphisms (RFLPs) in the toxin genes. To establish this diversity, parts of the toxin

genes were amplified and digested with selected restriction enzymes. Although the heterogeneity of Toxin A, as observed by RFLP analysis, is much less than Toxin B, deletions were observed in Toxin A. It was also noticed that genetic variants of Toxin A were seen in clinical isolates which may include hybrids that resulted from genetic exchange between different *Clostridia* strains.

Different serogroups were then subdivided into 10 toxinotypes based on subtle difference variations in the genes of the pathogenicity locus. Each toxinotype was designated with Roman numbers I to X where strain VPI 10463 was considered as toxinotype 0 (Table 1.1 and 1.2). No striking pathological differences were seen between variant strains and the classical *C. difficile* (Table 1.2). It has been speculated that molecular typing might be a useful tool not only in establishing toxinotypes and variants among the *C. difficile* toxins, but also in diagnosis and prediction of the degree of virulence of clinical isolates (Rupnik *et al.*, 1998).

Amongst the *C. difficile* strains that have been identified, some are more important than others because of their epidemic or virulence properties. For example, 027 strain isolated in Canada has been associated with a number of very important outbreaks and seems to create greater severity of illness than the classical *C. difficile* strains. It has been reported that some of the 027 isolates produced 20 times the level of Toxin A and B than other *C. difficile* strains, a property that has obvious implications for patient health and the costs of treatment (Eggertson, 2005).

Table 1.1: RFLP characteristics used for typing of toxin genes

Toxino-type	*Hin*cII and *Acc*I restriction pattern for B1 fragment[a]	*Hin*dIII and *Rsa*I restriction pattern for B3 fragment	*Eco*RI restriction pattern for A3 fragment	*Nsi*I restriction pattern for PL2 fragment	Standard strain	No. of strains found
0	1	1	1	1	VPI 10463	172
I	1	1	4d	1	EX 623	2
II	1	1	3d	1	AC 008	1
III	4	4	2	2	44027	6
IV	2	2	2	3i	55767	4
V	3	3	8	4i	SE 881	1
VI	3	3	5d	4i	51377	4
VII	3	3	6d	4i	57267	1
VIII	5	1	7d	3i	1470	25
IX	5	4	2	4i	51680	2
X	5	4	NA	4i	8864	1

[a] Restriction patterns were analysed and a number given to each profile. NA, not amplified; d, deletion; I, insertion.

(Rupnik *et al.*, 1998)

Table 1.2: *C. difficile* strains with variant toxin genes

Strain	Toxino-type	PFGE type	Serogroup	*tcdB*	*tcdA*	Date of isolation (day.mo.yr)	Patient birth date (yr)	Diagnosis
38544	I	NT[b]	C	+	+	1.7.1991	1944	PMC
EX 623	I	8	C	+	+	19.11.1990	Unknown	AAD
AC 008	II	9	A14	+	+	7.4.1994	Unknown	AAD
SE 844	III	2	A1	+	+	10.4.1995	1966	AAD
SE 847	III	2	A1	+	+	10.4.1995	1959	AAD
EX 482	III	2	A1	+	+	2.1.1990	1920	AAD
45129	III	2	A1	+	+	7.9.1992	1939	AAD
35004	III	2	A1	+	+	16.11.1989	1932	PMC
44027	III	ND[c]	A1	+	+	15.6.1992	1939	PMC
55538	IV	5a	A1	+	+	12.5.1995	1991	AAD
55767	IV	5b	A1	+	+	12.6.1995	1944	AAD
40067	IV	5b	A1	+	+	14.10.1991	1990	AAD
7701	IV	6	A5	+	+	23.7.1985	Unknown	AAD
SE 881	V	10	A15	+	+	30.8.1995	1951	AAD
48489	VI	3	A15	+	+	9.4.1993	1931	PMC
51377	VI	4	A15	+	+	11.2.1994	1926	PMC
BR 071	VI	3	A15	+	+	3.7.1995	Unknown	AAD
39696	VI	4	E6	+	+	19.9.1991	1980	AAD
57267	VII	4	E6	+	+	21.12.1995	1946	AAD
1470	VIII	7a	F	+	−	2.12.1981	1981	Asymptomatic
20376	VIII	7b	X	+	−	8.7.1988	1986	Asymptomatic
51680	IX	11a	A16	+	+	17.3.1994	1933	AAD
SE 938	IX	11b	A16	+	+	5.1.1996	1931	AAD
8864	X	12	Unknown	+	−	Unknown	Unknown	Unknown

[a] Brussels1, Brussels2, etc., represent different units or different hospitals in Brussels. [b] NT, nontypeable. [c] ND, not done. AAD, antibiotic associated disease. PMC, pseudomembranous colitis.

(Rupnik *et al.*, 1998)

1.7 Antibodies and CDR conformation

In response to materials that are foreign to the mammalian immune system, B-lymphocytes produce proteins which can recognize and bind to these immunogens. These proteins are known as antibodies. When antibodies are electrophoresed, their structure and molecular weight confers a migration pattern that is similar to the globulins, hence their designation as immunoglobulins. Immunoglobulins have a common basic structure but they can interact with different antigens due to the differences in sequence in regions known as the variable or complementarity determining regions (CDRs). The huge range of CDR variability allows binding to a very wide range of dissimilar protein antigens and non-peptide structures like phospholipids, sugar moieties or even metals (Amoroso *et al.*, 2003; Bosslet *et al.*, 1991; Cabrera *et al.*, 2006).

The natural response to an antigen is polyclonal as different features of the immunogen provoke the synthesis of individual, specific antibodies. During the last century, a major advance in the development of antibodies was the development of techniques for production of monoclonal antibodies. Originally developed by Kohler and Milstein (Kohler & Milstein, 1975), monoclonal technology allows the production of antibodies against just one feature (epitope) of an antigenic compound. Due to their specificity, sensitivity and the continuous supply, monoclonal antibodies have become used increasingly as tools for therapy and diagnosis by many researchers. Monoclonal antibody therapy is being use to treat variety of disease including cancers (Cheson, 2006; Furman *et al.*, 2006; Zhong *et al.*, 2006), pathogens (Casadevall, 1998; Zeitlin *et al.*, 1998) and autoimmune diseases (Amoura & Piette, 2006).

It was not until 1950 that the structure of antibodies became clear and the contributions of the heavy and light chains to the Fab and Fc regions of the molecule were understood through X-ray crystallography (Deisenhofer *et al.*, 1976; Poljak *et al.*, 1973). It was from these studies that the idea of T-shaped structure arose with each Fab component

independent in its orientation with respect to the Fc region. This is of importance because it means that antibody can recognize epitopes on a single or two different separate antigen molecules (Harris *et al.*, 1992). Antibodies display a structure known as the immunoglobulin fold that consists of two antiparallel β-sheets packed to form a β-sheet 'sandwich', the two halves of the sandwich being held together by a disulfide bond. Functionally, two regions of importance can be defined in the antibody. The first contains the CDRs which are formed by the variable domains of light and heavy chains (V_L and V_H). The second are the constant domains. The antigen binding domain is formed when six CDR loops (H1, H2 and H3 from heavy chain, and L1, L2 and L3 from light chain) are arranged in juxtaposition. Four of the loops are encoded in the V_L and V_H germline segments and the other two are formed at the junctions of the J segment for light chain and V, D and J for heavy chain. The segments fuse during the process of genomic rearrangment and somatic mutation during B-cell maturation (Wu & Kabat, 1970).

1.8 Engineered antibodies

Molecular biology has made possible the modification and manipulation of naturally existing proteins including antibodies. This has lead to the development of antibody engineering techniques. With proper primer design, it is possible to generate cDNA from mRNA isolated from spleen, lymph cells or hybridomas (Maynard & Georgiou, 2000). Since these cells will express a whole repertoire of transcripts, PCR is used to recover immunoglobulin-coding cDNAs. The V_H and V_L can be fused and expressed as single chain variable fragments (scFv) or the light and heavy chains can be expressed separately, allowing natural non-covalent association into a Fab fragment. Although the scFv is a synthetic form of antibody, fusion through the linker sequence prevents the dissociation of V_H and V_L domains (Winter *et al.*, 1994). These forms of antibodies are illustrated in Figure 1.2.

In addition to scFvs, other recombinant forms of antibodies like chimeric and humanized antibodies can also be generated. One aim of these manipulations is to reduce the response of human immune system when recombinant antibodies are introduced into patients. The other aim is to combine the characteristics of one antibody such as its specificity or sensitivity with the characteristics of another (for example efficiency of complement fixation)(Sandhu, 1992; Weiner, 2006). In recent years, fusion techniques have also been used to join the Fc domain of antibodies with non-immunoglobulin peptides. In this case, the variable region of the Ig molecule is replaced with a non-immunoglobulin structure that confers binding specificity upon the recombinant protein. These molecules are known as 'immunoligands' (Capon *et al.*, 1989; Landolfi, 1991; Traunecker *et al.*, 1989).

1.8.1 Humanization of antibodies

Laboratory aminals such as mice have been very valuable in modeling human disease and in developing methods for the manipulation of the immune response. The original development of monoclonal antibody technology was founded upon isolation of B-lymphocytes from mice and their manipulation *in vitro*. However, mouse monoclonal antibodies can not be used directly for human therapy as the proteins are quickly recognised as foreign, provoking a human anti-mouse response. Investigators have sought ways to modify mouse antibodies to overcome this problem.

1.8.1.1 Chimeric antibodies

In these forms of recombinant antibodies, the variable genes of a mouse antibody replace the equivalent human sequences to produce a complete immunoglobulin. The antibody will possess the specificity and avidity characteristics of the mouse immunoglobulin while two thirds of the antibody is of human origin to reduce the immunogenicity when introduced into the human immune system. These constructs are

illustrated in Figure 1.2. Studies have shown that immune adverse responses against chimeric antibodies occur rarely and some chimeric antibodies have been further developed as therapeutics (Brekke & Sandlie, 2003; Maynard & Georgiou, 2000; van Dijk & van de Winkel, 2001) .

1.8.1.2 CDR- grafted antibodies

The other route for creation of human-like antibodies from murine origins is the grafting of CDRs from the variable domains of rodent antibodies onto human variable domains (Jones *et al.*, 1986). This is shown in Figure 1.2. After manipulation, around 90 % of the recombinant antibody is human in sequence. However, this does not completely eliminate the risk of human anti-mouse responses. One challenging issue in CDR grafting is that the affinity of the protein might not be as high as the original mouse antibody. For this reason, it is critical to use a human antibody framework with high sequence homology to the original murine immunoglobulin to minimize the loss of affinity from sub-optimal conformation in the CDRs (Shearman *et al.*, 1991).

1.8.1.3 Transgenic Antibodies

One recent innovation for production of human antibodies is to use mice that have an impaired or inactivated immunoglobulin response but carry in their genome, repertoires of human heavy and light chains. These transgenic mice can be immunized with any target and the resulting antibody response can be captured and immortalized by creating hybridomas that produce complete functional human monoclonal antibodies (Fishwild *et al.*, 1996; Neuberger, 1996). An illustration of this approach has been described recently (Tomizuka *et al.*, 2000). Human chromosomal fragments containing the immunoglobulin heavy chain and κ light chain loci were introduced to a mouse after inactivation of the endogenous humoral response. Several human antibodies generated from transgenic mice are currently in clinical trials (van Dijk & van de Winkel, 2001). One further strategy

described the 'Trimera' mouse (Reisner & Dagan, 1998). The animal's immune system is disabled by exposure to radiation. Then bone marrow from an SCID mouse is transplanted to generate a partially reconstrituted immune system that can accept foreign cells or tissues. The B cell from a human source is then engrafted and monoclonal antibodies are produced. Antibodies against Hepatitis B produced in the Trimera mouse are currently under clinical trials (Dagan & Eren, 2003).

1.9 Recombinant Antibodies and phage display

Since 1985, when phage display was first suggested by Smith (Smith, 1985), recombinant antibody technology has witnessed significant developments reflected by the production of highly specific antibodies that can be employed in diagnostics, therapeutics, and research. In comparison with the traditional methods of creating polyclonal antibodies or even monoclonal antibodies, phage display technology is considered cheap, fast, and is not labor intensive. Furthermore, phage display technology does not require large numbers of animals, indeed in several applications, animals can be completely eliminated from the production of specific antibodies (Deng *et al.*, 2003). Antibody phage display can be divided into several distinct phases. Firstly, libraries of antibody-coding sequences are generated. These genes can be taken from immunized, non-immunized (naïve) lymphocytes (Amersdorfer *et al.*, 2002) or even synthetic constructs (Rauchenberger *et al.*, 2003). These coding sequences are cloned to create recombinant antibodies that can take different forms such as scFvs, Fabs or Fvs (McCafferty *et al.*, 1990). These are illustrated in Figure 1.2. The recombinant antibodies are displayed at the surface of viral particles that also carry the coding sequence for the recombinant antibody. The phage display library is then screened for the ability to bind to a target antigen through the specificity of the displayed antibody. By-passing the mammalian immune system in this way (Hoogenboom, 1997; Schier *et al.*, 1996) means that antibodies can be isolated against polypeptides that may be poorly immunogenic *in vivo*, substances like fatty acids (Gargir *et al.*, 2002), self-

antigens (Rauchenberger *et al.*, 2003; van Kuppevelt *et al.*, 1998) or toxic materials (Deng *et al.*, 2003). The specificity of the isolated antibodies can be confirmed with immunoassay methods based upon the production of further stocks of virus, or by expression and analysis of the recombinant antibody as separate, soluble proteins. If its affinity or specificity is sub-optimal, maturation or other modifications are possible, *in vitro*. Finally, the antibody can be expressed in prokaryotic or eukaryotic systems for more detailed analysis, or downstream applications. In the following sections, individual steps of this process are described in more detail.

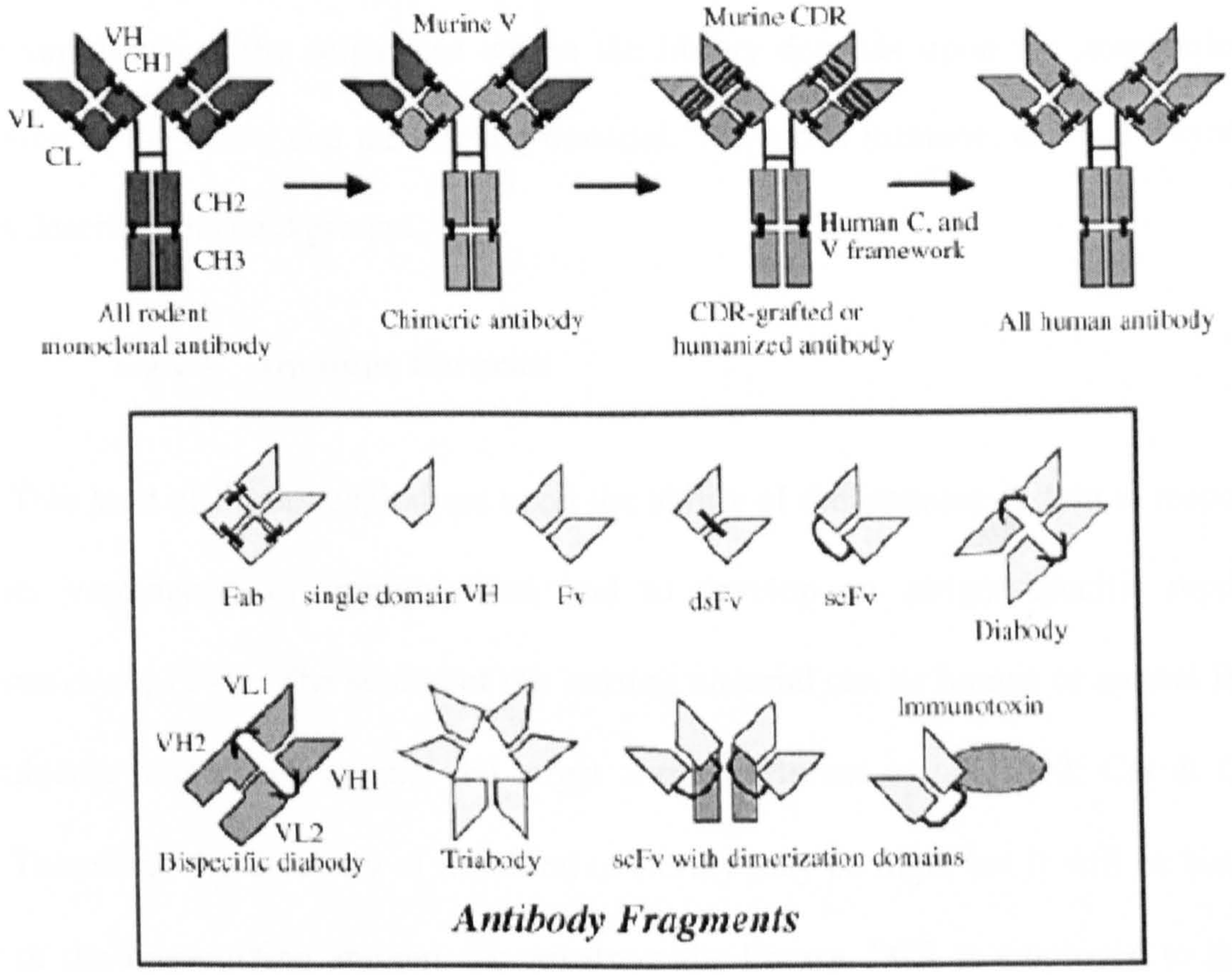

Figure 1.2: The various formats of engineered antibodies

IgG is composed of two identical heavy chains (V_H+CH1+CH2+CH3) and two identical light chains (V_L+V_H). Both chains are organized in domains containing about 110 amino acids with each domain possessing an intra-disulfide bond. Inter-disulfide bonds link the light chain to the heavy chain and the two heavy chains. The variable domains (V_L and V_H) contain the complementary determining regions (CDRs) which bind to the antigen. The Fv fragment V_L and V_H domains possess the binding activity. The scFv corresponds to the V_H linked to V_L by a flexible peptide linker. Dia-, tri- and tetrabodies can be obtained by using short linkers. The figure has been taken from the work of (Maynard & Georgiou, 2000).

1.9.1 Creation of antibody libraries

In antibody phage display, libraries of functional antibodies are displayed at the viral surface as a result of cloning of large numbers of immunoglobulin sequences into a display vector. Antibody genes can be recovered from different species (e.g. human, pig) and the variability of the sequences within the library depends upon the immunological background of the donor and the starting material. The terms immune, naïve and synthetic libraries describe this background.

1.9.1.1 Immune libraries

This kind of library capitalises upon the ability of the immune system to respond to infection, vaccination or immunization and to develop an antigen-specific repertoire (Clackson *et al.*, 1991). The source of the starting material can be human or animal B-cells from subjects immunized against the target antigen (Barbas *et al.*, 1993; Cai & Garen, 1995). Therefore, the diversity of this kind of library may be high, but it will be biased in favour of the immunizing antigen. To construct the library, PCR is employed to amplify immunoglobulin sequences from cDNA prepared from lymphoid cells or tissue (Welschof *et al.*, 1995). Since the immune system has already encountered the target antigen, the size of the library may not need to be large in order to extract antibodies against the material of interest. One obvious advantage of this library is that the antibody repertoire is rich with antigen specific antibodies which are of high affinity (Clackson *et al.*, 1991). However, since this kind of library is prepared from a natural source, it is likely that it will lack the ability to produce antibodies against self antigens

1.9.1.2 Non-immune libraries

This library is also known as a naïve library. The same source of starting material is used for cDNA isolation as the previously discussed library but the B-cells do come not from an immunized donor. B-cells may be pre-selected for expression of IgM reflecting

their naïve status. This means that antibodies against wider collection of antigens can be isolated including self antigens (Marks *et al.*, 1991) or toxins (Cardoso *et al.*, 2000; McElhiney *et al.*, 2002). The B-cell that is normally selected is the B-cell that expresses IgM or IgD (Glockshuber *et al.*, 1990; Philippa M. O'Brien, 2002) on its surface which indicates that this cell has not been activated. The V_H and V_L genes can be amplified from cDNA using PCR and randomly combined to form the library. When the affinity of the antibodies isolated from this kind of library is compared to those isolated from immune libraries, they may appear to be of lower affinity. To overcome this problem, larger libraries are created to increase the chance of isolating high affinity antibodies (Griffiths *et al.*, 1993; Marks *et al.*, 1991; Perelson, 1989).

1.9.1.3 Synthetic libraries

Synthetic and non-immune libraries are known as "single pot libraries" because antibodies against any given antigen can be isolated if the libraries are big enough (Nissim *et al.*, 1994). In synthetic libraries, investigators attempt to mimic the immune system by generating diversity after the rearrangement of V, D, and J segments (Winter, 1998). In the course of a natural immune respose, somatic mutations add more diversity to the CDRs (Rajewsky, 1996); in a synthetic library these regions of the antibody seqauences are diversified through molecular biology, *in vitro*.

This library concept was first applied by Hoogenboom (Hoogenboom & Winter, 1992) and Barbas (Barbas *et al.*, 1992) in studies where synthetic diversity was created in the CDRs especially CDR3 and bordering framework regions. This process allowed control of the location and the extent of diversity (e.g. the central loop CDR3 where the most diversity is found naturally) (Barbas *et al.*, 1992). In another synthetic library (Griffiths *et al.*, 1994) greater control was applied to the choice of framework sequences. Some scaffolds may be better suited to the integration of random sequences in the CDRs. In these kinds of libraries, problems linked to the expression of recombinant antibodies in

bacterial systems were also avoided (Knappik *et al.*, 2000) by choosing antibody sequences known to be successfully expressed.

One innovative advance in the development of synthetic libraries was the construction of the Human Combinatorial Library (HuCAL® GOLD). In this library, all six CDRs were diversified upon master frameworks generated from consensus sequences representing each of the Kabat V-gene subclasses. This library has a high percentage of functional antibody genes and contained more than 10^{10} clones. Further, the framework sequences used were very close to human germline sequences, raising the potential use of these antibodies in human therapy (Hoogenboom, 1997). Antibodies in this library are not displayed in the classical way through phage display. Usually, proteins are genetically fused to the phage surface proteins (see below). Instead, antibodies in the HuCal library were bound to phage by disulfide bond offering a convenient option for phage elution (http://www.morphosys.com).

1.9.2 Formats for antibodies in phage display

Antibodies can be of different forms in phage display i.e. scFv, Fabs, Fvs, single domain antibodies (McCafferty *et al.*, 1990). These forms are illustrated in Figure 1.2. Single domain antibodies have achieved popularity in commercial ventures, such as the library developed by Domantis (Holt *et al.*, 2003; Stockwin & Holmes, 2003). Fv antibodies have been found to dissociate into V_H and V_L components at low proteins concentrations and under physiological conditions. Recombinant Fab antibodies represent the nearest equivalent to natural immunoglobulins and have been very widely used in phage display (Hoogenboom, 1997). The group at the Scripps Institute, in particular, have had great success in creating immune libraries of Fab antibodies for studies of infectious diseases (Bowley *et al.*, 2007; Skerra & Pluckthun, 1988; Zhang *et al.*, 2006).

The most popular format for recombinant antibodies used in phage display is the single chain antibody, scFv. The scFv consists of cloned heavy V_H and light V_L chains, the binding domain, bridged by a peptide linker and is regarded as the minimal structural component of natural antibody required for antigen binding (Huston *et al.*, 1988; Maynard & Georgiou, 2000). It has been found that the linker does not obstruct the antigen binding site or impair domain folding. Also it was found that these small structures were stable after expression and were able to bind to their targets. The scFv sequence is encoded by one reading frame (Sheets *et al.*, 1998). After the cloning of V_H and V_L genes, they are used to build immune, naïve or synthetic libraries. The fusion of the antibody sequence to minor coat protein of a virus ensures that each phage in the library will carry a gene for an antibody and present this protein on its surface. Through use of phagemid vectors, the viral particle also carries the gene for the scFv. Any antibody can then be selected based on its ability to bind to a particular antigen (Vaughan *et al.*, 1996).

Recombinant antibodies constructed in this way can be expressed in *E. coli* (Skerra & Pluckthun, 1988) although there are some reports (Glockshuber *et al.*, 1990) of sub-optimal secretion of functional scFv. High levels of cytoplasmic or periplasmic expression of scFv are common but this can induce aggregation and limits the purification of proteins which might need further renaturation and purification steps. Yeast, plants, and mammalian cells have also been used for expression (Davis *et al.*, 1991; Dorai *et al.*, 1994; Makvandi-Nejad *et al.*, 2005; Tavladoraki *et al.*, 1993) have all been used. These eukaryotic hosts have often proved valuable for high level antibody expression. For library construction, phage display, screening, and initial characterization of recombginant antibodies, *E. coli* remains the most popular host.

1.10 Phage display

Displaying antibody molecules on the surface of bacteriophage particles (McCafferty *et al.*, 1990) is a powerful method for selecting a specific antibody from a

mixed antibody pool such as a library. Each phage particle contains the genes for the displayed antibody and hence attachment of phage to a target molecule through a specific antibody interaction recovers the coding sequence for that antibody from a diverse pool such as a library. This technique has thus been used to isolate antigen specific antibodies from un-immunized human libraries (Marks *et al.*, 1991) and from immunized mouse libraries (Clackson *et al.*, 1991). Antigen specific antibodies were also selected from semi-synthetic combinatorial antibody libraries (Barbas *et al.*, 1992).

The bacteriophage fd is most frequently involved in phage display. This is a filamentous ssDNA phage. The bacteriophage binds to sex pili present at the bacterial surface. This process is mediated by the gIII protein of the virion (Crissman & Smith, 1984; Glaser-Wuttke *et al.*, 1989). Fusion of peptides or folded proteins to the N-terminus of g3p of the phage (McCafferty *et al.*, 1990) ensures display on the surface of phage which then makes possible isolation by successive rounds of selection and enrichment (biopanning) (Parmley & Smith, 1988).

Antibodies displayed at the phage surface can be in the form of single chain Fv fragments, Fab fragments, or as multivalent bispecific or mono-specific constructs as described earlier (Holliger *et al.*, 1993; Marks *et al.*, 1991; McCafferty *et al.*, 1990). Many displayed proteins including antibody fragments, enzymes, hormones or other ligands retain their function and can be isolated on their binding specificity or catalytic functions (Hoogenboom *et al.*, 1993; Kang *et al.*, 1991a; McCafferty *et al.*, 1991).

For the construction of phage libraries, filamentous phage, phagemid vectors and excisable lambda phage have been used successfully (Hogrefe *et al.*, 1993). Phagemid vectors are superior and because they contain the filamentous phage origin of replication and the plasmid origin of replication. This increases the efficiency of transformation and the ease with which DNA can be purified and characterized. Many phagemids are designed to accept DNA inserts such as scFv coding sequences and to form fusions at the 5'

terminus of the gIII reading frame. For packing of the phagemid into phage particles, super-infection with a helper phage is required. This will be discussed in more details in following paragraphs (Hoogenboom *et al.*, 1991).

The phagemid pCANTAB-5 provides a useful illustration of the properties of these vecters. *Sfi*I/*Not*I sites enable protein fusion at the N-terminus of gIII. The translation products are directed to the periplasm by the synthetic leader sequence. One important development illustrated in pCANTAB-5 is the presence of an amber codon between the insert and the gIII seqeuence. This allows the expression of the fusion protein in amber suppressing strains (e.g. *E. coli* TG1) or the antibody alone in a soluble form in a non-suppressing *E. coli* such as HB2151 (Beckmann *et al.*, 1998; Harrison *et al.*, 1996; Tang *et al.*, 1995). As vectors like pCANTAB-5 do not carry all the genes required for assembly of a viral particle, transformed bacteria must be super-infected with a helper pahge such as M13K07 helper (Hoogenboom *et al.*, 1991). As phage progeny are extruded from the bacterial host, the phagemid is packaged into the virus and a mixture of wild type and antibody-gIII proteins complete assembly.

One further development in pCANTAB-5 was the insertion of c-myc and histidine-tags. This allows detection of soluble antibody with anti-c-myc monoclonal antibody. His-tag can be used for purification by affinity chromatography (McCafferty *et al.*, 1994; Munro & Pelham, 1986).

The small size, stability and convienient expression of scFv have encouraged widespread popularity for phage display (Glockshuber *et al.*, 1990). Two genetic arrangements are possible, V_H-linker-V_L or V_L-linker-V_H. The first orientation is the most common. If the linker is shortened, V_L-V_H constructs can be useful (George *et al.*, 1995), but if the linker length is reduced too far, the molecule is forced to dimerize or multimerize. Here, the V_H domain of one scFv is forced to pair with V_L domain of a different scFv molecule. This finding has been investigated as a function of linker length

(Desplancq *et al.*, 1994; Whitlow *et al.*, 1994). Since several sites often remain functional in such oligomers, some avidity effects can be observed, although this can be unpredictable and dependent on the primary sequence of the antibody (Desplancq *et al.*, 1994; Whitlow *et al.*, 1994). The miniantibody format (Pack *et al.*, 1993; Pack *et al.*, 1995) may be a more general solution to this problem. While these molecules have minimal oligomerization domains, many alternative fusions are possible that lead to dimers or multimers. This phenomenon of scFv association has also been utilized for constructing heterodimers, proteins that have been termed diabodies (Holliger *et al.*, 1993).

The diabody concept has quite a long history. The linkage of two antibody Fab fragments through disulphide bonds was described more than 40 years ago (Nisonoff & Mandy, 1962). Bispecific IgG have been developed, secreted from a 'hybrid-hybridoma' or 'quadroma' when two antibody-producing hybridoma lines are fused together (Milstein & Cuello, 1983). IgG-heterodimers are equivalent constructs in which two IgG antibodies are disulphide bonded together using a lysyl-reactive cross-linker (Segal *et al.*, 1986). The recent form of diabodies are scFv-scFv structures (Figure 1.2). The antigen binding sites are arranged in opposite directions, enabling them to cross-link two cells as confirmed by crystallography (Perisic *et al.*, 1994). These molecules were first introduced for enhanced killing of tumor cells in which one binding site of the antibody bound to a tumor cell antigen while the other binding site bound cytotoxic immune effector cells. Diabodies have also been labeled with chemotherapeutic drugs or radioisotopes through binding to one site of the diabody while the other site bound a specific cell antigen (Holliger *et al.*, 1996; Kikuchi *et al.*, 2005; Maynard & Georgiou, 2000; Schlenzka *et al.*, 2004). As these molecules are of 60 to 100 kDa, they have faster clearance rates when compared to the complete Ig molecules (Hudson & Kortt, 1999) but also better tissue penetration. Several antibodies for cancer therapy are already FDA approved (Farah *et al.*, 1998).

When the peptide linker length between V_H and V_L is decreased to two residues or less, it is possible to force the molecule to form triabodies or tetrabodies (tandem diabodies) which increase the avidity of antibody. One study has reported that tandem antibodies are more potent than the diabody for inducing T-cell-dependent killing of tumor cells which reflects the potency of recognition and binding (Kipriyanov *et al.*, 1999). These multimers may be monospecific or carry multiple specificities (Hudson & Kortt, 1999).

Among the large and small antibody molecules that can be expressed using phage display technology, scFv antibody has the advantage because of its distinctive physiological properties. scFv has a low retention time in non-targeted tissues, has rapid blood clearance and better tumor penetration (Nieroda *et al.*, 1995; Yokota *et al.*, 1992). Moreover, scFv can be expressed as a soluble protein with correct folding in the bacterial periplasm (Pugsley, 1993) providing a better chance for high volume expression (Kipriyanov *et al.*, 1997).

One study has reported the production of scFv from a human source against pertussis toxin. The patients were confirmed as seropositive. The starting material for the library was peripheral B lymphocytes from patients infected with *B. pertussis* and showing an antibody immune response. This study successfully produced four neutralizing scFv antibodies against the toxin. The author suggested that such antibodies could contribute to the treatment of pertussis especially in neonates who have high mortality and morbidity due to increasing antibiotic resistance (Williamson & Matthews, 1999).

Another study has reported the neutralization of BoNT/A neurotoxin of *C. botulinum*. This study employed oligoclonal antibodies consisting of three scFvs since individual monoclonal antibodies failed to neutralize the toxin *in vitro*. However, when three monoclonal antibodies were used together, a synergistic effect was observed and complete neutralization of the toxin was achieved in *vitro*. Animals treated with the scFvs were protected when challenged with high levels of toxin (Nowakowski *et al.*, 2002).

Deng *et. al.* (Deng *et al.*, 2003) have successfully combined the recombinant antibody cloning and phage display technology to produce scFv against *C. difficile* Toxin B for diagnostic purposes. This study utilized a mouse B-cell hybridoma line producing monoclonal antibodies against the toxin. RNA was isolated from the cells and from this, scFv was constructed. The recombinant antibody was able to detect Toxin B in a highly specific manner. The author suggested that this study will serve as a base for further investigations.

1.10.1 Selection of Phage antibody

Selection is the method used to isolate phage clones that have binding properties for a target from those which can not interact specifically. Several methods can be used for selection but the most common involves two steps: panning and screening. In panning, target antigens are adsorbed onto plastic, virus are added and those phage that fail to bind are removed with washing (Clackson *et al.*, 1991; Kang *et al.*, 1991b; Marks *et al.*, 1992). Despite its simplicity, there are some disadvantages of this method; purified antigens are required and high affinity antibodies are difficult to rescue because of the avidity effect. In alternative formats, the antigen can be biotinylated, phage and antigen interact in suspension and streptavidin-coated magnetic beads are used for selective capture of antigens with attached virus (Hawkins *et al.*, 1992). Other methods include bio-panning on solid supports, columns or BIA-core sensorchips to which antigen has been attached (Hawkins *et al.*, 1992), selection on fixed prokaryotic cells, eukaryotic or even tissues (Bradbury *et al.*, 1993; Parsons *et al.*, 1996; Van Ewijk *et al.*, 1997).

After incubating the phage libraries on target antigens and washing, antibodies are eluted. There are different elution methods. Several investigators (Kang *et al.*, 1991a; Roberts *et al.*, 1992) have used a single aliquot or gradients of acidic buffers to disrupt the antibody antigen interaction. Others (Griffiths *et al.*, 1993) have used chaotropic agents like dithiothreitol when biotin is linked to the target antigen by a disulphide bridge or basic

solutions such as triethylamine (Marks *et al.*, 1992). Others (Ward *et al.*, 1996) used enzymatic cleavage at an engineered site located between the antibody and gene III. Competition with high antigen concentration has also been reported as an elution method (Clackson *et al.*, 1991).

The most important factors that can affect the efficiency of panning are washing frequency, the stringency of washing and the concentration of the immobilized antigens. Low affinity antibodies cannot withstand aggressive washing therefore, it is very important to use limited number of washing cycles. On the other hand, high affinity antibodies may be best isolated after aggressive washing. These principles also apply to the immobilized antigen concentration, where a high concentration of coated antigen may favor isolation of low affinity antibodies and low concentration may help isolate high affinity antibodies.

Two to five rounds of selection are usually adequate for recovery of phage that carry specific antibody. However, the degree of enrichment of the antibodies after each round of selection is an important factor which decides how many rounds further to be taken. Normally, 5 to 1000 fold enrichment after each round of selection is expected (Griffiths *et al.*, 1993; Marks *et al.*, 1991).

1.10.2 Screening of phage antibodies

Since the selection process works on the principle of enrichment, it is predicted that it will recover a mixture of phage variants with differing properties. To identify the clones of interest, large numbers of clones are screened. The most commonly screening method is an ELISA based assay. This format of assay is fast, specific and it is suited to high throughput (Marks *et al.*, 1991), although other methods such as flow-cytometry have been described (Zaccolo *et al.*, 1997).

The control of the number of antibody fusions displayed at the phage surface has important impact on screening. Systems that favour monovalent display allow stringent

screening for variant phage antibodies with affinity for antigen as multiple display benefits from avidity effects. Phagemid systems designed with an amber codon between the antibody and gIII sequences (Hoogenboom *et al.*, 1991; Lowman *et al.*, 1991) can be convenient for analysis of isolated antibody fragments, and attached (e.g. c-myc or/and his-tag) tag sequences can be used for detection or purification (Lah *et al.*, 1994; Marks *et al.*, 1991).

An important part of the screening process is DNA sequencing after the identification of clones of interest (Marks *et al.*, 1991). This allows checks for defects on the genetic level and precise analysis of the similarity between clones that emerge from screening.

1.10.3 Antibody Expression

Once clones with useful properties have been identified, they must be expressed on a larger scale and to higher yield to allow further characterization. High protein expression of antibodies with the correct folding and function can be achieved if appropriate conditions are used (Better *et al.*, 1988; Pluckthun, 1994; Skerra & Pluckthun, 1988). Many approaches have been taken to meet these goals. The first approach has been to express soluble antibodies in the periplasm of *E. coli*. This can lead to successful expression of recombinant antibodies through the activity of periplasmic disulfide-forming proteins DsbA, DsbB, and DsbC. The second approach is the production of soluble recombinant antibodies which accumulate in the periplasm or culture medium through the use of specialized vectors with strong promoters (Colcher *et al.*, 1990; Gibbs *et al.*, 1991). The third approach is to induced the production of cytoplasmic inclusion bodies (George *et al.*, 1995; Knappik & Pluckthun, 1995) The yield of recombinant antibody can also depend to a significant degree upon the *E. coli* host (Strachan *et al.*, 2002).

1.10.4 Affinity maturation

In vivo, immunoglobulins undergo affinity maturation to elevate their affinity towards antigens. In this process, antibody sequences are diversified, resulting in the creation of variants of the original primary immunoglobulin that may show better recognition (affinity) of the target. Improvement arises from changes to critical amino acids in the CDRs. This process is known as affinity maturation (Adams & Schier, 1999).

Selection of antibodies from recombinant libraries by phage display usually recovers a mixture of antibodies of low and high affinities. If the affinity of antibodies selected in this way requires improvement, this can be achieved by generating a secondary phage library in a process that imitates the immune system. There are different approaches. One of the most popular is to modify the V-gene by mutagenesis, introducing substitutions at random throughout the sequence (Hawkins *et al.*, 1993). Variants generated in this way can be screened by phage display using a low antigen concentrations to isolate antibodies with affinities higher than that of the original immunoglobulin (Hawkins *et al.*, 1992). Thus the power of phage display can be applied to libraries of antibodies that contain many potential specificities, and equally to variants of an antibody in which the specificity is the same.

1.11 Aims

The first aim of this study was to isolate and characterize the carboxy-terminal region of Toxin A. The goals were to express the carboxy-terminal region as a recombinant protein in *E. coli*, to purify the material and to characterize the protein for reaction with antibodies raised against the native toxin and haemagglutinating activity *in vitro*. After isolating and characterizing the recombinant fragment, the purified toxin fragment would be used as substrate for recombinant antibody isolation by phage display.

The second aim was to isolate recombinant antibodies. It was proposed to the Tomlinson I and J scFv libraries against purified protein and to monitoring recoveries of phage through successive rounds of panning.

The third aim was to characterize the biological properties of these antibodies. Goals were to overexpress and purifiy the selected scFv antibodies, to characterize the ability of purified scFv antibodies to bind to their target antigen by ELISA and Western blotting and to determine if the scFv antibodies recognised common or unique epitopes.

The last aim was to seek antibodies with toxin neutralizing activity and evaluate their immunotherapeutic potential. This would involve testing if the recombinant antibodies possessed the ability to neutralize Toxin A using an *in vitro* cell cuture system.

Chapter 2

Materials and methods

2 Materials and methods

2.1 Bacterial strain

The plasmid pET767 was a generous gift of Dr. N. Fairwealter, Imperial College, London. The construct carried an insert of 1kb encoding 14 of the peptide repeat motifs present in the carboxy-terminal domain of Toxin A. The insert was cloned into pET28a (Novagen), placing expression of the insert under the control of the T7 promoter. DNA was transformed into *E. coli* DH5α for propagation and analysis.

2.2 Confirmation of insert encoding Toxin A C-terminal region

To confirm the presence of sequence encoding the terminal carboxy region of Toxin A, a fresh single colony of *E. coli* pET767 was inoculated to 5 ml of LB liquid medium (composition in Appendix) containing 50 μg/ml kanamycin and incubated overnight in a shaking incubator at 37° C. Next day, the culture was centrifuged at 4000 x g at 4° C for 10 min. DNA was extracted using a miniprep kit (QIA prep-miniprep, QIAGEN, UK.) following the recommended protocol and DNA then stored at 4° C. Purified plasmid DNA was then digested using the restriction enzymes *Nco*I and *Xho*I. Fifteen μl reaction mixtures were set up comprising 5 μl of plasmid DNA, 1 μl (10U) *Nco*I (Promega, UK), 1 μl (10U) *Xho*I (Promega, UK), 8.5 μl of distilled water and 1.5 μl of 10 x Buffer D (60 mM Tris-HCl, pH 7.9, 1.5 M NaCl, 60 mM $MgCl_2$ and 10 mM DTT). The reaction mixtures were incubated at 37° C for 1.5 hrs. Each reaction mixture was run on a 1 % agarose gel at 100 volts for 45 minutes and visualized with UV light. Plasmid DNA was sequenced using primers annealing to the T7 promoter T7 terminater and sequences

present in the pET28a vector sequences. Sequencing was carried out at the Sir Henry Wellcome Functional Genomics Facility, University of Galsgow.

2.3 Growth of bacteria and expression of the Toxin A carboxy-terminal region

E. coli pET767 was streaked on LB agar plates (Appendix) containing 50 μg/ml kanamycin (Sigma, UK) and incubated overnight at 37° C. The following day, a single bacterial colony was inoculated to 10 ml LB medium containing kanamycin and incubated in a shaking incubator overnight at 37° C. Next day, a larger volume (1 L) of LB containing 50 μg/ml kanamycin was inoculated with the overnight culture to an OD 600 nm between 0.07 – 0.1 and incubated in a shaking incubator at 37° C. When the OD 600 nm reached 0.9, the growing bacteria were induced by the addition of IPTG (Melford, UK.) to a final concentration of 1 mM and incubated for 19 hrs in a shaking incubator at 30° C. The culture was then centrifuged at 4000 x g at 4° C for 10 min. The supernatant was discarded and the pellet was resuspended in four aliquots each of 15 ml of cold PBS pH 7.4.

Bacterial cells were disrupted by sonication (Jencons Scientific, Inc. USA) for 20 min with 11 % sonication power using alternating of 10 sec cycles of sonication and cooling. The bacterial lysate was centrifuged at 4000 x g at 4° C for 10 min. The supernatant was collected and exchanged with column binding buffer (20 mM phosphate buffer, 0.5 M NaCl, pH7.2) using Amicon Ultra-4 centrifugal filter units with a 10 kDa cut-off (Millipore, UK).

2.4 Purification of the protein by affinity chromatography

A 5 ml HiTrap chelating HP column (Amersham Pharmacia Biotech, UK) was washed with 5 column volumes of distilled water. The column was then charged with 0.5

column volume of 0.1 M nickel sulfate and washed again with 5 column volumes of distilled water. The column was washed with 5 column volumes of binding buffer containing 50 mM imidazole (Sigma, UK) and again with 5 column volumes of unmodified binding buffer. Bacterial lysate in binding buffer was filtered though a 0.2 μm filter (Sartorius, Germany) and immediately loaded to the column. The column was then washed with 5 column volumes of binding buffer containing 20 mM imidazole and then with 5 column volumes of unmodified binding buffer. The column was then washed with 5 column volumes of 0.2 M citric acid-phosphate buffer (Appendix) at pH 6.0 and then with the same buffer at pH 5.0. The recombinant protein was eluted by stripping the column with 5 column volumes of 0.2 M citric acid-phosphate buffer at pH 3.4. Five, 5 ml fractions were collected into an equal volume of 2 x PBS.

2.5 Protein assay

To determine the concentration of the purified recombinant Toxin A, the BCA Protein Assay Kit (Pierce, USA) was used. BSA standards of known concentrations were prepared along with the working reagent. Samples of 0.1 ml each standard and the purified protein were added to 2 ml of the working reagent. Tubes were then incubated at 37° C for 30 min and then brought to room temperature. The colorimetric reaction in each tube was measured at 562 nm using a UNICAM UV/Vis spectrophotometer.

2.6 SDS-PAGE analysis

SDS-PAGE analysis carried out using standard methods (Laemmli, 1970). Polyacrylamide gels were cast with 5 % stacking and 12 % separating layers. Twenty μl of recombinant protein was boiled for 5 min in 10 μl of a 3 x sample buffer (0.24 M Tris-HCl pH 6.8, 6 % SDS, 30 % (v/v) glycerol, 16 % (v/v) β-mercaptoethanol and 0.6 mg/ml Bromophenol Blue). Twenty μl of each boiled sample was loaded to the polyacrylamide gel. Electrophoresis was carried out in 25 mM Tris, 0.2 M glycine buffer containing 0.1 %

(w/v) SDS at 200 Volts for 1 h. The gel was stained with Commassie Brilliant Blue solution (0.2 % stain in 45 : 45 : 10 % methanol : water : acetic acid) for 30 min. The staining solution was then discarded and the gel was destained overnight in 45 : 45 : 10 % methanol : water : acetic acid mix, with agitation.

2.7 Electroblotting

After electrophoresis, proteins were blotted to nitrocellulose membrane following a standard protocol for Western blotting (Sambrook, 1989). Briefly, 4 pieces of Watman filter paper were cut to the same size as the gel and soaked together with 2 fiber pads and a nitrocellulose membrane in transfer buffer for at least 20 min. The nitrocellulose membrane was placed on top of the gel and covered on both sides with two filter papers and one fiber pad. The sandwich was assembled into a blotting cassette and packed along with a frozen insert into a BioRad electrophoresis tank. The tank was filled with transfer buffer and was circulated with a magnetic stirrer. Transfer of proteins was carried out at 100 volts for 1 hr. after that, the membrane was washed 3 times with PBS and stained with Ponceau red dye (Sigma, UK) to demonstrate successful transfer. The membrane was washed 3 times with distilled water to remove the stain. The membrane was probed with goat anti-Toxin A antibody and, after washing, with an HRP conjugate. Finally, the blot was developed using chloronaphthol substrate (15 mg 4-chloronaphthol substrate dissolved in 5 ml methanol added to 25 ml of developing buffer (Appendix) and 15 μl 30 % hydrogen peroxide). The reaction was stopped by washing the membrane with distilled water.

2.8 Recognition in ELISA

To assess the recognition of the recombinant carboxy-terminal protein (hereafter designated recombinant Toxin A) by polyclonal antibody, two rows of an ELISA plate (Nunc, Denmark) were coated with 100 μl of purified protein in PBS, using doubling

dilutions from 20 μg/ml. The plate was incubated overnight at 4° C. Next day, the plate was washed 3 times with PBS. Then each well was blocked by addition of 200 μl of 3 % BSA in PBS and incubation for 2 hrs at 37° C. The plate was then washed 3 times with PBS. Subsequently, each well received 100 μl of goat anti-Toxin A antiserum (List Biological Laboratories, USA). The serum was diluted to 1:500 in blocking buffer and the plate was incubated for 1 h at 37° C. After that, wells were washed 3 times with PBS containing 0.05 % Tween-20 and probed by addition of 100 μl HRP-labeled rabbit anti-goat antibody (1:5000) in blocking buffer and incubation for 1 hr at 37° C. Wells were washed with PBS containing 0.05 % Tween-20 and developed with 100 μl of developing solution (5 mg o-phenylene-diamine (OPD), 15 ml of 0.1 M citri acid-citrate buffer pH 4.5 and 6 μl of 30 % hydrogen peroxide). The reaction was stopped after 10 min by adding 50 μl 1 M sulphuric acid and absorbance was measured at 590 nm using a microplate reader (ELx808 IU, Ultra Microplate Reader, Fisher Scientific, UK.).

2.9 Binding of native and recombinant Toxin A to cellular receptors

To assess the ability of the purified recombinant protein to bind to its receptor, the mouse embryonal carcinoma cell line F9 (European Collection of Cell Cultures, UK.) which possesses a high density of Toxin A cell receptors (Tucker *et al.*, 1990) was grown in DMEM medium (BioWhittaker, Belgium) with 10 % (v/v) fetal bovine serum and 1 mM L-glutamine supplemented with streptomycin-penicillin. Freshly trypsinized F9 cells were sub-cultured by transfer of 5 x 10^3 cells to the wells of 8-well chamber slides (Nunc, Denmark). The cells were allowed to recover for 18 h at 37° C in a 5 % CO_2 atmosphere. The medium was removed and the slides were then allowed to dry at room temperature for 30 min. After that, cells were fixed with 2 % paraformaldehyde in PBS for 20 min at room temperature. Cells were then saturated with 1 % BSA in PBS for 15 min. One hundred μl of 20 μg/ml native Toxin A (Biogenesis, UK) or 100 μl of 20 μg/ml recombinant Toxin A

was added onto the cells and incubated for 1 h at 4° C. After washing with PBS, the bound molecules of toxin were detected by addition of 100 μl of goat anti-Toxin A antiserum (1:400) in PBS and incubated for 1 hr at room temperature. After washing with PBS, 100 μl of FITC-labeled rabbit anti-goat antibody (1:400) was added and incubated as for the primary antibody. The slide was washed briefly 3 times with PBS and each well was mounted with 50 μl immunofluorescence mounting medium (90 % glycerol in phosphate-buffered saline) and inspected using an immunofluorescence microscope (Ziess, Germany) with illumination at 490 nm.

2.10 Haemagglutination activity of recombinant Toxin A

To assess the haemagglutination activity of the recombinant Toxin A, 25 μl of PBS was added to 4 rows of a U-shaped microtitre plate except the first well in each row. Fifty μl of recombinant Toxin A (256 μg/ml) or native Toxin A (51.2 μg/ml) was added to the first well in the row and 25 μl taken for two fold dilutions across the plate. The last four wells received only 50 μl PBS to serve as negative controls. All wells then received 25 μl of 2 % (v/v) washed rabbit erythrocytes and suspended in PBS. The plate was incubated for 2 hrs at 4° C. The end-point was taken as the last dilution of toxin that showed complete haemagglutination. The titer was defined as the reciprocal of that value.

2.11 Sensitivity of F9 and Vero cell lines to native Toxin A

To assess the ability of the native Toxin A to bind to its receptor and exerts its effect, the murine cell line F9 and Vero cells, were grown in DMEM medium with 10 % fetal bovine serum, 1 mM L-glutamine, supplemented with streptomycin-penicillin. Freshly trypsinized cells were sub-cultured into 100 μl of medium in flat bottom 96-well cell culture plates (Nunc, Denmark) at 5×10^3 cells per well. Cells were allowed to recover for 18 h at 37° C in a 5 % CO_2 atmosphere. The plate was then washed two times with complete growth medium. Native Toxin A was added in ten-fold serial dilutions starting

from 100 ng/well to 0.1 ng/well in 100 μl volumes to F9 and Vero cells. The experiment was carried out in duplicate for each cell line. The plate was then incubated for 5 hrs at 37° C in a 5 % CO_2. Cells were inspected by microscopy to find the minimum concentration of toxin able to elicit a cell rounding response.

2.12 Kinetics of action of native Toxin A on F9 and Vero cell

To assess the speed with which native Toxin A acted upon F9 and Vero cells, the cells were cultured as described earlier. After 18 hrs, the cells were washed once with complete medium. Eight wells of each cell line received 100 ng/ml of native Toxin A in 100 μl complete medium and were incubated at 37° C in a 5 % CO_2 atmosphere. Two wells from each cell line were washed after 4 min, 8 min, 16 min and 32 min. The plate was then incubated at 37° C in a 5 % CO_2 atmosphere. Cells were regularly observed over the following 3 hrs.

2.13 Inhibition of native Toxin A with polyclonal anti-Toxin A and recombinant Toxin A *in vitro*

To assess the ability of polyclonal anti-Toxin A antibody or recombinant Toxin A to block the activity of native Toxin A, the murine cell line F9 was sub-cultured in 100 μl volumes as described above. After 18 hrs, the cells were washed once with complete medium. In a separate microtitre plate, 10 ng of native Toxin A was mixed with 50 μl of 200 μg/ml recombinant Toxin A or Polyclonal anti-Toxin A polyclonal antibody diluted to 1:200 in PBS. The plate was then incubated for 30 min at room temperature. The mixtures were then transferred to a cell culture plate containing the growing F9 cells in 150 μl complete medium. The cells were then incubated at 37° C in a 5 % CO_2 atmosphere for 4 hrs. The plate was observed every 25 min.

2.14 Inhibition of native Toxin A with polyclonal Fab fragments

To assess the ability of anti-Toxin A Fab fragment to block the activity of native Toxin A, polyclonal IgG against the toxin antibody was digested with papain to release Fab fragment. Fab fragment was then used for inhibition experiments with native Toxin A *in vitro*.

2.14.1 Digestion of polyclonal anti-Toxin A antibody with papain

Ten μl of goat anti-Toxin A anti-serum was digested with papain 2.5 % v/v (Sigma, USA) in PBS. To start the reaction, a stock solution of L-cysteine (Sigma, USA) was added at 0.13 M to a final concentration of 10 % (v/v) and 5 mM EDTA (USP; Merck Speciality Chemicals) in PBS pH 7.5. The mixture was then incubated at 30° C for 7 hrs in a water bath. The reaction was stopped by the addition of a 0.15 M stock solution of E64 to 10 % (v/v) (Cresswell *et al.*, 2005).

2.14.2 SDS-PAGE gel electrophoresis

SDS-PAGE electrophoresis was carried out as described earlier. Twenty μl of goat anti-Toxin A digested with papain or undigested with the enzyme was boiled in 10 μl of 3 x non-reducing sample buffer for 2 minutes. Ten μl of each boiled sample was loaded to a lane of the polyacrylamide gel. Electrophoresis was carried out in 25 mM Tris, 0.2 M glycine buffer containing 0.1 % (w/v) SDS at 200 Volts for 1 h. The gel was stained and destained as described before.

2.14.3 Electroblotting

After SDS-electrophoresis had finished, proteins were blotted to nitrocellulose membrane following the standard protocol for Western blotting (Sambrook, 1989). The membrane was then probed with rabbit anti-goat IgG-Fab antibody (1:500) and detected

with HRP-labeled goat anti-rabbit antibody (1:500). The membrane was developed until clear bands were seen. The reaction was then stopped by washing the membrane with distilled water.

2.14.4 Detection of native Toxin A by Fab fragment in ELISA

After digesting anti-Toxin A IgG with papain the ability of Fab fragment to recognize native Toxin A was tested in ELISA. Briefly, an ELISA plate was coated with 100 μl/well Toxin A at 20 μg/ml in PBS. After incubation and washing as described before, wells were incubated with 100 μl digested or undigested goat anti-Toxin A polyclonal antibody. After washing, the binding of Fab fragments was detected with 100 μl rabbit anti-goat IgG-Fab fragment (Bethyl Laboratory, USA) at 1:500. After washing, rabbit antibodies were then detected with HRP-labeled goat anti-rabbit antibody (1:1000). The colour reaction was developed as previously described.

2.14.5 Inhibition of native Toxin A with polyclonal Fab fragments *in vitro*

The ability of Fab fragments of the polyclonal anti-Toxin A to block the activity of native Toxin A was tested using the murine cell line F9, grown as described above. After 18 hrs, the cells were washed. In a sterile microtitre plate (Iwaki, Japan), native Toxin A was mixed with the Fab preparation as shown in Table 2.1. The plate was incubated at room temperature for 30 min to allow toxin-antibody recognition. The mixtures were then transferred to a cell culture plate containing the growing F9 cells in 150 μl per well of complete medium. The culture was then incubated at 37° C in a 5 % CO_2 atmosphere for 4 hrs. The plate was observed every 25 min. The number of cells in each well undergoing a rounding reaction was counted every 25 min.

Table 2.1: Inhibition of native Toxin A with polyclonal Fab fragments *in vitro* experimental design:

Rows	50 µl PBS	1.0 µl nTA 10µg/ml	1.0 µl papain 19mg/ml	1.0 µl E64 0.15M	1.0 µl goat serum (1:1)	1.0µl undigested goat anti-Toxin A anti-serum (1:1)	1.3 µl papain digested goat anti-Toxin A anti-serum (1:1)
A	X	X			X		
B	X	X				X	
C	X	X					X
D	X	X		X			
E	X	X	X				

C: inhibition of the toxin with Fab fragments. B: positive control. A, D and E: Negative controls. nTA: native Toxin A.

2.15 Tomlinson scFv libraries

The Tomlinson I and J libraries are one of the most recent phage display resources to be released by the MRC HGMP Resource Center. Each contains over 100 million different scFv fragments incorporated into phagemid vector and transformed into *E.coli* TG1 cells. Phage can be produced from the stocks and used in display experiments to select specific binders to target molecules that are attached to the surface of a tube. This is termed "panning". After each round of panning, those scFv which fail to bind to the target are washed away and the phage that are bound are eluted and amplified by infection into fresh TG1 cells. Phage from the previous round of panning can be used again in further rounds of selection. Two or three rounds of panning are normally required to recover and enrich scFvs with the ability to bind to the target. The monoclonal scFvs can then be screened individually for binding and used for further analysis. Soluble scFv can be purified using an attached histidine tag, and detected with reagents against an attached c-myc epitope.

Library I and J are based on a single human framework for V_H (V3-23/DP-47 and J_H4b) and V_K (O12/ O 2/DPK9 and J_K 1). The structure (V_H: 1-3, V_k: 2-1-1) encoded by this framework is the most common in the human antibody repertoire. The CDR3 of the heavy chain was designed to be as short as possible while retaining the ability to form a binding surface for antigens. Diversity is synthetically incorporated at positions in the antigen binding site that are known to contact antigen in identified structures. Their positions are naturally diverse in the mature human repertoire. This strategy was intended to provide libraries that would be successfully expressed in bacteria.

The repertoire in these libraries was generated by diversification of 18 residues (H50, H52, H52a, H53, H 55, H56, H58, H95, H96, H97, H98, L50, L53, L91, L92, L93, L94 and L96). In each case, more than 1 x 10^8 clones are thought to make up the library with more than 95 % carrying full-length scFv inserts.

The libraries were constructed in the display vector pIT2 (Figure 2.1 and 2.2). This provides c-myc and histidine tags, and an amber stop codon separates the scFv sequence from gIII, the gene for a minor component of the phage coat. The V_H sequence can be excised by digestion with *Nco*I and *Xho*I, *Sal*I and *Not*I remove the V_L sequence.

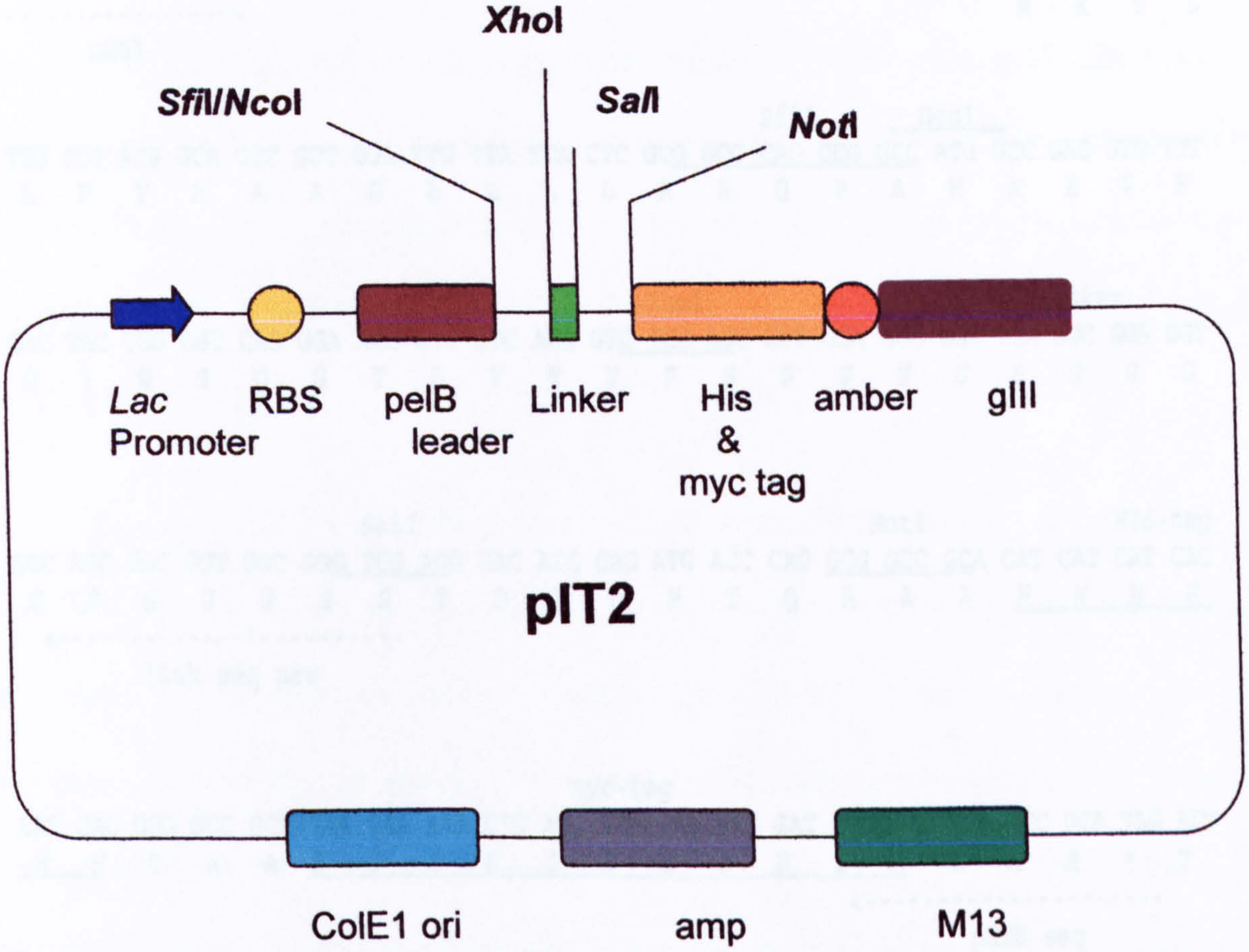

Figure 2.1: Structure of phagemid pIT2

Transcription starts at *lac* promotor and in a suppressor strain of *E. coli*, translated product comprises a PelB leader, the single-chain antibody created by insertion of V_H and V_L sequences at the sites indicated separated by a linker, peptide tags, and the phage pIII protein.

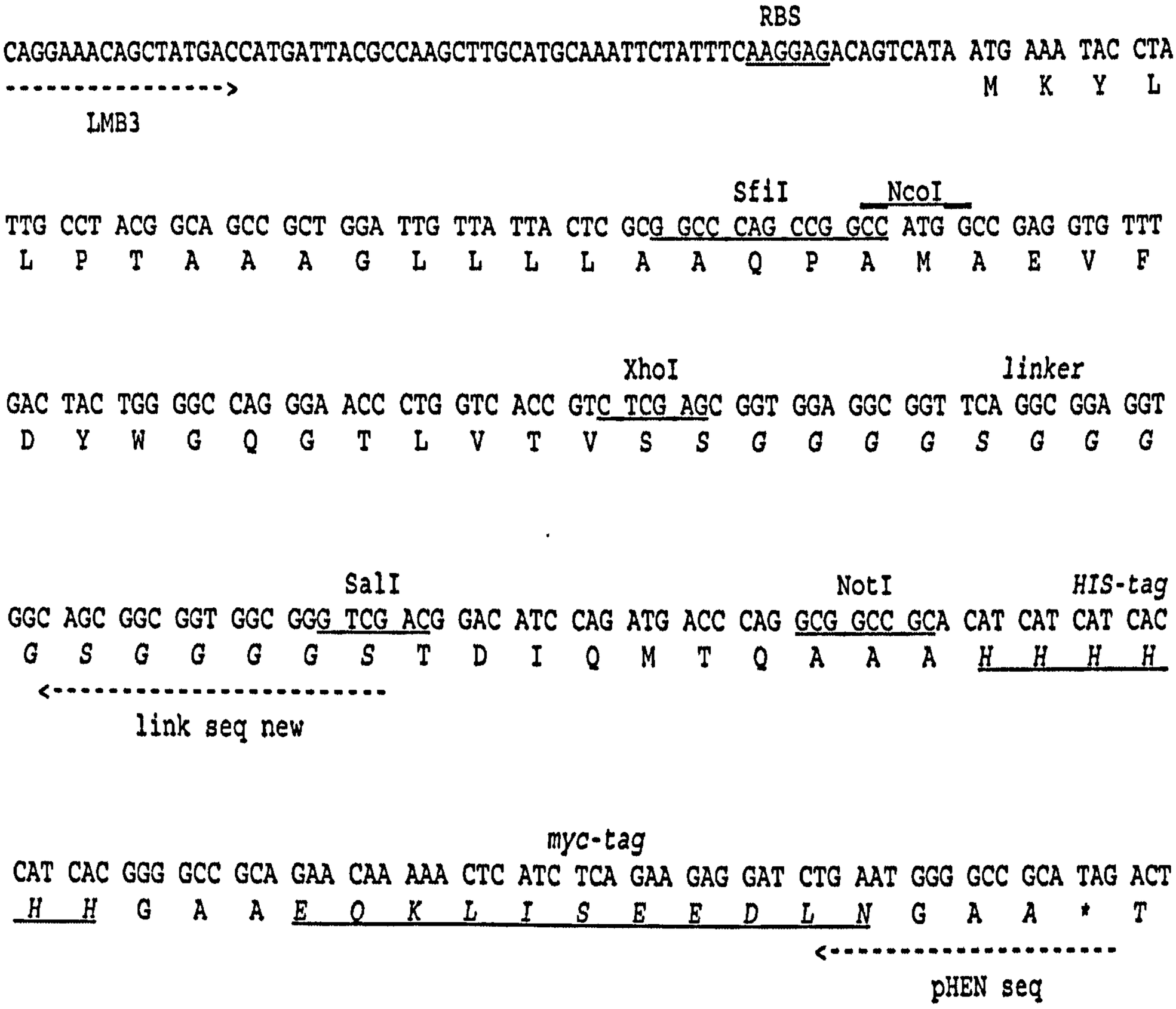

Figure 2.2: DNA sequence of pIT2 vector

Restriction sites are underlined. *Nco* I and *Xho* I sites confines the V_H sequence while *Sal* I and *Not* I sites confines V_L sequence. Dotted arrows indicate primers used for sequencing. The star indicates the stop codon. Histidine and myc tags are underlined.

2.15.1 KM13 helper phage

In order to produce virus for display and selection of scFv, bacterial cells carrying the phagemid must be infected with helper phage. As the viral progeny assemble, the phagemid is packaged into the particle. The capsid is capped with a mixture of pIII species, some derived from the helper phage genome, others from the phagemid. Control of the proportions of this mixture is difficult to achieve, and if only the helper phage-derived protein is incorporated, the specificity of the panning on a target-coated surface may be compromised. This problem has been tackled with the development of modified helper phage such as KM13.

Phage generated using KM13 are used as normal in a panning experiment. Once interaction with target has taken place and the surface has been thoroughly washed, viruses are eluted with trypsin treatment. Those viruses that are bound through the scFv component (potentially in specific interaction with target) are released by proteolytic cleavage between the scFv and pIII moieties (Figure 2.3, I). As pIII remains intact, these viruses can infect *E. coli*, undergo amplification and be used in the next round of selection. In contrast, any phage that lack a scFv-pIII protein (potentially a non-specific interaction with target) carry the pIII variant encoded by KM13. Trypsin treatment will cleave pIII between domains 2 and 3 (Figure 2.3, II) rendering these viruses non-infective. This should exclude them from further participation in the selection procedure.

The pIII protein contains 3 domains D1, D2 and D3. These three domains are all essential for infectivity of the filamentous bacteriophage (Riechmann & Holliger, 1997), but the attachment or insertion of peptide sequences between the domains does not lead to loss of phage infectivity. However, proteolytic cleavage of any part of pIII including the inserted peptides renders the filamentous phage non-infectious. KM13 helper phage was generated (Kristensen & Winter, 1998) to exploit this property.

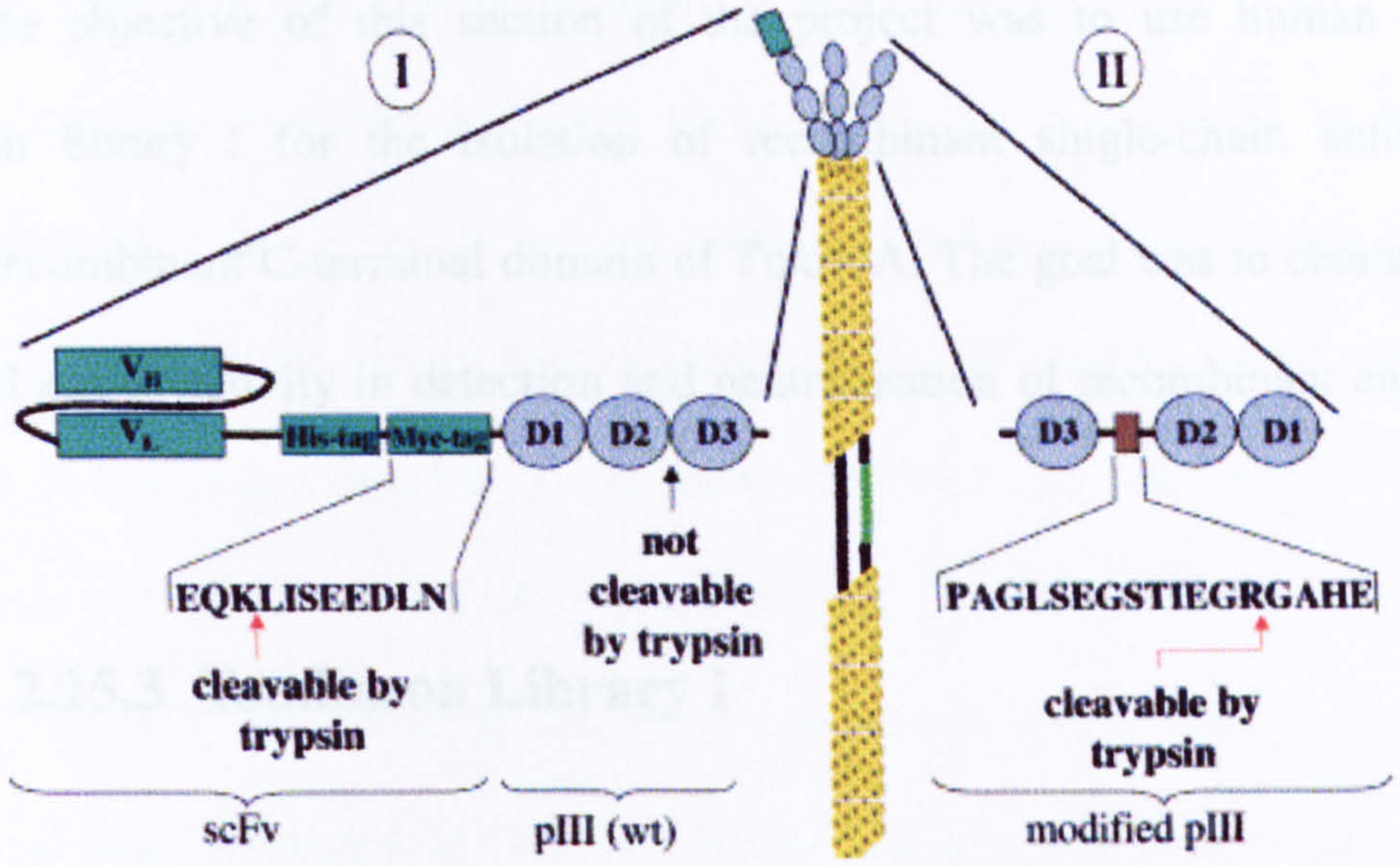

Figure 2.3 : Principle of proteolytic selection (Goletz *et al.*, 2002)

The figure shows a scFv-phage from a phagemid library using KM13 as helper phage During packaging and rescue of a phagemid scFv library, the pIII from the helper phage (II) competes with the phagemid-encoded scFv-pIII fusion protein (I) for incorporation into the phage particles (Goletz et al., 2002). The trypsin-sensitive site was inserted between D2 and D3. Infectivity of the phage was not affected by this insertion while the existence of the three domains was important. After trypsin treatment phage can still be infective because pIII fusion protein (I) is not cleavable (Goletz et al., 2002).

A protease cleavage site was introduced between the D2 and D3 domains to generate a pIII variant that could not mediate bacterial infection after treatment with trypsin. In contrast, the pIII translated from pIT2 and to which the scFv is attached is insensitive to trypsin. Therefore, the ability of this variant to mediate infection to bacteria is preserved.

2.15.2 Objectives

The objective of this section of the project was to use human semi-synthetic Tomlinson library I for the isolation of recombinant single-chain antibodies against purified recombinant C-terminal domain of Toxin A. The goal was to characterize isolated scFvs and assess activity in detection and neutralization of recombinant and native Toxin A.

2.15.3 Tomlinson Library I

Library of Tomlinson I was supplied by the MRC, Center for Protein Engineering, Cambridge, UK. The materials composed of 500 μl of library in *E. coli* TG1, glycerol stocks of positive control scFv against BSA and ubiquitin, a glycerol stock of T-phage resistant *E. coli*, TG1 for proliferation of phage (K12 ara Δ(lac-proAB) supE thi hsdD5/F' $proA^{+}B$ $lacI^{q}$ lacZ ΔM15), a glycerol stock of *E. coli* HB2151 for expression of antibody fragments (K12 ara Δ(lac-proAB) thi/F' $proA^{+}B$ $lacI^{q}$ lacZΔM15) and 100 μl KM13 phage (10^{7} pfu/ml). Materials were stored at -70° C.

2.15.3.1 Propagation of KM13 helper phage

A stock of KM13 helper phage was prepared by inoculating one colony of TG1 from a plate of minimal medium into 5 ml 2xTY broth (1.6 % tryptone, 1% yeast extract and 0.5 % sodium chloride) containing 100 μg/ml ampicillin and 1% glucose. This was incubated overnight at 37° C in a shaking incubator at 200 rpm. Next day, 500 μl from the overnight culture was added into 50 ml of 2xTY and grown until OD 600 nm reached 0.4.

Then 200 μl aliquots of the culture were infected with 10 μl of 10x serial dilutions of KM13 helper phage and incubated at 37° C in a water bath for 30 min. Then the content of each tube was mixed with 3 ml of H-top agar (Tryptone 10g, NaCl 8g, Agar 6g and distilled water up to 1 litre) warmed at 42° C and after mixing, the tubes were poured onto warm TYE agar plates (1.0 % tryptone, 0.5 % yeast extract, 0.8 % NaCl, 1.5 % agar) containing 100 μg/ml ampicillin and 1% glucose. The plates were incubated overnight at 37° C. The following day, a single plaque was inoculated into 5 ml of fresh TG1 at an OD 600 nm of 0.4 and incubated for 2 hrs in a shaking incubator at 37° C. The culture was then added to 500 ml of 2xTY in a large flask and incubated in a shaking incubator at 37° C for 1 hr. Before the culture was incubated overnight at 30° C, kanamycin was added to the culture to a final concentration of 50 μg/ml. the next day, the culture was pelleted by centrifugation at 10800x g for 15 min and 100 ml PEG/NaCl (20 % polyethylene glycol 6000, 2.5 M NaCl) was added to 400 ml of supernatant. The mixture was incubated on ice for 1 hr and then centrifuged at 10800x g for 30 min. The pellet was resuspended in 8 ml of PBS and 2 ml of PEG/NaCl. The mixture was incubated on ice for 20 min after which it was centrifuged at 11600x g for 10 min. To remove any remaining bacterial material, the pellet was redissolved in PBS 500 μl and recentrifuged. The supernatant was then stored at -20° C.

2.15.3.2 Titration of KM13 helper phage stock

A trypsin stock solution was prepared by dissolving the protease (Fluka, UK) in 50 mM Tris-HCl pH 7.4 and 1 mM $CaCl_2$. The final concentration of trypsin was 0.5 mg/500 μl PBS. Forty-five μl of phage was trypsinized by adding 5 μl trypsin stock solution. The mixture was incubated for 30 min at 37° C. An aliquot of 1 μl of the trypsinized phage was diluted into 1 ml of PBS and five serial 100 fold dilution were prepared in 1 ml of PBS. Fifty μl from each dilution was transferred to separate tubes containing 1 ml of TG1 grown

to an OD 600 nm 0.4. Then each tube received 3 ml of molten H-top agar. After mixing, tube contents were poured onto TYE plates.

2.15.3.3 Growing the library

Library stock (500 μl) was added to 200 ml pre-warmed 2xTY containing 100 μl/ml ampicillin and 1 % glucose and incubated in a shaking incubator at 37° C until OD 600 nm reached 0.4. Fifty ml of the culture was then taken and 2 x 10^{11} KM13 helper phage were added. The remaining 150 ml was used to make a secondary bacterial stock of the library for storage at -70° C in glycerol. The infected bacterial culture was incubated without shaking in a water bath for 30 min. The Bacteria were pelleted at 3000x g for 10 min and resuspended in 100 ml of 2xTY containing 100 μg/ml ampicillin, 50 μg/ml kanamycin and 0.1 % glucose. The culture was then incubated in a shaking incubator at 30° C overnight. Next day, the culture was spun at 3000x g for 30 min and 20 ml PEG/NaCl was added to 80 ml of the recovered supernatant. After mixing well, precipitating mixture was left for 1 hr one ice and then pelleted at 3300x g for 30 min. The supernatant was poured out. The pellet was then resuspended in 4 ml PBS and pelleted at 11,600x g for 10 min in a micro centrifuge to remove any debris. The supernatant was then stored at 4° C.

2.15.3.4 Titration of phage libraries

For titration of phage stock, 1 μl of phage was diluted in 100 μl PBS, 1 μl of this in 100 μl PBS and this was repeated to generate 6 serial 100 fold dilutions. Before incubation, *E. coli* TG1 was grown to an OD 600 nm of 0.4, and 900 μl was added to each tube at 37° C in a water bath for 30 min. Ten μl of each dilution was inoculated to a TYE plate containing 100 μg/ml ampicillin and 1 % glucose and spread for single colonies. The culture was incubated at 37° C and the number of colonies was counted the following day.

2.15.3.5 Growing TG1 and HB2151 cells

Secondary stocks of TG1 and HB2151 were generated by inoculating a loopful of each strain from the frozen glycerol suspensions onto minimal agar plates. The plates were incubated overnight at 37° C. A colony from each plate was picked and inoculated into 5 ml of 2xTY for overnight culture in a shaking incubator at 37° C. Next day, the cultures were pelleted at 3300x g for 10 min and the supernatant was discarded. The pellets were then resuspended in 1 ml 2xTY containing 15 % glycerol and stored at -80° C.

2.15.4 Experiments before selection

Before using the Tomlinson I library in selection, some preliminary experiments were performed.

2.15.4.1 Growing positive controls

A positive control scFv was prepared for the selection experiments. *E. coli* TG1 carrying anti-BSA and anti-ubiquitin antibodies were inoculated onto TYE containing 100 μl/ml ampicillin and 1 % glucose and grown overnight at 37° C. Next day, a single colony from each plate was inoculated into 5 ml of 2xTY containing 100 μl/ml ampicillin and 1 % glucose. The cultures were incubated at 37° C and 200 rpm in a shaking incubator. Plasmid DNA was extracted and 2 μl of each DNA sample was transformed into 100 μl of competent *E. coli* HB2151 (Sambrook, 1989). Cells were plated onto TYE plates containing 100 μl/ml ampicillin and 1 % glucose and incubated overnight at 37° C.

2.15.4.2 Production of soluble positive control

Single colonies of HB2151 containing anti-BSA and anti-ubiquitin scFvs were inoculated into 5 ml of 2xTY containing 100 μl/ml ampicillin and 1 % glucose and incubated overnight at 37° C in a shaking incubator at 200 rpm. One ml of each culture was inoculated into 100 ml of 2xTY containing 100 μg/ml ampicillin and 0.1 % glucose.

The cultures were incubated in a shaking incubator at 200 rpm and 37° C until OD 600 nm reached 0.9. IPTG was then added to a final concentration of 1 mM. The cultures were then transferred to a shaking incubator at 200 rpm and 37° C for 20 hrs. Next day, the cultures were centrifuged at 3000x g for 10 min. The supernatants were then concentrated about 20 times and buffer was exchanged to PBS using Amicon Ultra-4 Centrifugal Filter Units with a 10 kDa cut-off. The concentrated soluble scFvs were stored at -20° C.

2.15.4.3 Detection of anti-BSA and anti-ubiquitin scFvs by Western blot

To optimize the detection method for expressed scFv, concentrated culture supernatant were separated by SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane using a standard protocol for 1 hr at 200 Volts. The protocol is detailed earilier. The membrane was blocked with 3 % MPBS for 1 hr at room temperature. Monoclonal anti-c-myc antibody was then added onto the membrane at 1:3000 dilution in a 2 % solution of MPBS. Blots were probed for 90 min at 37° C and shaking at 150 rpm. The secondary antibody (anti-mouse IgG HRP conjugate [sigma]) was added at 1:1000 dilution in 2 % MPBS and incubated at 37° C with shaking at 150 rpm for 1 hr. After washing, the blot was developed with 4-chloro-naphthol substrate.

2.16 Antibody selection

2.16.1 Panning library I against purified recombinant C-terminal domain of Toxin A

2.16.1.1 First round of selection

An immunotube was coated with 4 ml of 50 μg/ml recombinant C-terminal domain from Toxin A and incubated overnight at room temperature. The next day, the tube was washed 3 times with PBS by pouring PBS in and out of the tube. The tube was then filled

to the top with 2 % MPBS and incubated vertically at room temperature for 2 hrs. The tube was washed 3 times with PBS. After that, approximately 10^{12} cfu of phage from library I were added to 4 ml of 2 % MPBS. To avoid the selection of phages against milk proteins, the suspension was incubated at room temperature for 30 min. The suspension was added to the coated immunotube and incubated for 1 hr at room temperature with continuous rotation. Then the tube was allowed to stand for a further 1 hr at room temperature. After that, the supernatant was discarded and the tube was washed 10 times with PBS containing 0.1 % Tween-20. The excess PBS was shaken out and the phages were eluted by adding 500 μl of trypsin-PBS solution and rotating for 10 min at room temperature.

Infecting TG1 cell with eluted phage antibodies

From the 500 μl eluate, 250 μl was used to infect *E. coli* TG1 grown to OD 600 nm of 0.4. The bacteria were prepared from growth on minimal medium, as described earlier. The infected culture was incubated at 37° C in a water bath for 30 min, and 10 μl samples were inoculated to TYE plates containing 100 μg/ml ampicillin and 1 % glucose. Aliquots were sampled directly from the infected culture or prepared at dilutions of 10^2 and 10^4. Plates were incubated overnight at 30° C and colonies counted to calculate the number of output phage for the first round of selection. The remaining 250 μl of phage were stored at 4° C for future use. Titration plates were stored at 4° C for future use.

The remaining culture of the infected TG1 bacteria was centrifuged at 11,600x g for 5 min, the pellet was resuspended in 50 μl of 2xTY and plated onto a TYE plate containing 100 μg/ml ampicillin and 1 % glucose. The plate was incubated overnight at 37° C. The growing colonies were then resuspended in 2 ml of 15 % glycerol and stored in 1 ml aliquots at -70° C. Fifty μl of the bacterial suspension was used in phage rescue for the next round of selection.

Rescue of selected phage antibodies

A 50 μl aliquot of the resuspended bacteria from the first round of selection was inoculated to 50 ml of 2xTY containing 100 μg/ml ampicillin and 1 % glucose. The culture was incubated at 37° C in a shaking incubator at 200 rpm. When the OD 600 nm reached 0.4, KM13 helper phage were added at a concentration of 5 x 10^{10} phage to 10 ml of culture. The mixture was incubated in a water bath for 30 min, and then pelleted at 3000x g for 10 min. The bacterial pellet was resuspended in 50 ml of 2xTY containing 100 μg/ml ampicillin, 50 μg/ml kanamycin and 0.1 % glucose then incubated overnight in a shaking incubator at 200 rpm and 30° C. Next day, the bacterial culture was centrifuged at 3300x g for 15 min. To precipitate the phage from the supernatant, 10 ml PEG/NaCl was added to 40 ml supernatant. After mixing well, the mixture was left for 1 hr on ice. The mixture was then centrifuged at 3300x g for 30 min, and the supernatant was then discarded. The precipitated phage were resuspended in 2 ml PBS. To remove the remaining bacterial debris, the suspension was pelleted at 11600x g for 10 min. One ml of the rescued phage supernatant was stored at 4° C and the remaining 1 ml was used for the next round of selection.

Titration of rescued phage

To titrate the phage amplified from the first round of selection, a series of 6, 100-fold dilutions were prepared and 100 μl samples were mixed with 900 μl of TG1 at an OD 600 nm of 0.4. Mixtures were incubated at 37° C for 30 min. Ten μl samples from each dilution were spotted onto a TYE plate containing 100 μg/ml ampicillin and 1 % glucose and incubated overnight at 30° C. Colonies were then counted to estimate the number of phage in the amplified stock.

2.16.1.2 Second round of selection

The approach of the second and third rounds of selections was based on the gradual reduction in the concentration of recombinant Toxin A coated to the immunotube in each round (Shadidi & Sioud, 2001). This approach has led to isolation of more specific antibodies with higher affinity. An immunotube was coated with protein at 50 μg/ml in PBS and blocked as for the first round of selection. The input phage (10^{12} cfu) in 2 % MPBS were added and incubated as before. The tube was then washed 20 times with PBS containing 0.1 % Tween-20. The steps of the second round of selection including elution of phage, infection of TG1 and recovery of phage were performed as described for the first round of selection.

2.16.1.3 Third round of selection

All stages of the third round of selection were carried out in the same way as for rounds one and two except that recombinant C-terminal domain of Toxin A was coated to the immunotube at a lower concentration (10 μg/ml).

2.16.2 Screening phage by ELISA

In phage display, each round of selection recovers a polyclonal population of phage particles. These can be checked for their overall recognition of target by polyclonal phage ELISA. Alternatively, phage can be rescued after each round of selection from single bacterial colonies and amplified virus can be tested for recognition of target (monoclonal phage ELISA).

2.16.2.1 Screening phage particles by ELISA (polyclonal ELISA)

Phage populations recovered from each round of selection were screened for binding to the C-terminal domain of Toxin A by ELISA. Two rows of an ELISA plate (Nunc) were divided for coating with either recombinant Toxin A or MPBS to check for

recovery of phage against milk proteins. Two wells coated with 20 μg/ml of purified recombinant Toxin A in 100 μl PBS and 2 wells coated with 20 μg/ml skimmed milk in PBS, were coated designated for each round of selection.

The plate was then incubated overnight at 4° C. The next day, the wells were washed 3 times with PBS and blocked with 3 % BSA in PBS for 2 hrs at room temperature. Wells were then washed 3 times with PBS. Ten μl of PEG precipitated phage from each round of selection were mixed with 100 μl 3 % BSA in PBS and added to the wells allocated for each round. The plate was incubated for 1 hr at room temperature after which the plate was washed 3 times with PBS containing 0.1 % Tween-20. Wells were then probed with a 1:5000 dilution of HRP-labeled anti-M13 (Amersham) in 3 % BSA in PBS and incubated for 1 hr at room temperature. After three washes with PBS containing 0.1 % Tween-20, the assay was developed by addition 100 μl of developing solution (5 mg OPD, 15 ml 0.1 M trisodium-citrate buffer, pH 4.5 and 6 μl of 30 % H_2O_2) to each well. The reaction was stopped after 10 min by adding 50 μl 1 M sulfuric acid and the absorbance was measured at 490 nm.

2.16.2.2 Screening phage particles by ELISA (monoclonal ELISA)

Monoclonal Phage ELISA

Sixteen individual colonies from the titration plate for round 1 and 40 colonies from plates prepared after rounds 2 and 3 were inoculated into 100 μl of 2xTY containing 100 μg/ml ampicillin and 1 % glucose in a 96 well culture plate (Nunc, Denmark). The plate was incubated overnight at 37° C in a shaking incubator. The following day, 2 μl was transferred from each well to another 96 well culture plate containing 200 μl of 2xTY with 100 μg/ml ampicillin and 1 % glucose per well. The plate was grown in a shaking incubator at 250 rpm and 37° C for 2 hrs. Then 25 μl 2xTY containing 100 μg/ml ampicillin, 1 % glucose and 10^9 helper phage was added to each well. Shaking was

continued at 37° C for 1 more hour. After that, the plate was spun at 1,800x g for 10 min, and supernatant was aspirated off all the wells. Pellets were then resuspended in 200 μl 2xTY containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. The plate was then incubated overnight at 30° C in a shaking incubator at 250 rpm. The following day, the plate was spun again at 1,800x g for 10 min. Fifty μl of the supernatant from each well was used in phage ELISA.

Each well in an ELISA plate was coated with 100 μl of 20 μg/ml of purified recombinant Toxin A in PBS. The plate was incubated in 4° C overnight. Next day, the plate was washed 3 times with PBS. Then each well was blocked with 200 μl of 3 % BSA in PBS and the plate was incubated for 2 hrs at room temperature. The plate was washed 3 times with PBS. After that, 50 μl of monoclonal phage stock was added to each well, followed by 50 μl of 6 % BSA in PBS. The plate was incubated for 90 min at room temperature. After the plate was washed 3 times with PBS, each well was probed with monoclonal anti-M13 antibody (1:5000) in the blocking buffer and plate was incubated at room temperature for 90 min. After washing with PBS containing 0.1 % Tween-20, each well was probed with HRP-labeled anti-mouse antibody (1:5000) in blocking buffer and plate was incubated at room temperature for 90 min. After washing with PBS containing 0.1 % Tween-20 each well was developed with 100 μl of developing solution. The reaction was stopped after 10 min by adding 50 μl 1 M sulfuric acid and light absorbance was measured at 490 nm.

2.17 Production of soluble antibody fragments

In *E. coli* TG1, the stop codon between scFv and gIII reading frames is suppressed and scFv is fused to pIII. Transfer of the phagemid to *E. coli* HB2151 (a non-suppressor strain) allows expression of soluble scFv for testing in ELISA experiments.

Ten μl of phage eluted from each round of selection was added to 200 μl HB2151 grown to an OD 600 nm of 0.4 and the mixture was incubated for 30 min at 37° C in a water bath. Serial, 100-fold dilutions were prepared and 50 μl of each were plated on TYE plates containing 100 μg/ml ampicillin and 1 % glucose and incubated at 37° C overnight. Then 18, 40 and 40 single colonies were picked from rounds 1, 2 and 3 respectively and inoculated into individual wells in a 96 well culture plate, each well containing 100 μl 2xTY with 100 μg/ml ampicillin and 1 % glucose and incubated at 37° C for overnight in a shaking incubator (250 rpm). The following day, 2 μl was transferred from each well to another 96 well culture plate containing 200 μl of 2xTY with 100 μg/ml ampicillin and 0.1 % glucose per well. The plate was grown in a shaking incubator (250 rpm) at 37° C until OD 600 nm was approximately 0.9. Then 25 μl of 2xTY containing 100 μg/ml ampicillin and 9.0 mM IPTG was added to give a final IPTG concentration of 1 mM. The plate was then incubated overnight at 30° C in a shaking incubator at 250 rpm. Then next day, the plate was spun at 1,800x g for 10 min. Fifty μl of the supernatant was used for ELISA.

The wells of an ELISA plate were coated with 20 μg/ml of purified recombinant Toxin A in 100 μl PBS and incubated at 4° C overnight. The following day, the plate was washed 3 times with PBS. Each well was then blocked with 200 μl 3 % of BSA in PBS and for 2 hrs at room temperature. Subsequently, the plate was washed 3 times with PBS. After that, each well received 50 μl of monoclonal scFv antibody and 50 μl of 6 % BSA in PBS. The plate was then processed as described previously for monoclonal phage ELISA. Rabbit anti-c-myc antibody (1:100) was used for probing and HRP-labeled goat anti-rabbit antibody (1:1000) was used for detection. The color reaction was developed as previously described and the final absorbance of each well was measured at 490 nm on an ELISA plate reader.

2.18 Genetic characterization of selected scFvs

Before further steps were taken to characterize the selected scFv, sequencing was used to verify the size and integrity of the inserts and their diversity. Twenty selected clones in *E. coli* HB2151 were chosen from rounds 2 and 3 on the basis of the strength of signal in ELISA with soluble scFv.

Plasmid DNA was purified by growing each clone in 5 ml of 2xTY containing 100 μg/ml ampicillin and 1.0 % glucose. The cultures were incubated overnight in a shaking incubator 200 rpm at 37° C. Plasmid DNA was extracted using miniprep reagents.

2.18.1 Plasmid digestion

Plasmid DNA was purified from the 20 clones and digested with restriction enzymes. In each digest, 3 μl of DNA was mixed with 1 μl of *Nco*I (Promega, UK.), 1 μl of *Not*I (Promega, UK.), 1 μl of buffer D (60 mM Tris-HCl (pH 7.9), 1.5 M NaCl, 60 mM $MgCl_2$ and 10 mM DTT) and the volume made up to 10 μl with water. The reactions were incubated at 37° C for 2 hrs. Five μl of each digest was analysed by electrophoresis on a 2 % agarose gel for 45 min at 100 V. The gel was visualized under UV light. SyberSafe (In Vitrogen) was used to stain the DNA fragments.

2.18.2 Sequence of selected clones

To predict amino acid sequence, diversity and integrity of the selected clones, sequencing was performed using the forward pelB and reverse gIII primers (Table 2.4). DNA sequencing was carried out at Sir Henry Wellcome Functional Genomics Facility, University of Galsgow. Big Dye (Applied Biosystems) and ET-Dye Terminator (Amersham Bioscience) were used. A MegaBACE 1000 sequencing machine was used to analyze the samples. DNA Star software was used for sequence data analysis. For

alignment, the DNAPLOT database (MRC Center for Protein Engineering, Cambridge, UK) was employed. Amino acid sequence was aligned using MEGALIN software.

2.19 SDS-PAGE and Western blot analysis of selected clones

Selected HB2151 clones were grown in 100 ml 2xTY containing 100 μg/ml ampicillin and 0.1 % glucose to an OD 600 nm of 0.9. Bacterial cultures were then induced by the addition of IPTG to a final concentration of 1 mM and transferred to a shaking incubator at 30°C for 18 to 22 hrs. Next day, the cultures were allowed to cool down on ice for 10 min and were then centrifuged at 3000x g for 10 min at 4°C. The supernatant was then concentrated 30 times with Amicon Ultra-4 centrifugal filters. The concentrate was then exchanged with PBS. Twenty μl of each sample was added to 10 μl SDS-loading buffer and heated in boiling water for 5 min. Sample were loaded to 12 % SDS-polyacrylamide gels. Gels were run at 200 Volts for 1 hr in a BioRad electrophoresis apparatus.

Gels were blotted to 4 nitrocellulose membranes by a standard protocol as outlined earlier. After blocking with 3 % MPBS for 1 hr, the membrane was washed 3 times with PBS and probed with anti-c-myc monoclonal antibody at a dilution of 1:3000 in 3 % MPBS for 1 hr at 37° C in a shaking incubator. The membrane was then washed 3 times with PBS containing 0.05 % Tween-20. The membrane was probed with HRP-labelled rabbit anti-mouse antibody in 3 % MPBS and incubated for 1 hr at 37° C with shaking. The membrane was then washed 3 times with PBS and color was developed with 4-chloro-1-naphthol substrate.

2.20 High level expression and purification of soluble scFv

In order to further study the properties of selected scFv antibodies, these antibodies were expressed in a soluble form and purified. One single HB2151 colony representing

each selected scFv was inoculated to 5 ml of 2xTY containing 100 µg/ml ampicillin and 1 % glucose. The culture was incubated at 37° C in a shaking incubator. The following day, 2 ml of the overnight culture was inoculated to 200 ml 2xTY containing 100 µg/ml ampicillin and 0.1 % glucose. The culture was incubated at 37° C in a shaking incubator at 200 rpm until the OD 600 nm reached 0.9. IPTG was then added to a final concentration of 1 mM. The cultures were then transferred to a shaker at 200 for rpm incubation at 30° C for 20 hrs. After centrifuging at 3000x g for 10 min, culture supernatants were concentrated about 20 times using Amicon Ultra-4 centrifugal filters with a 10 kDa cut-off. The devices were centrifuged at 4000x g at 4° C. The buffer was exchanged with column binding buffer (20 mM phosphate buffer, 0.5 M NaCl, pH 7.2).

2.20.1 Purification of the scFvs by affinity chromatography

A HiTrap Chelating HP column was prepared as described earlier before loading with scFv. Concentrated culture supernatant, exchanged into column binding buffer was filtered though a 0.2 µm filter and 25 ml was immediately loaded to the column. The column was then washed with 5 column volumes of binding buffer, containing 20 mM imidazole and then with 5 column volumes of binding buffer containing 70 mM imidazole. The scFv attached to the column was eluted by striping with binding buffer containing 200 mM imidazole. Five fractions of 5 ml were collected and concentrated 5 times with Ultra-4 centrifugal filters (10 kDa cut-off) at 4° C with centrifugation at 4000x g. The concentrated scFv was then exchanged into PBS at 4° C.

In all, nine selected scFv were prepared in this way, with anti-BSA scFv as a control. All preparations were quantified for total protein control using BCA Assay Kit (Pierce, USA) as described before.

2.21 Biological activity and immunochemical characterization of purified scFv

2.21.1 Recognition of native Toxin A by selected scFv

ELISA was carried out assess whether native Toxin A could be recognized by scFv antibodies isolated by phage display. Rows on an ELISA plate were coated with purified native Toxin A, starting with 100 μl of 20 μg/ml Toxin A and diluted in serial two-fold steps down the plate to generate 7 dilutions in total. The last well in each row received 100 μl of 20 μg/ml BSA in PBS. The plate was then incubated overnight at 4° C. Next day, the plate was washed 3 times with PBS and blocked with 200 μl 2 % MPBS. The plate was incubated for 2 hrs at room temperature. After the plate was washed 3 times with PBS, 100 μl of 10 μg/ml of purified monoclonal anti-Toxin A scFv antibody in blocking buffer was added to wells containing diluted toxin while 100 μl of 10 μg/ml of purified monoclonal anti-BSA scFv antibody was added to wells containing dilutions of BSA. The plate was then incubated at room temperature for 1.5 hrs. The plate was washed 3 times with PBS containing 0.1 % Tween-20 and all wells were probed with 100 μl of a 1:3000 dilution of monoclonal anti-c-myc. The plate was incubated at room temperature for 1 hr. The plate was then washed 3 times with PBS containing 0.1 % Tween-20. Finally, 100 μl of a 1:5000 dilution of HRP-labeled rabbit anti-mouse was added to each well. The plate was then incubated at room temperature for 1 hr and washed 3 times with PBS containing 0.1 % Tween-20. To generate signal, 100 μl of developing solution was added to each well. After 10 min, 50 μl of sulphuric acid was added to each well to stop the reaction and plate was then read with an ELISA reader at 492 nm.

2.21.2 Haemagglutination inhibition assay

It has been shown that rabbit erythrocytes carry a carbohydrate receptor for Toxin A on their surface (Krivan et al., 1986), and thus haemagglutination can be triggered in the

presence of the toxin. An inhibition assay was designed to test whether scFv antibodies isolated against the binding domain of recombinant Toxin A would prevent toxin binding to rabbit erythrocytes, and hence their agglutination.

2.21.2.1 Preparation of Rabbit erythrocytes

Fresh heparinized rabbit blood was obtained from the University animal facility. The blood was centrifuged at top speed in a microcentrifuge for 5 min, and after discarding the supernatant, the pellet was resuspended in PBS. Erythrocytes were washed with PBS until supernatant was clear.

2.21.2.2 Haemagglutination inhibition

Samples of purified scFv at 200 μg/ml were diluted in a two-fold series of 25 μl of PBS across the wells of a round bottom microtiter plate (Iwaki, Japan). Goat anti-Toxin A was prepared in the same way to serve as a positive control and a scFv against BSA was used as a negative control. The last well of each row contained only PBS to serve as a further control. Then 25 μl of native Toxin A at a concentration of 400 ng/ml was added to each well. The plate was incubated at 37° C for 30 min to allow interaction of antibody and toxin. Each well then received 25 μl of 2 % rabbit erythrocytes before incubation at 4° C. Samples were monitored for 2 hrs. After 2 hrs, the highest dilution of antibody able to inhibit haemagglutination was designated as the titre of inhibition.

2.21.3 Rabbit erythrocyte haemolysis assay

As an alternative to haemagglutination, haemolysis was adopted to provide a more sensitive assay for inhibition of the interaction of native Toxin A with rabbit erythrocytes. Samples of 200 μl native Toxin A at 30 μg/ml were diluted in PBS across an ELISA plate and incubated overnight at room temperature. Next day, the plate was washed 3 times with PBS and blocked with 3 % BSA in PBS for 2 hrs at room temperature. The plate was then

washed 3 times with PBS. Rows 1 and 2 of the plate received 200 μl of PBS to serve as the negative control. The first well of rows 3 and 4 received 200 μl of goat anti-Toxin A diluted 1:200 in PBS to serve as positive control. Serial, two-fold dilutions were prepared across the plate. The first well in rows 11 and 12 received 200 μl of 200 μg/ml of purified anti-BSA scFv antibody. Again, this was diluted in two-fold steps across the rows. The first well of the remaining rows received 200 μl of 200 μg/ml of purified scFv antibodies C5, D4, and a mixture (C5, D4 and D5) in duplicate. Two-fold serial dilutions were prepared. The plate was then incubated at 37° C for 1 hr to allow antibody-toxin interaction and washed 3 times with PBS containing 0.1 % Tween-20. Two hundred μl of 3 % rabbit erythrocytes were added to each well and incubated for 2 hrs at 4° C. The plate was then washed 3 times by submersion in a PBS buffer. Captured erythrocytes were haemolysed by adding 100 μl of distilled water to each well, mixing with a pipette and incubation for 30 min to allow complete haemolysis. The plate was read with an ELISA reader at 540 nm.

2.21.4 Indirect immunofluorescent antibody test with fixed cells

Murine F9 cells were used to assess the ability of purified scFvs antibodies to inhibit the binding of native Toxin A to its receptor. Cells were grown in DMEM medium (BioWhittaker, Belgium) with 10 % fetal bovine serum (BioWhittaker, Belgium) and 1 mM L-glutamine, supplemented with streptomycin-penicillin. Freshly trypsinized F9 cells were sub-cultured into 200 μl multi-well cell culture slides (BD Falcon Biosciences, USA) at 5000 cells per well and allowed to grow for 24 h at 37° C in a 5 % CO_2 atmosphere. Wells were then allowed to dry at room temperature for 30 min. After that, cells were fixed with fresh 2 % paraformaldehyde for 20 min at room temperature. Cells were then saturated with 1.0 % BSA in PBS for 15 min. One hundred μl of native Toxin A (50 μg/ml) was mixed with 100 μl (200 μg/ml) samples of 3 different purified anti-Toxin A scFvs. As a positive control, 100 μl of polyclonal goat anti-Toxin A antibody, diluted

1:400, was mixed with toxin. As a negative control, scFv anti-BSA was mixed with toxin. The antibody antigen mixtures were then incubated at 37° C for 1 hr. Then 100 μl of each mixture was added to 2 separate wells on the cell culture slide and incubated for 1 hr at 37° C. The slide was then washed 3 times with 1.0 % BSA in PBS. The slide was fixed with 4 % paraformaldehyde for 20 min at 4° C, washed 3 times with 1.0 % BSA in PBS and probed with 100 μl of diluted polyclonal goat anti-Toxin A antibody (1:400). Slides were incubated at 37° C for 1 hr and washed 3 times with 1.0 % BSA in PBS. Toxin was localized with 100 μl of FITC labeled rabbit anti-goat antibody 1:500 (Sigma). The slide was incubated at room temperature for 1 hr and washed 3 times with 1.0 % BSA in PBS. The slide was then mounted with immunofluorescence mounting medium (90 % glycerol in phosphate-buffered saline) and examined using an immunofluorescence microscope (Ziess, Germany) with illumination at 490 nm.

2.21.5 Indirect immunofluorescent antibody test with cells in suspension

To assess the ability of scFv antibodies to inhibit the binding of native Toxin A to its receptor, murine F9 were grown as before, scraped from the culture dishes, and adjusted to 50,000 cells/ml. Five 1.5 ml eppendorff tubes were coated with 3 % BSA in PBS and were then set up with 100 μl (200 μg/ml) of native Toxin A (200 μl/ml). Three tubes were then inoculated with 100 μl of the purified scFv antibodies (C5, D4 and D5) (200 μl/ml). A forth tube received 100 μl of polyclonal goat anti-Toxin A antibody as a positive control, and the fifth tube was set up with purified scFv against BSA (200 μg/ml) as a negative control. The antibody antigen mixtures were then incubated at 37° C for 1 hr. Aliquot of 100 μl of suspended F9 cells was added to each tube and incubated at 4° C for 45 min.

Cells were washed 3 times by cold centrifugation at 300x g. The supernatant was discarded and cells were resuspended in 100 μl of diluted polyclonal goat anti-Toxin A antibody 1:400. The tubes were then incubated at 4° C for 45 min. After washing 3 times

with centrifugation at 300x g, they were then probed with 100 μl of FITC-labeled rabbit anti-goat antibody 1:500. The tubes were then incubated at room temperature for 45 min and washed 3 times as before and resuspended in PBS. Fifty μl sample of cell suspension from each tube was finally spotted on a glass microscope slide covered with a cover slip and examined using an immunofluorescence microscope with illumination at 490 nm.

2.21.6 SDS-polyacrylamide gel electrophoresis and Western blotting

Western blotting was used to assess the ability of selected scFv to recognize Toxin A after its denaturation with SDS, β-mercaptoethanol and heat treatment. A crude bacterial lysate was prepared from *E. coli* pET767 and it was separated on an SDS-polyacrylamide gel electrophoresis as described before. The gel was then blotted to a nitrocellulose membrane following the standard protocol for Western blotting. Culture supernatant containing selected scFv was used to test for recognition of the carboxy-terminal domain of Toxin A. Supernatants for the experiment were prepared as follows.

Single colonies of *E. coli* HB2151 carrying the phagemid for each selected scFv were inoculated separately to 5 ml of 2xTY containing 100 μg/ml ampicillin and 1 % glucose. The cultures were incubated at 37° C overnight in a shaking incubator. The following day, 2 ml of the overnight incubated culture was inoculated to 200 ml 2xTY containing 100 μg/ml ampicillin and 0.1 % glucose. Each culture was incubated at 37° C in a shaking incubator at 200 rpm until OD 600 nm reached 0.9. IPTG was then added to a final concentration of 1 mM. The cultures were then transferred to a shaking incubator at 200 rpm with 30° C for 20 hrs. Next day, the cultures were centrifuged at 3000x g for 10 min. Twenty ml of each supernatant was then used to detect denatured recombinant Toxin A on Western blots. Toxin was separated by SDS-PAGE as described earlier. The binding of scFv to the blot was detected using mouse anti-c-myc monoclonal antibody, and HRP-labelled anti-mouse reagent as described earlier.

2.21.7 Expression levels of selected scFvs

In order to compare the level of expression of the scFvs chosen for further characterization and as an alternative method for concentration, each scFv was expressed in 100 ml cultures, spun down at 3000x g for 10 min at 4° C and concentrated to 25 times with exchange into PBS using Amicon Ultra-4 Centrifugal Filter Units with a 10 kDa cut-off (Millipore, UK). Aliquots of 20 μl from each sample were mixed with 40 μl of 3 sample buffer and heated for 5 min in boiling water. Samples of 18 μl were then loaded to lanes of duplicate polyacrylamide gels. Electrophoresis was run under the same conditions as described previously. For protein visualization, one gel was removed from the glass plate assembly and stained with Commassie Brilliant Blue for 30 min. The staining solution was discarded and gel was destained with destaining solution. The second gel was used from Western blotting.

Protein was transferred to a nitrocellulose membrane following the standard protocol for Western blotting. The membrane was then blocked with 3 % MPBS washed 3 times with PBS, and probed with monoclonal anti-c-myc and HRP-labeled rabbit anti-mouse antibodies.

2.22 Epitope analysis

Two approaches were employed to analyse the epitopes recognized in the carboxy-terminal domain of Toxin A. In the first, experiments assessed if scFvs recognized unique or common regions of Toxin A. Initial work established saturating levels for each scFv. Later experiments tested if ELISA signals could be increased by adding in a second scFv. To identify Toxin A epitopes, a series of recombinant fusion proteins were generated and tested for recognition by scFvs.

2.22.1 Epitope saturation curve

Before epitope sharing experiments were carried out, curve was first plotted for each selected purified scFv to determine the saturation point. Two rows of an ELISA plate were coated with 100 μl of 2 μg/ml native Toxin A to provide a low concentration of the target for analysis. After overnight incubation at 4° C, the plate was washed 3 times with PBS and blocked with 3 % BSA in PBS. After washing, well number 1 in both rows received 100 μl of 200 μg/ml purified scFv C5 in blocking buffer. Wells number 2 to 7 in both rows received only 200 μl of blocking buffer. The plate was incubated for 1 hr at 37° C. After washing the plate 3 times with PBS containing 0.05 % Tween-20, well number 1 and 2 in both rows received 100 μl of 200 μg/ml purified scFv C5 in blocking buffer. Wells number 3 to 7 in both rows received only 200 μl of blocking buffer. The plate was incubated for 1 hr at 37° C. After washing the plate 3 times with PBS containing 0.05 % Tween-20, wells 1 to 3 in both rows received 100 μl of 200 μg/ml purified scFv C5 in blocking buffer. Wells number 4 to 7 in both rows received only 200 μl of blocking buffer. The plate was incubated for 1 hr at 37° C. After washing the plate 3 times with PBS containing 0.05 % Tween-20, well number 1 to 4 in both rows received 100 μl of 200 μg/ml purified scFv C5 in blocking buffer. Wells number 5 to 7 in both rows received only 200 μl of blocking buffer. The plate was incubated for 1 hr at 37° C. After washing the plate 3 times with PBS containing 0.05 % Tween-20, wells number 1 to 6 in both rows received 100 μl of 200 μg/ml purified scFv C5 in blocking buffer. Well number 7 in both rows received only 200 μl of blocking buffer to serve as negative control. The plate was incubated for 1 hr at 37° C. After washing the plate 3 times with PBS containing 0.05 % Tween-20, all wells were probed with 100 μl of monoclonal anti-c-myc antibody 1:3000 in blocking buffer. The plate was then incubated at room temperature for 1 hr and washed with PBS containing 0.05 % Tween-20. All wells then probed with 100 μl of 1:500 rabbit anti-mouse antibody, incubated, and developed as described earlier. Absorbance was measured at 490 nm.

2.22.2 Epitope saturation experiment using two different scFvs

Having established the conditions required for saturation binding of a selected scFv, two different scFvs were used together in a similar experiment. The principle of this assay was that if the competing scFv bound to non-overlapping epitope, further elevation of the ELISA signal would arise. If however the scFvs bound to the same or adjacent epitope, no increase in the ELISA would be detectable. Two rows of an ELISA plate were coated with a low concentration of recombinant Toxin A as before. Next day, the plate was washed 3 times with PBS and blocked with 3 % BSA in PBS. After washing, the scFvs under test were added in 100 μl aliquots of (200 μg/ml) in blocking buffer in 3 cycles detailed below. Blocking buffer was added as (Table 2.2) indicated. For each cycle, the plate was incubated for 1 hr at 37° C and washed 3 times with PBS containing 0.05 % Tween-20. The experiment therefore allowed the binding of scFv D4 after 2 cycles of addition of C5 to be compared with 1, 2, or 3 cycles of addition of C5 (wells 4, 3 or 2 respectively). As controls, a single addition of D4 (well 5) was tested, with blanks (well 6 and 7). To detect scFv binding, the wells were then probed with 100 μl of monoclonal anti-c-myc antibody (1:3000). The plate was then incubated at room temperature for 1 hr and washed with PBS containing 0.05 % Tween-20. All wells then probed with 100 μl of rabbit anti-mouse antibody (1:500) washed and developed. Absorbance was measured at 490 nm.

Table 2.2 : Design of epitope saturation experiment

	Well number						
	1	2	3	4	5	6	7
Round 3	D4	C5	C5	C5	D4	Bb	Bb
Round 2	C5	C5	C5	Bb	Bb	Bb	Bb
Round 1	C5	C5	Bb	Bb	Bb	Bb	Bb

Bb, binding buffer; C5, scFv C5; D4, scFv D4.

2.22.3 Blocking of monoclonal phage antibody epitopes by scFv of the same specificity

Antibody saturation experiments proved unsatisfactory and so, a competitive ELISA was set up in which scFv and phage antibody of the same clone and specificity were mixed and allowed to compete for binding to the target (Boel *et al.*, 1998).

2.22.3.1 Preparation of phage antibodies

Plasmid DNA was extracted from 9 *E. coli* HB2151 clones by growing each clone in 5 ml of 2xTY containing 100 μg/ml ampicillin and 0.1 % glucose. The cultures were incubated overnight in a shaking incubator 200 rpm at 37° C. Plasmid DNA was extracted using reagent as described earlier.

E. coli TG1 was prepared for transformation using a calcium chloride method (Sambrook, 1989). Bacteria were grown on an LB plate without antibiotic overnight at 37° C. Next day, one colony was transferred to 100 ml of LB and incubated for 3 hrs at 37° C with brisk shaking at 300 rpm. Bacterial cells were transferred to an ice cold tube and left on ice for 10 min. Bacterial cells were then pelleted by centrifugation at 3200x g for 10 min at 4° C. The supernatant was discarded and the pellet was resuspended in 10 ml of cold 0.1 M $CaCl_2$. The tube was incubated on ice for 15 min. Bacterial cells were then centrifuged at 3200x g for 10 min at 4° C and the pellet was resuspended in 2 ml of ice cold 0.1 M $CaCl_2$. Two hundred μl aliquots of the resuspended bacterial cells were transferred to sterile microcentrifuge tubes using cooled sterile tips. Plasmid DNA (2.5 μl) was added to the tube and mixed. The tube was incubated on ice for 20 min and was then transferred to a warm water bath at 42° C for 60 sec After incubation on ice for 10 min, 800 μl of 2xTY medium was added to the tube and the cells were incubated in a shaking incubator at 37° C for 45 min. Samples of 50 μl were then spotted on TYE containing 100 μg/ml ampicillin and spread to allow growth of single colonies during overnight culture at

37° C. Next day, a single colony was picked from each plate and grown into 5 ml 2xTY. The following day, 500 μl of the transformed *E. coli* TG1 was used to produce phage antibodies as described previously. The supernatant containing phage was then stored at 4° C until required.

2.22.3.2 Optimization of epitope blocking by ELISA

To optimize the conditions for epitope blocking in ELISA, a single scFv C5 antibody was titrated against D5 and C5 phage antibodies. To perform the assay, 4 columns of an ELISA plate were coated with 100 μl of 2 μg/ml recombinant Toxin A in PBS. The plate was incubated overnight at 4° C. Next day, the plate was washed 3 times with PBS. The plate was then blocked with 200 μl of 2 % MPBS and incubated for 2 hrs at room temperature. The plate was then washed 3 times with PBS.

Before the addition of C5 or D5 scFvs to the toxin-coated plate, each were pre-diluted on a separate microtitre plate by a checkerboard titration as follows: 200 μl of 200 μg/ml of each scFv was added to each of four wells number (C5:1, 2, 3 and 4, D5: 5, 6, 7, and 8). Each sample was serially diluted in 4 two-fold steps. In another plate, 100 μl of 1:2 diluted phage antibody of C5 was prepared in two-fold steps. Each aliquot of phage was added to 8 wells, organized in the opposite orientation to the scFv samples and serially diluted in 4 two-fold steps. Ten μl of the diluted phage was then added to 90 μl of the diluted C5 and D5 scFvs in a checkerboard pattern. Phage-scFv mixes were then transferred to separate wells of a Toxin A-coated plate and incubated for 1 hr at room temperature after which the plate was washed 3 times with PBS containing 0.05 % Tween-20. Wells were then probed with dilution of HRP-labeled anti-M13 antibody (1:5000 in 2 % MPBS) and incubated for 1 hr at room temperature. Then wells were washed 3 times with PBS containing 0.05 % Tween-20 and developed. After 10 min, absorbance was measured at 450 nm.

2.22.3.3 Blocking of phage antibody by scFv

Seven monoclonal scFvs were prepared for use in a blocking ELISA with their phage antibody versions. To perform the test, 7 rows of an ELISA plate were coated with 100 μl of 2 μg/ml of recombinant Toxin A in PBS. The plate was incubated overnight at 4° C. Next day, the plate was washed 3 times with PBS. The plate was then blocked with 200 μl of 2 % MPBS and incubated for 2 hrs at room temperature. The plate was washed 3 times with PBS.

Ninety μl aliquots of the selected scFvs (B9, C5, D4, D5, D6, E4 and G9) at 50 μg/ml in 2 % MPBS were set up in competition with 10 μl of 1:8 diluted phage antibodies 2 % MPBS, in all combinations. Mixtures were transferred to the toxin coated plate after which the plate was washed, probed, and developed as described earlier to assess binding of phage antibodies.

2.23 Assay for toxin neutralizing activity

To characterize the ability of recombinant antibodies to neutralize Toxin A, F9 cells were cultured and tested in flow cytometry and cell culture experiments.

2.23.1 Flow cytometric analysis of toxin binding

To assess the ability of purified anti-Toxin A scFvs to inhibit the binding of the toxin to its receptor, murine F9 cells were grown as described earlier, trypsinized and grown on in 20 ml full culture medium. After 3 hrs incubation the cells were scraped, washed into washing buffer (2.5 mM Hepes, 2 % FBS and 4 mM $CaCl_2$ in PBS) and adjusted to 5 x 10^4 cells/ml (Demarest et al., 2005).

Five tubes were coated with 3 % BSA in PBS to block protein binding and each received 100 μl of native Toxin A (50 μg/ml), anti-Toxin A scFvs antibodies (C5, D4 and

D5) or 100 μl of diluted polyclonal goat anti-Toxin A antibody as a positive control. A 100 μl aliquot of purified anti-BSA scFv (200 μl/ml) served as a negative control. The antibody antigen mixtures were then incubated at 37° C for 1 hr before addition of 100 μl of suspended F9 cells. Tubes were incubated at 4° C for 45 min.

Cells were then washed 3 times with washing buffer and centrifuged at 300x g, 4° C. Supernatants were discarded and cells were resuspended in 100 μl of diluted polyclonal goat anti-Toxin A antibody (1:400). The tubes were then incubated at 4° C for 45 min. The cells were washed as before and were then probed with 100 μl of FITC-labeled rabbit anti-goat antibody (1:500). The tubes were incubated at room temperature for 45 min, washed as before and resuspended in 1 ml of cold washing buffer. Cells were analysed on a FACSCAN flow cytometer (Becton Dickenson) using Cell Quest Software.

2.23.2 Inhibition of Toxin A cytotoxicity *in vitro*

2.23.2.1 Preparation of F9 cell line

F9 cells was grown and maintained as described above. Freshly trypsinized cells were sub-cultured into 100 μl volumes in flat bottom 96-well cell culture plates (Nunc, Denmark) at 5×10^3 cells per well and allowed to recover for 18 hrs at 37° C in a 5 % CO_2 atmosphere. The plate was then washed two times with complete growth medium and 85 μl of complete medium was added to each well.

2.23.2.2 Addition of Toxin A - antibody mixtures

Twenty-five μl aliquots of the scFvs under test (C5, D4, D5, and a mixture these scFvs each at 200 μg/ml; anti-BSA scFv) were added to 2 wells in a 96-well microtitre plate (Iwaki, Japan). A 25 μl sample of BSA (200 μg/ml) in PBS was added to 2 wells containing polyclonal anti-Toxin A (1:400) as a positive control, and 2 empty wells as negative control. Native Toxin A was added to a final concentration of 2 ng/well to all wells. The plate was incubated for 30 min at room temperature in a humid atmosphere.

After that, each antibody-Toxin A mixture was transferred into a separate well in the cell culture plate. The plate was then incubated for 2 hrs at 37° C in a 5 % CO_2 atmosphere. Cell morphology was monitored and cells were counted at 25 min intervals over a 2 hr test period.

This experiment was repeated with some modification (Sauerborn et al., 1997). After the addition of antibody-Toxin A mixtures to the cell culture plate, the plate was incubated at 4° C for 30 min and wells were then washed with chilled PBS. Each well then received 100 μl of complete medium and plate was shifted to 37° C in a 5 % CO_2 atmosphere. Rounding up of cells was monitored and counted every 25 min over a 2 hrs period.

2.24 Epitope mapping

2.24.1 Materials and methods

General bacteriological procedures

2.24.1.1 Bacteria and plasmids

Bacterial strains used in this section of the study are in Table 2.3.

2.24.1.2 Preparation of pET767

E. coli carrying the plasmid was sub-cultured on LB agar plates containing kanamycin at 50 μg/ml. Colonies were transferred to liquid culture and grown in Universal containers overnight and plasmid DNA was extracted using Qiagen reagents. DNA was eluted from the QIAprep column with distilled water and stored at -20° C.

The plasmid pET767 was used as the starting point for epitope mapping since it carried that part of the Toxin A carboxy-terminal domain used in phage display. As

described later, regions of the sequence were recovered by PCR. The products were cloned into pCR 2.1-TOPO (Invitrogen) for analysis before excision by restriction digest and ligation into pCG806 (di Guan *et al.*, 1988). This is a vector that allows the convenient fusion of foreign sequence to the carboxy-terminus of MalE, the maltose-binding protein of *E. coli*. With the exception of pET767, all plasmids carried an ampicillin resistance marker.

2.24.2 Polymerase chain reaction

2.24.2.1 PCR primers

PCR primers were obtained from Sigma-Genosys, UK. PCR primers were checked for strong secondary structure, and pairs were chosen to provide Tms that were compatible. The primers were redissolved in sterile distilled water to give stock concentrations of 100 μM. Table 2.4 shows the primers used in this study.

Table 2.3: Bacterial strains used for epitope mapping

Strain	Genotype	Source/Remarks
E. coli DH5α	F- 80*lac*Z.M15.(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd* R17 (rk-, mk+) *pho*A *sup*E44 ⊾- *thi*-1 *gyr*A96 *rel*A1	Invitrogen
Topo10	F- *mcr* A .(*mrr-hsd* RMS-*mcr* BC) 80 *lac* Z.M15 .*lac*×74 *rec* A1 *ara*D139. (*ara*/*eu*) 7697 *gal* U *gal* K *rps* L (StrR) *end* A1 *nup* G	Invitrogen
E. coli DH5α pET767	See above	Plasmid from Dr. N. Fairwealter, Imperial college, London
E. coli DH5α pCG806	See above	Plasmid from Dr. P. Riggs, New England BioL

Table 2.4: List of primers used for epitope mapping and characterization of scFv genes

Primer name	Primer sequence 5' to 3'	Primer position †
rTA rev 1	ATATAAGCTTCTACTAAGGGCTTTTACTCC	995 b
rTA rev 2	ATATAAGCTTCTAAGCTGTATTAGGCTCGAAG	684 b
rTA rev 3	ATATAAGCTTCTAAGAAGTGTTAGTATTAAAG	306 b
rTA for 1	TGATATCGCCTCAACTGGTTATACAAG	36 b
rTA for 2	TGATATCAAAGCAGTTACCGGACTG	247 b
rTA for 3	TGATATCAAAGCGGCTACTGGTTGG	586 b
ToxA repeat rev (RR1)	GATCCTTAAGTATCAGGCATAAAGTAATATACTTTAC-CATTAATAGTTTGCCATCCAGTAACTGCTTTCGAGCT	921 b
ToxA repeat for (FR1)	CGAAAGCAGTTACTGGATGGCAAACTATTAATG-GTAAAGTATATTACTTTATGCCTGATACTTAAG	859 b
gIII	CCCTCATAGTTAGCGTAACG	-
pelB	ATGAAATACCTATTGCCTACGGCAGC	-

Underlined sequences indicate inserted restriction sites. †Primers position on rTA DNA sequence.

2.24.2.2 PCR reactions

The reactions were set up to a total volume of 25 μl containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTPs) 100 pmol each primer, 1.0 U of Taq DNA polymerase (Promega, USA) and 2.5 μl of template DNA preparation.

Amplifications were performed using a Hybaid thermal cycler (Hybaid Ltd, Middlesex, UK). Conditions used were denaturation at 94° C for 5 min, followed by 30 cycles of denaturation 94° C for 1 min, annealing 52° C to 60° C (for different primer sets) for 1 min, extension at 72° C for 1 min. A final extension step at 70° C for 5 min was included. PCR products were stored at -20° C or used immediately.

Samples from the PCR reactions were mixed with 6x loading buffer (40% (w/v) sucrose and 0.25% (w/v) Bromophenol blue dissolved in sterile distilled water) and electrophoresed on 0.7-1.0 % (w/v) agarose gels. The 1 Kb DNA ladder (Promega, USA) was used as a DNA molecular weight marker.

Agarose was prepared in Tris-Acetate-EDTA buffer and 5 μl of SYBR Safe DNA gel stain (Invitrogen, USA) was added to each 100 ml of the mixture.

Gels were run at a voltage of 1-5 volts/cm^2. Electrophoresis continued until the marker dye had migrated an appropriate distance.

A UV transilluminator was used to visualize the SYBR Safe stained DNA. Images of the gel were either stored electronically or printed out for further analysis.

2.24.3 Elution and purification of DNA from agarose gels

Routinely, DNA fragments were eluted and purified from agarose gels by QIAquick extraction reagents.

The DNA band was visualized and excised from a SYBR Safe stained gel with a scalpel under UV light (Chromato VUE, C-70 Viewing system, UVP. Inc., USA). Briefly, to purify DNA from the gel, the gel slices were weighed in a microcentrifuge tube. About 3 volumes of buffer QG were added to each tube and incubated at 56° C for 10 min in a water bath. The tubes were shaken every 2 min until the agarose was dissolved. The tubes were then centrifuged. After that, 1 gel volume of isopropanol was added to the tubes and mixed gently. The dissolved gel was then loaded to QIAquick spin column and centrifuged as recommended. The flow-through was discarded and the column was washed with 0.5 ml of buffer QG. After centrifugation, the column was washed with 0.75 ml of buffer PE and the column was centrifuged as recommended. The flow-through was discarded and the column was put onto a 1.5 ml sterile microcentrifuge tube. DNA was then eluted with 30 μl of sterile distilled water and recovered by centrifugation.

2.24.4 Restriction enzyme digestion and ligation

Restriction enzyme digests were routinely performed according to the manufacturer's instruction unless otherwise stated. The set of restriction enzymes used are listed in Table 2.5. Vector and insert fragments were ligated using T4 DNA ligase (promega and New England Biolabs). Ligation mixtures were transfomed into competent *E. coli* DH5α (Invitrogen) and transformant were selected on LB agar with ampicillin 100 μg/ml as described earlier.

2.24.5 DNA sequencing

Plasmid DNA was purified from selected clones of *E. coli* DH5α by growing each clone in 5 ml of LB containing 100 μg/ml ampicillin. The cultures were incubated overnight in a shaking incubator 200 rpm at 37° C. Plasmid DNA was extracted using Qiagen reagents. Sequencing was performed using the primers appropriate sets at Sir Henry Wellcome Functional Genomics Facility, University of Galsgow as described earlier. For data analysis, Chromas (versions 2.3 & 1.15) and DNA star software were utilized.

Table 2.5: List of restriction enzymes and their target sequences

Restriction Enzyme	Target sequence	Source
Sac I	5'…GAGCT↓C…3' 3'…C↑TCGAG…5'	Promega, USA
Hin dIII	5'…A↓AGCTT …3' 3'…T TCGA↑A …5'	Promega, USA
Eco RV	5'…GAT↓ATC…3' 3'…CTA↑TAG …5'	Promega, USA
Bam HI	5'…G↓GATC↓C…3' 3'…C↑CTAG ↑G…5'	Promega, USA
Sma I	5'…CCC↓GGG…3' 3'…GGG↑CCC …5'	BioLabs, UK

All digests were carried out at 37° C except for *Sma* I (25° C).

2.24.6 Cloning

2.24.6.1 Cloning into pCR 2.1-TOPO vector

PCR products were cloned using the pCR 2.1-TOPO vector system (Invitrogen). PCR products were purified from the gel and resuspended in sterile distilled water to a concentration of 50 ng/μl. Ligation step was done according to the manufacturer's instructions. Briefly, 4 μl of PCR product was added to 1 μl of TOPO vector. The vector solution contains topoisomerase, the linearised vector and ligation buffer. The mixture was incubated for 5 min at room temperature after which it was transferred to ice. After thawing one vial of TOP10F One Shot competent cells on ice, 2 μl of the ligation mix was added with gentle mixing using pre-cooled tips. After the mixture was incubated on ice for 30 min, the cells were heat-shocked at 42° C for 30 sec in water bath. The cells were then incubated on ice for 2 min before addition of 250 μl of warm SOC. Tubes were incubated with shaking at 37° C for 2 hrs. After that, 50 μl was spread on an LB plate containing 100 μg/ml ampicillin and incubated overnight at 37° C.

2.24.6.2 Cloning into pCG806

Vector and insert DNA were routinely digested with restriction endnucleases to generate complimentary cohesive overhangs or blunt ends. The resultant fragments were gel purified and DNA concentrations were measured. DNA fragments were mixed to provide high molar excess of insert over vector.

2.24.6.3 Ligation

Ligation reactions were performed in a total volume of 20 μl. Briefly, compatible vector and insert fragments were prepared as above with 300 ng of vector DNA and an appropriate quantity of insert DNA for each reaction. After that, 2 μl of 10 x buffer, and 1 μl T4 DNA ligase (400,000 U/ml; New England BioLabs) were added. The reaction volume was completed to 20 μl with sterile distilled water. After snap centrifugation, tubes

were incubated at room temperature for 20 min. After incubation the ligation mixture was then stored at -20° C if not used immediately in transformation.

2.24.6.4 Transformation

One vial of commercially prepared *E. coli* DH5α was thawed on wet ice. Cells were mixed gently and 50 μl aliquots were transferred into sterile chilled polypropylene tubes. Samples of ligated DNA were added to the cells and mixed gently. The tubes were then incubated on ice for 30 min. Cells were then heat-shocked for 20 sec at 37° C in water bath without shaking. The tubes were then returned ice for 2 min and then received 950 μl of pre-warmed SOC. The tubes were then incubated at 37° C for 1 hour in a shaking incubator at 225 rpm. After incubation, 100 μl of each transformation was spread on an LB agar plate containing 100 μg/ml ampicillin. Plates were incubated overnight at 37° C.

2.24.6.5 Preparation of synthetic DNA duplex

Oligonucleotides (FR1 and RR1) were resuspended in sterile distilled water to 100 μM. 16 μl of the mixed oligonucleotides was added to a PCR tube with of an annealing buffer (50 mM KCl, 10 mM, Tris-HCl pH 9.0, 1.5 mM $MgCl_2$ and 0.1 Triton X-100) and 2 μl of sterile distilled water. The annealing mixture was then incubated in a small volume water bath at 96° C. The water was left to return gradually to room temperature.

2.24.6.6 Phosphorylation of annealed oligonucleotides

In order to enable the DNA duplex to ligate to the digested vector, samples were phosphorylated using T4 polynucleotide kinase. Briefly, 2 μl of the annealed duplex was mixed with 2 μl of 5 x forward buffer (350 mM Tris-HCl 7.6 pH, 50 mM $MgCl_2$, 500 mM KCl and 5 mM 2-mercaptoethanol), 2 μl of 10 U/μl T4 phosphokinase, 2 μl of 10 mM ATP and 12 μl sterile distilled water. The phosphorylation mixture was then mixed, centrifuged briefly and incubated in a water bath at 37° C for 15 min. The reaction was

then stopped by heating at 65° C for 10 min. The mixture was then purified using a PCR purification kit (Qiagen, UK). Phosphorylated linker DNA was then eluted in warmed 30 μl sterile distilled water and its DNA concentration was determined. Ligation and transformation were carried out as described earlier.

2.25 Protein expression

Bacteria were grown in LB medium with 100 μg/ml ampicillin. When the OD at 600 nm reached 0.4, expression of MalE fusion proteins was induced by the addition of IPTG to a concentration of 1 mM and incubated for 3 hrs in a shaking incubator at 37° C. The culture was recovered by centrifugation at 4000x g at 4° C for 10 min. The supernatant was discarded and pellet was resuspended in 10 ml aliquots of cold PBS pH 7.4.

Bacterial cells were sonicated (Status US200, Philip Harris Scientific, Germany) for 5 min with 20 % sonication power using 10 sec cycle sonication and cooling. The bacterial lysate was centrifuged at 10000x g at 4° C for 10 min. Supernatant was collected and stored at -20° C or used immediately.

2.25.1 SDS-PAGE and electrobloting

SDS-electrophoresis was carried out as described earlier. On completion, proteins were blotted to nitrocellulose membrane following the standard protocol for Western blotting described earlier. The membrane was then blocked, probed with appropriate reagents and developed using 4-chloronaphthol substrate. The reaction was stopped by washing the membrane with distilled water.

For enhanced sensitivity, blots were also developed using ECL substrate (Pierce Biotechnology, USA). Briefly, working solutions of the substrates were prepared according to the manufacturers' instructions and added to the membranes for 1 minute. The membranes were removed from the substrates and placed between plastic sheets. Each

membrane was exposed to CL-XPosure film for 20 seconds. The CL-Xposure film was developed with M35-XO mart processor.

Chapter 3

Results

3 Results

3.1 Confirmation of sequences encoding Toxin A C-terminal binding domain

The presence of the coding sequence for the C-terminal domain of Toxin A in pET767 was assessed using plasmid DNA sequencing (Figure 3.1) and restriction analysis of the extracted plasmid. The insert encoded a reading frame for 320 amino acids from Toxin A, preceeded by a repeat of 6 histidine residues. The Toxin A sequence carried 14 of the peptide repeat sequences prominent in the carboxy-terminal domain of the toxin. This includes regions recently analysed at the structural level by Ho and collegues (Ho *et al.*, 2005). In this study, the folding of peptide sequence into short repeats (SRs) and long repeats (LRs) was characterized. Locations of SRs and LRs sequences are shown with colour codes in Figure 3.1. The SRs and LRs of Toxin A have been shown to form interactions with the carbohydrate receptor on target cells (Greco *et al.*, 2006), explaining the receptor binding activity of this part of the toxin.

Translation of insert (5'->3')

```
          10        20        30        40        50        60        70        80        90
          |         |         |         |         |         |         |         |         |
  ATGGGCAGCAGCCATCATCATCATCATCATCACGCCTCAACTGGTTATACAAGTATTAATGGTAAACATTTTTATTTTAATACTGATGGTATTATGC 100
1 M  G  S  S  H  H  H  H  H  H  H  H  A  S  T  G  Y  T  S  I  N  G  K  H  F  Y  F  N  T  D  G  I  M  Q
          |         |         |         |         |         |         |         |         |
  AGATAGGAGCGTTTAAAGGACCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAACATAGAAGGTCAAGCTATACTTTACCAAAATAA 200
35   I  G  A  F  K  G  P  N  G  F  E  Y  F  A  P  A  N  T  D  A  N  N  I  E  G  Q  A  I  L  Y  Q  N  K
          |         |         |         |         |         |         |         |         |
  ATTCTTAACTTTGAATGGTAAAAAATATTACTTTGGTAGTGACTCAAAAGCAGTTACCGGACTGCGAACTATTGATGGTAAAAAATATTACTTTAATACT 300
68  F  L  T  L  N  G  K  K  Y  Y  F  G  S  D  S  K  A  V  T  G  L  R  T  I  D  G  K  K  Y  Y  F  N  T
          |         |         |         |         |         |         |         |         |
  AACACTGCTGTTGCAGTTACTGGATGGCAAACTATTAATGGTAAAAAATACTACTTTAATACTAACACTTCTATAGCTTCAACTGGTTATACAATTATTA 400
90 N  T  A  V  A  V  T  G  W  Q  T  I  N  G  K  K  Y  Y  F  N  T  N  T  S  I  A  S  T  G  Y  T  I  I  S
          |         |         |         |         |         |         |         |         |
  GTGGTAAACATTTTTATTTTAATACTGATGGTATTATGCAGATAGGAGTGTTTAAAGGACCTGATGGATTTGAATACTTTGCACCTGCTAATACAGATGC 500
124   G  K  H  F  Y  F  N  T  D  G  I  M  Q  I  G  V  F  K  G  P  D  G  F  E  Y  F  A  P  A  N  T  D  A
          |         |         |         |         |         |         |         |         |
  TAACAATATAGAAGGTCAAGCTATACGTTATCAAAATAGATTCCTATATTTACATGACAATATATATTATTTTGGTAATAATTCAAAAGCGGCTACTGGT 600
158  N  N  I  E  G  Q  A  I  R  Y  Q  N  R  F  L  Y  L  H  D  N  I  Y  Y  F  G  N  N  S  K  A  A  T  G
          |         |         |         |         |         |         |         |         |
  TGGGTAACTATTGATGGTAATAGATATTACTTCGAGCCTAATACAGCTATGGGTGCGAATGGTTATAAAACTATTGATAATAAAAATTTTTACTTTAGAA 700
192 W  V  T  I  D  G  N  R  Y  Y  F  E  P  N  T  A  M  G  A  N  G  Y  K  T  I  D  N  K  N  F  Y  F  R  N
          |         |         |         |         |         |         |         |         |
  ATGGTTTACCTCAGATAGGAGTGTTTAAAGGGTCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAATATAGAAGGTCAAGCTATACG 800
235   G  L  P  Q  I  G  V  F  K  G  S  N  G  F  E  Y  F  A  P  A  N  T  D  A  N  N  I  E  G  Q  A  I  R
          |         |         |         |         |         |         |         |         |
  TTATCAAAATAGATTCCTACATTTACTTGGAAAAATATATTACTTTGGTAATAATTCAAAAGCAGTTACTGGATGGCAAACTATTAATGGTAAAGTATAT 900
268   Y  Q  N  R  F  L  H  L  L  G  K  I  Y  Y  F  G  N  N  S  K  A  V  T  G  W  Q  T  I  N  G  K  V  Y
          |         |         |         |         |         |         |         |         |
  TACTTTATGCCTGATACTGCTATGGCTGCAGCTGGTGGACTTTTCGAGATTGATGGTGTTATATATTTCTTTGGTGTTGATGGAGTAAAAGCCCCTTAGT 1000
302 Y  F  M  P  D  T  A  M  A  A  A  G  G  L  F  E  I  D  G  V  I  Y  F  F  G  V  D  G  V  K  A  P  *  *

  AGCTC
336   L
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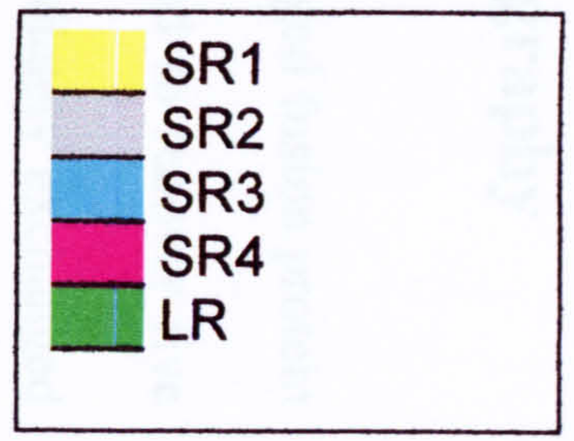

Figure 3.1: Complete nucleic acid sequence encoding recombinant Toxin A from pET767

3.2 Purification of the protein by affinity chromatography

The C-terminal domain of Toxin A was expressed as histidine tagged fusion protein and immobilized on a nickel-chelation column. Acid elusion was used to recover five fractions that were collected into 2 x PBS. Fractions were then immediately exchanged with cold PBS using centrifugal filter units and stored at -20° C until further analysis. In initial experiments, protein was eluted from the column using 200 mM imidazole buffer. Problems of protein precipitation became evident on prolonged storage that were overcome by acid elution. The concentration of protein in the pooled acid elutions was 400 μg/ml.

3.3 SDS-electrophoresis

The C-terminal domain of Toxin A representing 14 repetitive sequences of the binding domain was successfully expressed and purified in recombinant form. The bacterial extract (Figure 3.2; lane 1) showed several dominant proteins of 10-220 kDa. The expected molecular weight of the recombinant protein was 37 kDa. The extract was exchanged into column binding buffer, filtered through a membrane of 0.2 μm and loaded to the affinity column. Washing with binding buffer containing 20 mM imidazole removed many of the proteins seen in the bacterial extract (lane 3), and further washing with unmodified binding buffer (not shown) further depleted the column. The column was then washed with buffer of pH 6.0 and then pH 5.0 (lane 4 and 5). Rather few proteins eluted from the column at pH 6.0 (lane 4), but reduction to pH 5.0 removed several species, the most prominent being of 45 kDa molecular weight. Having done this, the pH was then lowered to pH 3.4. This eluted a protein of the expected size for recombinant Toxin A (lane 6). No other protein contaminants were present (Figure 3.2; lane 6).

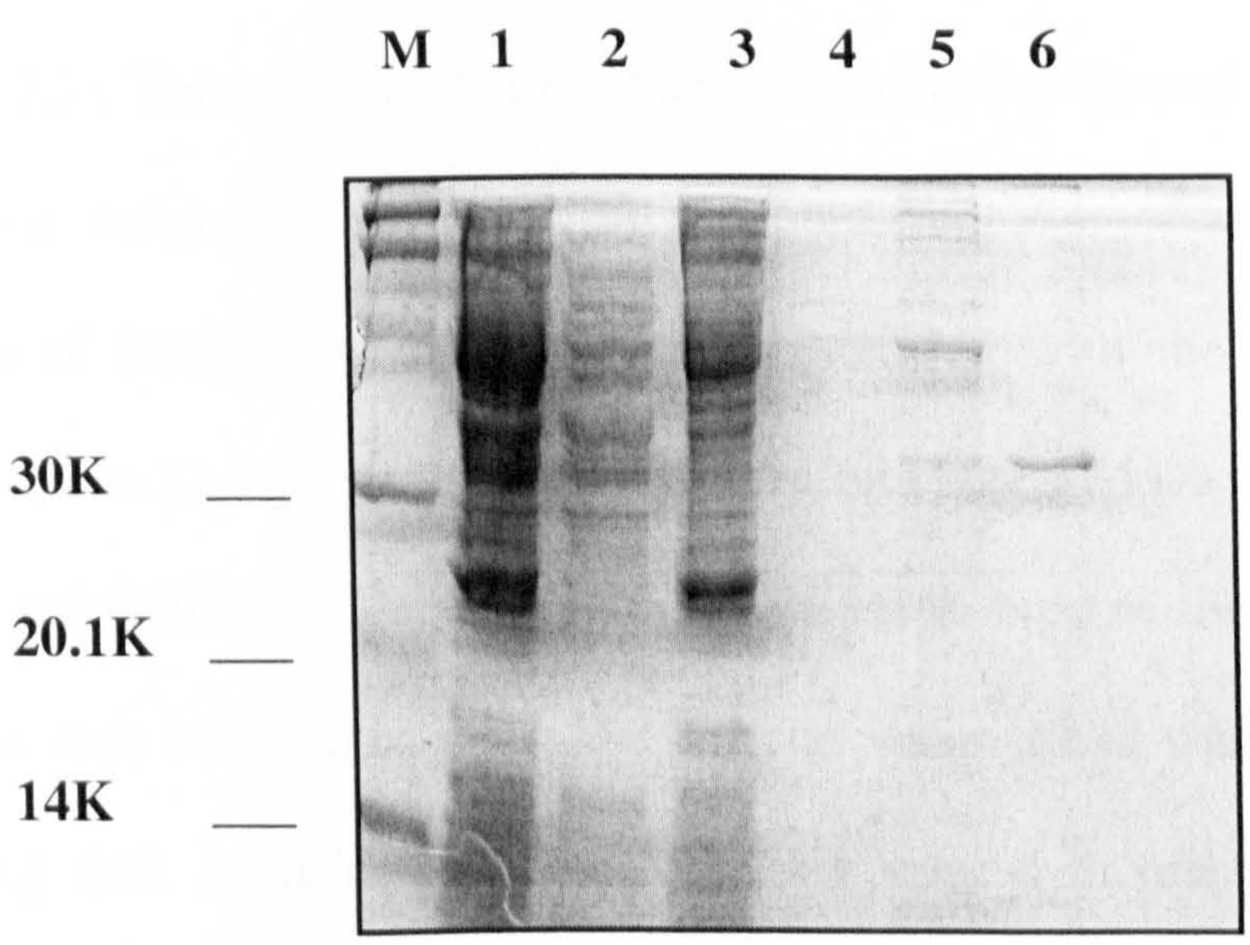

Figure 3.2: SDS-PAGE analysis of recombinant Toxin A by affinity chromatography

Lysate from induced bacterial culture was exchanged into binding buffer (lane 1) and applied to the column. Flow through (lane 2) was discarded. Washings with imidazole buffer (lane 3) and buffer of pH 6.0 (lane 4) and pH 5.0 (lane 5) were discarded. Protein bound to the column was eluted with buffer of pH 3.4 (lane 6). Lane M shows the migration of protein molecular weight marker/size as indicated.

3.4 Electroblotting analysis

The identity of protein recovered by affinity chromatography was determined by Western blotting. These experiments confirmed that the commercial polyclonal anti-Toxin A raised against native toxin would recognise recombinant Toxin A after its preparation for SDS-PAGE with boiling and reduction. Proteins were separated by SDS-PAGE (Figure 3.3) and transferred to a nitrocellulose membrane for probing with polyclonal anti-Toxin A (Figure 3.3). This showed that the bacterial extract contained a prominent band of 37 kDa molecular weight, consistent with the predicted size of recombinant Toxin A. Other proteins of lower molecular weight also reacted with the antibody. The flow-through fraction (lane 2) showed that some protein of a size 37 kDa consistent with that predicted for the recombinant C-terminal domain failed to bind to the column. A similar range of proteins were also eluted when the column was washed with 20 mM imidazole (lane 3). Washing with a buffer of pH 6.0 did not appear to release protein reactive with the antiserum from the column (lane 4). Elution started when the column was washed with buffer of pH 5.0 (lane 5) and high yields of the recombinant Toxin A was achieved by further reduction of the pH to 3.4 (lane 6)

3.5 Recognition in ELISA

ELISA was used to assess recognition of undenatured purified recombinant Toxin A by the polyclonal serum raised against native Toxin A (Figure 3.4). The concentration of recombinant protein in initial wells was 225 μg/ml. The protein was serially diluted and remained detectable to concentrations less than 480 ng/ml. The specificity of the ELISA was confirmed by the negative controls (wells 11 and 12) which were coated with BSA instead of recombinant protein.

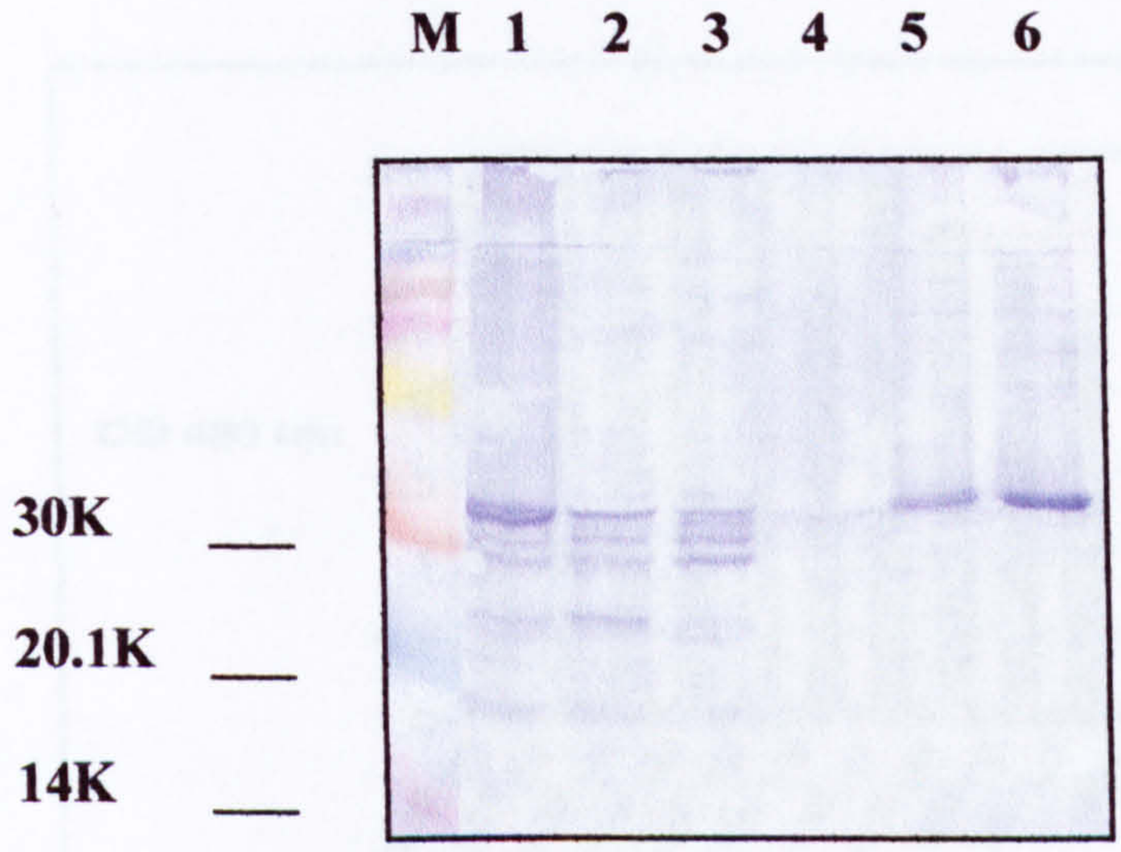

Figure 3.3: Western blotting analysis of recombinant Toxin A purified by affinity chromatography

Fractions from the affinity column were prepared as in Figure 3.2. After separation by SDS-PAGE, proteins were blotted to nitrocellulose and probed with a goat anti-Toxin A antiserum. Fractions: bacterial extract (lane 1), flow-through (lane 2), washing with 20 mM imidazole (lane 3), washing with buffer of pH 6.0 (lane 4), washing with buffer of pH 5.0 (lane 5), recombinant protein eluted with buffer of pH 3.4 (lane 6). Lane M shows the migration of protein molecular weight marker/size as indicated.

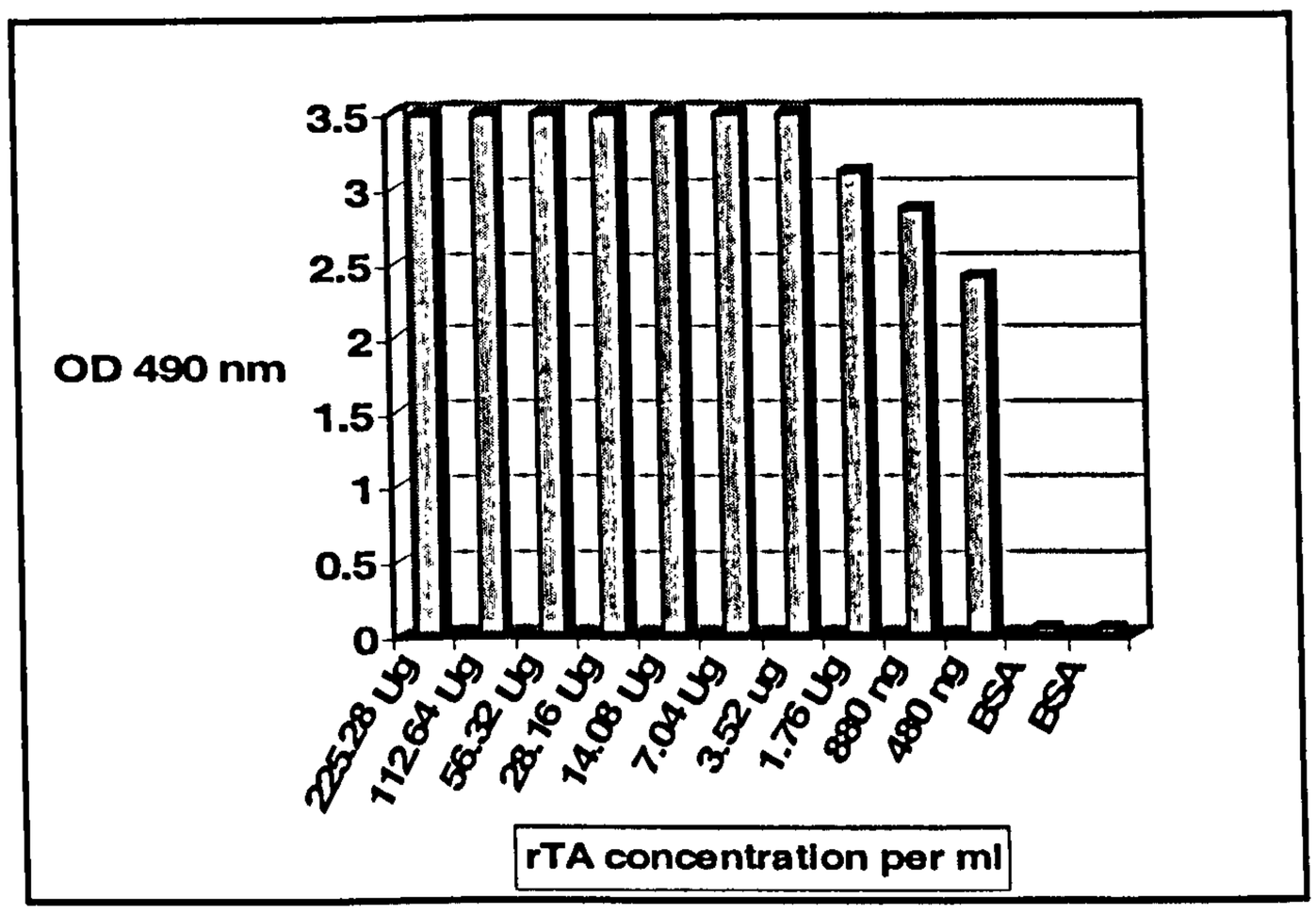

Figure 3.4: Detection of recombinant Toxin A in ELISA.

Purified recombinant Toxin A was serially diluted and coated to an ELISA plate and then probed with goat anti-Toxin A antibody and HRP-conjugated antibodies. Negative control wells were coated with 3 % BSA in PBS. The coating concentrations of toxin are shown on the x-axis. ELISA signal at 490 nm is shown on the y-axis.

3.6 Binding of native and recombinant Toxin A to cellular receptors

The binding activity of native and recombinant Toxin A was tested using paraformaldehyde-fixed F9 cells immobilized on a slide (Figure 3.5). Strong immunofluorescence signal was observed out-lining the cell membrane reflecting the degree of binding activity of Toxin A to its receptor. When recombinant Toxin A was used (Figure 3.5; B), a positive immunofluorescence reaction was seen out-lining the cell membrane of F9 cell line but of lower intensity. This batch of recombinant protein was purified only early in the project using imidazole elution from an affinity column.

3.7 Haemagglutination activity of recombinant Toxin A

Native Toxin A is known to possess the ability to agglutinate rabbit erythrocytes, through interaction with surface carbohydrates moieties. The recombinant Toxin A was tested for this property (Figure 3.6). Assays with rabbit red blood cells showed that haemagglutinating activity was detectable and that after serial dilutions, this persisted to a concentration of 32 μg/ml and 0.2 μg/ml for recombinant and native Toxin A respectively. These finding are consistant with previous studies (Ward *et al.*, 1999a).

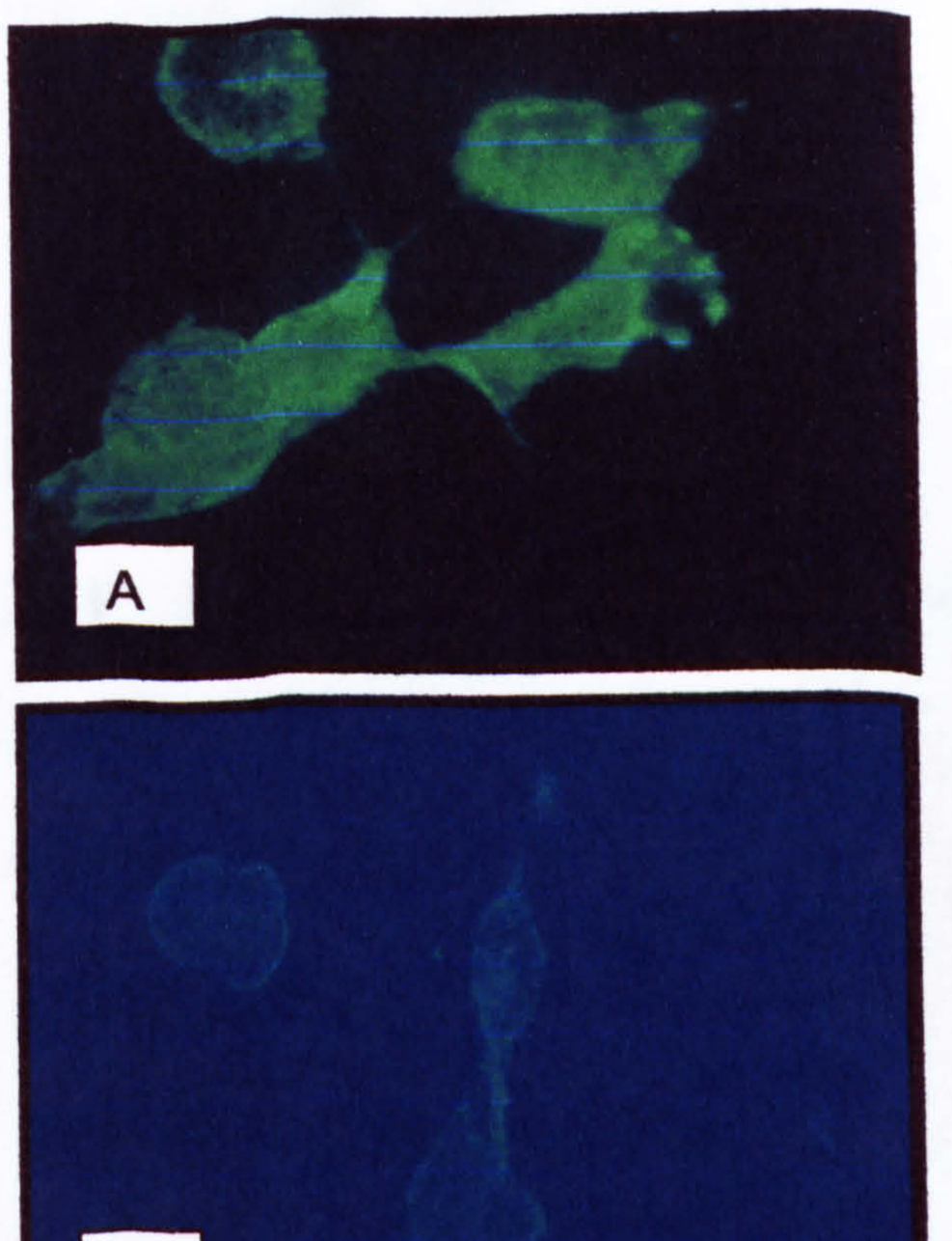

Figure 3.5: Immunofluorescence assay to detect the binding of recombinant and native Toxin A to receptors on F9 cells

F9 cells were grown, fixed on 8-well chamber slides and incubated with native (A) or recombinant Toxin A (B). After washing, the cells were incubated with polyclonal anti Toxin A antibody and FITC-labeled anti-goat antibody. In the negative control (C), cells were incubated with FITC-labeled anti-goat antibody. Cells were then examined under immunofluorescence microscope.

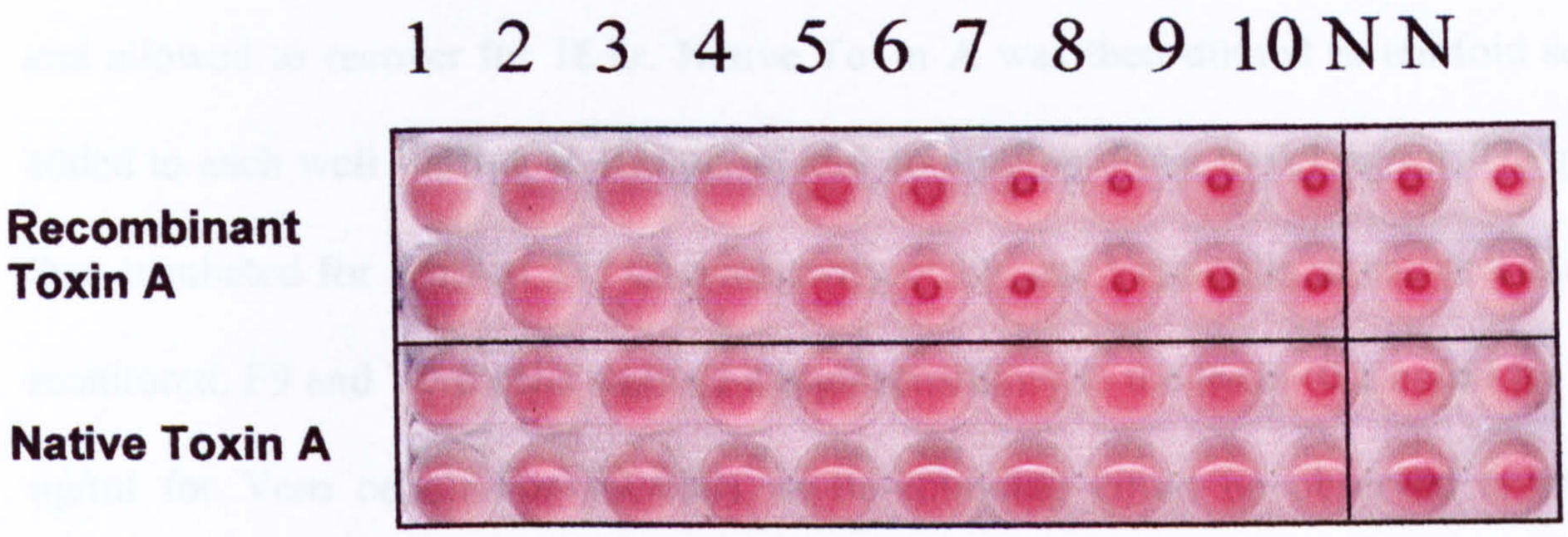

Figure 3.6: Haemagglutination activity of recombinant Toxin A

Rabbit red blood cells were added to two-fold serially diluted recombinant Toxin A in a microtiter plate and incubated at 4° C. Haemagglutination became evident after 2 hrs. PBS was used as a negative control (N). The titer was defined as the final dilution in which haemagglutination was evident. The initial concentration of recombinant Toxin A was 256 µg /ml (well 1). For native Toxin A, the starting concentration was 51.2 µg/ml (well 1).

3.8 Sensitivity of F9 and Vero cell lines to native Toxin A

To assess the ability of the native Toxin A to bind to its receptor and exerts its effect, tests were carried out with the murine cell line F9 and Vero cells. The F9 cell line is thought to carry a high density of receptors for Toxin A (Tucker *et al.*, 1990). Cells were sub-cultured in 100 μl volumes in flat bottom cell culture plates at 5×10^3 cells per well and allowed to recover for 18 hr. Native Toxin A was then diluted in ten-fold series and added to each well starting at 100 ng/ml and continuing down to 0.1 ng/ml. The plate was then incubated for 5 hrs at 37° C. Sensitivity of F9 and Vero cells to native Toxin A was monitored. F9 and Vero cells showed a high sensitivity to the toxin (0.1 ng/ml for F9 and 1 ng/ml for Vero cells). For F9 cells, some rounding could be observed at the lowest concentration tested whereas for Vero cells 1 ng/ml was required to elicit a response. All cells were observed rounding at 10 ng/ml for both F9 and Vero cells (Figure 3.7, 3.8 and 3.10). However, this cytopathic reaction was achieved after 4 hrs for F9 cells and 5 hrs for Vero cells. At low toxin concentrations (0.1 ng/ml) Vero cells did not show any reactivity after 5 hrs of incubation whereas some F9 cells observed undergoing a rounding response. The response of F9 cells to toxin was also faster than for Vero cells. For example, when cells were challenged at the highest toxin dose, 60 % of F9 cells had undergone rounding within 1 hr of addition (Figure 3.8). By comparison, Vero cells had failed to respond to their dose of toxin after 1 hr (Figure 3.9). After 2 hrs, about 70 % had rounded in response to Toxin A.

3.9 Kinetics of action of native Toxin A on F9 and Vero cell lines

To assess the time required for native Toxin A to bind and enter F9 and Vero cells, a time course experiment was set up. Cells were sub-cultured as described earlier. Eight wells of each cell line were inoculated with Toxin A at 100 ng/ml and incubated at 37° C. Two wells from each cell line were washed with PBS after 4 min, 8 min, 16 min and 32

min. After replacement with complete medium, the plate was then incubated at 37° C and observations were taken every 10 min for 3 hrs (Figure 3.10)

Results showed that F9 cells responded to Toxin A after a short exposure whereas longer contact with Vero cells was required to elicit an equivalent response. F9 cells also responded to toxin more quickly than Vero cells after identical exposure. For example, a 2 min exposure of F9 cells to toxin was sufficient to elicit 50 % rounding with 2 hrs. In contrast, toxin had to be in contact with Vero cells for 16 hrs to achieve this biological response within a 2 hrs period. Similarly, F9 cells showed 100 % rounding with 16 and 32 min exposures and reached this response after 160 and 100 min respectively. In contrast, Vero cells showed 100 % rounding up after only 32 min of exposure to toxin and 180 min of incubation was required for complete rounding (Figure 3.10).

3.10 Inhibition of the action of native Toxin A

Experiments were carried out to assess the ability of polyclonal anti-Toxin A and recombinant C-terminal domain of Toxin A to block the activity of native toxin. Recombinant Toxin A or anti-Toxin A polyclonal antibody were incubated with Toxin A in PBS in a microtiter plate. After incubation, the mixtures were added to F9 cells culture plate as described earlier. The response of cells to the toxin was then observed.

Results showed that polyclonal goat anti-Toxin A was a very effective inhibitor of the toxin. When diluted to 1:200, and mixed with toxin at a final dose of 10 ng/ml, fewer than 7 % of cells underwent a rounding response over a 2 hr period (Figure 3.11). This was in marked contrast to the recombinant C-terminal domain. Mixing this with active toxin did not block the response of F9 cells although the recombinant protein was present in approximately 1000-fold more excess.

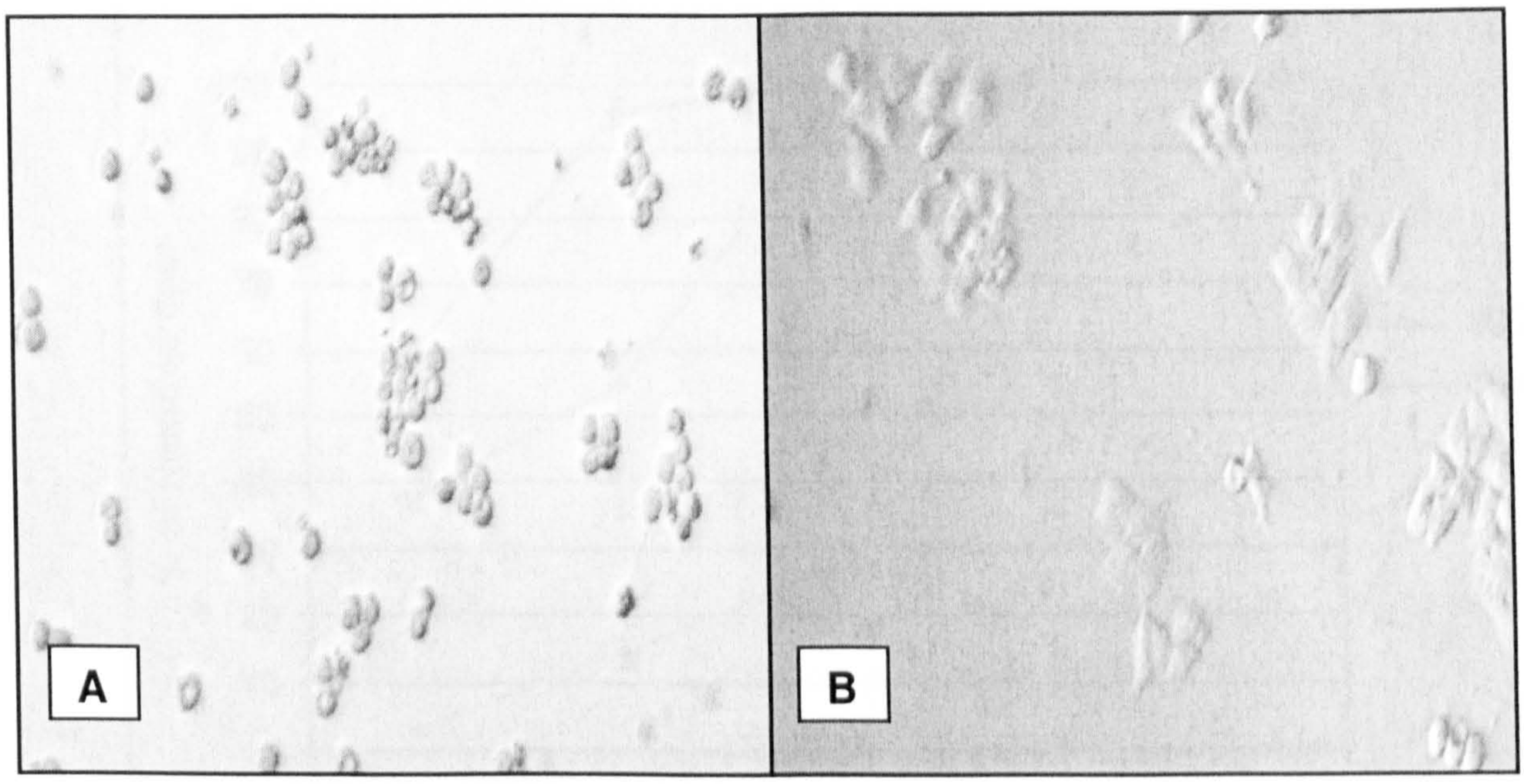

Figure 3.7: Sensitivity of F9 cell lines to native Toxin A

Native Toxin A was prepared in a series of 10–fold dilutions and added to growing F9 cells in culture. The plate was then incubated for 5 hrs at 37° C. Sensitivity of F9 cells to native Toxin A was monitored by examining the cells for evidence of loss of their normal shape and change to a rounded appearance. 100 % cell rounding was observed with toxin doses as low as 10 ng/ml (A). PBS was used as negative control (B).

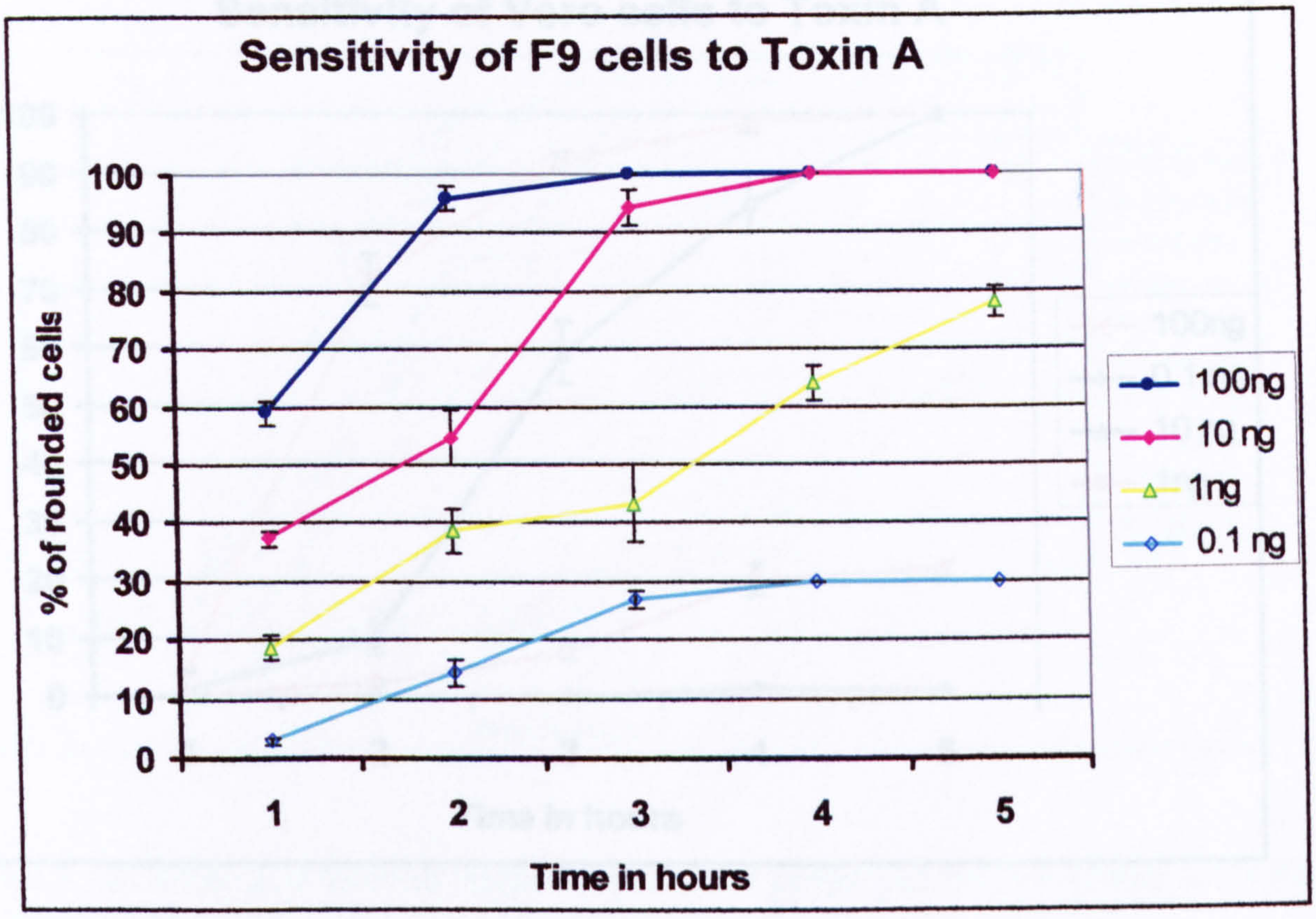

Figure 3.8: Sensitivity of F9 cell lines to native Toxin A

Native Toxin A was serially diluted and added to growing F9 cells. The plate was then incubated for 5 hrs at 37° C. The reaction of F9 cells to Toxin A was monitored. Cell rounding being taken as a sign of toxin action. Fields were examined and the mean percentage of rounded cells is shown in the figure. Error bars show Standard Deviation Error STDE. PBS was used as negative control, and no cell rounding was observed during the experiment (data not shown). Key shows the amout of toxin per well added to the cultures

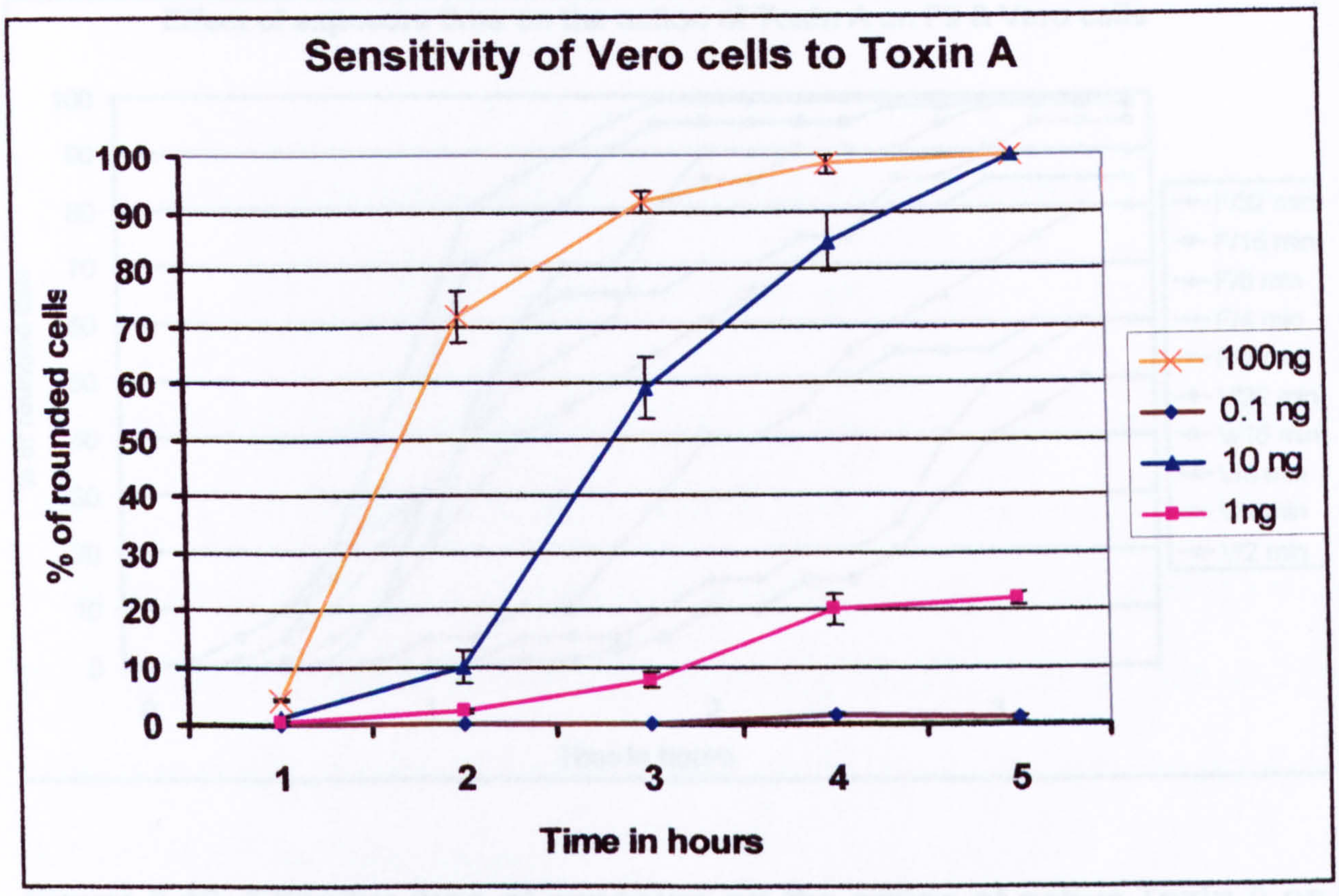

Figure 3.9: Sensitivity of Vero cell lines to native Toxin A

Native Toxin A was serially diluted and added to growing Vero cells. The plate was then incubated for 5 hrs at 37° C. The reaction of Vero cells to Toxin A was monitored. Cell rounding being taken as a sign of toxin action. Fields were examined and the mean percentage of rounded cells is shown in the figure. Error bars show Standard Deviation Error STDE. PBS was used as negative control, and no cell rounding was observed during the experiment (data not shown). Key shows the amout of toxin per well added to the cultures

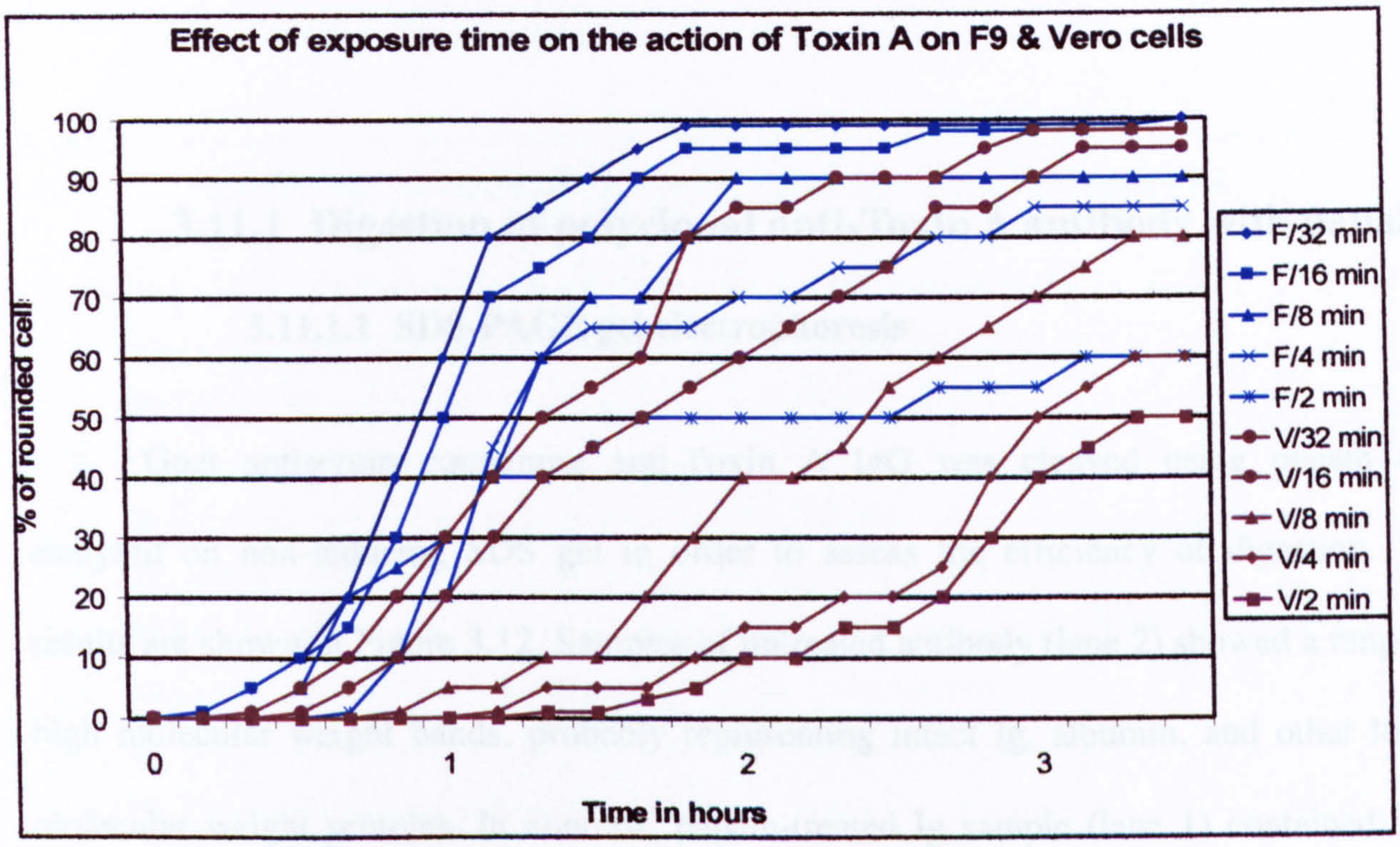

Figure 3.10: Effective time of exposure on the action of native Toxin A on F9 and Vero cells.

Native Toxin A (100 ng/ml) was added to wells of growing F9 or Vero cells. The plate was incubated at 37° C. Two wells from each cell line were washed with PBS after 4 min, 8 min, 16 min and 32 min exposure to remove toxin that had failed to bind or enter the cells. Cells were inspected at intervals thereafter and the mean percentage of rounded cells was calculated. In the key, the letter indicates the cell line tested and the number to exposure time to toxin.

3.11 Inhibition of native Toxin A with polyclonal Fab fragments

To assess the ability of Fab fragments from an anti-Toxin A antibody to block the activity of native Toxin A, a polyclonal IgG antibody was digested with papain. The Fab preparation was then used in neutralization experiments *in vitro*.

3.11.1 Digestion of polyclonal anti-Toxin A antibody with papain

3.11.1.1 SDS-PAGE gel electrophoresis

Goat antiserum containing anti-Toxin A IgG was cleaved using papain and analysed on non-reducing SDS gel in order to assess the efficiency of digestion. The results are shown in Figure 3.12. Samples of untreated antibody (lane 2) showed a range of high molecular weight bands, probably representing intact Ig, albumin, and other lower molecular weight proteins. In contrast, papain-treated Ig sample (lane 1) contained only bands of lower molecular weight.

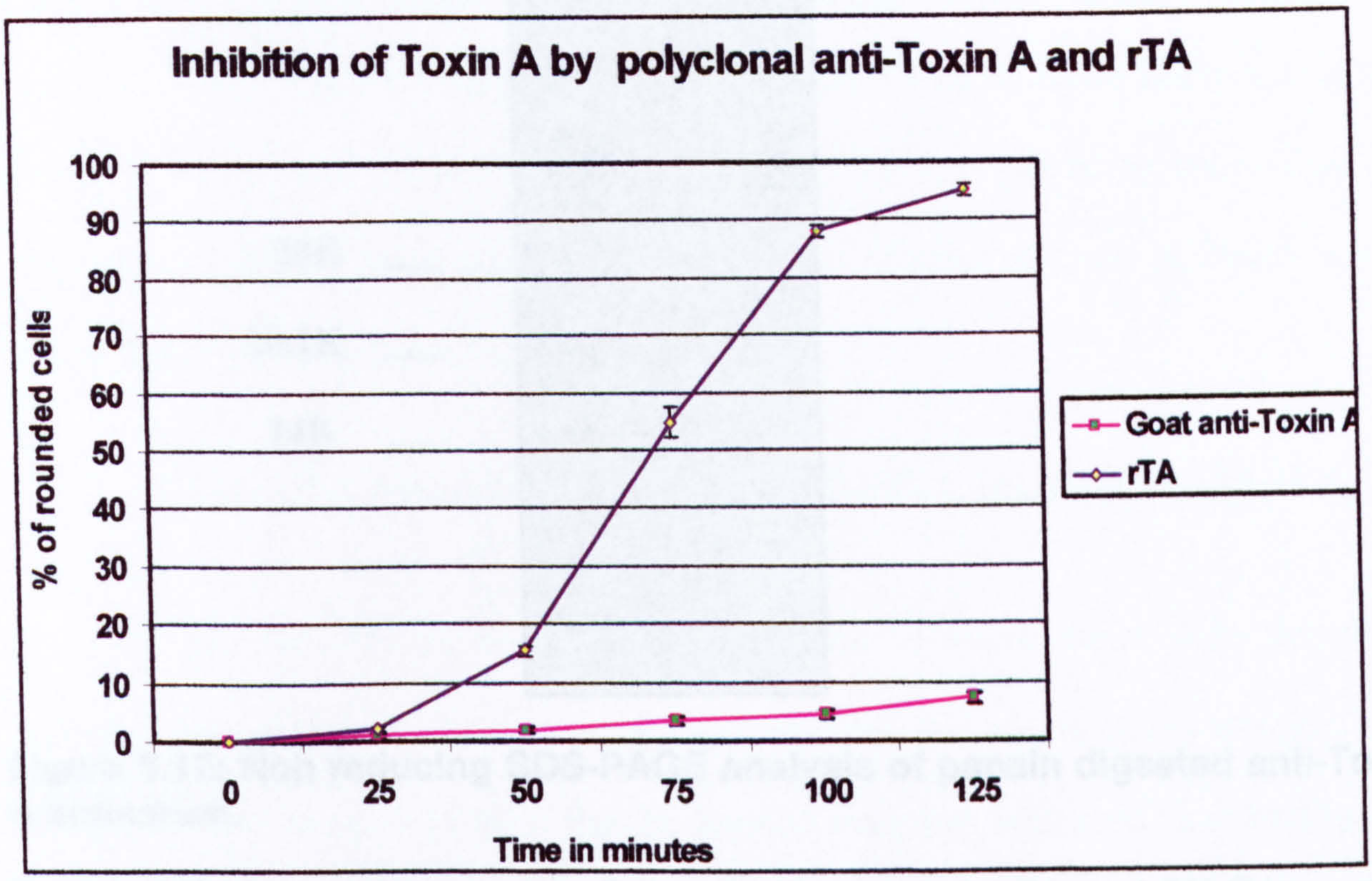

Figure 3.11: Inhibition of Toxin A by polyclonal anti-Toxin A and rTA

In a sterile microtitre plate, native Toxin A 10 ng/ml was mixed with 50 µl of (1:200) polyclonal anti-Toxin A or 50 µl of 200 µg/ml recombinant C-terminal domain (rTA) in PBS. After incubation for 30 min at room temperature, the mixture was transferred to a cell culture plate containing growing F9 cells in 150 µl of complete medium. The cells were incubated at 37° C in a 5 % CO_2 atmosphere for 2 hrs. The plate was observed every 25 min and the percentage of cells undergoing a rounding response was calculated.

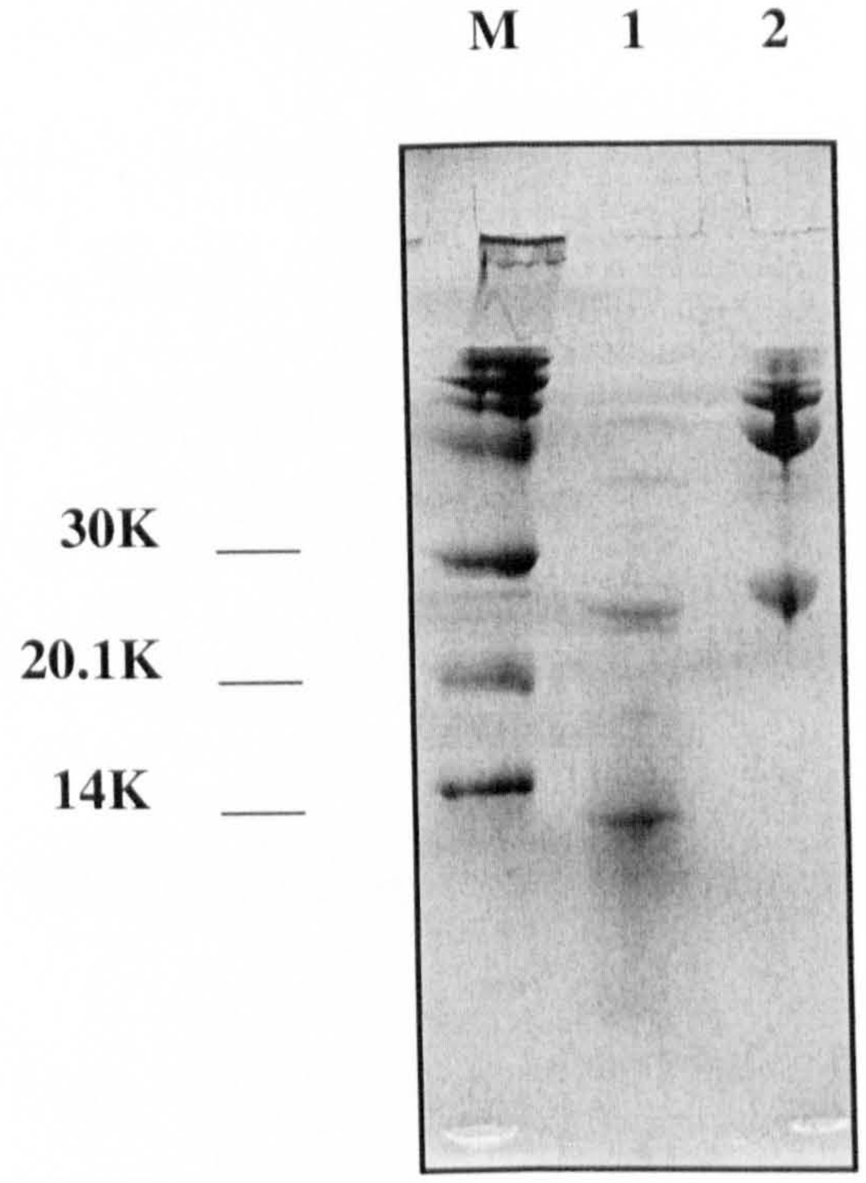

Figure 3.12: Non reducing SDS-PAGE analysis of papain digested anti-Toxin A antiserum.

Goat anti-Toxin A antiserum was digested with papain for 7 hrs at 30º C in a water bath and a sample was loaded to the gel (lane 1). Undigested goat anti-Toxin A was also analysed (lane 2). An equal volume of each sample was run on 12 % plyacrylamide gel and stained with Commassie Brilliant Blue. The migration of Rainbow protein molecular weight markers is shown in lane M.

3.11.2 Electroblotting

The identity of Fab fragments was determined by Western blotting. A non-reducing SDS gel was prepared as described earlier and proteins were transferred to a nitrocellulose membrane. The blot (Figure 3.13) was then probed with anti-goat IgG Fab fragments antiserum raised in rabbit (Bethyl Laboratories, Inc. USA). The blot showed an intensely staining high molecular weight band in untreated samples (lane 3). This was likely to be intact IgG. After digestion with papain for 4 hours (lane 1), traces of this material were still detectable. In contrast, digestion with papain for 7 hours removed all traces (lane 2), leaving only bands of lower molecular weight. Other proteins of lower molecular weight also reacted with the antibody.

3.11.3 Detection of native Toxin A by Fab fragment in ELISA

ELISA was used to assess if the recognition of native Toxin A by polyclonal antibody was retained after conversion to Fab fragments. Wells were coated with 100 μl of 20 μg/ml native Toxin A in PBS. Papain digested antiserum was added and binding was detected as described before. Untreated antiserum was tested for comparison. The data are shown in Figure 3.14. This experiment showed that both goat anti-Toxin A polyclonal antibody and its Fab fragments were reactive with the native Toxin A. However, slightly weaker signal was observed with the preparation of Fab fragments.

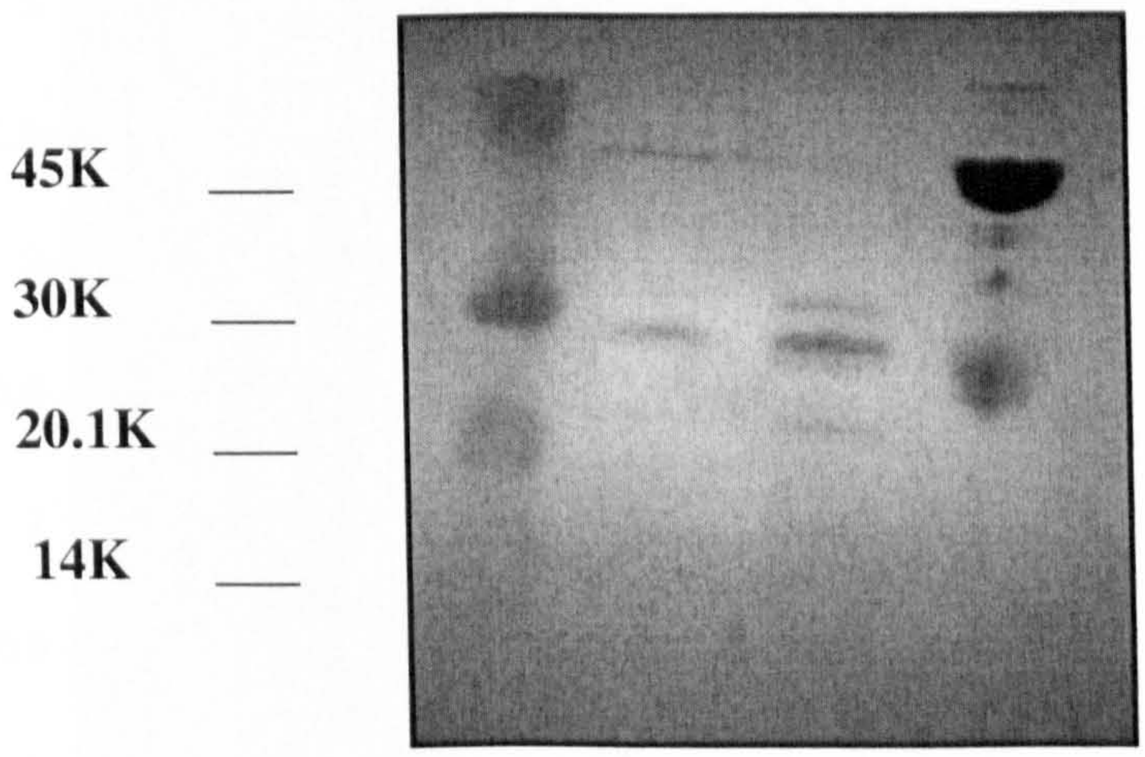

Figure 3.13: Western blotting analysis of papain digested anti Toxin A antiserum

Proteins from a non-reducing SDS gel were transferred to nitrocellulose membrane by Western blotting. The membrane was then probed with anti-Fab rabbit antiserum. Goat anti-Toxin A antiserum was digested with papain for 4 hrs (lane 1) or 7 hrs (lane 2) at 30º C. Undigested goat anti-Toxin A antiserum was also analysed (lane 3). Rainbow protein molecular weight markers are shown in lane M.

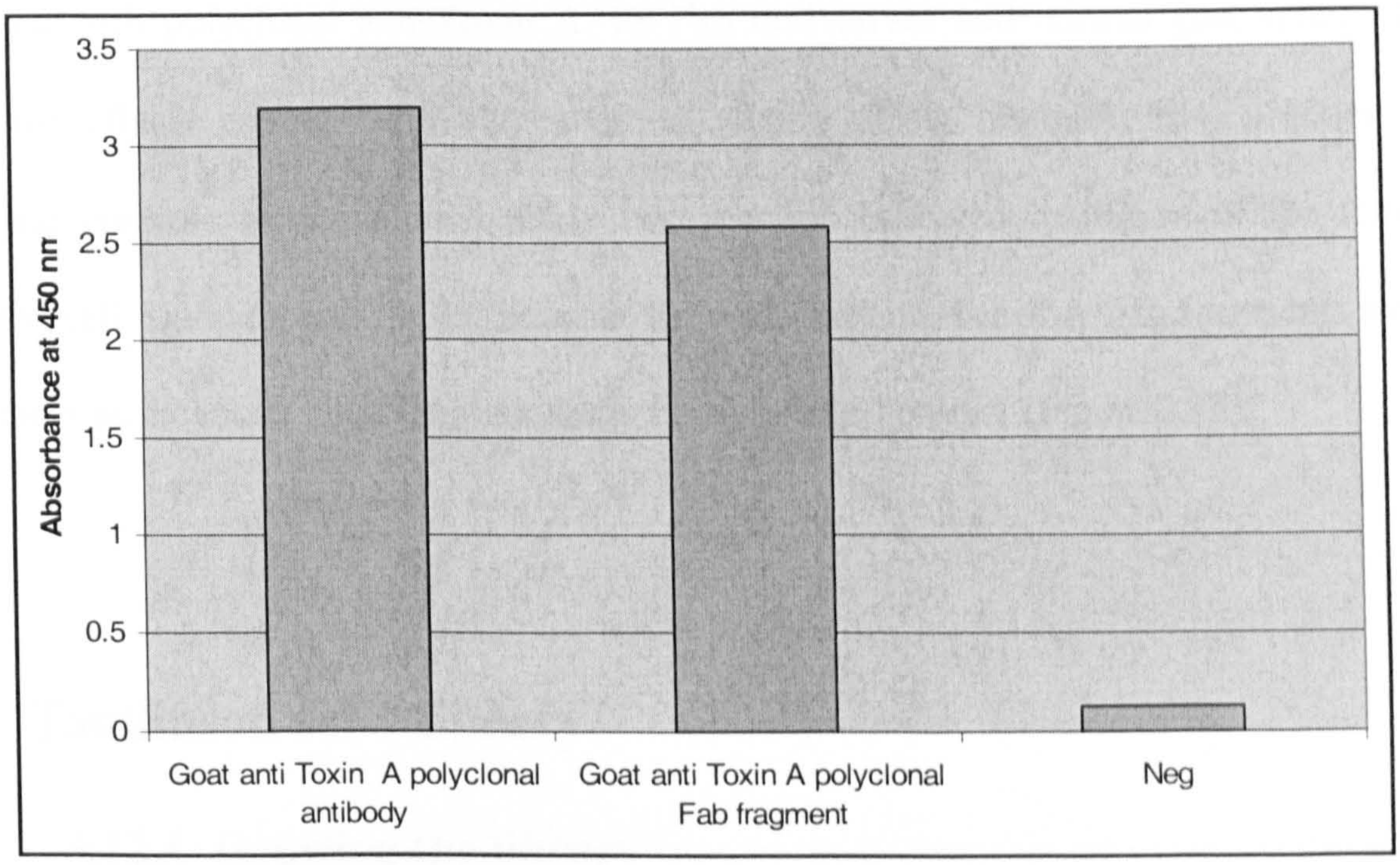

Figure 3.14: Reaction of intact IgG and Fab fragments with native Toxin A in ELISA

Native Toxin A was coated to an ELISA plate and probed with intact and papain digested anti-Toxin A antiserum. Binding was detected with an HRP-labeled antibody. Negative control wells were coated with 2 % MPBS. Samples were tested in duplicate and the mean value is presented.

3.11.4 Inhibition of native Toxin A with polyclonal Fab fragments *in vitro*

To assess the ability of polyclonal anti-Toxin A Fab fragments to inhibit the activity of native Toxin A, F9 cells were grown as described earlier and challenged with toxin-antibody mixtures. To ensure thorough control of the experiment, toxin was incubated with polyclonal anti-Toxin A, its Fab derivatives and normal goat serum. To check for effects arising from carry-over of papain or its inhibitor E64, a series of additional controls were included. Only two reactions showed inhibition of the toxin-mediated cell rounding and the experiment showed that anti-Toxin A Fab fragments were as efficient as the intact polyclonal antibody in inhibiting Toxin A (Figure 3.15).

3.12 Tomlinson scFv library

3.12.1 Growing the library

Library I in *E. coli* TG1 was grown according to the protocol provided with the materials and infected with KM13 helper phage. The resulting phage particles carrying scFv were precipitated by PEG/NaCl, diluted and used to infect exponentially growing TG1. After plating to selective medium, the colonies were counted and the titre of the phage library was estimated to be 5×10^{13} cfu per ml.

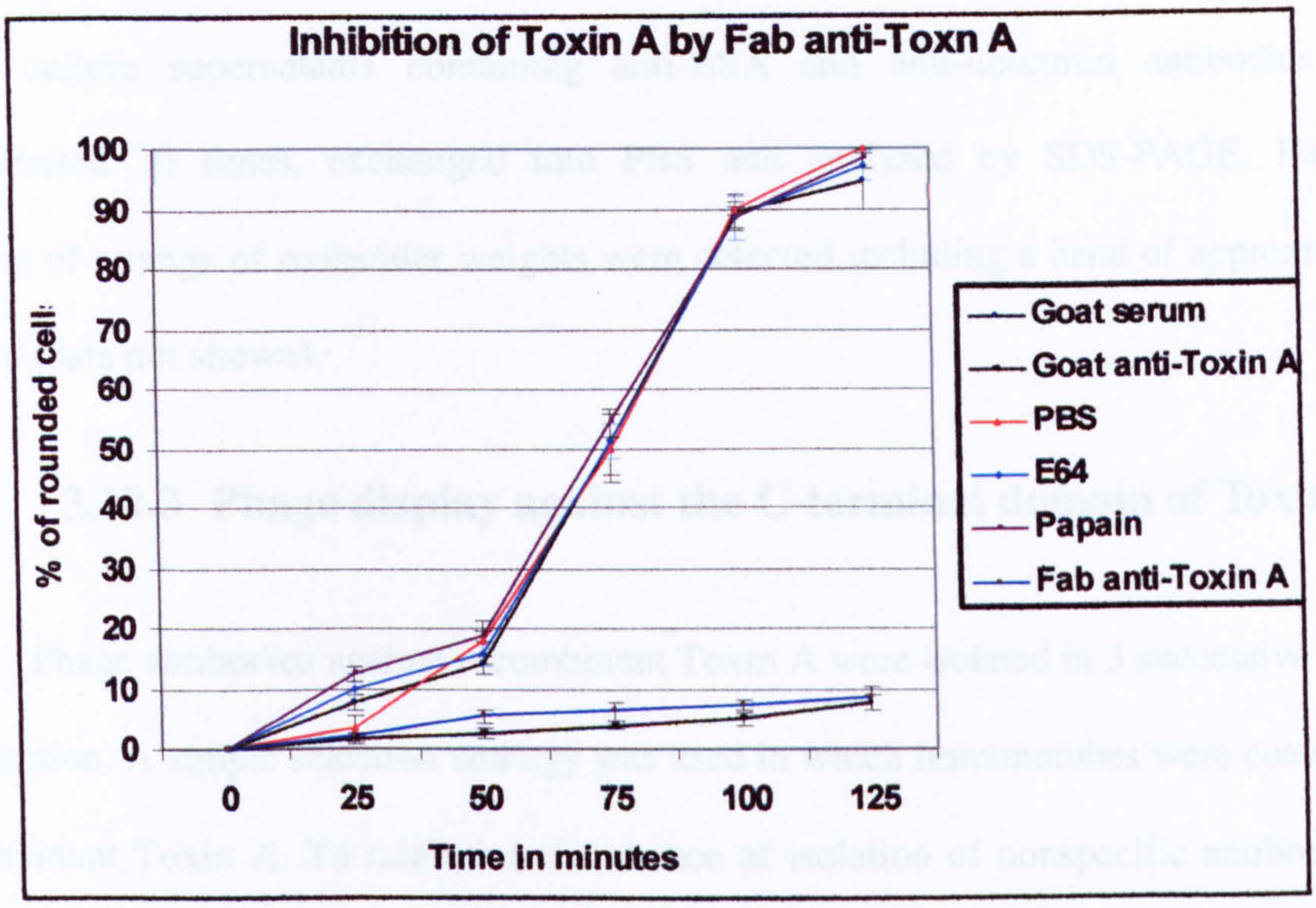

Figure 3.15: Inhibition of Toxin A by papain digested polyclonal anti-Toxin A antiserum

In a microtitre plate, 10 ng/ml of native Toxin A was mixed with 50 µl of (1:50) anti-Toxin A antiserum, the same value of the Fab fragments, components of the same proteolysis reaction or normal goat serum. The plate was then incubated for 30 min at room temperature. The mixtures were then transferred to a cell culture plate containing growing F9 cells in 150 µl complete medium. The cells were incubated at 37° C for 4 hrs. The plate was observed every 25 min and the proportion of cells that had undergone rounding was recorded.

3.12.2 Detection of anti-BSA and anti-ubiquitin scFvs by Western blot

The detection of scFv on a nitrocellulose membrane following Western blotting had been tested by others in the laboratory (Golchin, 2004) using anti-c-myc antibody, Protein L and Protein A. Anti-c-myc antibody was found to be superior for detection. In this study, culture supernatants containing anti-BSA and anti-ubiquitin antibodies were concentrated 20 times, exchanged into PBS and analysed by SDS-PAGE. Bacterial proteins of a range of molecular weights were detected including a band of approximately 29 kDa (data not shown).

3.12.3 Phage display against the C-terminal domain of Toxin A

Phage antibodies against recombinant Toxin A were isolated in 3 successive rounds of selection. A simple selection strategy was used in which immunotubes were coated with recombinant Toxin A. To minimize the chance of isolation of nonspecific antibodies the concentration target was decreased progressively in the second and third rounds of selection and an absorption step was included before each selection round by incubating the input phage with the blocking buffer (2% MPBS). For each round of selection, the input and output phage titers were measured and used to calculate the fraction of phage recovered at each stage. This is shown in Table 3.1.

Input and output of phage were calculated by titration. The % recovery shows the number of phage recovered from a selection step as a proportion of the input.

At round 1, a significant number of phage were recovered from the Toxin A-coated immunotube, rather more than is typical given the wide diversity of the Tomlinson library (estimated to be over 1×10^8 different specificities). Phage eluted from the tube with trypsin were amplified to form the input for round 2. Compared to initial selection, approximately equivalent number of phage were used for selection, but recovery increased

by about 1000-fold. At round 3, a very high number of phage were added to the selection. In absolute terms, the recovery at round 3 was roughly equivalent to that from round 2 but because of the size of the input, the percentage recovery appeared to fall to a value similar to that for round 1. To test whether Toxin A-specific scFvs were recovered from the selection, ELISA was carried out initially on the mixed population of virus, then on individual clones.

Table 3.1: Recoveries of phage during selection

	Tomlinson Library I		
Selection	Input [a]	Output [a]	% Recovery [b]
Round 1	1.36×10^{11}	2.28×10^{5}	1.67×10^{-4}
Round 2	3.4×10^{11}	4.44×10^{8}	1.3×10^{-1}
Round 3	2×10^{14}	2.16×10^{8}	1.08×10^{-4}

[a] Input and output phage titers were determined by transduction of *E. coli* to ampicillin resistance using serial dilutions of sample.
[b] Percent recovery was determined by division of output phage titre by input titer and multiplication by 100.

3.12.4 Screening polyclonal phage by ELISA

Phage antibody particles recovered from each round of selection were tested against the recombinant C-terminal domain of Toxin A in ELISA. The initial reaction of the library stock to Toxin A was low, and signal of equal strength was detected against a control target, BSA. Phage recovered from a single round of selection showed a higher response in ELISA, and this reaction was noticeably higher than against BSA. In parallel with the viral recoveries shown in Figure 3.16, a very large jump in signal strength resulted when phage from round 2 were tested, and the signal was sustained for virus from round 3 in spite of the apparent fall in percent recovery at the end of selection. Reactions against BSA remained steady and low for phage recovered throughout the screening.

3.12.5 Screening monoclonal phage by ELISA

TG1 colonies were picked up at random from titration plates from rounds 1, 2 and 3 and were inoculated to a 96-cell culture plate. Colonies were grown and infected with helper phage to prepare single phage stocks carrying scFv at the viral surface. Phage were then added to an ELISA plate that had been coated with recombinant Toxin A and blocked with 3 % BSA in PBS. The binding of phage was then detected with HRP-labeled antibody. Surprisingly, 31 % of phage antibodies from the unselected library showed some reaction with the antigen coated surface (Figure 3.17). After a single round of panning, this rose to 37 %. A sharp increase occurred after round 2 with 91 % and of phage binding to Toxin A. This rise was sustained after round 3 (96 %) in spite of the lower percentage recovery of phage at this stage in the process (Table 3.1). Signal strength in the ELISA was variable (Figure 3.17) but there was a trend towards stronger reaction with Toxin A at rounds 2 and 3. This and the leap in frequency of reaction suggested that enrichment for Toxin A-specific antibodies was taking place.

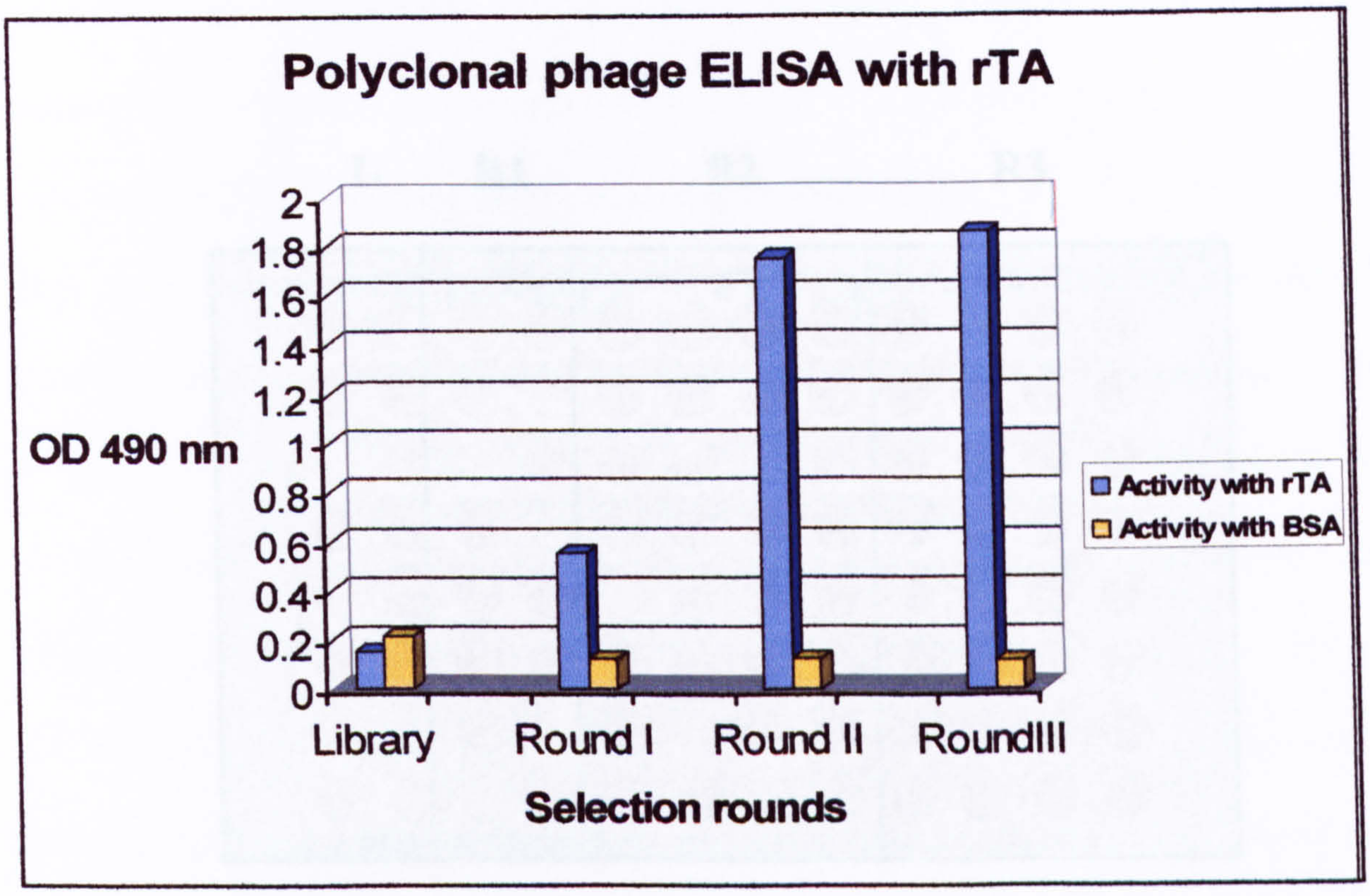

Figure 3.16: Polyclonal phage ELISA of Tomlinson library I during panning against recombinant Toxin A

Phage from the original library and recovered at each round of selection were tested in ELISA against purified recombinant Toxin A and 2 % BSA. Recognition was detected using monoclonal anti-M13-HRP conjugate. Phage samples were tested against each antigen in duplicate and the mean value is presented.

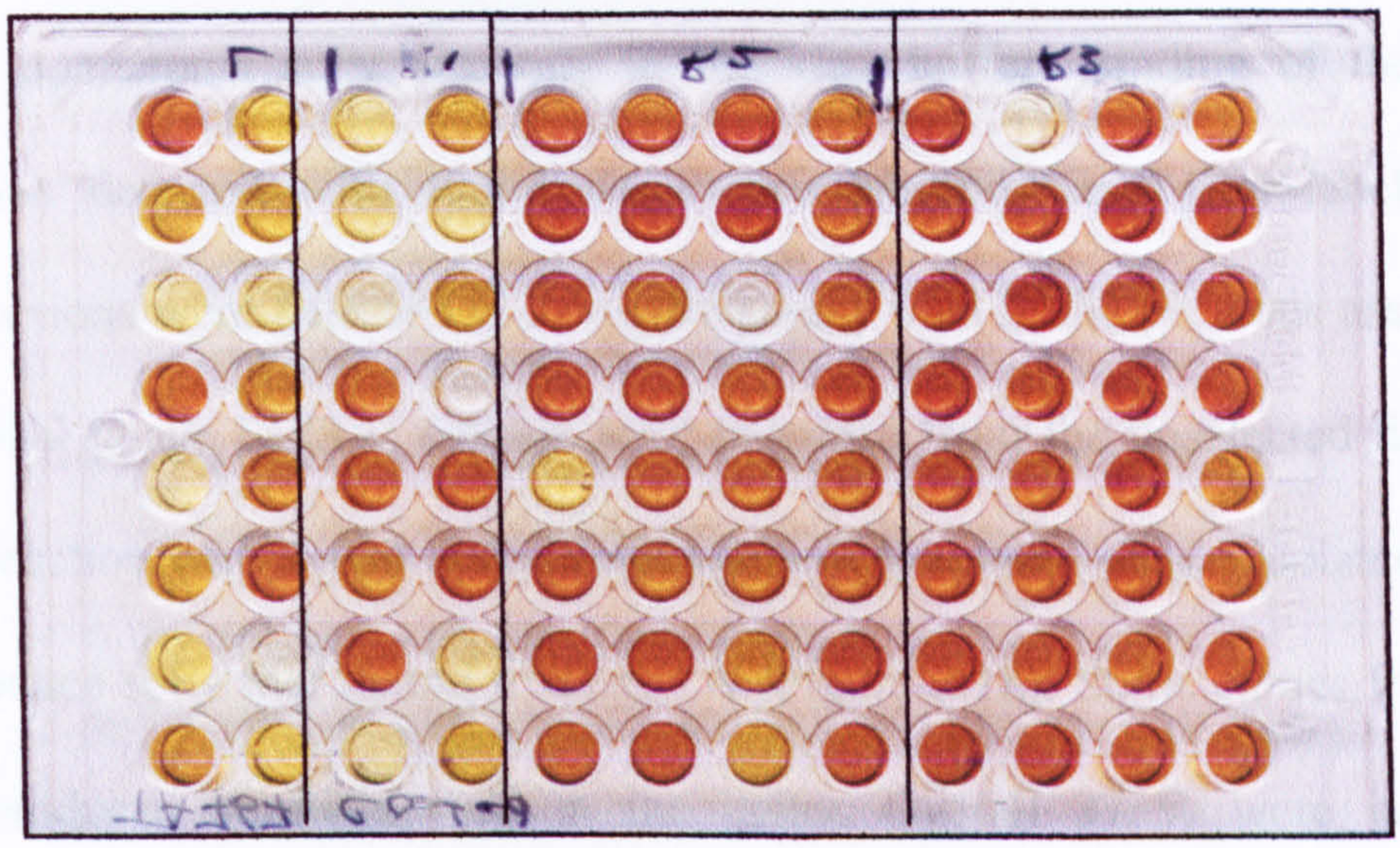

Figure 3.17: Monoclonal phage ELISA against recombinant Toxin A

Individual phage clones from the original library and each round of selection were tested in ELISA against purified recombinant Toxin A. Recognition was detected using an anti-M13-HRP conjugate.

Phage were applied as follow:

Section L: 16 clones picked at random from unselected library

Section R1: 16 clones picked at random after round 1 of selection

Section R2: 32 clones picked at random after round 2 of selection

Section R3: 32 clones picked at random after round 3 of selection

3.12.6 Screening monoclonal soluble scFv antibodies by ELISA

E. coli HB2151 were grown to an OD 600 nm of 0.4 and infected with phage eluted from each round of selection. Colonies were selected on TYE plates containing ampicillin, single colonies were picked from unselected library and rounds 1, 2 and 3 grown, and the production of soluble scFv was induced with IPTG. Samples of individual culture supernatant, were used for monoclonal ELISA. The ELISA plate was coated with purified recombinant Toxin A at 20 μg/ml and blocked with 3% BSA in PBS. Each well received 100 μl of monoclonal scFv antibody in blocking buffer. Binding of the recombinant antibody was detected with rabbit anti-c-myc antibody and HRP-labeled conjugate. Overall, reactions were detected at lower frequency than in the previous assay. In contrast to monoclonal phage ELISA, soluble scFv prepared from the unselected library failed to show any reaction with Toxin A (data not shown). Similarly, clones isolated after round 1 did not produce scFv that bound to Toxin A (Figure 3.18). Some clones from round 2 of selection produced strong signals in the assay. Overall 37 % were judged positive, indicating the successful enrichment of scFv at this round. Given the result in phage ELISA, it was surprising to find that the number of clones expressing reactive scFv in round 3 were very few.

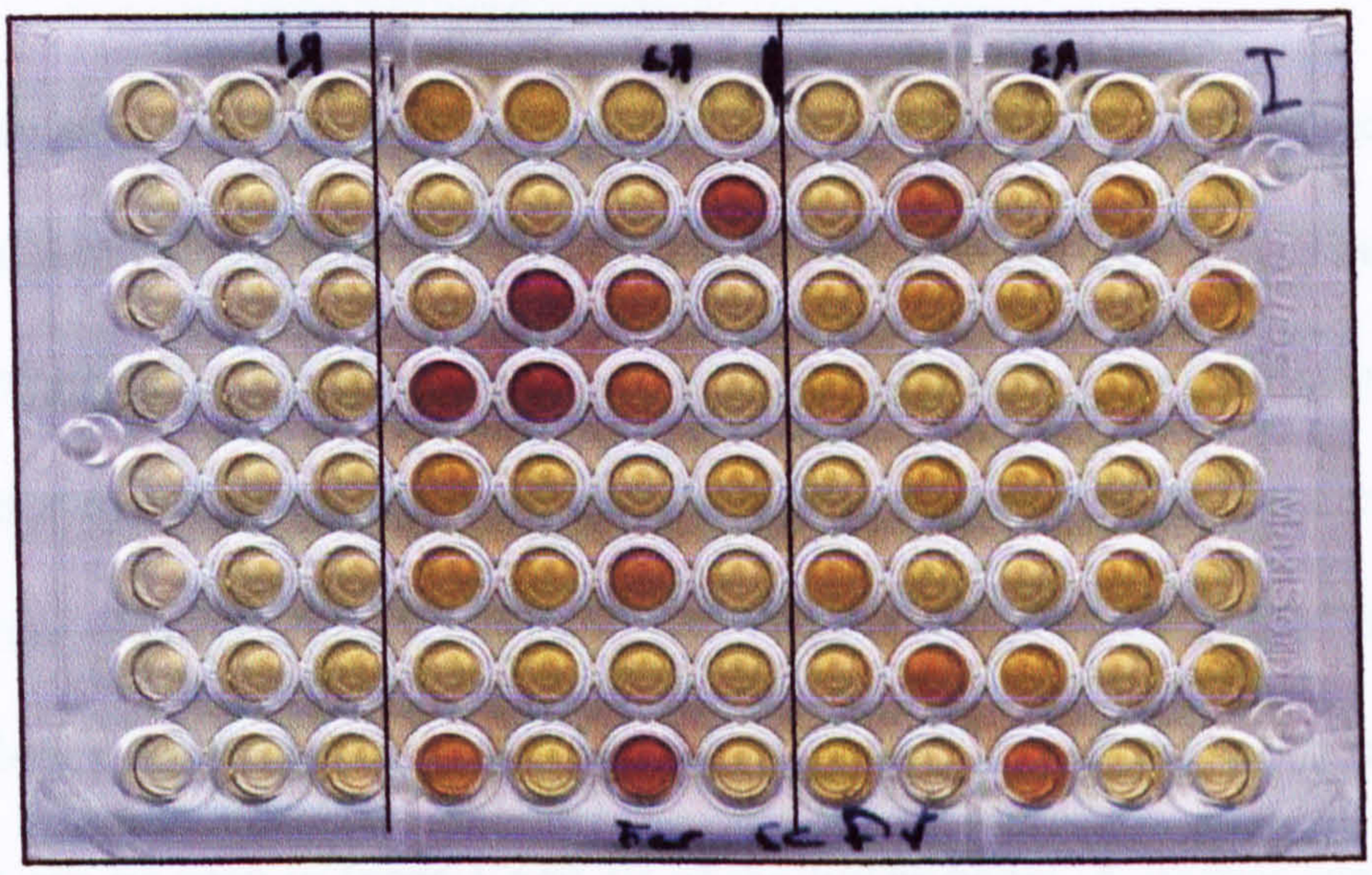

Figure 3.18: Monoclonal scFv ELISA against recombinant Toxin A

scFv prepared from colonies from the original library (data not shown) and each round of selection were tested in ELISA against purified recombinant Toxin A. scFv were detected by an anti-c-myc antibody and HRP-labeled antibody conjugate.

scFv were applied as following:

Section R1: 24 clones picked at random after round 1 of selection

Section R2: 32 clones picked at random after round 2 of selection

Section R3: 40 clones picked at random after round 3 of selection

3.13 Genetic characterization of selected scFvs

During library construction, some clones arise that lack V_H or V_L components of the scFv reading frame. Bacteria that carry these short inserts can grow more quickly than these with full scFvs and if the antibodies possess affinity for the target, they can emerge from selection. Therefore, genetic characterization was used to verify the size and integrity of the inserts.

Twenty selected clones of *E. coli* HB2151 were picked from round 2 and 3 based upon their signal in monoclonal ELISA against recombinant Toxin A. The 20 clones were grown, plasmid DNA was prepared, and this was digested with restriction enzymes *Nco*I and *Not*I, enzymes that excise the full scFv sequence from pIT2 (Figure 3.21). Reaction mixtures were electrophoresed on an agarose gel. Figure 3.19 revealed that 19 out of 21 (90.4 %) clones contained the full-length scFv genes. Two clones contained smaller scFv genes. This can be seen in lanes 1 and 10 of the lower panel. A further analysis is presented in Figure 3.20, confirming the presence of full-length inserts in scFvs against Toxin A.

3.14 Sequence of selected clones

To predict amino acid sequence, diversity and integrity of seven selected clones, sequencing was performed as described earlier. The sequence results were initially checked for the presence of a complete V_H-linker-V_L sequence. This is illustrated for clones 2D4, 2D5 and 2E4 in Figures 3.22 and 3.23. Since Tomlinson library I is based on single human V_H and V_L frameworks, the sequences are identical for the most part. At construction, diversity was confined to the heavy chain complementarity determining regions CDR2 (residues H50, H52, H52a, H53, H55, H56, H58), and CDR3 (H95, H96, H97, H98) for the heavy chain component, and CDR2 (L50, L53) and CDR3 (L91 L92, L93, L94, L96) for the light chain component. The data indicate diversity of the selected clones in these regions. The predicted amino acid sequences of the CDRs for more clones are presented in

Table 25. Two clones showed identical amino acid sequences in most of CDR2 of the heavy chain (B9 and C5) (Table 25) but carried quite different light chain CDRs. Overall, sequencing showed a high diversity among the selected clones.

3.15 Detection of native Toxin A by selected scFv

To assess the binding activity of four of the selected scFv against native Toxin A, native Toxin A was diluted across 96-well ELISA and blocked with 3 % milk in PBS. Equal concentrations of the purified scFvs were added to each well across the plate. scFv were then detected with anti-c-myc antibody. Figure 3.25 shows that the scFvs chosen for analysis recognized recombinant Toxin A across 5 serial dilutions and were therefore able to recognize toxin from 2 μg/well down to 62 ng/well. Beyond this lower limit, reactivity was indistinguishable from background. Polyclonal anti-Toxin A antibody was substantially more sensitive as previously demonstrated. This reagent was able to detect coating concentrations of toxin at less than 48 ng/well. scFv anti-BSA antibody showed very low reactivity with Toxin A, confirming the specificity of the assay.

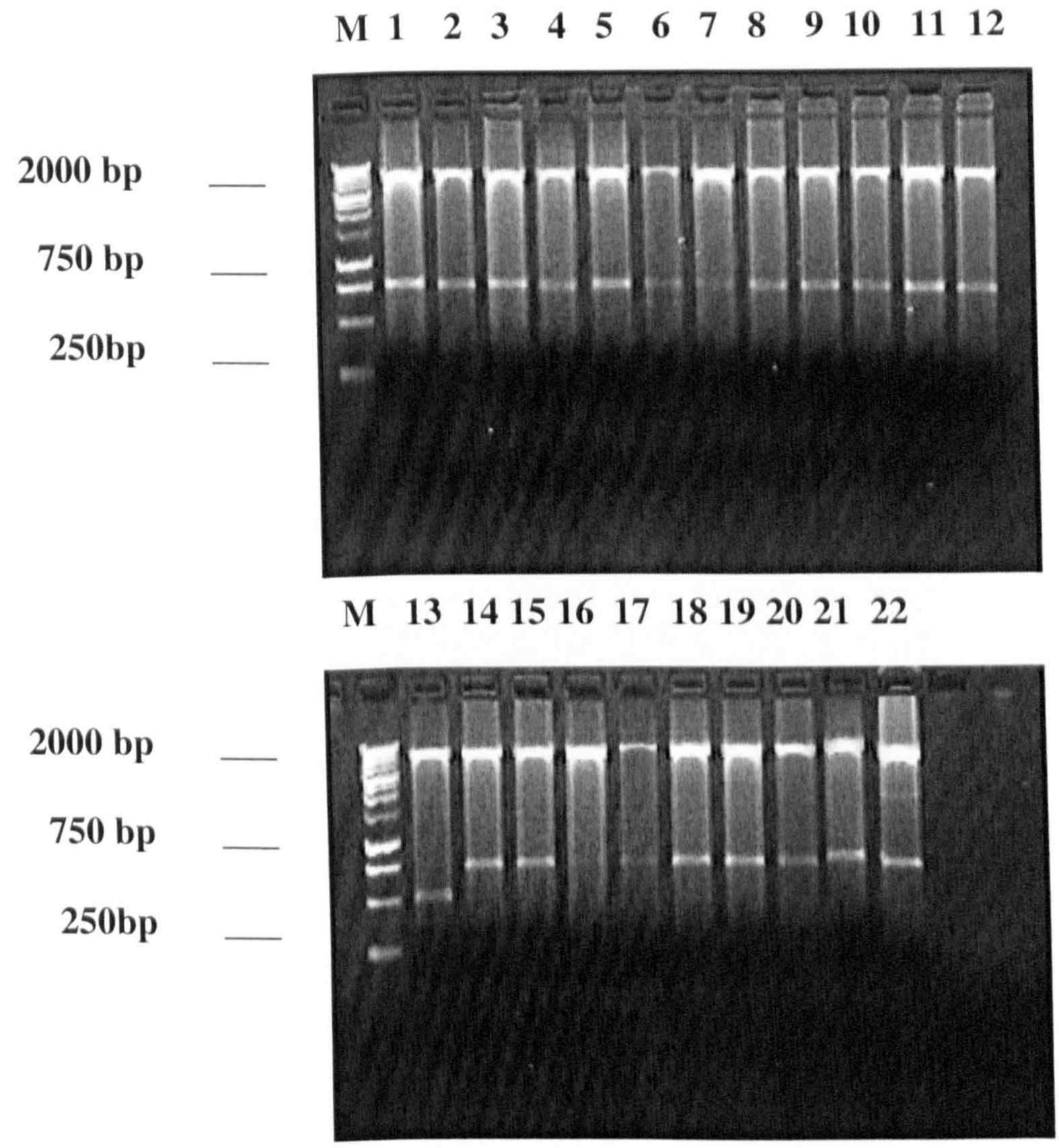

Figure 3.19: Restriction analysis of clones from Tomlinson library I

Twenty one clones were chosen randomly from Tomlinson library I. Plasmid DNA from each clone was digested with *Nco* I and *Not* I. The expected size of the small fragment released is 708 bp.

M: 1 kb DNA Ladder (Promega, USA)

Control 1: positive control anti-BSA scFv.

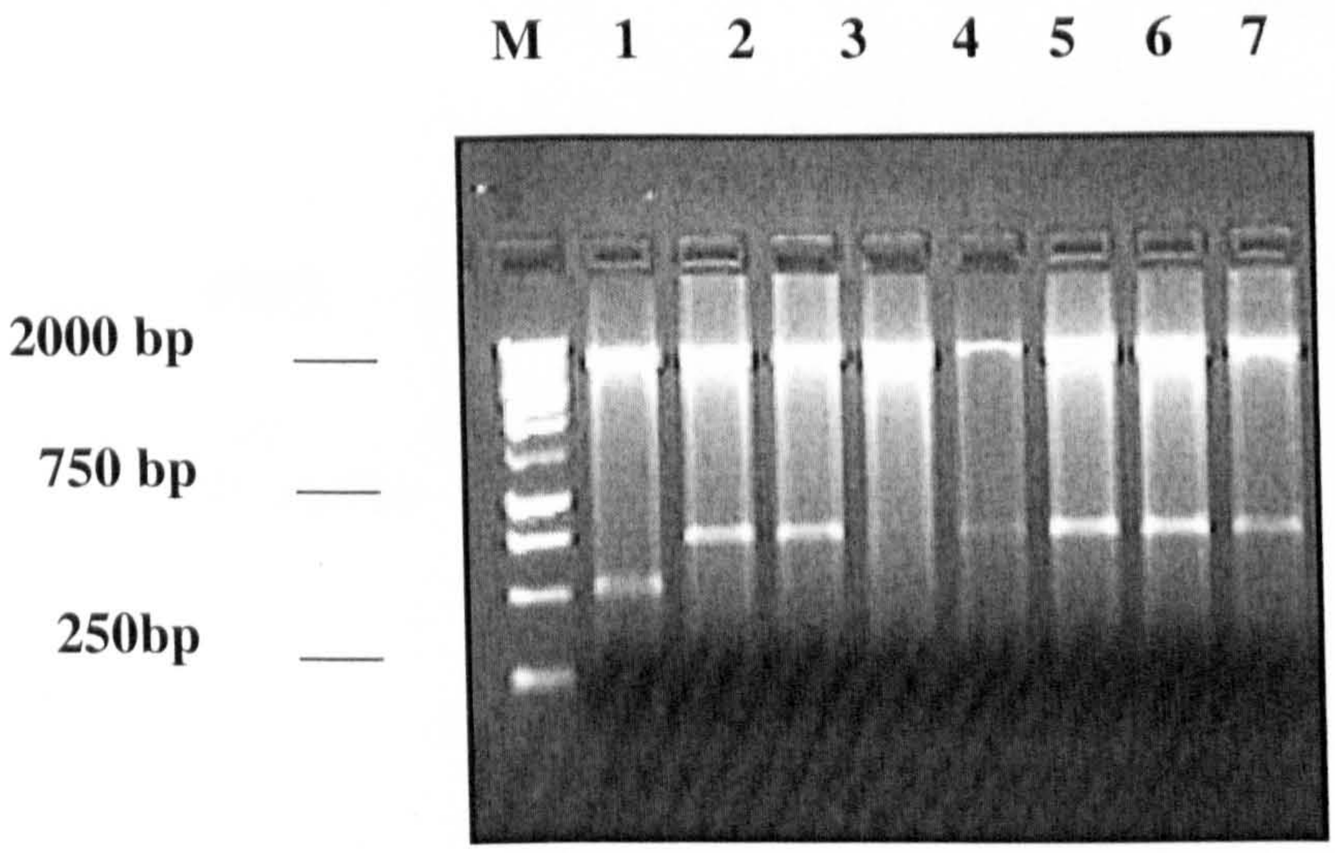

Figure 3.20: Restriction analysis of 7 anti-Toxin A Tomlinson library I

Seven clones were chosen after screening of the Tomlinson library I on the basis of their reaction to recombinant Toxin A in ELISA of soluble scFv. Plasmid DNA from each clone was digested with *Nco*I and *Not*I. The expected size of the small fragment release is 708 bp

M: 1 kb DNA Ladder (Promega, USA)

Lane 1: A negative sample from the previous experiment was used in this experiment as a negative control.

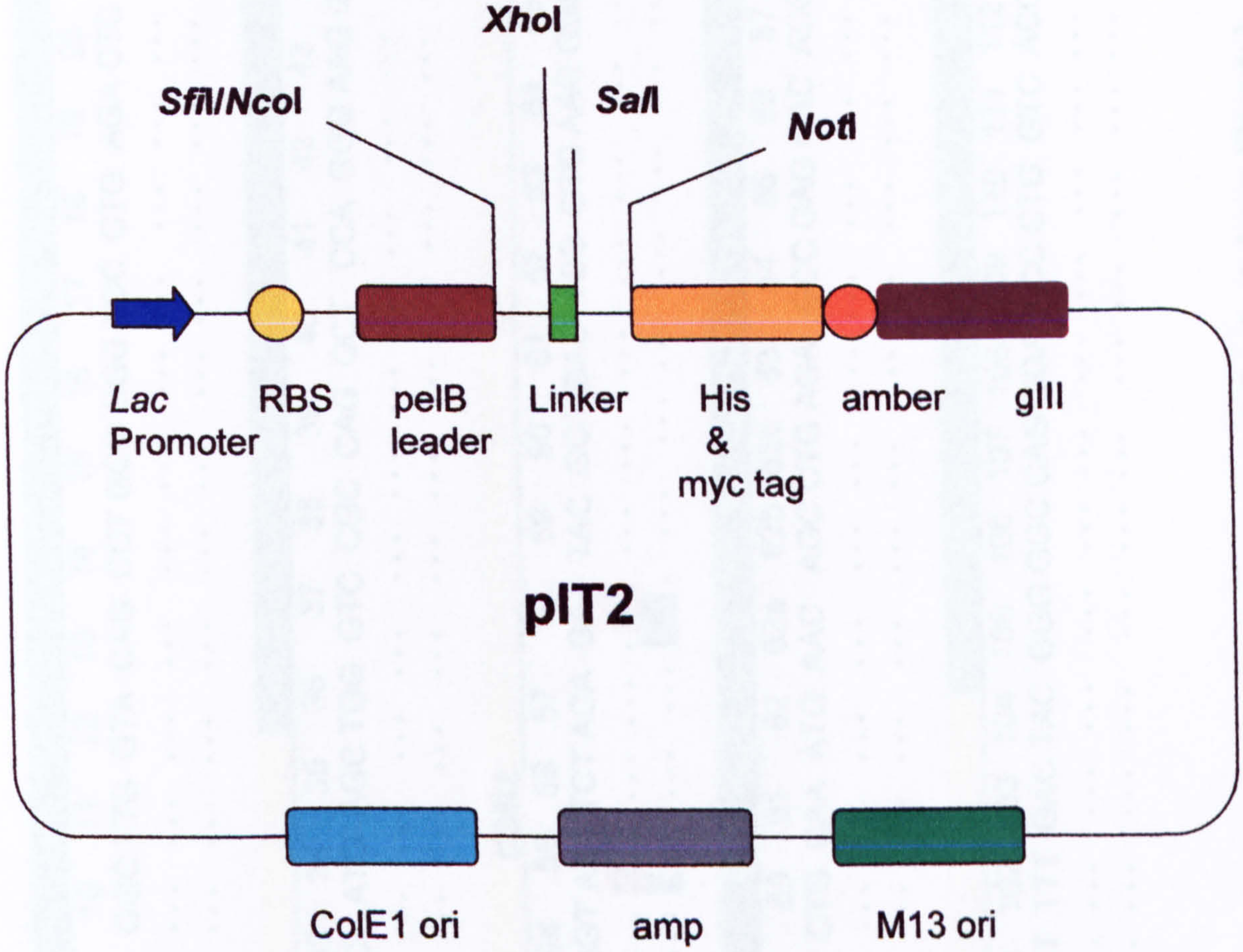

Figure 3.21: Structure of phagemid pIT2

V_H inserts are cloned between *Nco*I and *Xho*I sites, whereas V_L inserts lie between *Sal*I and *Not*I sites. Digestion with *Nco*I and *Not*I therefore excises the complete scFv insert.

FR1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
2D4-	GAG	GTG	CAG	CTG	TTG	GAG	TCT	GGG	GGA	GGC	TTG	GTA	CAG	CCT	GGG	GGG	TCC	CTG	AGA	CTC	TCC	TGT	GCA	GCC	TCT
2D5-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2E4-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

CDR1 FR2

	26	27	28	29	30	31	31a	31b	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
2D4-	GGA	TTC	ACC	TTT	AGC	AGC	...	...	TAT	GCC	ATG	AGC	TGG	GTC	CGC	CAG	GCT	CCA	GGG	AAG	GGG	CTG	GAG	TGG
2D5-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2E4-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

CDR2

	48	49	50	51	52	52a	52b	52c	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
2D4-	GTC	TCA	GGT	ATT	AAT	TCT	...	...	AAT	GGT	ACT	TCT	ACA	GAT	TAC	GCA	GAC	TCC	GTG	AAG	GGC	CGG	TTC	ACC	ATC	TCC
2D5	...	...	A..	...	GG.	G..	...	...	GC.	...	G..	...	...	.CC	...	...	...	...	...	...	...	...	...	...	...	...
2E4-	...	...	AA.	...	TC.	AA.	...	...	TC.	...	T..	...	...	GG.	...	...	...	...	...	...	...	...	...	...	...	...

FR3

	71	72	73	74	75	76	77	78	79	80	81	82	82a	82b	82c	83	84	85	86	87	88	89	90	91	92
2D4-	AGA	GAC	AAT	TCC	AAG	AAC	ACG	CTG	TAT	CTG	CAA	ATG	AAC	AGC	CTG	AGA	GCC	GAG	GAC	ACG	GCC	GTA	TAT	TAC	TGT
2D5-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2E4-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

CDR3 FR4

	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113
2D4-	GCG	AAA	TGT	GCG	AAA	AGT	AGT	GCT	ACT	TTT	GAC	TAC	GGG	GGC	CAG	GGA	ACC	CTG	GTC	ACC	GTC
2D5-	...	...	...	...	...	...	.C.	TA.	..G	...	...	...	...	...	...	...	...	...	...	...	...
2E4-	...	...	...	...	...	GC.	TC.	.A.	TT.	...	...	...	...	...	...	...	...	...	...	...	...

Figure 3. 22: complete nucleic acid sequence of 3 selected clones of anti-Toxin A from library I - Heavy chain

FR1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
2D4-	GAC	ATC	CAG	ATG	ACC	CAG	TCT	CCA	TCC	TCC	CTG	TCT	GCA	TCT	GTA	GGA	GAC	AGA	GTC	ACC	ATC	ACT	TGC	CGG	GCA
2D5-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2E4-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

CDR1 FR2

	26	27	28	29	30	31	31a	31b	31c	31d	31e	31f	32	33	34	35	36	37	38	39	40	41	42	43	44
2D4-	CAG	AGC	ATT	ATT	AGC	AGC	...	...	...	...	...	...	TAT	TTA	AAT	TGG	TAT	CAG	CAG	AAA	CCA	GGG	AAA	GCC	CCT
2D5-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2E4-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

CDR2

	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
2D4-	AAG	CTC	CTG	ATC	TAT	TCT	GCA	TCC	AAT	TTG	CAA	AGT	GGG	GTC	CCA	TCA	AGG	TTC	AGT	GGC	AGT	GGA	TCT	GGG	ACA	GAT
2D5 -	...	...	...	...	...	GG	.	...	...	GG	.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2E4-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

FR3

	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92
2D4-	TTC	ACT	CTC	ACC	ATC	AGC	AGT	CTG	CAA	CCT	GAA	GAT	TTT	GCA	ACT	TAC	TAC	TGT	CAA	CAG	ACT	TGT
2D5-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2E4-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

CDR3 FR4

	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
2D4-	CAA	CAG	ACT	ACT	ACT	AGT	CCT	TCT	ACG	TTC	GGC	CAA	GGG	ACC	AAG	GTG	GAA	ATC	AAA	CGG
2D5-	...	...	...	...	...	C	.	...	AG	.	...	...	...	...	...	...	...	...	...	...
2E4-	...	...	...	...	...	C	.	...	...	...	...	...	...	...	...	...	...	...	...	...

Figure 3.23: complete nucleic acid sequence of 3 selected clones of anti-Toxin A from library I - Light chain

	V_H CDR2							V_H CDR3				V_L CDR2			V_L CDR3			
Clone	H 50	H 52	H 52a	H 53	H 55	H 56	H 58	H 95	H 96	H 97	H 98	L 50	L 53	L 91	L 92	L 93	L 94	L 96
D6	G	A	T	S	A	T	A	A	N	G	N	T	N	T	T	T	S	S
E4	N	S	N	S	S	S	G	A	D	S	D	S	N	T	T	T	T	S
D5	S	G	A	A	A	S	A	S	T	Y	S	G	G	T	T	T	T	S
D4	G	N	S	N	T	S	D	S	S	A	T	S	N	T	T	T	S	S
G9	T	A	G	T	G	G	S	G	Y	Y	G	N	A	S	T	S	S	S
C5	Y	N	N	D	D	N	S	N	L	L	Y	S	S	S	G	T	A	Y
B9	Y	N	N	D	D	N	A	A	N	G	N	D	S	T	T	T	C	S
αBSA	T	Y	Y	A	S	N	Y	G	Y	Y	T	Y	N	S	D	T	S	T

Figure 3.24: Amino acid sequence diversity of 7 selected clones of anti-Toxin A scFvs from library I

3.16 Haemagglutination inhibition

To assess if the scFvs chosen for analysis were able to inhibit the attachment of native Toxin A to rabbit erythrocytes, evidence of inhibition of haemagglutination was sought. scFv antibodies at a fixed concentration were mixed with serial dilutions of toxin and after incubation, a 2 % suspension of rabbit erythrocytes was added. The plate was monitored for 2 hrs. Surprisingly, there was no significant inhibition of haemagglutination with any of the selected scFvs, nor was inhibition observed with the control polyclonal antibody. This assay was repeated in several different ways (with different buffer concentrations, different buffer pH, different components, different incubation temperatures and different length of incubations) but the original result was observed consistently.

3.17 Haemolysis assay

As an alternative to haemagglutination, haemolysis was adopted as a more sensitive test for the inhibition of recombinant Toxin A binding to rabbit erythrocytes. To perform the test, fixed amounts of recombinant Toxin A was coated to an ELISA plate and serial dilutions of the scFvs under test were added. An anti-BSA scFv served as a negative control, with polyclonal anti-Toxin A as a positive control. After allowing interaction, rabbit erythrocytes, were added, washed and attached cells were lysed. The data are shown in Figure 3.26. This assay showed an improvement in the positive control compared to the haemagglutination assay.

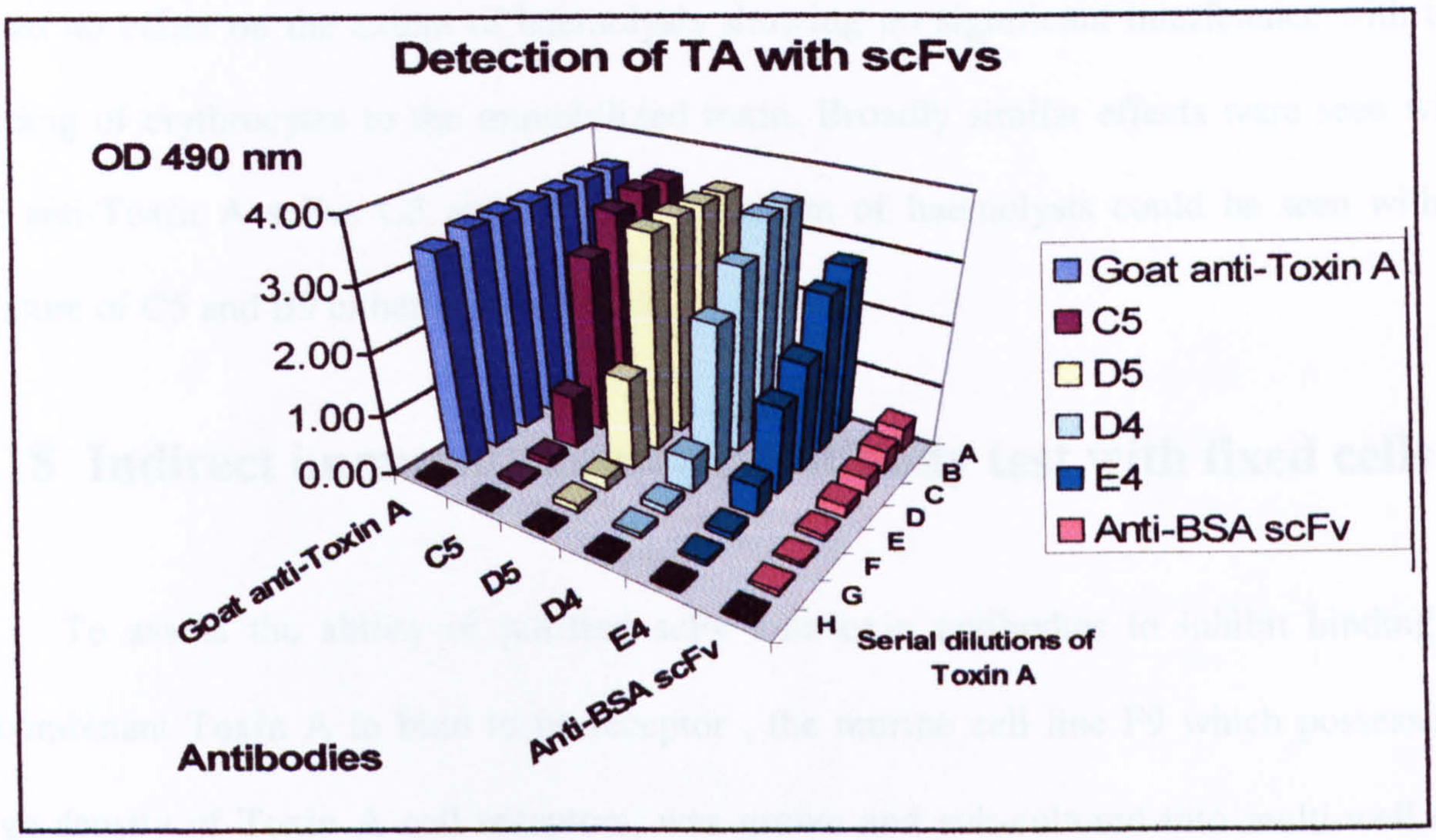

Figure 3.25: Reactivity of scFvs with native Toxin A in ELISA

Native Toxin A was serially diluted and coated to an ELISA plate (A to G). Each well received monoclonal anti-Toxin A scFvs as indicated by the clone designation, or polyclonal goat antibody to the toxin, or a scFv against BSA. Row H was coated only with blocking reagent. After washing, wells were probed with anti c-myc and HRP-conjugated reagent. Positive control wells were probed with anti-goat reagents. Samples were tested in duplicate and the mean value is presented.

As the polyclonal anti-toxin antibody was diluted (A to F), increasing absorbance values were obtained. This indicated that more erythrocytes could bind to the toxin-coated surface as the amount of antibody fell. A reverse profile was seen with Toxin A at progressively lower concentrations (A to F; Figure 3.26). When an anti-BSA scFv was used in the assay, it had no effect on the extent of haemolysis showing no significant interference with the binding of erythrocytes to the immobilized toxin. Broadly similar effects were seen with the anti-Toxin A scFvs C5 and B9. No inhibition of haemolysis could be seen with a mixture of C5 and B9 either (Figure 3.26).

3.18 Indirect immunofluorescent antibody test with fixed cells

To assess the ability of purified scFv anti-toxin antibodies to inhibit binding of recombinant Toxin A to bind to its receptor , the murine cell line F9 which possesses a high density of Toxin A cell receptors, was grown and sub-cultured into multi-well cell culture slides. These allowed experimental conditions to be varied conveniently and cells to be examined by microscopy to assess effects. Cells were fixed and mixtures of native Toxin A and purified anti-Toxin A scFvs antibodies were added. The binding of the toxin to cellular receptors was assessed using polyclonal antibody and FITC reagents. In Figure 3.27, panel A shows a bright florescence visible at the cell surface indicated that toxin successfully bound to receptor after mixing with anti-BSA scFv, and could be detected at this location with high sensitivity. In contrast, when Toxin A was pre-incubated with polyclonal anti-Toxin A, panel B shows no sign of toxin binding. Panels C and D show the outcome of experiments in which scFvs D4 and D5 were used, respectively. A level between that seen in panels A and B suggested that toxin was bound to receptor at an intermediate level.

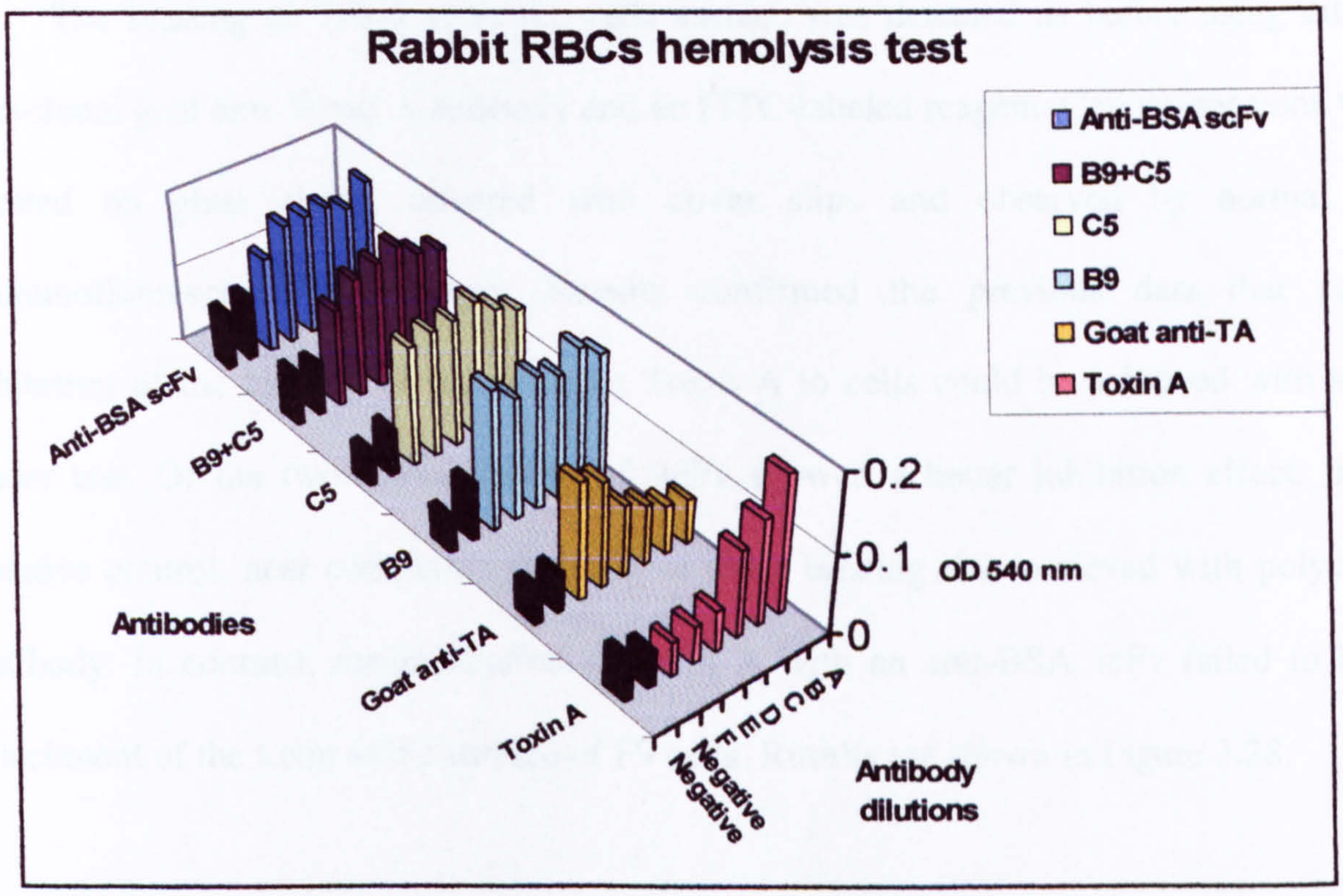

Figure 3.26: Haemolysis test

Most wells were coated with an amount of recombinant Toxin A and blocked with 3 % BSA. scFvs and polyclonal anti-Toxin A as positive control were serially diluted (A to F) and added as indicated. After incubation and washing, rabbit erythrocytes were added to the wells and the plate was incubated at a cold temperature. The wells were then washed and the attached cells were lysed. Absorbance values were measured at 540 nm. In wells designated "Toxin A", the coating concentration of toxin was serially diluted and no antibodies were added. In wells designated "negative", no Toxin A was coated to the plate.

3.19 Indirect immunofluorescent antibody test with suspended cells

As an alternative to the immunofluorescent antibody assay with slide-fixed cells, F9 cells were used in suspension. The antibody-antigen mixtures were incubated at 37° C before the addition of F9 cells and further incubation at 4° C.

The binding of Toxin A to the cells surface was detected as before using diluted polyclonal goat anti-Toxin A antibody and an FITC-labeled reagent. Cell suspensions were spotted on glass slides, covered with cover slips and observed by normal and immunofluorescence microscopy. Results confirmed the previous data that partial inhibition of the binding of recombinant Toxin A to cells could be achieved with scFvs under test. Of the two scFvs tested, D4 scFv showed a better inhibition effect. In the positive control, near complete inhibition of toxin binding was achieved with polyclonal antibody. In contrast, pre-incubation of toxin A with an anti-BSA scFv failed to block attachment of the toxin to the surface of F9 cells. Results are shown in Figure 3.28.

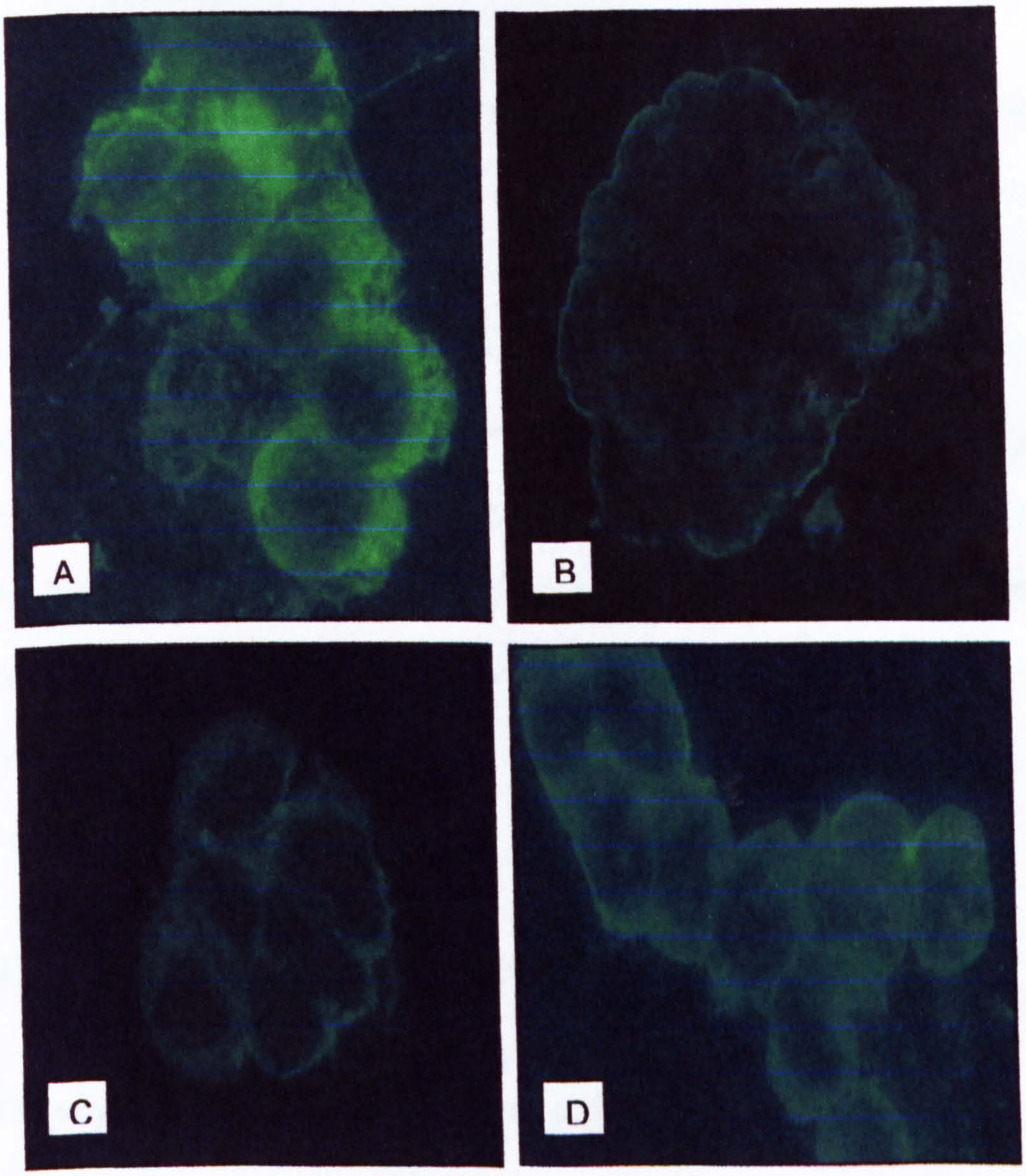

Figure 3.27: Immunofluorescence assay binding activity of Toxin A to F9 cells.

Recombinant Toxin A was pre-incubated with purified scFvs diluted polyclonal anti-Toxin A antibody (positive control) or purified anti-BSA scFv (negative control). The mixtures were then added to paraformaldehyde-fixed F9 cells and the binding of toxin was visualized by immuno-cytochemistry. Antibodies chosen for pre-incubation with toxin were scFv anti-BSA (A); polyclonal anti-Toxin A (B); scFv D4 (C); scFv D5 (D).

3.20 Characterization of selected scFvs

3.20.1 SDS-polyacrylamide gel electrophoresis and Western blotting

Western blotting was used to assess the specificity of selected scFv and their ability to detect Toxin A after treatment with SDS and heat. A crude bacterial lysate from *E. coli* pET767 was separated on an SDS-polyacrylamide gel and blotted to a nitrocellulose membrane. The blot was divided into a set of strips and each was probed with an HB2151 culture supernatant containing the scFv chosen for analysis. Binding of scFvs to the blot was detected with monoclonal anti-c-myc and HRP-labeled reagent. The results are shown in Figure 3.29. All the scFvs selected for analysis were reactive with heat-treated recombinant Toxin A except scFv H10. As the blots also carried many *E. coli* proteins present in the lysate, the Figure indicates the specificity of the scFvs for the recombinant, carboxy-terminal domain of Toxin A.

3.21 Expression levels of selected scFvs

SDS polyacrylamide gel electrophoresis and Western blotting was used to assess the expression of selected scFvs. *E. coli* HB2151 cultures for each antibody were grown in 100 ml of medium, induced, and the culture supernatant was concentrated 25 fold. The buffer was also exchanged with PBS. After boiling in buffer, aliquots were loaded to the lanes of duplicate polyacrylamide gels. After separation, one gel was stained for protein visualization and proteins from the second gel were transferred to nitrocellulose membrane. After blocking, the membrane was probed with monoclonal anti-c-myc and HRP-labeled reagent.

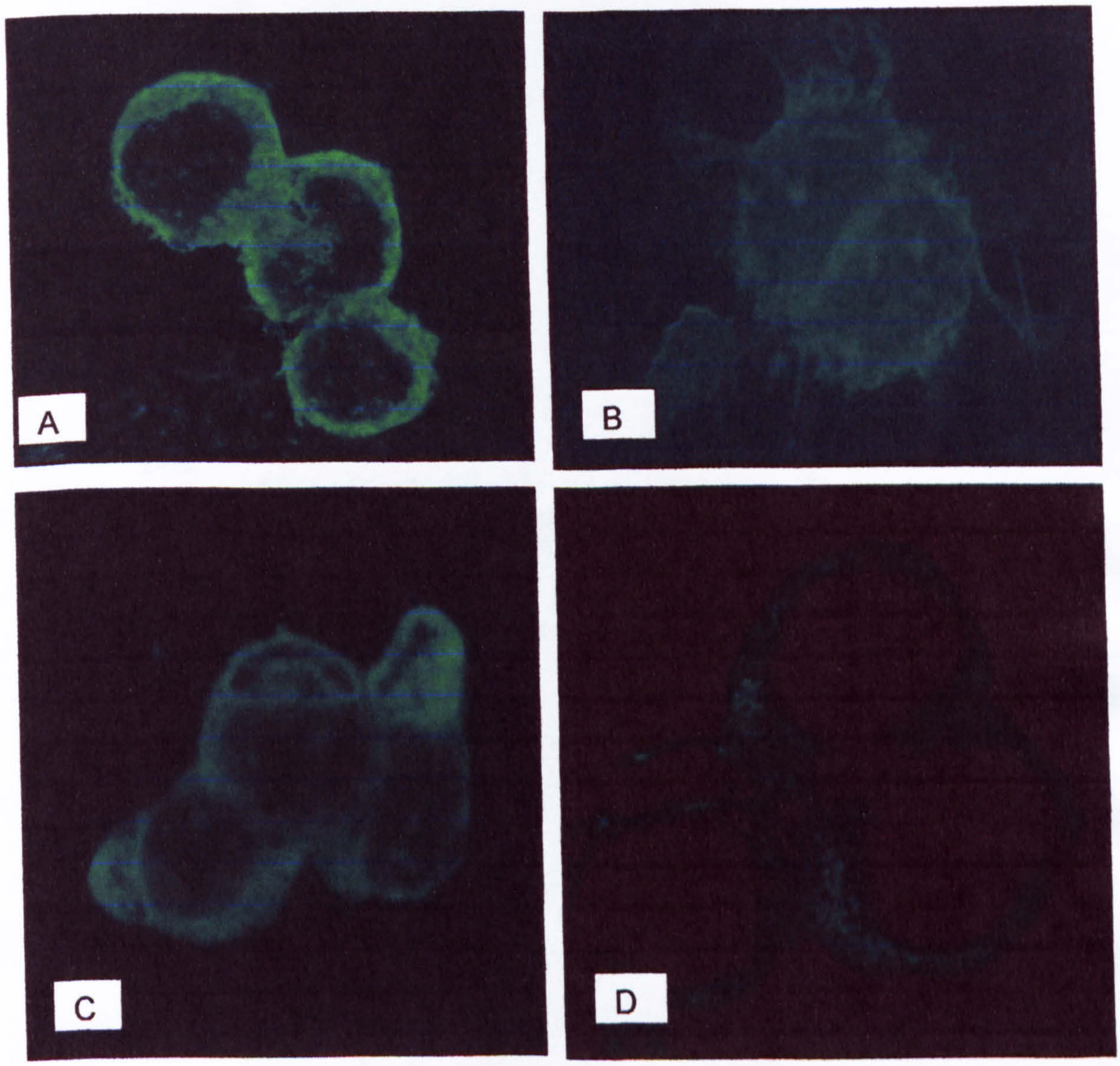

Figure 3.28: Immunofluorescence assay for binding activity of Toxin A to F9 cells in suspension.

Recombinant Toxin A was pre-incubated with purified scFvs, diluted polyclonal anti-Toxin A antibody (positive control) or purified anti-BSA scFv (negative control). The mixtures were then added to F9 cells and the binding of toxin was visualized by immunohistochemistry. Antibodies chosen for pre-incubation with toxin were scFv anti-BSA (A); polyclonal anti-Toxin A (B); scFv D4 (C); scFv D5 (D).

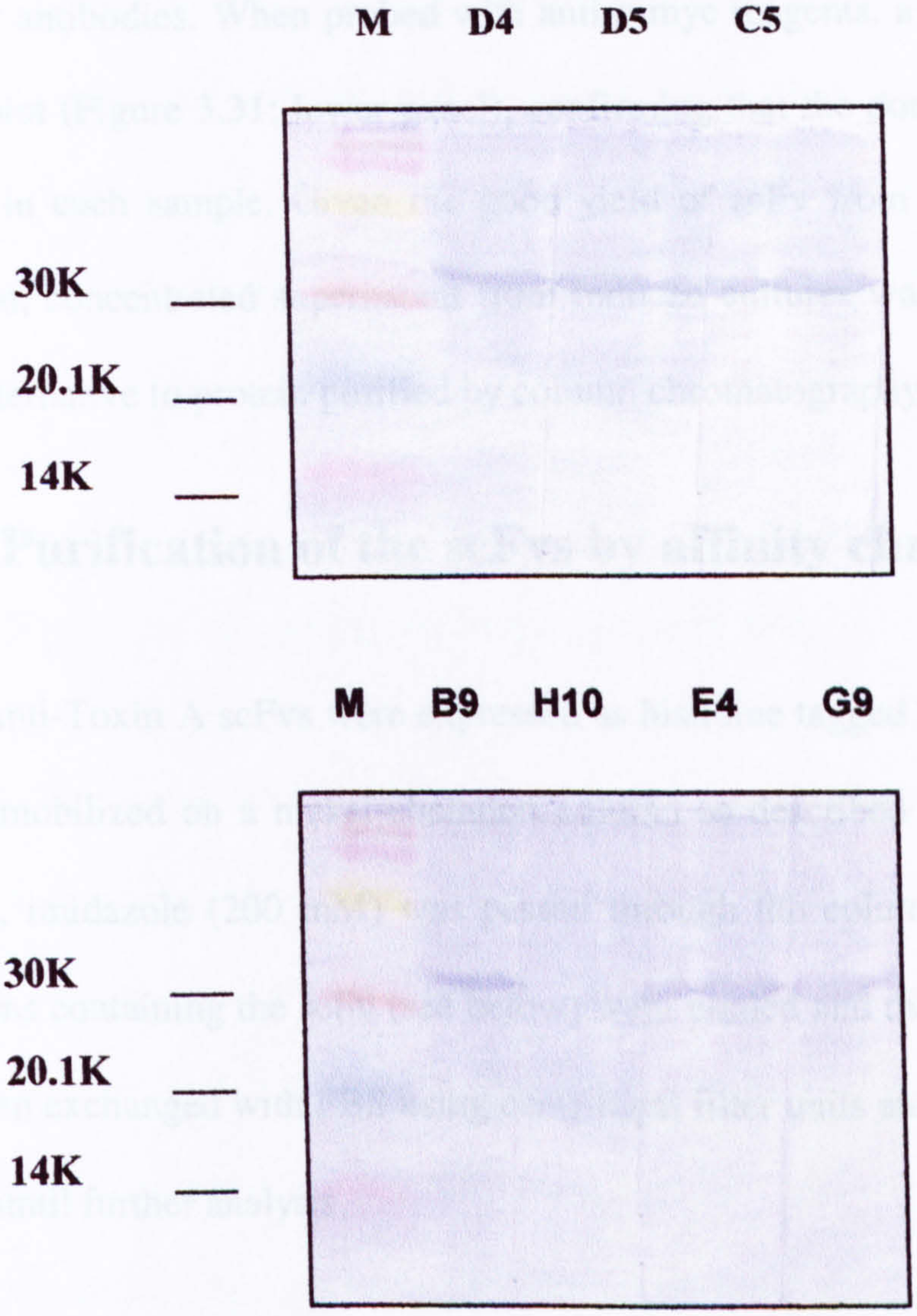

Figure 3.29: Recognition of recombinant Toxin A in Western blotting using scFv

Lysate from an induced culture of *E. coli* pET767 was separated by SDS-PAGE and blotted to nitrocellulose. Individual strips from the blot were probed with scFv as indicated. The binding of scFv was detected with anti-c-myc antibody and HRP-labelled reagent. M: Rainbow protein molecular weight markers (Pharmacia).

The results are shown in Figure 3.31. The total proteins composition of the concentrated supernatants was complex (upper panel) reflecting the leakage of periplasmic proteins from the bacterial cells on induction. It is also likely that some lysis of bacteria taken place. The most abundant single protein in the samples was of a size consistent with the scFv antibodies. When probed with anti-c-myc reagents, a single protein was detected on the blot (Figure 3.31; lower panel), confirming that the dominant protein was the scFv present in each sample. Given the good yield of scFv from each clone and its specific detection, concentrated supernatant from induced cultures was used in some experiments as an alternative to protein purified by column chromatography.

3.22 Purification of the scFvs by affinity chromatography

Anti-Toxin A scFvs were expressed as histidine tagged fusion proteins, concentrated and immobilized on a nickel-chelation column as described earlier. In order to elute the protein, imidazole (200 mM) was passed through the column to generate five fractions. Fractions containing the scFv (see below) were pooled and the buffer containing imidazole was then exchanged with PBS using centrifugal filter units and the samples were stored at -20° C until further analysis.

The scFvs chosen for further analysis were successfully expressed and purified. Supernatant from induced culture (Figure 3.30; lane 1) contained several prominent proteins of about 45 kDa. The expected molecular weight of the scFv being 30 kDa, this corresponding to the most abundant single protein in the supernatant. The culture supernatant was exchanged into column binding buffer, filtered through a membrane of 0.2 μm and loaded to the affinity column. Binding buffer containing imidazole at 20 and 70 mM was passed through to remove many of the proteins seen in the culture supernatant (lanes 3 and 4). scFv was eluted with binding buffer containing imidazole 200 mM (Lanes 6 and 7) . No other protein contaminants were present (Figure 3.30).

3.23 Epitope saturation curve

To assess if the scFvs chosen for further analysis recognized the same or over-lapping epitopes on Toxin A, a series of competition ELISAs were devised. The first attempted to saturate Toxin A with one antibody using cumulative binding with successive rounds of incubation and then with another scFv, to observe whether the signal could be increased further. This experiment showed that saturation was incomplete even after 6 rounds of addition and incubation. The ELISA signal continued to rise even after 6 rounds of incubation with the same scFv, evidence suggested that a plateau was not reached (Figure 3.32).

3.24 Epitope saturation experiment using two different scFvs

To test the result of the previous experiment, two different scFvs were used in an antibody saturation experiment. Two rows of an ELISA plate were coated with a low concentration of recombinant Toxin A. After washing and blocking, wells received single applications of scFvs C5 or D4, and double or triple applications of C5 or D4 was added after double applications of C5. The binding of scFv was detected with monoclonal anti-c-myc antibody and then probed with HRP-labeled reagent. The results are shown in Figure 3.33. Signals from wells probed with single applications of C5 or D4 were comparable and further testing would have to be done to establish if the slight difference evident in (Figure 3.32) was meaningful. A higher final absorbance was obtained after two or three applications of C5, consistent with findings from the previous experiment (Figure 3.32). However, no difference was detectable between wells probed with three applications of C5 and those probed twice with C5 and once with D4. Hence, it could not be established if C5 and D4 recognized unique or over-lapping epitopes on toxin A.

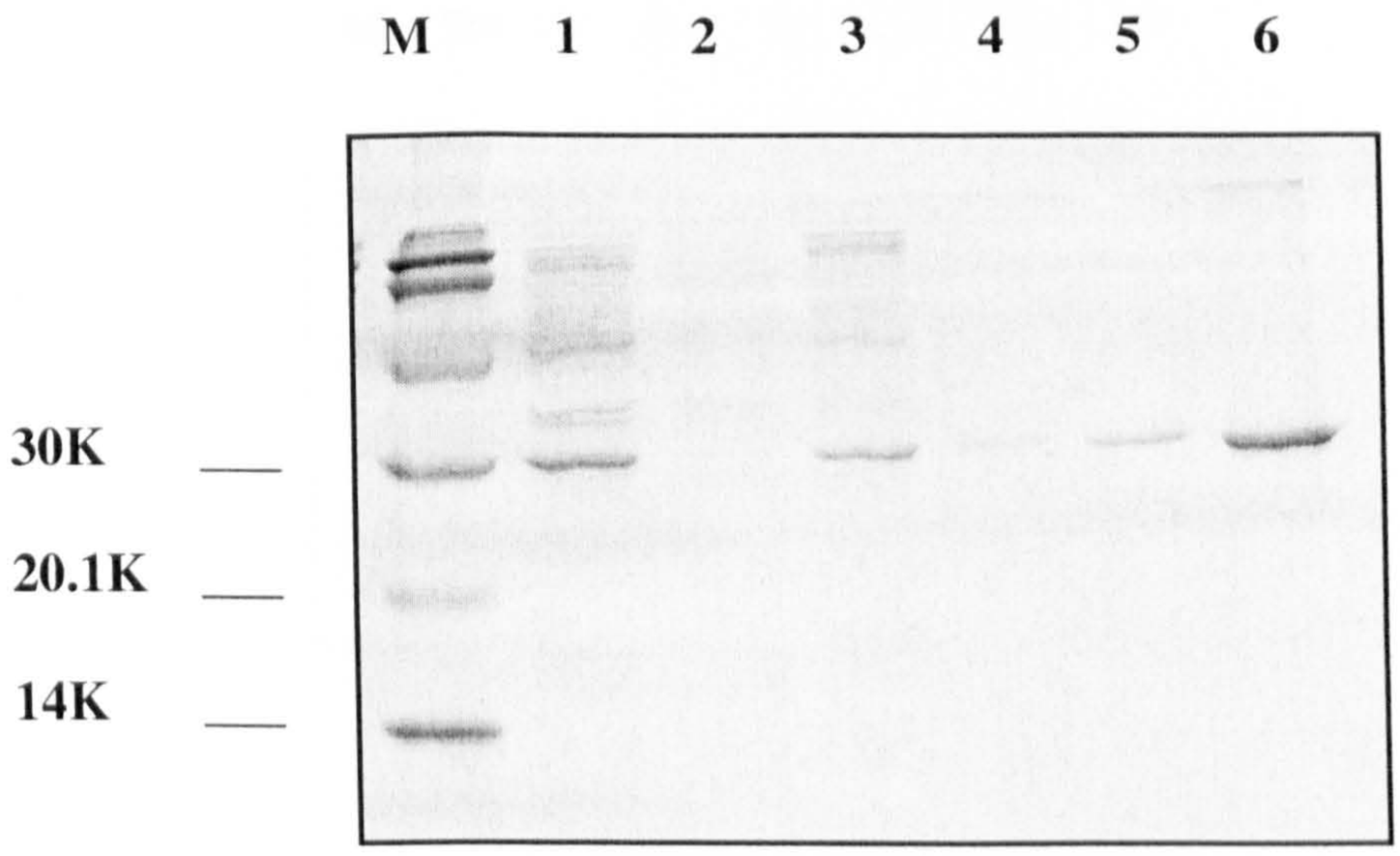

Figure 3.30: Purification of anti-Toxin A scFv D5.

Concentrated supernatant from induced cultures (lane 1) was applied to a column of chelated nickel. Flow through (lane 2) and washings with binding buffer containing 20 mM (lane 3) and 70 mM (lane 4) Imidazole were collected for analysis. scFv was eluted by pumping binding buffer containing 200 mM imidazole through the column, the scFv appearing in fractions one and two (lanes 5 and 6).

M: Rainbow protein molecular weight markers (Pharmacia).

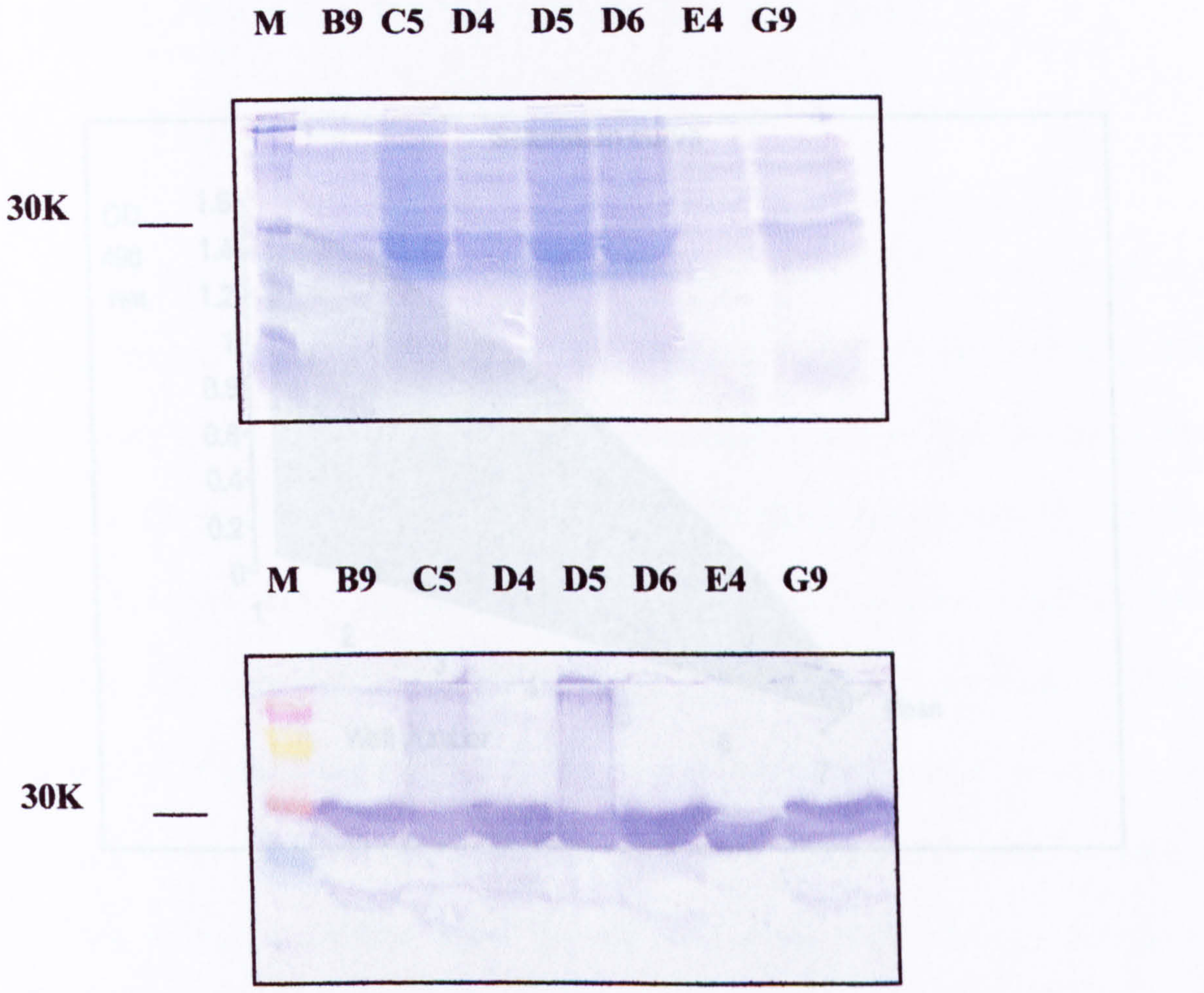

Figure 3.31: Expression of the panel of scFvs.

Concentrated supernatants from induced cultures of *E. coli* HB2151 were run on SDS-PAGE, blotted and probed with an anti-c-myc antibody. The upper panel shows a gel stained with Commassie Blue. The lower panel shows a Western blot of duplicate samples. scFv clones are indicated over each lane. M: Rainbow protein molecular weight markers (Pharmacia).

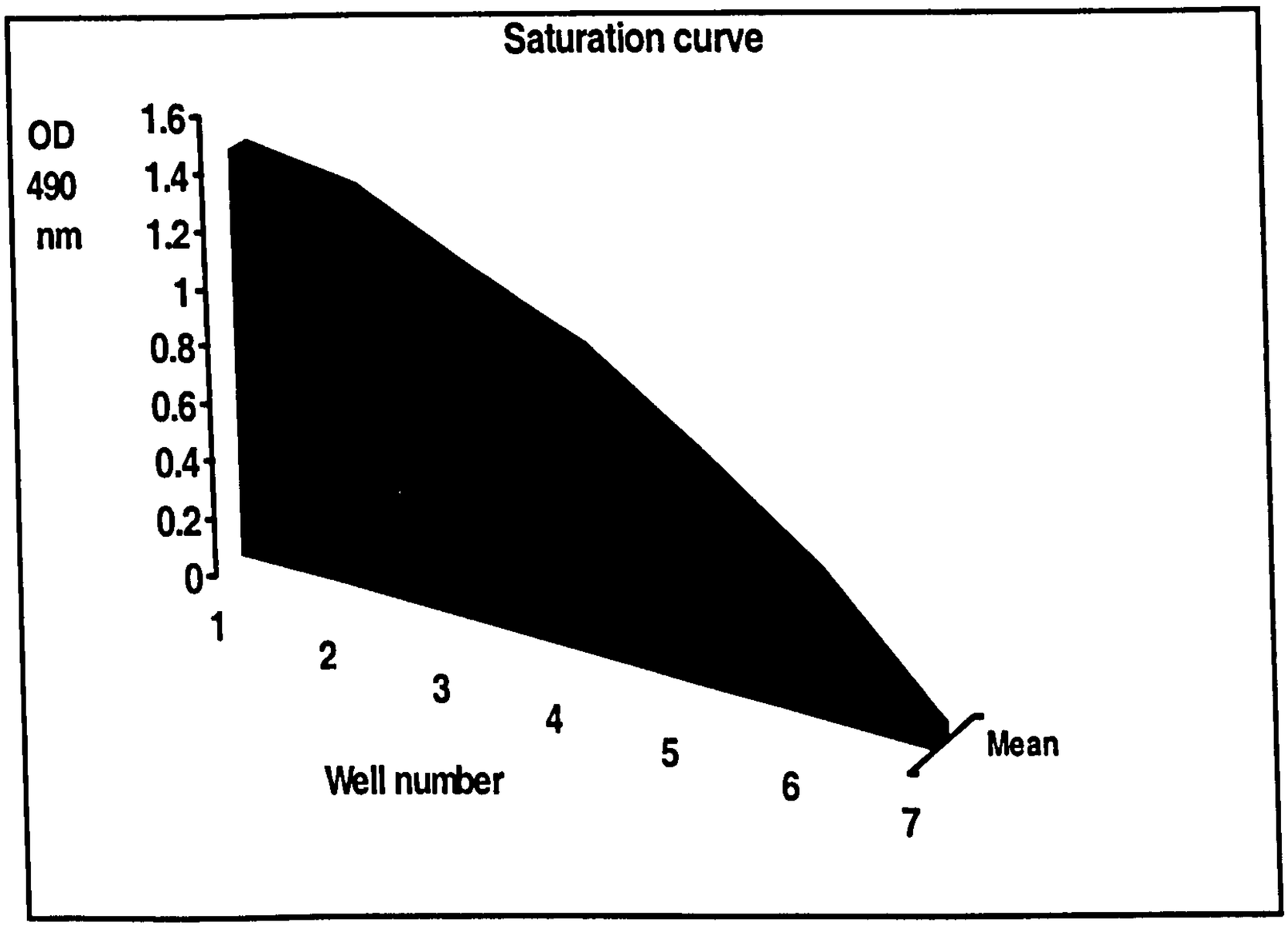

Figure 3.32: Epitope saturation with scFv

An ELISA plate was coated with a low concentration of recombinant Toxin A. Wells number 1 to 7 were exposed to 7, 6, 5, 4, 3, 2 or 1 rounds of scFv addition and incubation respectively. Samples were tested in duplicate and the mean value is presented. scFv binding was detected with anti-c-myc and HRP-labelled reagents.

3.25 Competitive ELISA with monoclonal phage antibodies and scFv

Because of the unsatisfactory result of the saturation experiments, a competitive assay was set up in which scFv and phage antibodies of the same specificity were mixed and allowed to compete on their target antigen (Boel *et al.*, 1998).

3.25.1 Preliminary experiments

The experimental system was optimized by titration of a single phage antibody (C5) against its scFv derivative and against a different scFv (D5). Wells of an ELISA plate were coated with a low concentration of recombinant Toxin A. The two scFvs were pre-diluted in a separate microtitre plate and mixed with the phage antibody in a checkerboard pattern. Mixtures were then transferred to the coated ELISA plate. After incubation and washing, wells were probed with HRP-labeled anti-M13. Results are shown in Figure 3.34.

The titration of phage antibody C5 and its scFv showed a weak signal in all wells (area A1 to C4) indicating low binding of C5. However, wells titrated with phage antibody C5 and scFv D4 showed a stronger signal. In this area of the plate, readings decreased in the direction of phage dilutions (wells 1 to 4) and dilution of the scFv had little impact. This indicated that phage antibody C5 and scFv C5 competed for the same epitope and as a result, was phage inhibited from binding to the toxin-coated plate. However, there was no evidence that phage antibody C5 and scFv D5 competed and a strong signal was obtained in the assay. This result indicated that competitive ELISA could be employed to study whether antibodies isolated by phage display recognised the same or overlapping epitopes on Toxin A, or if each antibody recognised a unique feature of the target protein.

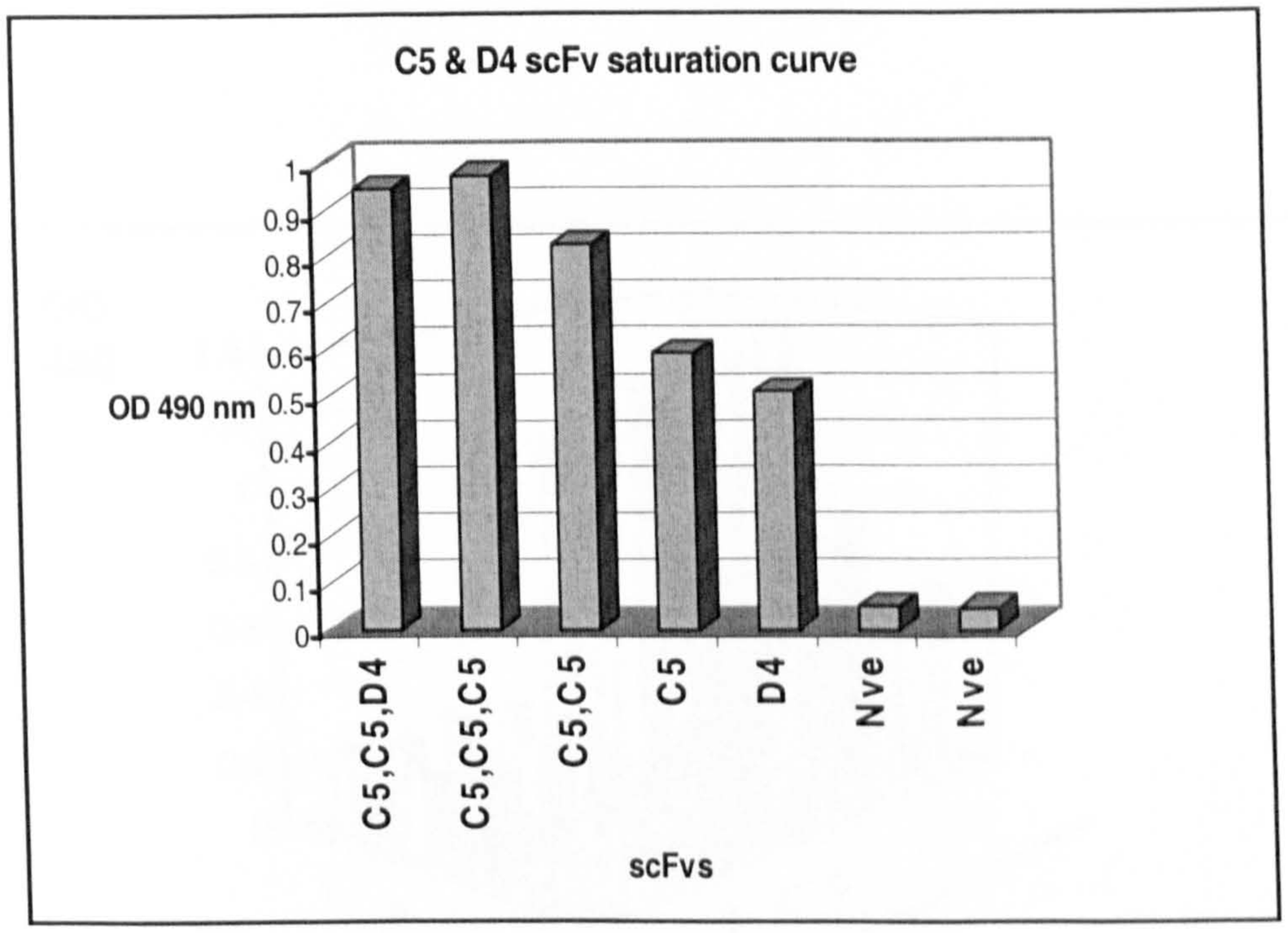

Figure 3.33: Epitope saturation with two scFvs

An ELISA plate was coated with a low concentration of recombinant Toxin A. scFv antibodies were added in 1, 2, or 3 rounds as indicated. Negative control wells were coated with BSA. scFv binding was detected with anti-c-myc and HRP-labelled reagents. Samples were tested in duplicate and the mean value is presented.

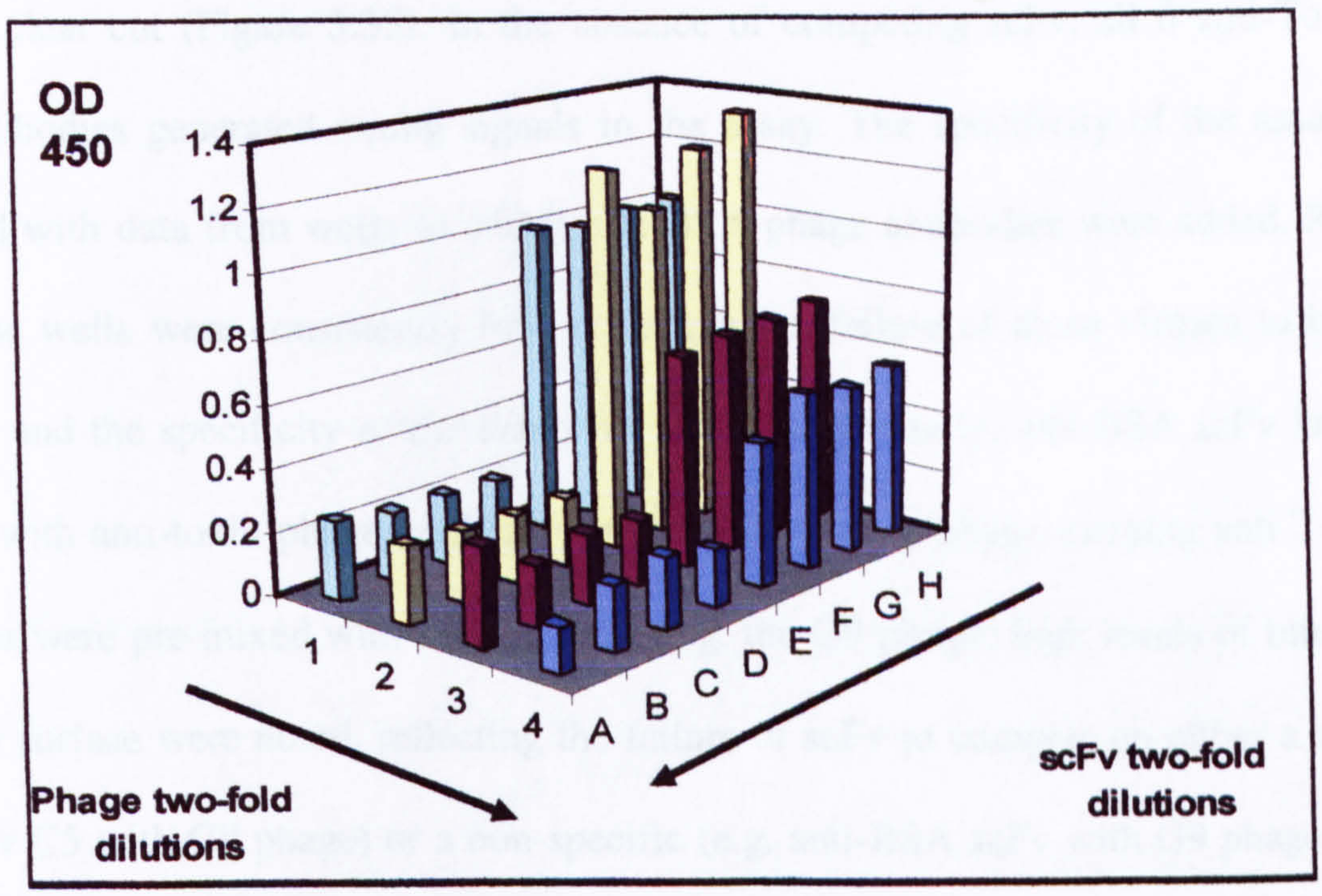

Figure 3.34: Competitive ELISA with monoclonal phage antibodies scFv preliminary experiment

The plate was coated with recombinant Toxin A and mixtures of phage antibodies and scFvs were added to wells after checkerboard dilutions were prepared. scFv C5:1, 2, 3 and 4 were titrated against phage antibody C5: A, B, C and D. scFv D5:1, 2, 3 and 4 were titrated against phage antibody C5: E, F, G and H. Phage-scFv mixes were then transferred to separate wells of Toxin A-coated plate. After incubation phage were detected by HRP-labelled anti-M13 antibody. Samples were tested in duplicate and the mean value is presented

3.25.2 Competitive ELISA experiments

Clones chosen for analysis were expressed as monoclonal scFvs and as monoclonal phage antibodies for use in the competition ELISA. The test was performed by coating an ELISA plate with a low concentration of recombinant Toxin A and adding pre-diluted mixtures of scFv and phage antibodies. The binding of the phage antibodies was detected as described earlier. The complexity of the assay meant that phage and the competing scFvs could only be tested at single concentrations. In spite of this, the data obtained were relatively clear cut (Figure 3.35). In the absence of competing scFv, all 6 anti-Toxin A phage antibodies generated strong signals in the assay. The specificity of the assay was confirmed with data from wells to which anti-BSA phage antibodies were added. Results from these wells were consistently low, reflecting the failure of these viruses to bind to Toxin A, and the specificity of the detecting reagents. Similarly, anti-BSA scFv failed to compete with anti-toxin phage antibodies in the assay. when phage carrying anti-Toxin A antibodies were pre-mixed with soluble scFv (e.g. the G9 phage) high levels of binding to the assay surface were noted, reflecting the failure of scFv to compete on either a specific (e.g. scFv C5 with G9 phage) or a non-specific (e.g. anti-BSA scFv with G9 phage) basis. The consistent exception was when the phage antibodies were mixed with their soluble scFv derivatives (e.g. scFv G9 with G9 phage); in these assays, results were suppressed to near-background levels, indicating the efficiency of competition. In one instance, some evidence suggestive of cross-competition was obtained: the E4 phage appeared to bind to Toxin A less well in the presence of soluble scFvs G9, D6 and D5. However, the soluble form of E4 showed no ability to block bind of D5, D6 or G9 phage, so the apparent inhibition was most likely to be due to variation within the assay. Overall, the assay indicated that all recombinant anti-Toxin A antibodies probably recognized non-overlapping epitopes.

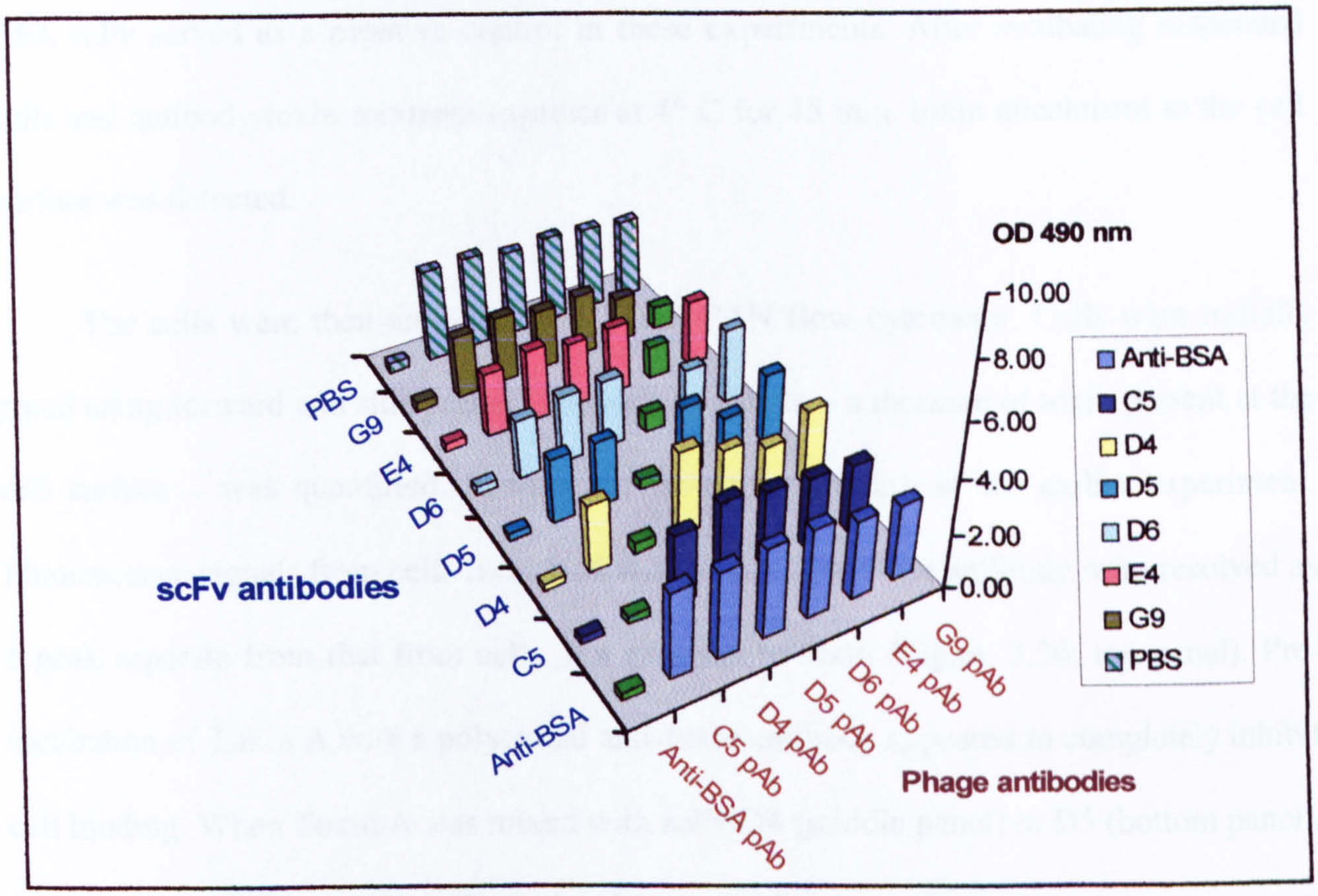

Figure 3.35: Toxin A competitive ELISA using phage antibodies and their soluble scFv derivatives

The plate was coated with recombinant Toxin A and mixtures of phage antibody (designated pAb) and scFv were added. Bound phage were detected by HRP-labelled anti-M13 antibody. Samples were tested in duplicate and the mean value is presented

3.26 Flow cytometric analysis of Toxin A-receptor interaction

Flow cytometry was used to assess if purified anti-Toxin A scFvs were able to inhibit recombinant Toxin A from binding to its receptor. F9 cells were trypsinized and sub-cultured in a complete medium for 3 hrs. Cells were then scraped, washed, and mixtures of recombinant Toxin A and different purified scFvs antibodies were added. Polyclonal goat anti-Toxin A antibody was used as a positive control and purified anti-BSA scFv served as a negative control in these experiments. After incubating suspended cells and antibody-toxin mixtures together at 4° C for 45 min, toxin attachment to the cell surface was detected.

The cells were then analysed on a FACSCAN flow cytometer. Cells were initially gated using forward and side scatter. Their fluorescence – a measure of toxin present at the cell surface – was quantified. Results confirmed the finding of the earlier experiment. Fluorescence signals from cells incubated with Toxin A without antibody were resolved as a peak separate from that from cells, not exposed to toxin (Figure 3.36; top panel). Pre-incubation of Toxin A with a polyclonal anti-toxin antibody appeared to completely inhibit cell binding. When Toxin A was mixed with scFv D4 (middle panel) or D5 (bottom panel), it failed to bind to cells efficiently, but neutralization of receptor interaction was incomplete. A scFv against BSA failed to reduce attachment of toxin to the surface of F9 cells (data not shown).

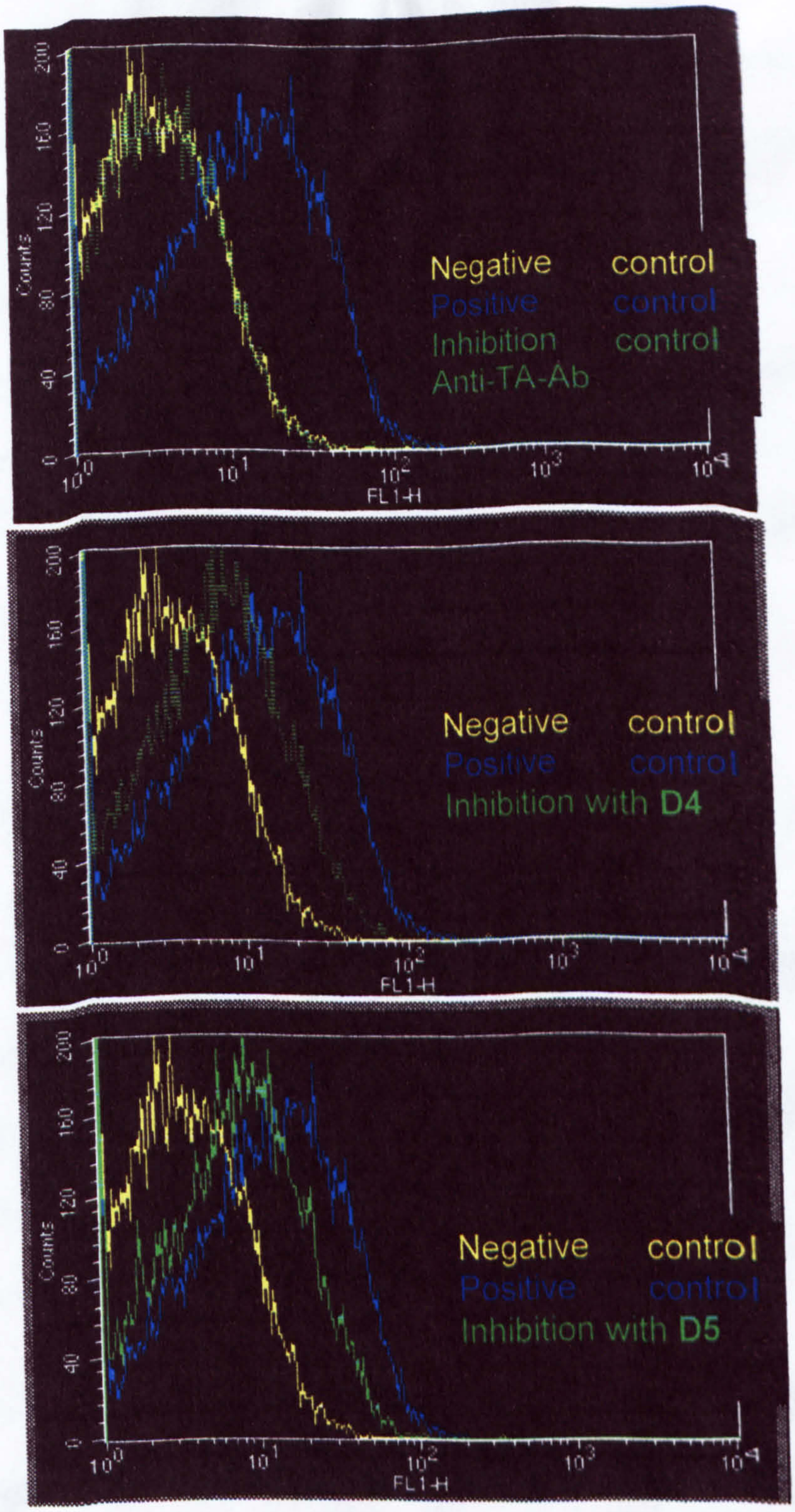

Figure 3.36: Flow cytometry assay for the binding of Toxin A to F9 cells.

F9 cells were incubated with mixtures of Toxin A and antibodies, and toxin binding detected by immunofluorescence. Controls included cells with Toxin A and no inhibitory antibody (positive control; all panels) and cells not exposed to toxin (negative control; all panels). Pre-incubation of Toxin A with polyclonal antibody confirmed successful inhibition of binding to cells (top panel, inhibition control). scFvs D4 (middle panel) and D5 (bottom panel) were pre-incubated with toxin before application to F9 cells to test their receptor-blocking activity.

3.27 Inhibition of Toxin A cytotoxicity by scFvs

As the scFvs showed some ability to block the interaction of Toxin A with its receptor at the surface of F9 cells, assays, were set up to test if the antibodies could block cytotoxic activity. F9 cells were grown and sub-cultured into flat bottom 96-well plates. In separate plates, scFvs were set out and full-length Toxin A was added to a final concentration of 2 ng/well. After incubation to allow scFv interaction, each antibody-Toxin A mixture was transferred into the cell culture plate, and cell morphology was monitored. The proportion of cells undergoing a rounding response was used as an indication of successful toxin binding, entry and intoxication of the F9 cells. The data are shown in Figure 3.37.

In each panel of the Figure, data are shown for control reactions. When cells were challenged with Toxin A that had been pre-incubated with a scFv against BSA, rapid cell rounding was observed. This was first detectable 25 minutes after addition of the toxin and after 75 minutes, approximately half of all cells observed were rounded in their morphology. Intoxication was essentially complete after 100 minutes. The kinetics of this assay previously, the key feature being the rapid increase in rounding between 50 and 100 minutes post-challenge.

When toxin was mixed with polyclonal antibody, near-complete neutralization of cytotoxicity was observed. Again, the data are shown in each panel of Figure 3.37. Modest proportion of cells underwent rounding and even after prolonged incubation, no more than 10 % had undergone morphological change. When toxin was pre-incubated with scFv C5, a shift in the cell rounding response was evident (Figure 3.37; top panel). The response of F9 cells to Toxin A could not be detected at 25 minutes post-challenge, but by 50 minutes, the proportion of rounded cells was indistinguishable from the anti-BSA control. Thereafter, however, the rate of cell intoxication was slower than in the control and complete cell rounding was achieved with a delay of about 25 minutes.

The obstruction of toxin action rather than its neutralization, could also be seen in tests with scFv D4 (Figure 3.37; middle panel). In these assays, early effects of the scFv were not apparent and rounding of F9 cells proceeded in a way very similar to that seen in the BSA control. The assays diverged beyond 50 minutes post-challenge and as with scFv C5, a delay in the main phase of the intoxication response could be seen.

Given that scFvs C5 and D4 recognised different features of Toxin A, tests were carried out to see if their effects on cytotoxicity were additive. The data are shown at the foot of (Figure 3.37). Interestingly, a mixture of three scFvs (C5, D4 and D5) generated a delay in the cytotoxic response to Toxin A that was most similar to the effect of D4 alone, but no enhancement in neutralizing activity could be seen.

This experiment was repeated with some modification (Sauerborn et al., 1997). After the addition of antibody-Toxin A mixtures to the cell culture plate, the plate was incubated at 4° C for 30 min and wells were then washed with chilled PBS. Each well received full medium. The plate was then incubated for 2 hrs at 37° C. Cell morphology change was monitored and counted for statistical purposes every 25 min. This experiment showed similar results (data not shown).

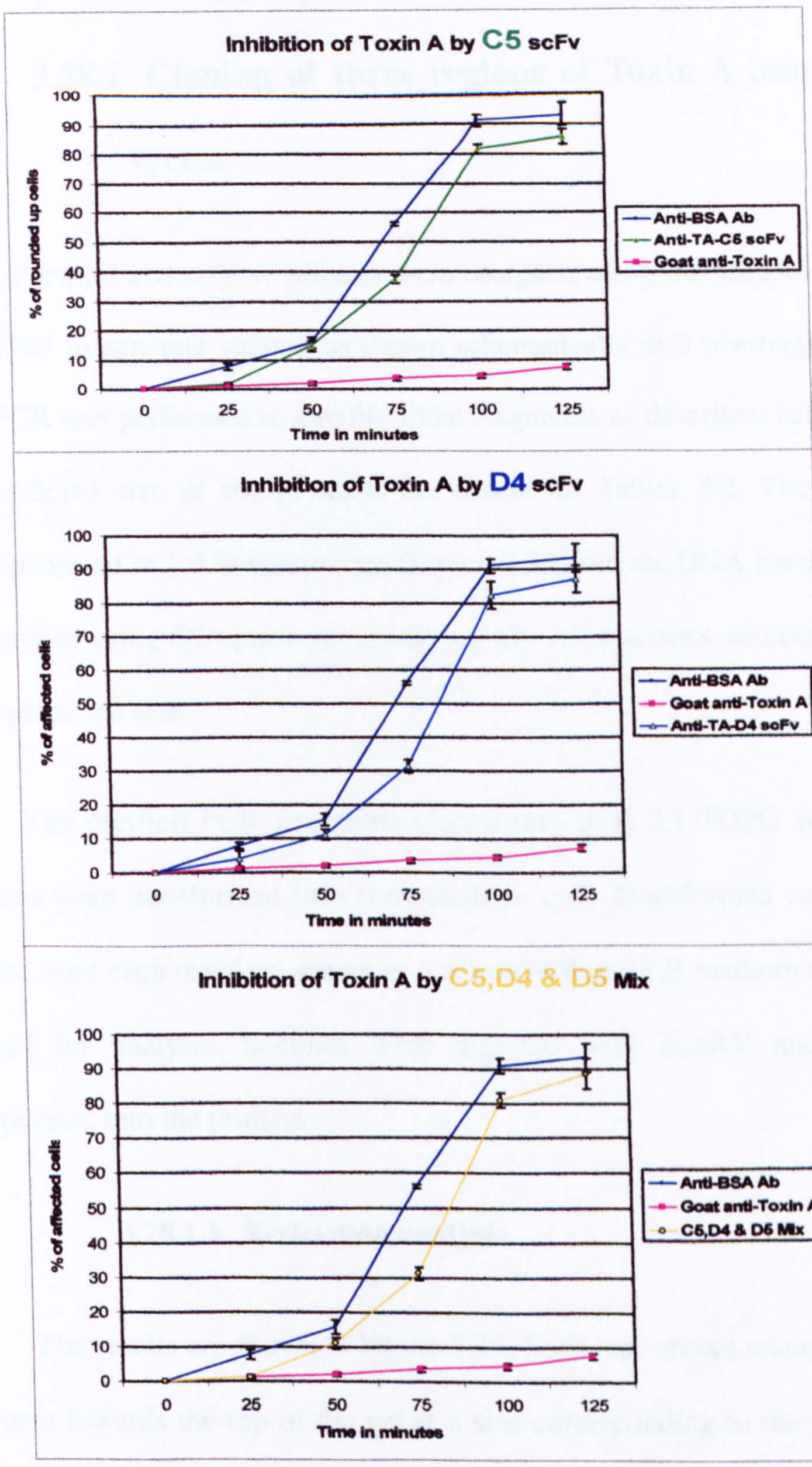

Figure 3.37: Inhibition of Toxin A cytotoxicity by scFvs

Toxin A and purified anti-Toxin A scFv antibody were incubated together prior to transfer to a culture plate containing growing F9 cells. The culture was then incubated at 37° C for 4 hrs. The plate was observed every 25 min, and the proportions of cells that had undergone rounding was determined by eye. Polyclonal goat anti-Toxin A antibody and purified anti-BSA scFv were used as positive and negative controls respectively.

3.28 Epitope mapping

3.28.1 Cloning of three regions of Toxin A into pCR 2.1-TOPO vector

Forward and reverse primers were designed using the sequence of Toxin A carried in pET767 to generate shorter, as shown schematically in 3 overlapping fragments Figure 3.40. PCR was performed to amplify three fragments as described before. The primer pairs and predicted size of the products are shown in Tables 3.2. The PCR products were electrophoresed in 1.0 % agarose gel (Figure 3.38) and the DNA bands cut out from the gel and purified using QIAquick gel extraction kit. All reactions successfully amplified DNA of the predicted size.

The purified PCR fragments cloned into pCR 2.1-TOPO vector and the ligation mixtures were transformed into competent *E. coli*. Transformed colonies were picked at random from each reaction, grown in a selective liquid LB medium and plasmid DNA was isolated for analysis. Samples were digested with *Eco*RV and *Hin*dIII using sites incorporated into the primers.

3.28.1.1 Restriction analysis

The results are shown in Figure 3.39. Each lane shows release of a prominent DNA fragment towards the top of the gel at a size corresponding to the pCR 2.1 vector. Lower down, insert fragments can be seen. Since products I and II only differed by 11 bp, the gel was unable to resolve a difference in their sizes. Inserts were however clearly present in all clones picked for analysis. Product III is predicted to be 270 bp shorter than product II and the smaller size of the release insert is evident in Figure 3.39.

To express the protein encoded by each region of the Toxin A sequence, fusions to maltose-binding protein were created. The vector pCG806 was chosen for this purpose.

Table 3.2 : List of the primer pairs and predicted size of the products used in cloning of four regions of Toxin A

Fragment	Primer sets	bp
I	3For-1Rev	412
II	2For-2Rev	403
III	1For-3Rev	270
R1	R1 for & R1 rev	60

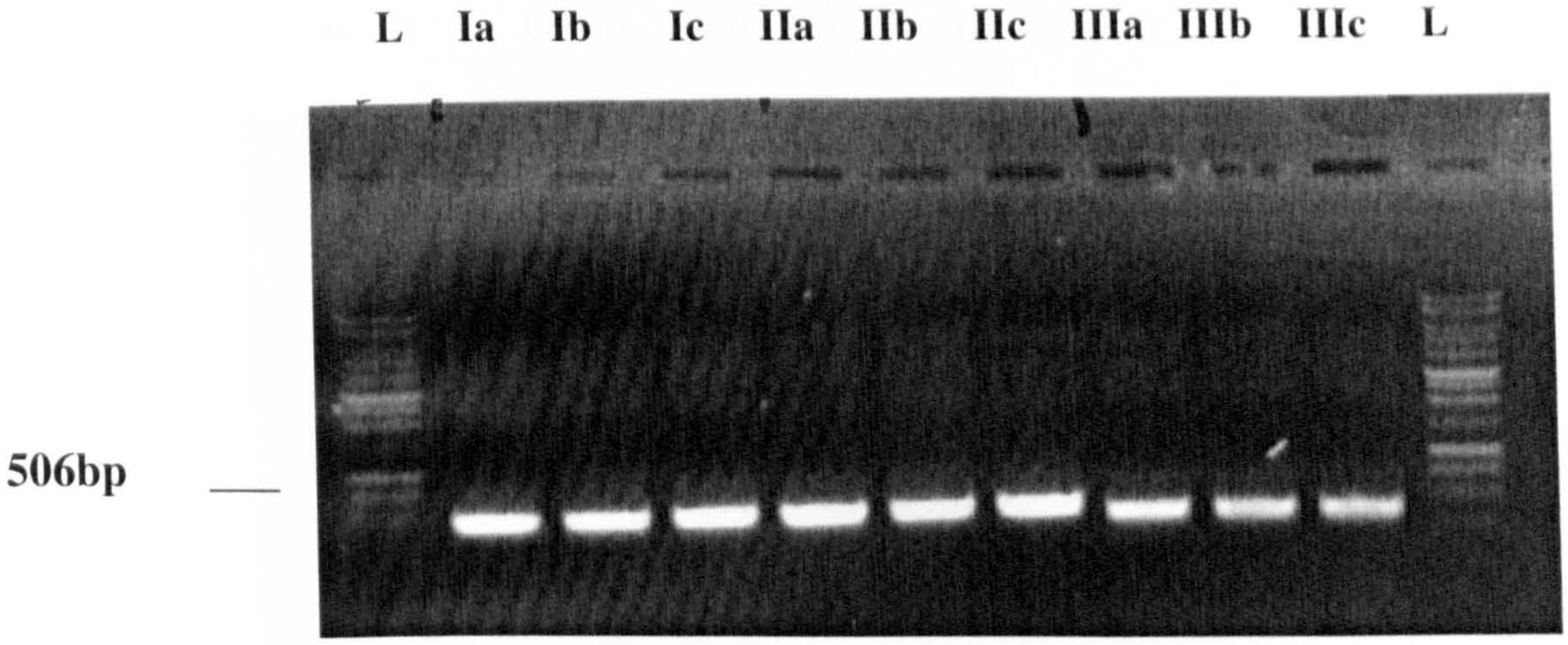

Figure 3.38: PCR amplification of fragments I, II and III from pET767.

Plasmid DNA was extracted from *E. coli* pET767 and amplified by PCR using 3 pairs of primers Table (3.2). Amplicons were analysed by agarose gel electrophoresis. Ia, Ib and Ic represent separate reactions for product I (Figure 3.40); IIa, IIb, IIc, IIIa, IIIb and IIIc represent reactions for products fragment II and III respectively. L: indicates the migration of a 1kb DNA ladder (Invitrogen, UK).

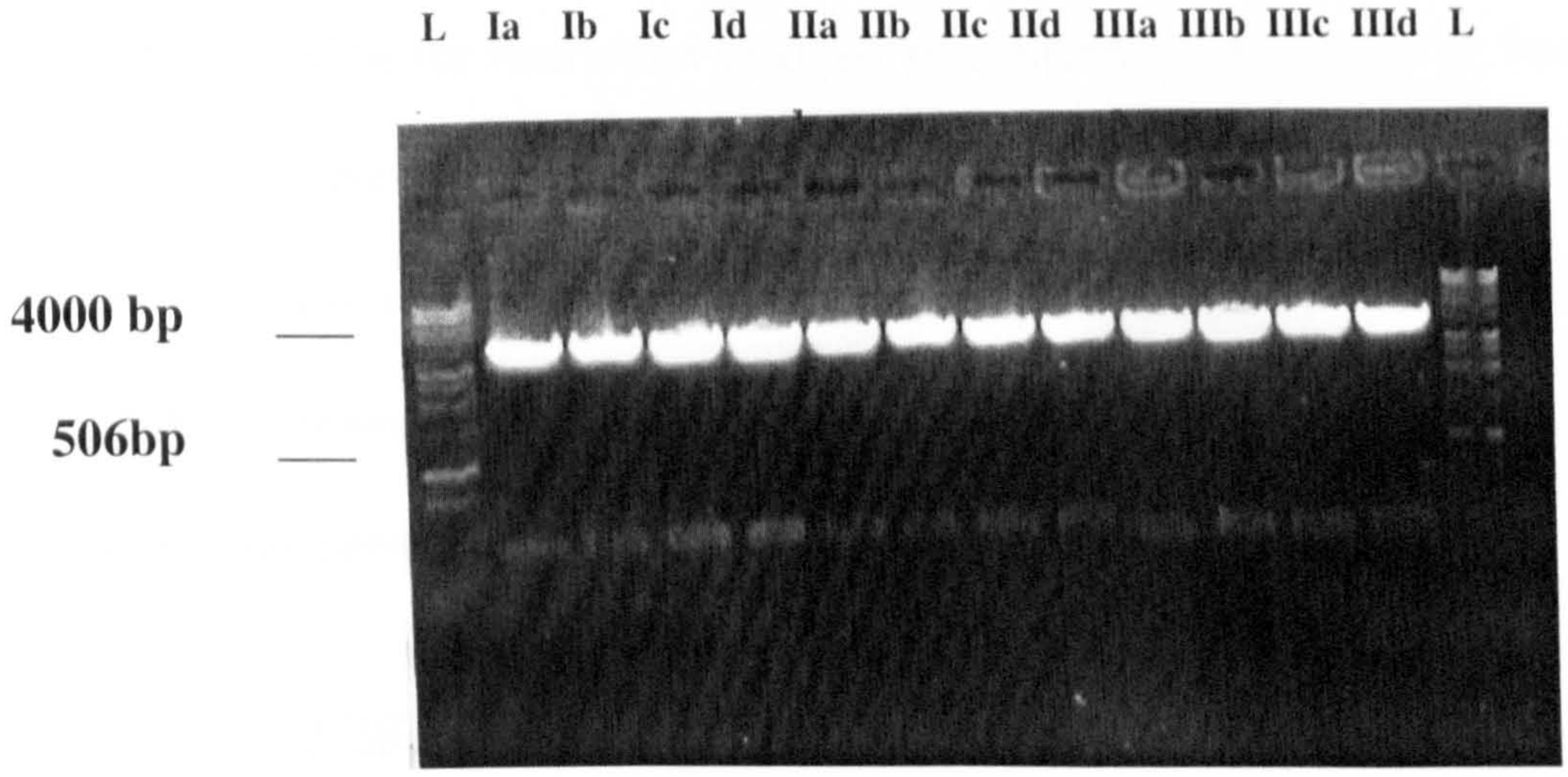

Figure 3.39: Restriction analysis of recombinant pCR2.1 plasmids.

Plasmid DNA was extracted form colonies transformed with each ligation (as indicated) and digested with *Eco*RV and *Hin*dIII. Ia, Ib, Ic Id represent separate digestion reactions for clone I; IIa, IIb, IIc, IId, IIIa, IIIb, IIc and IIId represent digestion reactions for products fragment II and III respectively, L: indicates the migration of a 1kb DNA ladder (Invitrogen).

3.28.2 Cloning Toxin A fragments into a maltose-binding protein vector

DNA fragments generated by restriction digestion in the previous experiment were isolated. The MPB fusion vector was purified and digested with *Sma*I and *Hin*dIII to create compatible termini in the appropriate reading frames. The ligated products were then transformed into competent *E. coli* DH5α. Colonies were picked at random from each transformation for restriction analysis to confirm the presence of Toxin A sequences I, II, and III. To confirm the presence of the correct insert in each construct and ensure the MBP and Toxin A reading frames were fused as intended, DNA samples were sequenced using an M13 forward primer. This anneals to a site downstream of the polylinker in pCG806. Data from sequencing are shown in Figures 3.41, 3.42 and 3.43.

3.28.3 Cloning of DNA linker into pCG806 MBP vector

Following from analysis of MBP fusions IA, IIW and IIIF, a smaller construct taking DNA from one of the repetitive sequences of the Toxin A C-terminal domain was cloned into pCG806. In this experiment, complimentary primers were annealed to generate a synthetic DNA duplex with overhangs. This enabled ligation to pCG806 after digestion with *Bam*HI and *Hin*dIII. As the insert was very small (60 bp), successful ligation into pCG806 was initially confirmed by loss of the *Kpn*I site located in the vector between *Bam*HI and *Hin*dIII sites. The construct was then confirmed by sequencing (Figure 3.44).

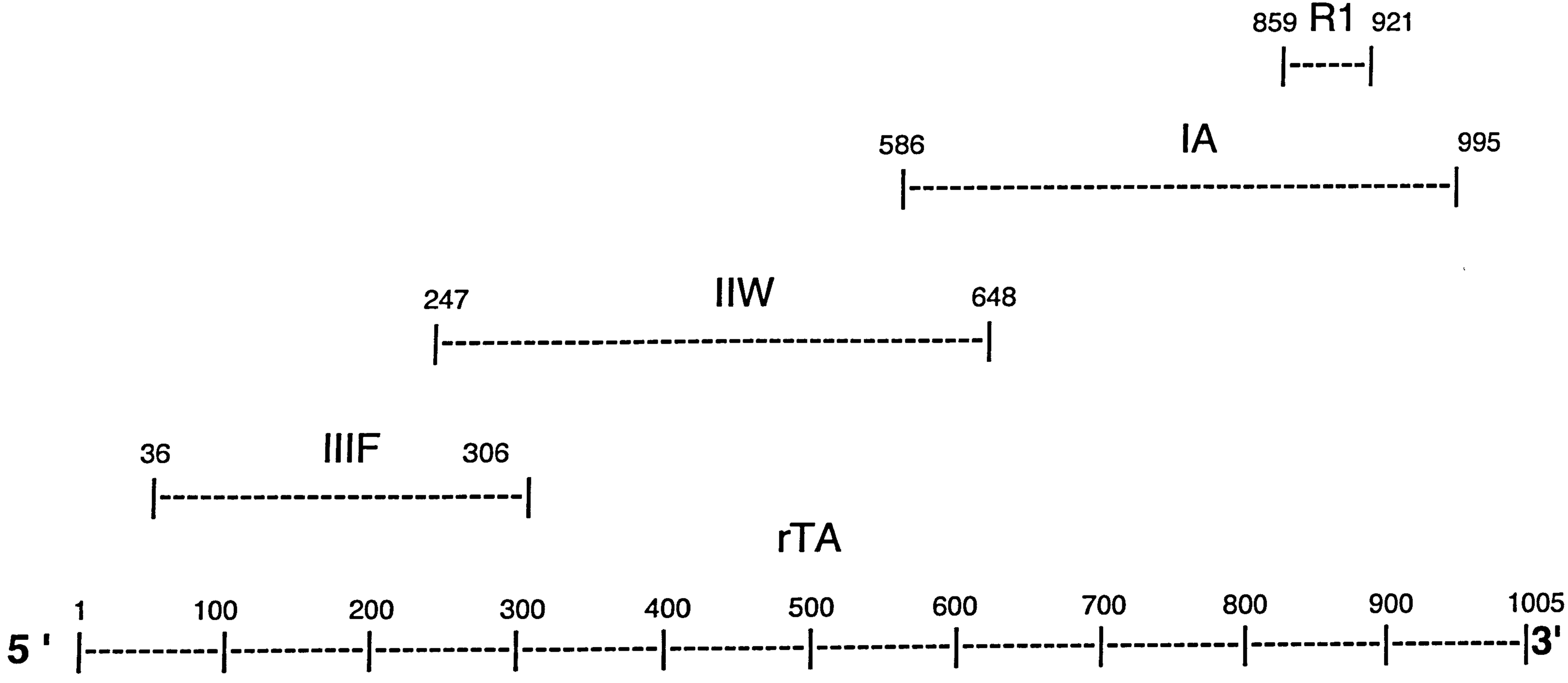

Figure 3. 40: Line digram showing locations of fragments used in epitope mapping

Clone IA
Translation (5'->3')

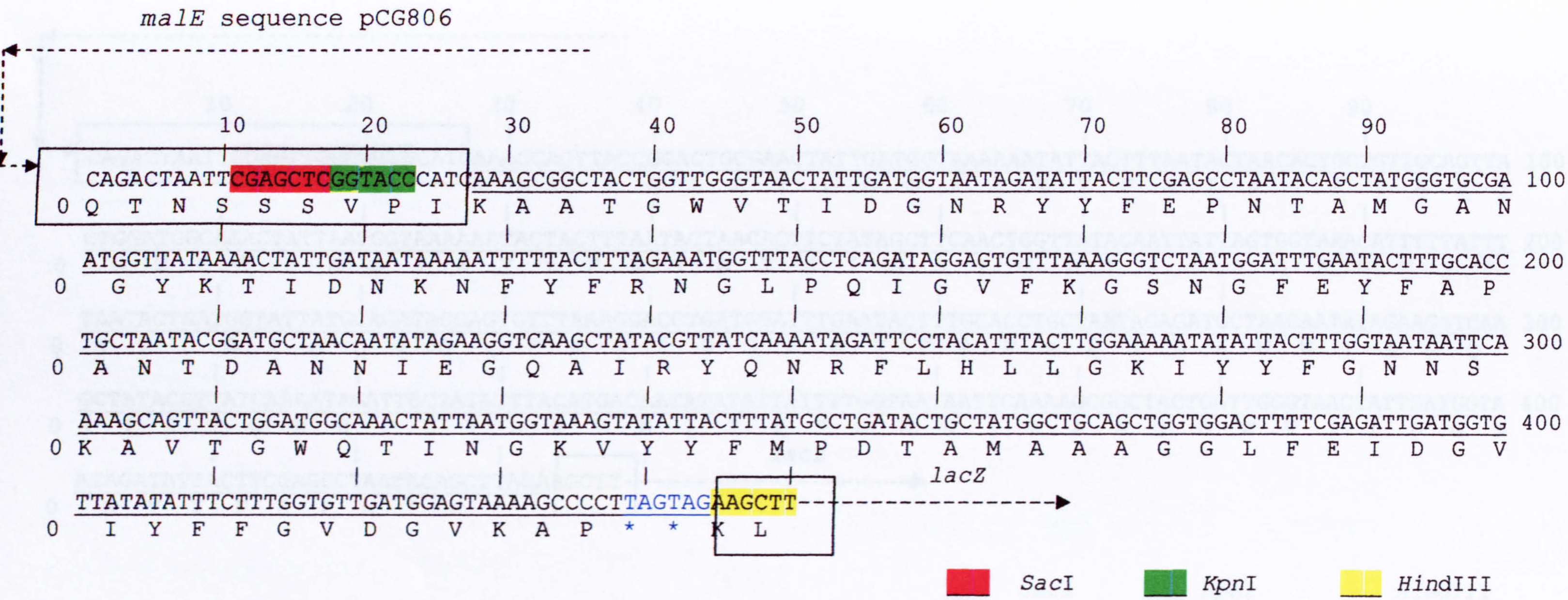

Figure 3. 41: DNA sequencing of IA fragment

The figure presents part of the DNA sequence of the pCG806 for IA construct carrying Toxin A sequence (underlined). The restriction enzyme sites are shown in colours and stop codons are presented as asterisks. Vector DNA is presented within rectangular area.

Clone IIW
Translation (5'->3')

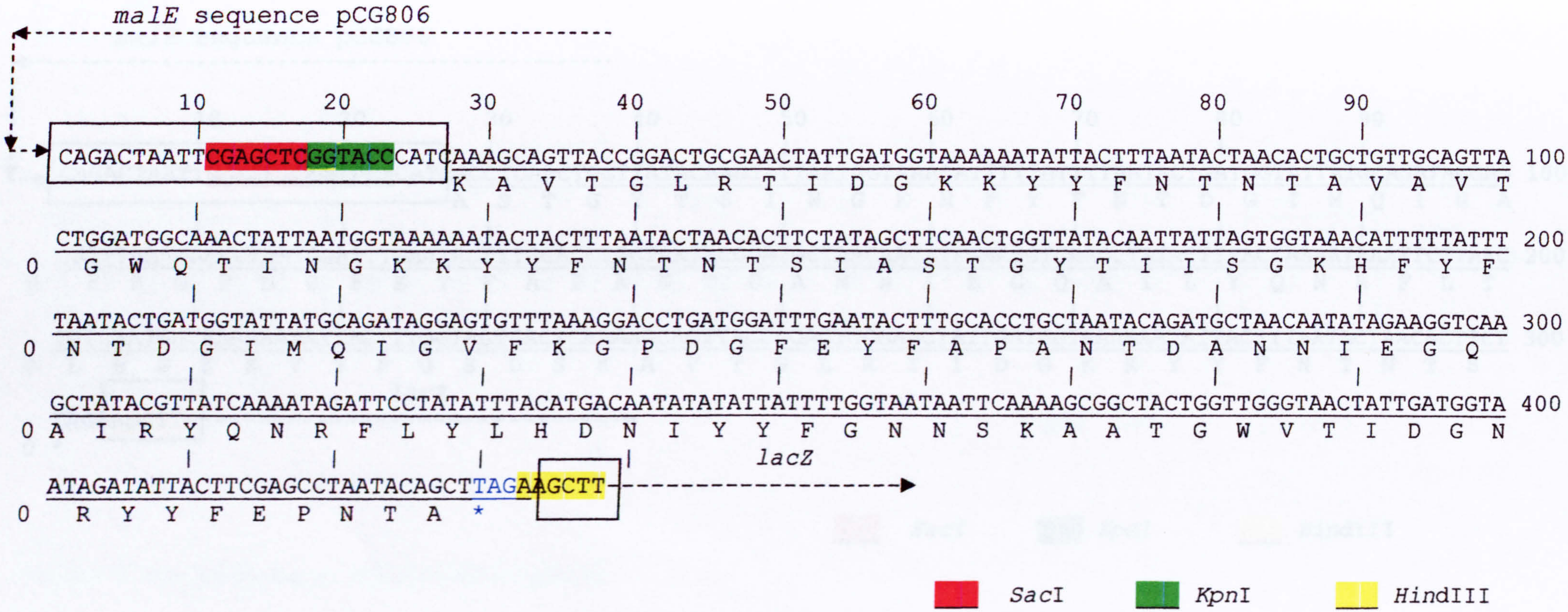

Figure 3. 42: DNA sequencing of IA fragment

The figure presents part of the DNA sequence of the pCG806 for IIW construct carrying Toxin A sequence (underlined). The restriction enzyme sites are shown in colours and stop codons are presented as asterisks. Vector DNA is presented within rectangular area.

Clone IIIF

Translation (5'->3')

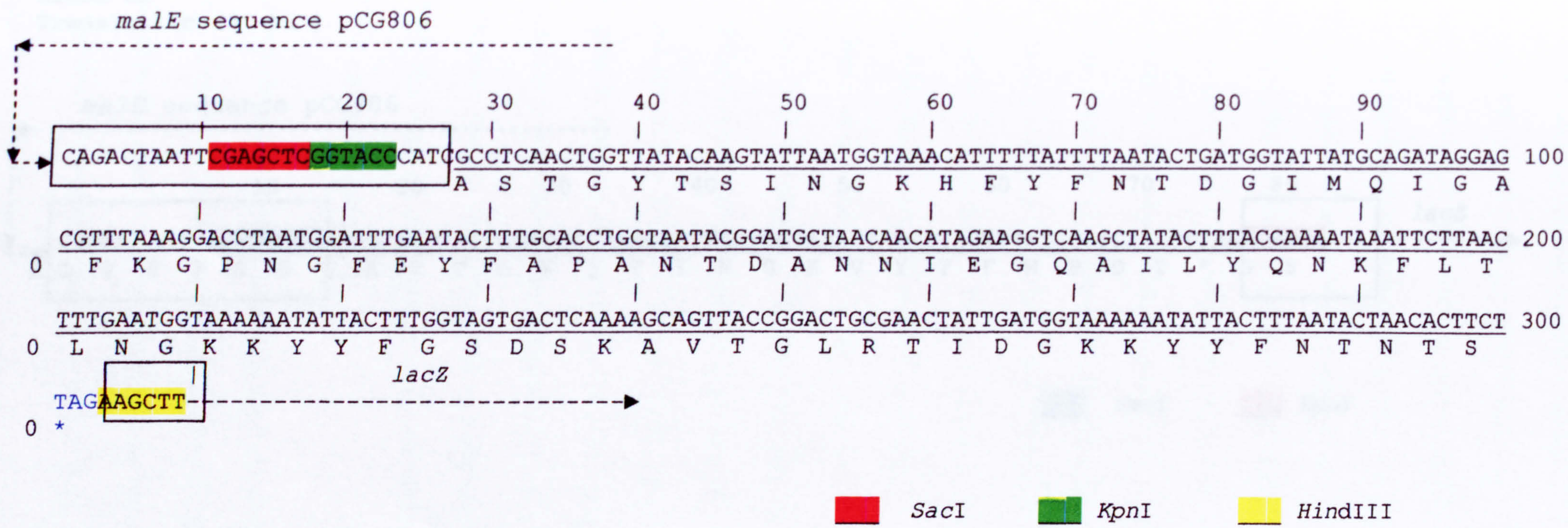

Figure 3. 43: DNA sequencing of IIIF fragment

The figure presents part of the DNA sequence of the pCG806 for IIIF construct carrying Toxin A sequence (underlined). The restriction enzyme sites are shown in colours and stop codons are presented as asterisks. Vector DNA is presented within rectangular area.

Clone R1
Translation (5'->3')

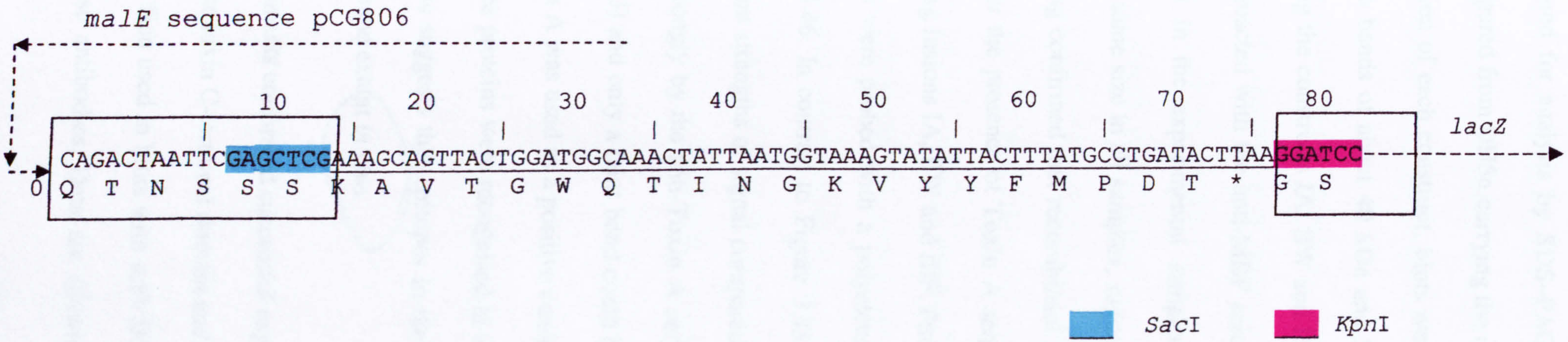

Figure 3. 44: DNA sequencing of R1 fragment

The figure presents part of the DNA sequence of the pCG80 for R1 construct carrying Toxin A sequence (underlined). The restriction enzyme sites are shown in colours and stop codons are presented as asterisks. Vector DNA is presented within rectangular area.

3.29 Characterisation of MBP fusions

E. coli DH5α carrying fusions IA, IIW, and IIIF were grown, induced and bacterial lysates were prepared for analysis by SDS–PAGE and Western blotting. As a control, lysate was also prepared from DH5α carrying the unmodified pCG806 vector. To check for successful expression of each construct, blots were first probed with an anti-MBP serum (Figure 3.45). Two bands of about 40 kDa and 50 kDa were seen in the 3 lysates from plasmids expressing the constructs IA, IIW and IIIF (lanes 1, 2, 3). In the control (lane 4) two proteins also reacted with the anti-MBP serum. The upper band was smaller in size that its equivalent in the experimental samples. In contrast, the lower band was of approximately the same size in all samples, suggesting that it could be the wild type MBP from DH5α. Having confirmed that recombinant MBPs were expressed, the analysis then sought evidence for the presence of Toxin A sequence. Blots were prepared with lysates from DH5α carrying fusions IA, IIW and IIIF. Purified recombinant Toxin A was included as a control. Blots were probed with a polyclonal serum against Toxin A. The data are shown in Figure 3.46. In contrast to Figure 3.45, single bands were recognized in the 3 lysates. But different strengths of signal compared with anti-MBP were seen. Construct IA was recognised strongly by the anti-Toxin A serum (lane 1). Signal from the IIIF lysate was weaker (lane 3) and only a faint band could be developed from IIW (lane 2). Purified recombinant Toxin A was used as a positive control (lane 4) and reacted strongly on blots. Given that the three proteins were recognised at equivalent intensities by anti-MBP serum in Figure 3.45, this suggests that epitopes in the C-terminal domain of Toxin A are not recognised to the same extent *in vivo*.

These experiments confirmed successful expression of the three MBP fusion proteins carrying parts of the toxin C-terminal domain and the stability of the recombinant proteins. The proteins were then used in blots with scFv D5 and D4 to attempt to locate the epitope recognised by these antibodies. Data are shown in Figure 3.47. When membrane was

probed with scFv D4, faint signal was seen with IA (panel B, lane 1). When membrane was probed with scFv D5, all three MBP fusions IA, IIW and IIIF (panel A, lanes 1, 2 and 3), were recognized. Recognition of construct IA showed more prominent signal (panel A and B lane 1). Similar analyses were carried out with scFv C5 and D6. This revealed that C5 recognised Toxin A epitopes present in MBP fusion IA, whereas scFv D6 reacted with IIW. The data from western blotting analysis are summarized in Table 3.3.

Whilst this analysis went same way to identifying the epitopes recognised by scFvs from phage display, one observation of particular interest was the apparent recognition of all three MBP fusion proteins by scFv D5. One feature common to fusions IA, IIW and IIIF was the peptide repeats motifs (Figure 3.47; panel A). To test if one of the motifs might explain the apparent cross-reaction shown by scFv D5, the fusion protein R1 was generated (Figure 3.44). Bacterial lysate was prepared from induced culture of *E. coli* DH5α carrying a fusion R1 (Table 3.2) and analysed by SDS–PAGE and Western blotting with scFv D5. The membrane was probed with scFv anti-c-myc reagents and a light-emitting substrate before exposure to an X-ray film (Figure 3.48). Two bands IA and R1 (≈ 55 kDa) were seen (lanes 1 and 3). Negative control lysate from *E. coli* DH5α carrying pCG806 failed to produce any signal (lane 2 and 4).

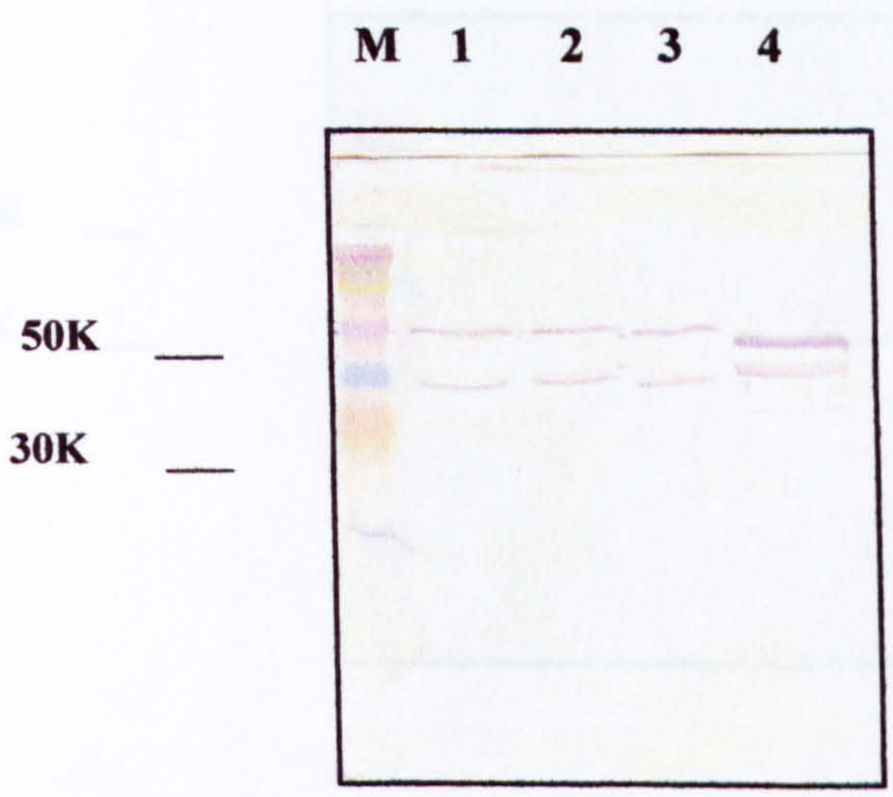

Figure 3.45: Recognition of maltose-binding protein in Western blotting with anti-MBP antiserum.

Bacterial lysates from induced cultures of clones IA (lane 1), IIW (lane 2), IIIF (lane 3) and unmodified pCG806 Vector as a control (lane 4) were separated by SDS-PAGE, blotted and probed with anti-MBP antiserum. M: Rainbow protein molecular weight markers (Amersham Biosciences).

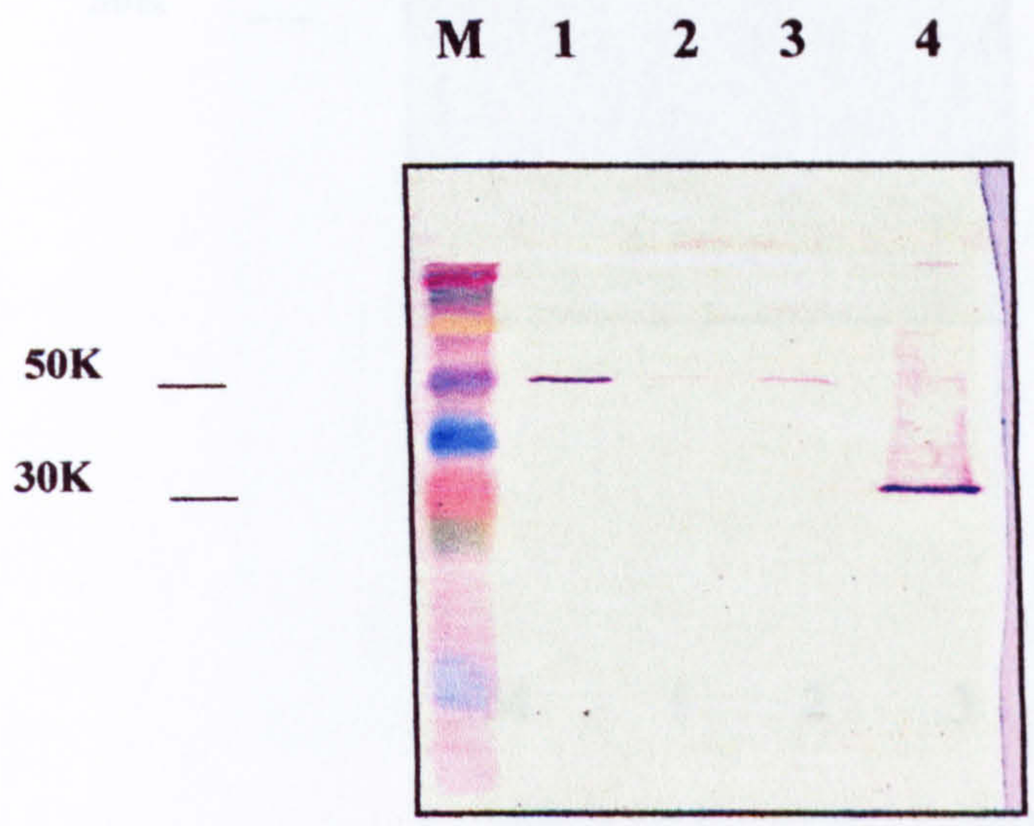

Figure 3.46: Recognition of recombinant Toxin A fragments in Western blotting with anti-Toxin A antiserum.

Bacterial lysate from induced cultures of clones IA (lane 1), IIW (lane 2), IIIF (lane 3) and purified rTA (lane 4) were separated by SDS-PAGE, blotted and probed with anti-Toxin A antiserum. M: Rainbow protein molecular weight markers (Amersham Biosciences).

Figure 3.47: Recognition of recombinant Toxin A fragments in Western blotting with anti Toxin A scFv.

Bacterial lysate from induced cultures of clones IA (lane 1), IIW (lane 2), IIIF (lane 3) were separated by SDS-PAGE, blotted and probed with anti-Toxin A scFv D5 (panel A) and scFv D4 (panel B). M: Rainbow protein molecular weight markers (Amersham Biosciences).

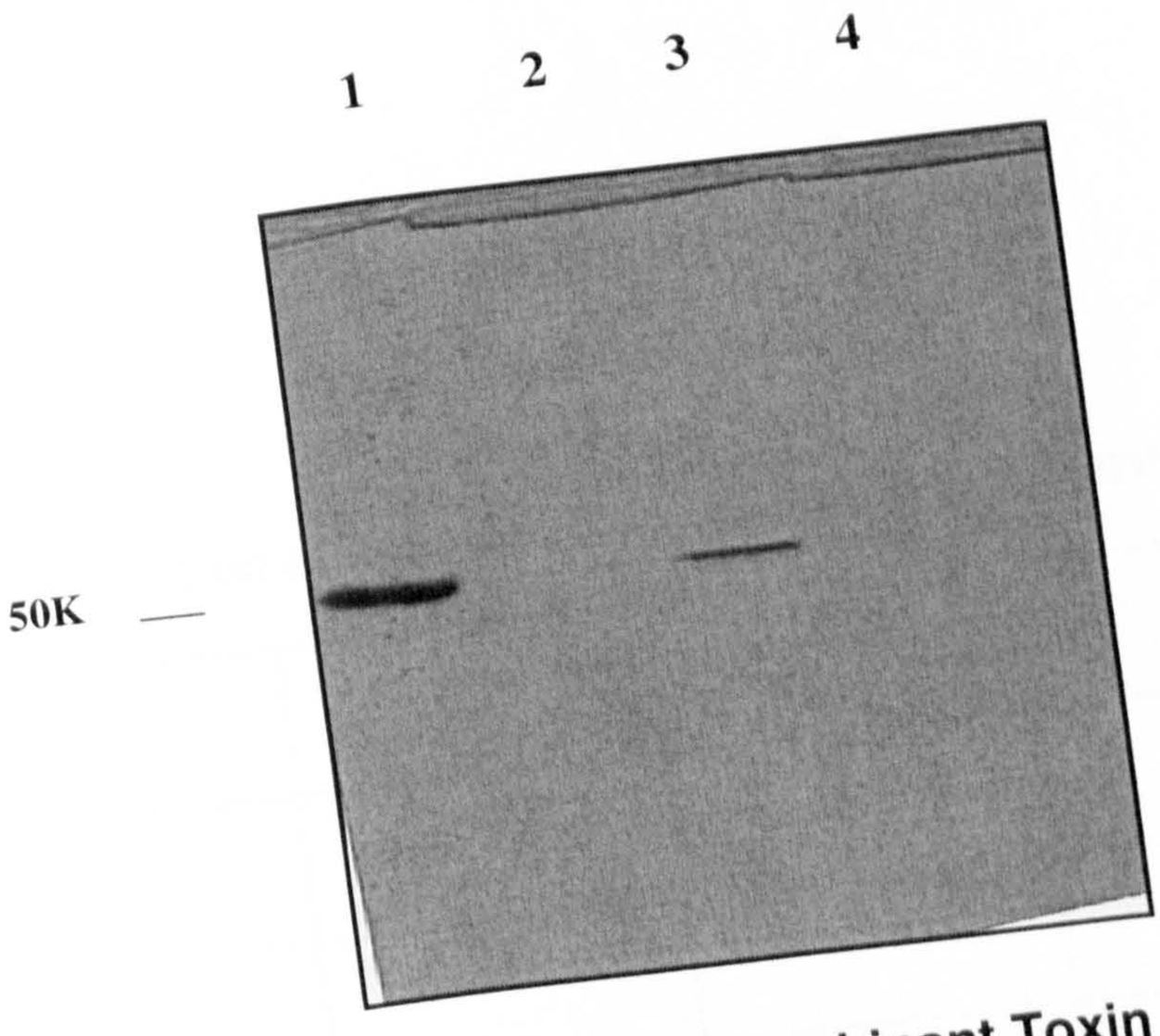

Figure 3.48: Recognition of recombinant Toxin A fragment repeat (generated by a linker) in Western blotting with anti Toxin A scFv D5.

Bacterial lysates from induced culture expressing the MBP fusion IA (lane 1), MBP from pCG806 (lanes 2 and 4), and MBP fusion R1 (lane 3) were separated by SDS-PAGE, blotted and probed with scFv D5. scFv binding was detected with ECL reagents.

Table 3.3: List of scFvs and the detected fragments

scFv	IA	IIW	IIIF	R1
D4	x			
D5	x	x†	x†	x†
C5	x			
D6		x		

† weak signal in Western blotting

Chapter 4

Discussion

4 Discussion

4.1 Purification and characterization of recombinant C-terminal domain of Toxin A

The exotoxin A is expressed by pathogenic *Clostridium difficile* that cause) an antibiotic associated diarrhea. The aims of this part of the project were to express the C-terminal domain of Toxin A as a recombinant protein, check the biological activity of the purified protein and use it as a target for isolation of antibodies from phage display libraries.

Initially, the presence of the gene encoding the recombinant Toxin A in a T7 expression system was confirmed by DNA sequencing. The insert encodes for 14 repetitive sequences of the 38 naturally present in the C-terminal receptor binding domain of the toxin. Other groups (Frey & Wilkins, 1992; Frisch *et al.*, 2003; Kink & Williams, 1998; Wren *et al.*, 1991) have reported successful cloning and expression of recombinant Toxin A or Toxin B fragments of different sizes (3, 5, 6, 9, 19, or 32 repeats), derived from the C-terminal domain, in *E. coli* or *Salmonella typhimurium* (Ward *et al.*, 1999b). These fragments were expressed as fusion proteins or independent stable proteins (Demarest *et al.*, 2005; Mathieu *et al.*, 2003). The expressed recombinant fragments were used as active or passive vaccines in animal trials. These recombinant fragments were also used *in vitro* to study the binding behavior of regions of the Toxin A C-terminal domain to its receptor or as an epitope mapping tool for localization of epitopes recognized by monoclonal antibodies raised against the C-terminal domain of the toxin. For example, to localize epitopes recognized by monoclonal antibody PCG-4 which inhibits the cytotoxicity *in vitro*, a series of fragments representing different parts of the C-terminal domain of Toxin A were successfully sub-cloned, expressed and tested for their reactivity with monoclonal

PCG-4 antibody (Frey & Wilkins, 1992). The results of this study showed that monoclonal PCG-4 antibody recognised epitopes on some fragments generated by sub-cloning of repeating units of Toxin A. Others (Frisch *et al.*, 2003) have reported successful sub-cloning and expression of recombinant proteins from different parts of the C-terminal domain of Toxin A. These recombinant proteins were expressed as GST-fusion proteins and used as ligands to bind to their receptor and to compete with the native Toxin A for the receptor. The results showed that the complete C-terminal domain could compete with native Toxin A, when present in 100-fold molar excess and thereby protect cells against rounding. However, if the fusion contained only the amino-terminal half of the receptor-binding domain, the recombinant protein was completely incapable of competing at the toxin receptor. It also showed that the addition of a small part of the hydrophobic domain to the C-terminal recombinant proteins can indirectly improve the binding efficiency by maintaining correct folding of the molecule.

Protein purification using affinity chromatography recovered a protein of 37 kDa as shown by SDS-PAGE and Western blot. This corresponds to the expected size of the target recombinant protein. Column elution by imidazole-containing buffer showed that a low degree of protein precipitation occurred immediately after elution but precipitation increased dramatically over time in storage. Similar effect was reported when recombinant proteins were expressed with a his-tag sequence (Hamilton *et al.*, 2003). To overcome this problem, acidic elution of protein was used and fractions were collected into 2 x concentrated PBS buffer for pH neutralization. The activity of the purified recombinant protein was retained after acidic elution and problems of precipitation on extended storage were eliminated. This indicates that this is an effective way for purification of acidic-tolerant recombinant proteins that carry his-tags. The concentration of the recombinant protein (> 200 μg/ml) in a soluble form made possible comprehensive characterization and study of its biological activity.

Preliminary identification of the recombinant protein as the C-terminal domain of Toxin A was made by Western blotting using polyclonal antibodies raised against the native toxin. These conclusions were supported by the high titer of the goat anti-Toxin A or anti-*C. sordelii* toxin in ELISA using the purified recombinant protein as target. This possibly reflected correct folding of the purified recombinant protein after the acidic elution. The data also suggest that the presence of the his-tag has no substantial effect on the conformation of the proteins, exposure of the epitopes and consequently on its reactivity with antibody raised against the native Toxin A. In contrast, the presence of TETC-tag (nontoxic binding domain from tetanus toxin) was shown to induce conformational changes within the Toxin A repeats, possibly masking or destroying toxin A epitopes (Ward *et al.*, 1999a). These results encouraged further study of the biological activity of the purified recombinant protein.

The repeating units of amino acids in Toxin A are thought to represent the binding domain forming a multivalent lectin able to recognise the trisaccharide Galα1-3Galβ1-4GlcNAc. This carbohydrate is found on the surface of rabbit erythrocytes, bovine thyroglobulin, a wide variety of Ig and non-Ig molecules in human milk, and is expressed on brush border membranes of hamster ileum (Ho *et al.*, 2005; Tucker *et al.*, 1990). Recently, it was reported that Galα1-3Galβ1-4GlcNAc may not be the only receptor for the toxin (Ho *et al.*, 2005). It has been widely reported that the C-terminal part of the toxin consists of short and long repeats of a series of peptide sequences (Florin & Thelestam, 1983). Each repeat consists of a β-hairpin and a loop structure as shown by crystal structures of protein derived from the binding site of the toxin. The sequence conservation in the short and long repeats are thought to play a role in the formation of kinks in the protein structure. It has been speculated that this conformation interferes with receptor recognition and cell entry. It was found that some repeats, between 5 to 15 repeats, form stable secondary structures without the interference with other structures. The folded

secondary structures may augment the binding affinity of this domain with its receptor (von Eichel-Streiber *et al.*, 1992b; Weis & Drickamer, 1996). Recently, it was reported that adjacent pairs of short repeats leads to a rotation of 120° in a screw-axis structure. In contrast, short repeats followed long repeats leads to a rotation of 90° in the screw-axis structure. This property leads to the formation of straight segments of ß-solenoid structure comprising short repeats, interrupted by kinks (Ho *et al.*, 2005). It was reported that a recombinant fragment of the binding domain of Toxin A containing 11 repetitive sequences was able to bind to receptor and that high concentration of the recombinant protein was also able to inhibit the cytotoxicity of native Toxin A (Frisch *et al.*, 2003; Sauerborn *et al.*, 1997). Other bacterial proteins which have solenoid-like structures and which contain tandem repeats of residues (e.g. pneumococcal virulence factor LytA) are often found in bacterial surface proteins. This feature allows the interaction with proteins or carbohydrates (Fernandez-Tornero *et al.*, 2001). Non-bacterial proteins like lectins also share the similar property of carbohydrate recognition (Ho *et al.*, 2005)

Given this background and the known involvement of the C-terminal domain in receptor binding, an important goal was to determine if the purified recombinant Toxin A retained the ability to interact with receptor. Recombinant Toxin A was not as effective as the native toxin in agglutination of rabbit erythrocytes which was in agreement with a previous report (Ward *et al.*, 1999a). This finding suggests that firm binding to the receptor can occur when complete the binding domain of the toxin is used, as was suggested by (Florin & Thelestam, 1983) in studies that showed that 50 % of the receptor-binding domain of Toxin A failed to bind firmly and compete at the toxin receptor (Florin & Thelestam, 1983). Similar effects were also reported by others (Frisch *et al.*, 2003; Roberts & Shone, 2001). This finding was also substantiated by an immunofluorescence assay for capture of the toxin on its receptor. This showed a weaker immunofluorescence signal when recombinant Toxin A was used compared to native toxin. Other studies (Genth *et al.*, 2000) indicated that the hydrophobic control domain has importance in the binding activity

which suggests that the complete or partial absence of this domain could reduce the efficiency of binding of the recombinant protein possibly by a failure to maintain the correct three dimensional structure of the receptor-binding domain. This was supported recently (Frisch *et al.*, 2003) in studies that showed that the addition of amino acids from the hydrophobic domain to the recombinant binding domain enhanced the blocking of the Toxin A from binding to its receptor. This shows that the hydrophobic domain has an importance in the binding activity. Their study has also shown that complete recombinant binding domain can initiate endocytosis while deletion of the C-terminal binding domain prevented the binding completely (Frisch *et al.*, 2003). However, there is some inconsistency here with earlier reports that a recombinant fragment of the binding domain of Toxin A containing only 11 repetitive sequences was able to bind to the receptor of F9 cells in high concentrations and compete with native toxin (Sauerborn *et al.*, 1997).

It has been reported that the antiserum raised against a short synthetic peptide from the repetitive domain can interfere with the binding activity of Toxin A and can inhibit the haemagglutination and the cytotoxic activities of the toxin (Wren *et al.*, 1991). Haemagglutination has been used quite widely as a measure of binding but inconsistencies in the literature hint that the assay might only measure one aspect of the interaction between Toxin A and its receptor or receptors. It is possible, for example, that erythrocytes lack glycoproteins that are expressed on other cell types, to which toxin can bind. In this thesis, antiserum raised against native Toxin A and pre-incubated with Toxin A effectively neutralized the cytotoxicity of Toxin A in cell culture. However, pre-incubation of antiserum with Toxin A failed to show an effective inhibition of the haemagglutination activity of the toxin. This problem was not solved even when different buffer concentrations, components, pHs, or different incubation periods were used. In addition, no haemagglutination inhibition was observed when higher concentrations of antiserum or lower concentrations of rabbit RBC were used. Moreover, in some instances, higher

concentration of antiserum caused a slight augmentation to the toxin haemagglutination activity. These observations could be explained in several ways. For example, antibodies might bind to and block regions of the toxin that are not linked to receptor-binding, but thereby inhibit cytotoxic action (e.g. the N-terminal enzyme domain). However, cytometry data in the thesis clearly showed that anti-Toxin A antibodies reduced protein binding to the surface of nucleated cells. This lends support to a more complex model for receptor interaction than is observed in haemagglutination. Data from wren's paper show some features in common with work presented in this thesis. For example, when antiserum raised against Toxin A was used to inhibit the haemagglutination activity of Toxin A or recombinant Toxin A, higher serum concentrations approved to have the reverse effect (Wren *et al.*, 1991). When anti-TIDGKKYYFN polyclonal antibody was pre-incubated with native or recombinant Toxin A, haemagglutination activity was efficiently inhibited. One of the earlier reports of a monoclonal reagent against Toxin A (Lyerly *et al.*, 1986) showed that the antibody (PCG-4) also recognised peptide repeat motifs at the C-terminal domain (Frey & Wilkins, 1992). The monoclonal antibody failed to block cytotoxicity *in vitro*, yet was able to protect hamsters against challenge with *C. difficile* (Frey & Wilkins, 1992). Taken together, studies of haemagglutination, the inhibition of haemagglutination and cytotoxicity experiments reflect continuing uncertainty about the identity of the host receptor for Toxin A and about the nature of the toxin-receptor interaction.

Given this uncertainty, one important goal of this project was to investigate if pre-incubation of Toxin A with monovalent, proteolytic fragment of anti-Toxin A antibody could inhibit the biological activities of Toxin A *in vitro*. This was important for two reasons. Firstly, it would establish if the neutralizing effect of polyclonal anti-toxin antibodies could be attributed to cross-linking and agglutination effects made possible because of the bivalency of the antibody molecules. Secondly, the overall goal of the project was to isolate recombinant antibodies against Toxin A and to test their biological activity. The recombinant antibodies would be monovalent scFv proteins. If the protective

effects of a polyclonal serum could be retained when these antibodies were converted to smaller, monovalent derivatives, it would demonstrate that recombinant scFvs might be expected to possess toxin neutralizing activity if they were directed against appropriate regions of Toxin A.

Hence, an anti-Toxin A antiserum was digested with papain to release functional Fab fragments (Cresswell *et al.*, 2005). Digestion of the gamma-globulin region was shown to be complete by SDS-PAGE and Western blotting. The capacity of Fab fragments to bind Toxin A was confirmed by ELISA. Given the nature of the Fab fragment, this would be predicted, but in support of this, It has been reported (Aharonov *et al.*, 1974) that Fab fragments generated by papain digestion were still functional and could block binding of intact antibodies in a competitive experiment. When Fab fragments were tested with active Toxin A in a cell culture challenge experiment, cytotoxicity was inhibited effectively. The outcome of this experiment therefore indicated that small molecular weight antibodies can retain their affinity and neutralizing activity against a complex target. This is further supported by competitive ELSIA experiments between scFv and the parent antibody (Deng *et al.*, 2003). This finding gave an indication that monoclonal scFv antibodies isolated against Toxin A might inhibit the biological activities of the toxin *in vitro*.

scFv antibodies with the ability to detect and neutralize toxins have been isolated by other investigators. It was reported that scFvs against pertussis toxin were able to recognize and neutralize the toxin in cell culture using CHO cells (Williamson & Matthews, 1999). Anti-pertussis toxin scFvs were generated from an immune phage library that was produced from the peripheral blood lymphocyte mRNA of patients with *B. pertussis* infection. scFv antibodies inhibited *in vitro* glucan synthesis catalyzed by β-1,3-glucan synthase of *S. cerevisiae* as well as that of *C. albicans*. These scFvs were also able to inhibit β-1,3-glucan synthase in four *Candida* species *in vitro* (Selvakumar *et al.*, 2006). It was reported that scFv inhibited the potent neurotoxins AahI and AahII of *Androctonus*

australis scorpion from binding to their receptor. This lead to protection against the toxin in animal trials (Juste *et al.*, 2007). scFv raised against the protective antigen of anthrax toxin were found to recognize and neutralize the cytotoxicity of the lethal toxin in cell culture (Chen *et al.*, 2006). There are some instances where individual scFv proteins failed to neutralize their target antigen but a pool of several monoclonal scFvs recognizing different epitopes showed improved potency in toxin neutralization. This effect was reported by Amersdorfer and colleages (Amersdorfer *et al.*, 2002) when the neutralization potency of single monoclonal scFvs were compared to a combination of different monoclonal scFvs against botulinum type-A toxin. These reports highlight the potential of recombinant antibody fragments in recognition and neutralization of different toxin systems from different origins. This strategy against Toxin A and was adopted in the present work and has recently been proposed by Babcock (Babcock *et al.*, 2006).

Overall, the initial work reported in this thesis fulfilled the aim to prepare a pure and biologically active form of Toxin A for the isolation of recombinant antibodies. As part of this, an improved method for the purification of the recombinant Toxin A has been devised that produces concentrated and purified protein that has potential in several applications. It is clear from the literature that the C-terminal domain of Toxin A can elicit protective response against infection. Studies in this thesis show that protective antibodies retain activity as monovalent fragments. The next phase of the project was to isolate scFv against Toxin A and then assess the biological activity of these recombinant antibodies.

4.2 Screening of the Tomlinson library

The Tomlinson I semi-synthetic library was used to isolate soluble monoclonal scFv antibodies against Toxin A. To check the quality of this library before and after the selection, the length of inserts was assessed in clones from the library. This experiment confirmed that full-length inserts were present in almost all clones tested, in contrast to

Griffin I library that showed a steady decrease of insert size during amplification cycles (de Bruin *et al.*, 1999). This finding was supported by the expression of soluble scFv from Tomlinson library at a later stage and comparison to positive control clones. This effect was also observed by a previous postgraduate student (Golchin, 2004). The expression of recombinant antibodies by bacteria can be toxic and therefore a selective pressure can drive the progressive deletion of sequence from scFvs during selection and amplification. One strength of the Tomlinson system is its use of antibody framework sequence that are well-tolerated by bacteria.

The outcome of selection was consistent with the expected enrichment of specific clones. This could be observed from the number of phage recovered from each round of selection. The increase in recovery rate at round 2 was shown to be the most successful and round 3 did not appear to increase significantly the recovery of phage. It is possible that a plateau was reached in enrichment and that the selection system had simply recovered as many binding phage as was possible. Alternatively, the competition between specific and non-specific interactions on the selecting surface may have reached equilibrium. Whatever the reason, an increase in the proportion of phage recovered during panning cycles is not always constant and variations have been reported by others (de Bruin *et al.*, 1999; Li *et al.*, 2003). Increases in the number of panning cycles from 3 to 5 have been reported but this does not necessarily increase the specificity of the screen or the quality of the antibodies that emerge. Whatever the bases for this observation, the screen provided ample numbers of phage for analysis and characterization. Clones isolated from the Tomlinson library were shown to be specific for Toxin A in polyclonal phage ELISA and reaction with the skimmed milk blocking reagent was not seen. Monoclonal scFv antibodies picked from each round of selection bound to Toxin A with high frequency when tested in phage ELISA, but the frequency was lower when soluble scFv antibodies were tested. This anomaly may be due to the multivalent display of scFv at the phage surface. Filamentous

phage carry three copies of scFv antibodies which increases the overall avidity of each phage antibody for its target (de Wildt *et al.*, 2002). This might result in the binding of phage particles in ELISA and a positive signal in ELISA, but the inability of the monovalent scFv protein to replicate this result. It is also perhaps possible that nonspecific reactions between the phage particles and the target antigen explain the observation. Similar effect has been reported while using Tomlinson I and J libraries (Wu *et al.*, 2007). To overcome this problem, some modifications were introduced to the panning cycles i.e. the use of immunotubes with two kinds of surface materials (maxisorp and polysorp), alternation of two different blocking reagents between panning cycles and the gradual increase in the concentration of Tween-20 after each cycle of panning to enhance the stringency of washing. It is also possible that scFvs were not actually expressed successfully in monoclonal scFv ELISA experiment or that some scFv proteins could not form stable structures during the expression process (Lorimer *et al.*, 1996).

The diversity of Tomlinson library I is created by synthetic variation of codons at certain positions in the scFv inserts. Whatever the anomalies in screening, phage display with this resource has facilitated the isolation and the expression of specific monoclonal anti-Toxin A scFv antibodies. From the panel of antibodies that was generated, several scFv antibodies were chosen for further study based on the strength of reactivity in ELISA. Western blot showed specific binding of all chosen scFvs to recombinant Toxin A except for one scFv which reacted with the toxin in ELISA or in dot blot only. This suggested that the majority of scFvs could react with linearized peptide sequences while one scFv reacted with a conformational structure. DNA sequencing of the clones showed diversified sequences with similarity confined to the light chain. V_L sequence showed a high frequency of serine and threonine amino acids. This could indicate a bias towards these residues in the light chain component of library. Similar observation was reported by others (Carzaniga *et al.*, 2002). The result might also have arisen because of selection

pressure imposed by the general nature of the target protein as both serine and threonine share the same physical and chemical properties (neutral-polar/hydrophilic amino acids). The diversity evident from DNA sequencing also suggests that the chosen scFvs may recognize different epitopes on the toxin. This was supported by blocking experiments in which phage and scFv were run in competition ELISA. These data showed that anti-Toxin A monoclonal scFv antibodies were only able to inhibit the binding of monoclonal phage antibodies of the same clone and that little blocking was evident between different clones (Boel *et al.*, 1998).

The Tomlinson library therefore provided a convenient source of recombinant antibodies against the carboxy-terminal region of Toxin A. The convenience of phage display as a method provided a significant advantage over conventional hybrodoma mehtods for the generation of these monoclonal reagents.

4.3 Biological activity of anti-Toxin A scFvs.

The next series of objectives were to assess the biological activities of the anti-Toxin A scFvs. One test that was used was to assess if purified anti-Toxin A scFv could inhibit the haemagglutination activity of recombinant Toxin A. As described earlier haemagglutination by Toxin A has been described as a model for receptor binding (Krivan *et al.*, 1986; Lyerly *et al.*, 1986). There are uncertainties about its relevance but it remains widely used in this context. One issue of concern was the failure of an anti-Toxin A antiserum with neutralizing activity activity to block haemagglutination mediated by the recombinant carboxy-terminal protein. Similar unexpected result was also seen when anti-Toxin A monoclonal scFv were used. Furthermore, some anti-Toxin A scFv antibodies selected clones in this study were shown to increase the haemagglutination reaction. Similar unpredicted phenomenon was reported by (Krivan *et al.*, 1986) during the evaluation of haemagglutination assay. In this assay, when anti-Toxin A antibody was incorporated, it was noticed that the sensitivity of the haemagglutination was increased

from 0.8 μg/ml of Toxin A to about 50 ng/ml. As an attempt to overcome this problem, a haemolysis assay was set up with surface-immobilized Toxin A to substitute for the haemagglutination test. This technique revealed an improved result with polyclonal anti-Toxin A antibody but not with anti-Toxin A monoclonal scFv antibodies. Others (Babcock *et al.*, 2006; Kamiya *et al.*, 1991) have reported that full-length monoclonal antibodies raised against Toxin A were able to inhibit cytotoxicity but not haemagglutination activity. Despite these inconsistent findings *in vitro*, some of these antibodies were able to inhibit enterotoxicity in animal trials. This highlights the lack of robust *in vitro* tests for assessment of the properties of the anti-Toxin A monoclonal scFv antibodies.

Further studies examined the inhibition activity of the scFv antibodies for toxin binding to F9 cells. In these experiments, when Toxin A was pre-incubated with polyclonal anti-Toxin A, no sign of toxin binding to the cells was seen. When scFvs were pre-incubated with Toxin A, incomplete inhibition of toxin binding was evident. These experiments showed that the anti-Toxin A monoclonal scFv antibodies were partially effective in blocking the binding of Toxin A to its receptor on F9 cells. These results were gathered using immunofluorescence microscopy. To validate these results, a quantitative assay using a Facscan flow-cytometer was employed. Facscan was used successfully by Cirino and colleages to show disruption of the receptor-mediated binding of PA of anthrax Toxin with scFv (Cirino *et al.*, 1999). In the research reported here, results were consistent with immunofluorescence microscopy: pre-incubation of Toxin A with a polyclonal anti-toxin antibody appeared to completely inhibit cell binding, whereas pre-incubation with scFv lead to incomplete neutralization of interaction of the toxin with receptor. This suggested that anti-Toxin A monoclonal scFv antibodies may interfere in the binding of the recombinant protein to its receptor but not to an extent to cause effective inhibition even when high concentrations (100 μg/ml) of scFv antibodies were used. This represents an approximate molar ratio of 100 scFv to 1 Toxin A in the assay. Increasing the pre-

incubation period of recombinant protein with scFvs from 30 min to 2 hrs in the cold may lead to more convincing results as has been the case in other studies (Cirino *et al.*, 1999).

To corroborate these results, cytotoxicity assays were carried out with F9 cells. These possess a high density of Toxin A cell receptors (Tucker *et al.*, 1990). This experiment revealed that anti-Toxin A scFv antibodies (100 μg/ml) or the recombinant C-terminal domain (100 μg/ml) were unable to inhibit Toxin A cytotoxicity. However, it was observed that the antibodies caused a consistent delay of about 25 min in the activity of Toxin A. If this is the case, higher concentration of monoclonal scFvs might enhance the inhibition to Toxin A in cell culture. Surprisingly, no enhanced effect was observed when pools of two or three different monoclonal scFvs were used against the toxin as was reported for botulinum toxin (Amersdorfer *et al.*, 2002). The positive control, anti-Toxin A antiserum, showed near complete inhibition of the toxin even after an extended period of incubation. The data suggest that binding of one or more scFvs to the toxin impairs the process of receptor-binding or entry of toxin to the target cell, but that this is insufficient to completely block toxin action. Given that the scFvs appear to recognised different epitopes in the carboxy-terminal domain, the results lend weight to the concept that receptor interaction can take place through several independent processes: inhibition of any one, or indeed several, serves to delay but not prevent toxin action. The other observation of interest is that conversion of the polyclonal antibody to Fab fragments did not result in loss of toxin-neutralizing activity. This suggests that an appropriate combination of monoclonal antibody fragments can, at least in principle, succeed in this assay. The specificity of these fragments remains undefined, however.

The other observation of importance is the inability of the recombinant carboxy-terminal protein used in screening to block cytotoxicity. Recent data (Frisch *et al.*, 2003) have suggested that residues from the toxin translocation domain may influence the conformation of the carboxy-terminal domain and its repeats. Recombinant protein containing only the carboxy-terminal residues failed to block toxin action, but better

blocking activity was achieved if part of the translocation domain was included in the construct. Perhaps some epitopes crucial for neutralization are only formed under these circumstances.

One of the most striking features of the receptor binding carboxy-terminal region of Toxin A is the presence of 38 repeating sequences (Dove *et al.*, 1990). It is possible that the multiple repeats help to amplify binding affinity by increasing the avidity. This increased avidity effect might lead to quick but gradual replacement of anti-Toxin A scFv (Ho *et al.*, 2005). This effect is also seen with some other enzymes and receptors (von Eichel-Streiber *et al.*, 1992b).

Studies on animals showed that death due to Toxins A and B is correlated to *in vitro* neutralization of the toxins and not to the level of antibodies measured by ELISA (Corrado *et al.*, 1990).

To our knowledge, only one monoclonal anti-Toxin A antibody has been isolated that has an inhibitory effect against the cytotoxic activity, haemagglutination and enteric toxicity in animals (Babcock *et al.*, 2006). In this study, antibodies were produced in a transgenic mouse carrying human immunoglobulins HuMAb genes by immunization with inactivated Toxin A and Toxin B. The antibodies were then tested for their cytotoxic and haemagglutination activities. Of the antibodies isolated, only one antibody showed neutralization activity against cytotoxicity, haemagglutination and enterotoxicity. This antibody was found to recognize an epitope within the C-terminal binding domain. This suggests that screening for scFv antibodies that have three inhibitory activities against the toxin might be warranted.

Screening anti-Toxin A scFv might increase the chances of isolating antibodies that recognize critical epitopes on the toxin (Frey & Wilkins, 1992) and which have inhibitory effects, but the complexity of this process needs to be considered. In the work reported in

this thesis, a modest number of scFv clones that were reactive with Toxin A were chosen for closer analysis. They were chosen largely upon the basis of the strength of reaction in ELISA. If much greater numbers of clones were to be processed, it must be noted that scFv purification, characterization and neutralization experiments are time consuming and laborious to carry out. Therefore, a more efficient and quicker method is required to screen a large number of scFvs needs to be employed, such as array technology. In the system described by antibodies were prepared and screened in an array system in which antibodies showing specificity to the target antigen were selected and grown for further analysis (Rubinstein *et al.*, 2003). This method made possible a selection based on the biological activity of the recombinant antibodies and not on a random basis, using immunoassay data as the primary feature.

The structural differences between native immunoglobulin and scFv antibodies should not be disregarded when seeking to improve on the results reported in this thesis. An important difference between native immunoglobulin and scFv is that native immunoglobulin has a multivalent interaction with antigen whereas scFv is monovalent because of the significant difference in molecular weight. Stubbe *et al.* reported that polymeric IgA was superior to monomeric IgA and IgG carrying the same variable domain in neutralizing *Clostridium difficile* Toxin A (Stubbe *et al.*, 2000). Therefore, divalent, trivalent or even quadravalent formats for scFv expression should also be considered to overcome the limitations of the monovalency of the scFv antibody. This could be explored with the scFvs reported here to see if the change affects their capacity to block the action of Toxin A. However, it is worth noting that inhibition of Toxin A cytotoxicity using Fab fragments from polyclonal anti-toxin showed comparable neutralizing activity to the complete anti-Toxin A antibodies. This suggested that there are some sites on Toxin A that are blocked by monovalent antibody. One strategy would therefore be to use Fab fragments from a polyclonal serum in competitive elution of phage antibodies from a Toxin A target.

This could be used to isolate scFvs from a library that react with epitopes recognised by Fabs with neutralizing activity. This form of competitive elution has been used by others (Meulemans *et al.*, 1994). As part of the research in this thesis, the molecular weight of anti-Toxin A scFv fragments was increased by cross-linking to anti-c-myc antibody (Wang *et al.*, 2004). This added a significant mass to the scFv fragment by assembling it into an immune complex. The samples were used in cytotoxicity tests with the toxin but no inhibition was observed. Although the peptide tag is located at the C-terminus of the scFv, it is possible that anti-c-myc antibody might interfere with the scFv function or more optimization of the technique is required.

4.4 Epitope mapping of anti-Toxin A scFv

It was discussed previously that the carboxy-terminal part of Toxin A is thought to form a solenoid like structure that consists of short and long repeats (Florin & Thelestam, 1983). Each repeat consists of a β-hairpin and a loop structure and at least some of these features represent the receptor binding site of the toxin (Greco *et al.*, 2006). The sequence conservation in the short and long repeats are thought to play a role in the formation of kinks which is speculated to interfere with receptor recognition and cell entry (von Eichel-Streiber *et al.*, 1992b; Weis & Drickamer, 1996). Because of this, some regions in the C-terminal binding domain of the toxin might have more importance in receptor recognition than other regions. This was supported by crystal structure analysis (Ho *et al.*, 2005). Therefore, an important goal for the research reported in this thesis was to identify more precisely the regions that are recognised by the scFvs on the C-terminal binding domain of Toxin A. Potentially, the binding characteristics of these antibodies might explain the outcome of the *in vitro* inhibition experiments.

To identify the regions recognized by the monoclonal anti-Toxin A scFv antibodies, partially overlapping fragments of the C-terminal domains of Toxin A were

isolated by PCR for sub-cloning. To facilitate their expression and to assist the successful folding of these small proteins, they were fused with maltose-binding protein and expressed in *E. coli* (di Guan C, 1988). The three constructs (IA, IIW and IIIF) were predicted to have molecular weights of about 58, 58 and 52 kDa proteins respectively. The molecular weight of the maltose-binding proteins carriers in each fusion was 43 kDa. Monoclonal anti-Toxin A scFv antibodies were first tested in Western blots against the recombinant C-terminal domain. This and other experiments showed that the scFvs recognised linearized epitopes. A similar approach then revealed that monoclonal scFv antibodies reacted with the sub-cloned fragments from the carboxy-domain of Toxin A. This enabled localization of epitopes recognised by the scFvs. This approach was recently used by Bacock and colleages (Babcock *et al.*, 2006). Interestingly, the reaction of scFv D5 with all three MBP fusions enabled further, more precise epitope mapping. DNA sequencing and alignment of the three sub-cloned fragments highlighted the repetitive sequences common to all three sub-clones. The hypothesis that D5 was cross-reactive with one of these repetitive sequences was tested by creation of another MBP fusion containing one of these sequences. This R1 protein contained only 3 kDa of Toxin A sequence (20 amino acids) comprising SR3 short repeat motif (Ho *et al.*, 2005). This approach proved that the monoclonal scFv D5 antibody recognized a short linear sequence within R1 protein and which might exist partially or completely in scattered regions throughout the C-terminal domain of Toxin A. Further sub-cloning, deletion or peptide synthesis could be used for precise definition of the epitope recognised by scFv D5 antibody. It also would be valuable to sub-clone short sequences from IIW and IIIF regions for epitope mapping of the other selected anti-Toxin A scFvs.

Wren *et al.* reported that a short peptide sequence (TIDGKKYYFN) from the C-terminal region of Toxin A induced the production of anti-Toxin A antibodies when injected into rabbits (Wren *et al.*, 1991). The antibodies were shown to neutralize the haemagglutination and cytotoxicity activity of Toxin A. It would be valuable to isolate a

monoclonal scFv antibody against this peptide sequence and test if similar biological properties were evident. This short peptide sequence is present in the MBP fusions IIW and IIIF, therefore, selection based upon panning on alternating coated surfaces might generate more phage antibodies against this and other repeat sequences.

Another observation of potential relevance to the outcome of this project was the failure of the recombinant C-terminal protein to compete with, and inhibit the action of Toxin A, *in vitro*. The protein used in this thesis carried 14 of the 38 peptide repeats present in the native toxin. This was sufficient to create haemagglutination and the cross-reaction of scFvs with native toxin confirmed the presence of relevant epitopes. Recent work has shown that the complete C-terminal domain can inhibit the action of native toxin, presumably through competition for receptor binding (Frisch *et al.*, 2003). Interestingly, the inhibition effect is increased when parts of the toxin translocation domain are also present. Perhaps this improves the folding of the receptor binding domain or creates novel receptor binding interactions that are different to those in haemagglutination. Isolation of antibodies against these forms of the toxin might produce scFvs with more effective biological properties.

4.5 Conclusion

Overall, the use of the Tomlinson libraries enabled successful isolation of anti-Toxin A scFvs. When the isolated scFv were tested against Toxin A, none were able to neutralize the toxin. When different combinations of scFvs were used to neutralize the toxin no improvement was achieved even with high concentrations of the scFv antibodies. However, a consistent delay of toxin action was observed. This effect might be explained by the multivalent binding of the toxin with its receptor. The experiments used a recombinant form of the C-terminal domain containing 14 of the peptide repeats present in

this part of the toxin. The recombinant protein did not have the capacity to inhibit the complete Toxin A *in vitro*.

Although isolated anti-Toxin A scFv antibodies did not have the capacity to inhibit Toxin A, they were able to detect the toxin with high specificity and sensitivity compared to a commercial monoclonal antibody (Fu *et al.*, 2004). This sensitivity could be increased further by combining phage display technology and PCR. Simply, phage antibodies which carry scFv antibodies can be used in a sandwich assay to detect a captured antigen and then DNA from the phage can be amplified by PCR (Guo *et al.*, 2006). This can increase detection limit of an immunoassay to 10 pg/ml. This is more sensitive than other ELISA formats.

scFv antibodies have many other advantages over conventional monoclonal antibodies. The use of bacteria for scFv production eliminates the need for animals, adds speed, flexibility and removes boundaries on yield. Being recombinant proteins, scFvs can be modified and expressed with reporters such as fluorescent molecules possible which can make one-step detection of target proteins.

The failure of scFvs characterized in this thesis to neutralize Toxin A highlights our continuing ignorance of the nature of interaction between Toxin A and its receptor. The production of polyclonal anti-Toxin A antibodies which have inhibitory effects against the toxin was definitely a major step towards more understanding of the disease caused by this toxin. However, it remains uncertain if the benefits of recombinant antibody technology can be applied to the production of therapeutic proteins for *C. difficile* infection.

5 Appendices

5.1 Luria-Bertani (LB) broth or agar

Tryptone (Duchefa biochemi)	10.0g
Yeast extract (Duchefa biochemi)	10.0g
NaCl (Analar, BDH)	10.0g
Distilled water	to 1 litre

The solutes were dissolved in distilled water by shaking and the pH was adjusted to 7.0 with 1 M NaOH. For solid medium, 1.2 % (w/v) agar was added before autoclaving.

5.2 Preparation of antibiotic solutions

Stock solutions of antibiotics (ampicillin (1.0 mg/ml), kanamycin (0.5 mg/ml)) were dissolved in distilled water and filter sterilised by passing to solution through a filter of 0.2 μm pore size. All antibiotic solutions were stored at -20° C.

5.3 SOC medium

A) SOC base

	Per liter of dH2O	Final conc.
Tryptone	20.0 g	2 % (w/v)
Yeast extract	5.0 g	0.5 % (w/v)
NaCl	0.58 g	10 mM
KCl	0.18 g	2.5 mM

The solutes were dissolved by shaking and the pH was adjusted to 7.0 with NaOH. The broth was sterilised by autoclaving and stored at 4° C.

B) SOC additive (10x concentration):

	Per 100 ml of dH2O	Final conc.
Glucose	2.0 g	20 mM
$MgCl_2$ $6H_2O$	2.0 g	10 mM
$MgSO_4$ $7H_2O$	2.46 g	10 mM

The solutes were dissolved by using a magnetic stirrer, filter sterilised and stored at 4° C. The SOC additive was added to SOC base in a ratio of 1:10 just before use.

5.4 Citric acid phosphate buffer

To prepare citric acid phosphate buffer pH values of 3.4, 5.0, and 6.0, citric acid and disodium phosphate were mixed as shown in the following table. After mixing, NaCl was added to a final concentration of 0.5 M and the solutions were filtered though a 0.2 μm filter.

Target pH	0.1 M citric acid	0.2 M disodium phosphate
3.5	65 ml	35 ml
5	45 ml	55 ml
6	33 ml	67 ml

6 Bibliography

Aboudola, S., Kotloff, K. L., Kyne, L., Warny, M., Kelly, E. C., Sougioultzis, S., Giannasca, P. J., Monath, T. P. & Kelly, C. P. (2003). *Clostridium difficile* vaccine and serum immunoglobulin G antibody response to toxin A. *Infect Immun* **71**, 1608-1610.

Adams, G. P. & Schier, R. (1999). Generating improved single-chain Fv molecules for tumor targeting. *J Immunol Methods* **231**, 249-260.

Aharonov, A., Gurari, D. & Fuchs, S. (1974). Immunochemical characterization of Naja naja siamensis toxin and of a chemically modified toxin. *Eur J Biochem* **45**, 297-303.

Aktories, K. & Just, I. (1995). Monoglucosylation of low-molecular-mass GTP-binding Rho proteins by clostridial cytotoxins. *Trends Cell Biol* **5**, 441-443.

Alcantara, C., Stenson, W. F., Steiner, T. S. & Guerrant, R. L. (2001). Role of inducible cyclooxygenase and prostaglandins in *Clostridium difficile* toxin A-induced secretion and inflammation in an animal model. *J Infect Dis* **184**, 648-652.

Amersdorfer, P., Wong, C., Smith, T., Chen, S., Deshpande, S., Sheridan, R. & Marks, J. D. (2002). Genetic and immunological comparison of anti-botulinum type A antibodies from immune and non-immune human phage libraries. *Vaccine* **20**, 1640-1648.

Amoroso, A., Mitterhofer, A. P., Del Porto, F., Garzia, P., Ferri, G. M., Galluzzo, S., Vadacca, M., Caccavo, D. & Afeltra, A. (2003). Antibodies to anionic phospholipids and anti-beta2-GPI: association with thrombosis and thrombocytopenia in systemic lupus erythematosus. *Hum Immunol* **64**, 265-273.

Amoura, Z. & Piette, J. C. (2006). [New therapeutic approaches to autoimmune diseases]. *Presse Med* **35**, 709-713.

Anand, A. & Glatt, A. E. (1993). *Clostridium difficile* infection associated with antineoplastic chemotherapy: a review. *Clin Infect Dis* **17**, 109-113.

Aronsson, B., Mollby, R. & Nord, C. E. (1985). Antimicrobial agents and *Clostridium difficile* in acute enteric disease: epidemiological data from Sweden, 1980-1982. *J Infect Dis* **151**, 476-481.

Ashkenazi, A. & Dixit, V. M. (1998). Death receptors: signaling and modulation. *Science* **281**, 1305-1308.

Babcock, G. J., Broering, T. J., Hernandez, H. J. Mandell RB, Donahue K, Boatright N, Stack AM, Lowy I, Graziano R, Molrine D, Ambrosino & DM, Thomas WD Jr. (2006). Human monoclonal antibodies directed against toxins A and B prevent *Clostridium difficile*-induced mortality in hamsters. *Infect Immun* **74**, 6339-6347.

Barbas, C. F., 3rd, Bain, J. D., Hoekstra, D. M. & Lerner, R. A. (1992). Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. *Proc Natl Acad Sci U S A* **89**, 4457-4461.

Barbas, C. F., 3rd, Collet, T. A., Amberg, W. Roben P, Binley JM, Hoekstra D, Cababa D, Jones TM, Williamson RA, & Pilkington GR, (1993). Molecular profile of an antibody response to HIV-1 as probed by combinatorial libraries. *J Mol Biol* **230**, 812-823.

Barroso, L. A., Moncrief, J. S., Lyerly, D. M. & Wilkins, T. D. (1994). Mutagenesis of the *Clostridium difficile* toxin B gene and effect on cytotoxic activity. *Microb Pathog* **16**, 297-303.

Bartlett, J. G. (1981). Antimicrobial agents implicated in *Clostridium difficile* toxin-associated diarrhea of colitis. *Johns Hopkins Med J* **149**, 6-9.

Bartlett, J. G. (1992). Antibiotic-associated diarrhea. *Clin Infect Dis* **15**, 573-581.

Bartlett, J. G. (1994). *Clostridium difficile*: history of its role as an enteric pathogen and the current state of knowledge about the organism. *Clin Infect Dis* **18 Suppl 4**, S265-272.

Beckmann, C., Haase, B., Timmis, K. N. & Tesar, M. (1998). Multifunctional g3p-peptide tag for current phage display systems. *J Immunol Methods* **212**, 131-138.

Beloosesky, Y., Grosman, B., Marmelstein, V. & Grinblat, J. (2000). Convulsions induced by metronidazole treatment for *Clostridium difficile*-associated disease in chronic renal failure. *Am J Med Sci* **319**, 338-339.

Better, M., Chang, C. P., Robinson, R. R. & Horwitz, A. H. (1988). *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* **240**, 1041-1043.

Boel, E., Bootsma, H., de Kruif, J., Jansze, M., Klingman, K. L., van Dijk, H. & Logtenberg, T. (1998). Phage antibodies obtained by competitive selection on complement-resistant Moraxella (Branhamella) catarrhalis recognize the high-molecular-weight outer membrane protein. *Infect Immun* **66**, 83-88.

Borriello, S. P., Wren, B. W., Hyde, S., Seddon, S. V., Sibbons, P., Krishna, M. M., Tabaqchali, S., Manek, S. & Price, A. B. (1992). Molecular, immunological, and biological characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile*. *Infect Immun* **60**, 4192-4199.

Bosslet, K., Steinstraesser, A., Hermentin, P., Kuhlmann, L., Bruynck, A., Magerstaedt, M., Seemann, G., Schwarz, A. & Sedlacek, H. H. (1991). Generation of bispecific monoclonal antibodies for two phase radioimmunotherapy. *Br J Cancer* **63**, 681-686.

Bowley, D. R., Labrijn, A. F., Zwick, M. B. & Burton, D. R. (2007). Antigen selection from an HIV-1 immune antibody library displayed on yeast yields many novel antibodies compared to selection from the same library displayed on phage. *Protein Eng Des Sel* **20**, 81-90.

Bradbury, A., Persic, L., Werge, T. & Cattaneo, A. (1993). Use of living columns to select specific phage antibodies. *Biotechnology (N Y)* **11**, 1565-1569.

Brekke, O. H. & Sandlie, I. (2003). Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat Rev Drug Discov* **2**, 52-62.

Brito, G. A., Fujji, J., Carneiro-Filho, B. A., Lima, A. A., Obrig, T. & Guerrant, R. L. (2002). Mechanism of *Clostridium difficile* toxin A-induced apoptosis in T84 cells. *J Infect Dis* **186**, 1438-1447.

Buggy, B. P., Fekety, R. & Silva, J., Jr. (1987). Therapy of relapsing *Clostridium difficile*-associated diarrhea and colitis with the combination of vancomycin and rifampin. *J Clin Gastroenterol* **9**, 155-159.

Cabrera, O., Cuello, M., Soto, C. R., Martinez, M. E., del Campo, J. M., Perez, O., Infante, J. F. & Sierra, G. (2006). New method for obtaining conjugated vaccines. *Vaccine* **24 Suppl 2**, S2-76-78.

Cai, X. & Garen, A. (1995). Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. *Proc Natl Acad Sci U S A* **92**, 6537-6541.

Capon, D. J., Chamow, S. M., Mordenti, J. Marsters SA, Gregory T, Mitsuya H, Byrn RA, Lucas C, Wurm FM, & Groopman JE (1989). Designing CD4 immunoadhesins for AIDS therapy. *Nature* **337**, 525-531.

Cardoso, D. F., Nato, F., England, P., Ferreira, M. L., Vaughan, T. J., Mota, I., Mazie, J. C., Choumet, V. & Lafaye, P. (2000). Neutralizing human anti crotoxin scFv isolated from a nonimmunized phage library. *Scand J Immunol* **51**, 337-344.

Carneiro, B. A., Fujii, J., Brito, G. A., Alcantara, C., Oria, R. B., Lima, A. A., Obrig, T. & Guerrant, R. L. (2006). Caspase and bid involvement in *Clostridium difficile* toxin A-induced apoptosis and modulation of toxin A effects by glutamine and alanyl-glutamine in vivo and in vitro. *Infect Immun* **74**, 81-87.

Cartmill, T. D., Panigrahi, H., Worsley, M. A., McCann, D. C., Nice, C. N. & Keith, E. (1994). Management and control of a large outbreak of diarrhoea due to *Clostridium difficile*. *J Hosp Infect* **27**, 1-15.

Carzaniga, R., Fiocco, D., Bowyer, P. & O'Connell, R. J. (2002). Localization of melanin in conidia of Alternaria alternata using phage display antibodies. *Mol Plant Microbe Interact* **15**, 216-224.

Casadevall, A. (1998). Antibody-mediated protection against intracellular pathogens. *Trends Microbiol* **6**, 102-107.

Castagliuolo, I., Keates, A. C., Wang, C. C., Pasha, A., Valenick, L., Kelly, C. P., Nikulasson, S. T., LaMont, J. T. & Pothoulakis, C. (1998a). *Clostridium difficile* toxin A stimulates macrophage-inflammatory protein-2 production in rat intestinal epithelial cells. *J Immunol* **160**, 6039-6045.

Castagliuolo, I., Riegler, M., Pasha, A., Nikulasson, S., Lu, B., Gerard, C., Gerard, N. P. & Pothoulakis, C. (1998b). Neurokinin-1 (NK-1) receptor is required in *Clostridium difficile*- induced enteritis. *J Clin Invest* **101**, 1547-1550.

Castagliuolo, I., Sardina, M., Brun, P., DeRos, C., Mastrotto, C., Lovato, L. & Palu, G. (2004). *Clostridium difficile* toxin A carboxyl-terminus peptide lacking ADP-ribosyltransferase activity acts as a mucosal adjuvant. *Infect Immun* **72**, 2827-2836.

Cavalcante, I. C., Castro, M. V., Barreto, A. R. Sullivan GW, Vale M, Almeida PR, Linden J, Rieger JM, Cunha FQ, Guerrant RL, Ribeiro RA, & Brito GA.(2006). Effect of novel A2A adenosine receptor agonist ATL 313 on *Clostridium difficile* toxin A-induced murine ileal enteritis. *Infect Immun* **74**, 2606-2612.

Chang, T. W., Lin, P. S., Gorbach, S. L. & Bartlett, J. G. (1979). Ultrastructural changes of cultured human amnion cells by *Clostridium difficile* toxin. *Infect Immun* **23**, 795-798.

Chardin, P., Boquet, P., Madaule, P., Popoff, M. R., Rubin, E. J. & Gill, D. M. (1989). The mammalian G protein rhoC is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. *Embo J* **8**, 1087-1092.

Chen, Z., Moayeri, M., Zhou, Y. H. Leppla S, Emerson S, Sebrell A, Yu F, Svitel J, Schuck P, St Claire M, & Purcell R. (2006). Efficient neutralization of anthrax toxin by chimpanzee monoclonal antibodies against protective antigen. *J Infect Dis* **193**, 625-633.

Cheson, B. D. (2006). Monoclonal antibody therapy for B-cell malignancies. *Semin Oncol* **33**, S2-14.

Cho, W. (2001). Membrane targeting by C1 and C2 domains. *J Biol Chem* **276**, 32407-32410.

Ciesla, W. P., Jr. & Bobak, D. A. (1998). *Clostridium difficile* toxins A and B are cation-dependent UDP-glucose hydrolases with differing catalytic activities. *J Biol Chem* **273**, 16021-16026.

Cirino, N. M., Sblattero, D., Allen, D., Peterson, S. R., Marks, J. D., Jackson, P. J., Bradbury, A. & Lehnert, B. E. (1999). Disruption of anthrax toxin binding with the use of human antibodies and competitive inhibitors. *Infect Immun* **67**, 2957-2963.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D. & Winter, G. (1991). Making antibody fragments using phage display libraries. *Nature* **352**, 624-628.

Colcher, D., Bird, R., Roselli, M. Hardman KD, Johnson S, Pope S, Dodd SW, Pantoliano MW, Milenic DE, & Schlom J. (1990). In vivo tumor targeting of a recombinant single-chain antigen-binding protein. *J Natl Cancer Inst* **82**, 1191-1197.

Corrado, O. J., Mascie-Taylor, B. H., Hall, M. J. & Bolton, R. P. (1990). Prevalence of *Clostridium difficile* on a mixed-function ward for the elderly. *J Infect* **21**, 287-292.

Corthier, G., Muller, M. C., Wilkins, T. D., Lyerly, D. & L'Haridon, R. (1991). Protection against experimental pseudomembranous colitis in gnotobiotic mice by

use of monoclonal antibodies against *Clostridium difficile* toxin A. *Infect Immun* **59**, 1192-1195.

Cresswell, C., Newcombe, A. R., Davies, S., Macpherson, I., Nelson, P., O'Donovan, K. & Francis, R. (2005). Optimal conditions for the papain digestion of polyclonal ovine IgG for the production of bio-therapeutic Fab fragments. *Biotechnol Appl Biochem* **42**, 163-168.

Crissman, J. W. & Smith, G. P. (1984). Gene-III protein of filamentous phages: evidence for a carboxyl-terminal domain with a role in morphogenesis. *Virology* **132**, 445-455.

Dagan, S. & Eren, R. (2003). Therapeutic antibodies against viral hepatitis. *Curr Opin Mol Ther* **5**, 148-155.

Daubener, W., Leiser, E., von Eichel-Streiber, C. & Hadding, U. (1988). *Clostridium difficile* toxins A and B inhibit human immune response in vitro. *Infect Immun* **56**, 1107-1112.

Davis, G. T., Bedzyk, W. D., Voss, E. W. & Jacobs, T. W. (1991). Single chain antibody (SCA) encoding genes: one-step construction and expression in eukaryotic cells. *Biotechnology (N Y)* **9**, 165-169.

de Bruin, R., Spelt, K., Mol, J., Koes, R. & Quattrocchio, F. (1999). Selection of high-affinity phage antibodies from phage display libraries. *Nat Biotechnol* **17**, 397-399.

de Wildt, R. M., Tomlinson, I. M., Ong, J. L. & Holliger, P. (2002). Isolation of receptor-ligand pairs by capture of long-lived multivalent interaction complexes. *Proc Natl Acad Sci U S A* **99**, 8530-8535.

Deisenhofer, J., Colman, P. M., Epp, O. & Huber, R. (1976). Crystallographic structural studies of a human Fc fragment. II. A complete model based on a Fourier map at 3.5 A resolution. *Hoppe Seylers Z Physiol Chem* **357**, 1421-1434.

Demarest, S. J., Salbato, J., Elia, M. Zhong J, Morrow T, Holland T, Kline K, Woodnutt G, Kimmel BE, & Hansen G. (2005). Structural characterization of the cell wall binding domains of *Clostridium difficile* toxins A and B; evidence that Ca2+ plays a role in toxin A cell surface association. *J Mol Biol* **346**, 1197-1206.

Deng, X. K., Nesbit, L. A. & Morrow, K. J., Jr. (2003). Recombinant single-chain variable fragment antibodies directed against *Clostridium difficile* toxin B produced by use of an optimized phage display system. *Clin Diagn Lab Immunol* **10**, 587-595.

Desplancq, D., King, D. J., Lawson, A. D. & Mountain, A. (1994). Multimerization behaviour of single chain Fv variants for the tumour-binding antibody B72.3. *Protein Eng* **7**, 1027-1033.

di Guan, C., Li, P., Riggs, P. D. & Inouye, H. (1988). Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. *Gene* **67**, 21-30.

di Guan C, L. P., Riggs PD, Inouye H. (1988). Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. *Gene 1988 Jul* **67**, 12-30.

Dillon, S. T., Rubin, E. J., Yakubovich, M., Pothoulakis, C., LaMont, J. T., Feig, L. A. & Gilbert, R. J. (1995). Involvement of Ras-related Rho proteins in the mechanisms of action of *Clostridium difficile* toxin A and toxin B. *Infect Immun* **63**, 1421-1426.

Dodd, R. B. & Drickamer, K. (2001). Lectin-like proteins in model organisms: implications for evolution of carbohydrate-binding activity. *Glycobiology* **11**, 71R-79R.

Doral, H., McCartney, J. E., Hudziak, R. M., Tai, M. S., Laminet, A. A., Houston, L. L., Huston, J. S. & Oppermann, H. (1994). Mammalian cell expression of single-chain Fv (sFv) antibody proteins and their C-terminal fusions with interleukin-2 and other effector domains. *Biotechnology (N Y)* **12**, 890-897.

Dove, C. H., Wang, S. Z., Price, S. B., Phelps, C. J., Lyerly, D. M., Wilkins, T. D. & Johnson, J. L. (1990). Molecular characterization of the *Clostridium difficile* toxin A gene. *Infect Immun* **58**, 480-488.

Eggertson, L. (2005). *C. difficile* strain 20 times more virulent. *Cmaj* **172**, 1279.

Farah, R. A., Clinchy, B., Herrera, L. & Vitetta, E. S. (1998). The development of monoclonal antibodies for the therapy of cancer. *Crit Rev Eukaryot Gene Expr* **8**, 321-356.

Faust, C., Ye, B. & Song, K. P. (1998). The enzymatic domain of *Clostridium difficile* toxin A is located within its N-terminal region. *Biochem Biophys Res Commun* **251**, 100-105.

Fekety, R. (1997). Guidelines for the diagnosis and management of *Clostridium difficile*-associated diarrhea and colitis. American College of Gastroenterology, Practice Parameters Committee. *Am J Gastroenterol* **92**, 739-750.

Fekety, R., McFarland, L. V., Surawicz, C. M., Greenberg, R. N., Elmer, G. W. & Mulligan, M. E. (1997). Recurrent *Clostridium difficile* diarrhea: characteristics of and risk factors for patients enrolled in a prospective, randomized, double-blinded trial. *Clin Infect Dis* **24**, 324-333.

Fernandez-Tornero, C., Lopez, R., Garcia, E., Gimenez-Gallego, G. & Romero, A. (2001). A novel solenoid fold in the cell wall anchoring domain of the pneumococcal virulence factor LytA. *Nat Struct Biol* **8**, 1020-1024.

Fernie, D. S., Thomson, R. O., Batty, I. & Walker, P. D. (1983). Active and passive immunization to protect against antibiotic associated caecitis in hamsters. *Dev Biol Stand* **53**, 325-332.

Fiorentini, C., Malorni, W., Paradisi, S., Giuliano, M., Mastrantonio, P. & Donelli, G. (1990). Interaction of *Clostridium difficile* toxin A with cultured cells: cytoskeletal changes and nuclear polarization. *Infect Immun* **58**, 2329-2336.

Fiorentini, C., Donelli, G., Nicotera, P. & Thelestam, M. (1993). *Clostridium difficile* toxin A elicits Ca(2+)-independent cytotoxic effects in cultured normal rat intestinal crypt cells. *Infect Immun* **61**, 3988-3993.

Fiorentini, C., Fabbri, A., Falzano, L., Fattorossi, A., Matarrese, P., Rivabene, R. & Donelli, G. (1998). *Clostridium difficile* toxin B induces apoptosis in intestinal cultured cells. *Infect Immun* **66**, 2660-2665.

Fishwild, D. M., O'Donnell, S. L., Bengoechea, T. Hudson DV, Harding F, Bernhard SL, Jones D, Kay RM, Higgins KM, Schramm SR, & Lonberg N. (1996). High-avidity human IgG kappa monoclonal antibodies from a novel strain of minilocus transgenic mice. *Nat Biotechnol* **14**, 845-851.

Flegel, W. A., Muller, F., Daubener, W., Fischer, H. G., Hadding, U. & Northoff, H. (1991). Cytokine response by human monocytes to *Clostridium difficile* toxin A and toxin B. *Infect Immun* **59**, 3659-3666.

Florin, I. & Thelestam, M. (1983). Internalization of *Clostridium difficile* cytotoxin into cultured human lung fibroblasts. *Biochim Biophys Acta* **763**, 383-392.

Frey, S. M. & Wilkins, T. D. (1992). Localization of two epitopes recognized by monoclonal antibody PCG-4 on *Clostridium difficile* toxin A. *Infect Immun* **60**, 2488-2492.

Frisch, C., Gerhard, R., Aktories, K., Hofmann, F. & Just, I. (2003). The complete receptor-binding domain of *Clostridium difficile* toxin A is required for endocytosis. *Biochem Biophys Res Commun* **300**, 706-711.

Fu, S. W., Zhang, Y. L. & Zhou, D. Y. (2004). Development of an ELISA kit using monoclonal antibody to *Clostridium difficile* toxin A. *World J Gastroenterol* **10**, 2747-2749.

Fujisawa, K., Madaule, P., Ishizaki, T., Watanabe, G., Bito, H., Saito, Y., Hall, A. & Narumiya, S. (1998). Different regions of Rho determine Rho-selective binding of different classes of Rho target molecules. *J Biol Chem* **273**, 18943-18949.

Furman, R. R., Coleman, M., Muss, D. & Leonard, J. P. (2006). Monoclonal antibodies in the treatment of non-Hodgkin's lymphoma. *Cancer Treat Res* **131**, 221-250.

Gargir, A., Ofek, I., Meron-Sudai, S., Tanamy, M. G., Kabouridis, P. S. & Nissim, A. (2002). Single chain antibodies specific for fatty acids derived from a semi-synthetic phage display library. *Biochim Biophys Acta* **1569**, 167-173.

Genth, H., Selzer, J., Busch, C., Dumbach, J., Hofmann, F., Aktories, K. & Just, I. (2000). New method to generate enzymatically deficient *Clostridium difficile* toxin B as an antigen for immunization. *Infect Immun* **68**, 1094-1101.

George, A. J., Titus, J. A., Jost, C. R., Kurucz, I., Perez, P., Andrew, S. M., Nicholls, P. J., Huston, J. S. & Segal, D. M. (1995). Redirection of cellular cytotoxicity. A two-step approach using recombinant single-chain Fv molecules. *Cell Biophys* **26**, 153-165.

Giannasca, P. J. & Warny, M. (2004). Active and passive immunization against *Clostridium difficile* diarrhea and colitis. *Vaccine* **22**, 848-856.

Gibbs, R. A., Posner, B. A., Filpula, D. R., Dodd, S. W., Finkelman, M. A., Lee, T. K., Wroble, M., Whitlow, M. & Benkovic, S. J. (1991). Construction and characterization of a single-chain catalytic antibody. *Proc Natl Acad Sci U S A* **88**, 4001-4004.

Glaser-Wuttke, G., Keppner, J. & Rasched, I. (1989). Pore-forming properties of the adsorption protein of filamentous phage fd. *Biochim Biophys Acta* **985**, 239-247.

Glockshuber, R., Malia, M., Pfitzinger, I. & Pluckthun, A. (1990). A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry* **29**, 1362-1367.

Golchin, M. (2004). Recombinant antibodies against the K99 colonisation factor of *E. coli.* 194.

Goletz, S., Christensen, P. A., Kristensen, P., Blohm, D., Tomlinson, I., Winter, G. & Karsten, U. (2002). Selection of large diversities of antiidiotypic antibody fragments by phage display. *Journal of Molecular Biology* **315**, 1087-1097.

Gorbach, S. L., Chang, T. W. & Goldin, B. (1987). Successful treatment of relapsing *Clostridium difficile* colitis with Lactobacillus GG. *Lancet* **2**, 1519.

Greco, A., Ho, J. G., Lin, S. J., Palcic, M. M., Rupnik, M. & Ng, K. K. (2006). Carbohydrate recognition by *Clostridium difficile* toxin A. *Nat Struct Mol Biol* **13**, 460-461.

Green, D. R. & Amarante-Mendes, G. P. (1998). The point of no return: mitochondria, caspases, and the commitment to cell death. *Results Probl Cell Differ* **24**, 45-61.

Griffiths, A. D., Malmqvist, M., Marks, J. D. & Bye JM, Embleton MJ, McCafferty J, Baier M, Holliger KP, Gorick BD, Hughes-Jones NC (1993). Human anti-self antibodies with high specificity from phage display libraries. *Embo J* **12**, 725-734.

Griffiths, A. D., Williams, S. C., Hartley, O. Tomlinson IM, Waterhouse P, Crosby WL, Kontermann RE, Jones PT, Low NM, & Allison TJ (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *Embo J* **13**, 3245-3260.

Guo, Y. C., Zhou, Y. F., Zhang, X. E., Zhang, Z. P., Qiao, Y. M., Bi, L. J., Wen, J. K., Liang, M. F. & Zhang, J. B. (2006). Phage display mediated immuno-PCR. *Nucleic Acids Res* **34**, e62.

Hall, A. (1990). The cellular functions of small GTP-binding proteins. *Science* **249**, 635-640.

Hamilton, S., Odill, J., Pacifico, M. D., Wilson, G. D. & Kupsch, J. M. (2003). Effect of imidazole on the solubility of a his-tagged antibody fragment. *Hybrid Hybridomics* **22**, 347-355.

Harris, L. J., Larson, S. B., Hasel, K. W., Day, J., Greenwood, A. & McPherson, A. (1992). The three-dimensional structure of an intact monoclonal antibody for canine lymphoma. *Nature* **360**, 369-372.

Harrison, J. L., Williams, S. C., Winter, G. & Nissim, A. (1996). Screening of phage antibody libraries. *Methods Enzymol* **267**, 83-109.

Hawkins, R. E., Russell, S. J. & Winter, G. (1992). Selection of phage antibodies by binding affinity. Mimicking affinity maturation. *J Mol Biol* **226**, 889-896.

Hawkins, R. E., Russell, S. J., Baier, M. & Winter, G. (1993). The contribution of contact and non-contact residues of antibody in the affinity of binding to antigen. The interaction of mutant D1.3 antibodies with lysozyme. *J Mol Biol* **234**, 958-964.

He, D., Hagen, S. J., Pothoulakis, C., Chen, M., Medina, N. D., Warny, M. & LaMont, J. T. (2000). *Clostridium difficile* toxin A causes early damage to mitochondria in cultured cells. *Gastroenterology* **119**, 139-150.

Hecht, G., Pothoulakis, C., LaMont, J. T. & Madara, J. L. (1988). *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. *J Clin Invest* **82**, 1516-1524.

Hecht, J. R. & Olinger, E. J. (1989). *Clostridium difficile* colitis secondary to intravenous vancomycin. *Dig Dis Sci* **34**, 148-149.

Ho, J. G., Greco, A., Rupnik, M. & Ng, K. K. (2005). Crystal structure of receptor-binding C-terminal repeats from *Clostridium difficile* toxin A. *Proc Natl Acad Sci U S A* **102**, 18373-18378.

Hogrefe, H. H., Mullinax, R. L., Lovejoy, A. E., Hay, B. N. & Sorge, J. A. (1993). A bacteriophage lambda vector for the cloning and expression of immunoglobulin Fab fragments on the surface of filamentous phage. *Gene* **128**, 119-126.

Holliger, P., Prospero, T. & Winter, G. (1993). "Diabodies": small bivalent and bispecific antibody fragments. *Proc Natl Acad Sci U S A* **90**, 6444-6448.

Holliger, P., Brissinck, J., Williams, R. L., Thielemans, K. & Winter, G. (1996). Specific killing of lymphoma cells by cytotoxic T-cells mediated by a bispecific diabody. *Protein Eng* **9**, 299-305.

Holt, L. J., Herring, C., Jespers, L. S., Woolven, B. P. & Tomlinson, I. M. (2003). Domain antibodies: proteins for therapy. *Trends Biotechnol* **21**, 484-490.

Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P. & Winter, G. (1991). Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res* **19**, 4133-4137.

Hoogenboom, H. R. & Winter, G. (1992). By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. *J Mol Biol* **227**, 381-388.

Hoogenboom, H. R., Marks, J. D., Griffiths, A. D. & Winter, G. (1993). Building antibodies from their genes. *Rev Fr Transfus Hemobiol* **36**, 19-47.

Hoogenboom, H. R. (1997). Designing and optimizing library selection strategies for generating high-affinity antibodies. *Trends Biotechnol* **15**, 62-70.

Hudson, P. J. & Kortt, A. A. (1999). High avidity scFv multimers; diabodies and triabodies. *J Immunol Methods* **231**, 177-189.

Huston, J. S., Levinson, D., Mudgett-Hunter, M. Tai MS, Novotny J, Margolies MN, Ridge RJ, Bruccoleri RE, Haber E, & Crea R (1988). Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli. Proc Natl Acad Sci U S A* **85**, 5879-5883.

Jarvis, W. R. (1998). Epidemiology, appropriateness, and cost of vancomycin use. *Clin Infect Dis* **26**, 1200-1203.

Jobe, B. A., Grasley, A., Deveney, K. E., Deveney, C. W. & Sheppard, B. C. (1995). *Clostridium difficile* colitis: an increasing hospital-acquired illness. *Am J Surg* **169**, 480-483.

Johnson, S., Kent, S. A., O'Leary, K. J., Merrigan, M. M., Sambol, S. P., Peterson, L. R. & Gerding, D. N. (2001). Fatal pseudomembranous colitis associated with a variant *clostridium difficile* strain not detected by toxin A immunoassay. *Ann Intern Med* **135**, 434-438.

Jones, P. T., Dear, P. H., Foote, J., Neuberger, M. S. & Winter, G. (1986). Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* **321**, 522-525.

Just, I., Wilm, M., Selzer, J., Rex, G., von Eichel-Streiber, C., Mann, M. & Aktories, K. (1995). The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. *J Biol Chem* **270**, 13932-13936.

Juste, M., Martin-Eauclaire, M. F., Devaux, C., Billiald, P. & Aubrey, N. (2007). Using a recombinant bispecific antibody to block Na+ -channel toxins protects against experimental scorpion envenoming. *Cell Mol Life Sci* **64**, 206-218.

Kamiya, S., Yamakawa, K., Meng, X. Q., Ogura, H. & Nakamura, S. (1991). Production of monoclonal antibody to *Clostridium difficile* toxin A which neutralizes enterotoxicity but not haemagglutination activity. *FEMS Microbiol Lett* **65**, 311-315.

Kang, A. S., Barbas, C. F., Janda, K. D., Benkovic, S. J. & Lerner, R. A. (1991a). Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc Natl Acad Sci U S A* **88**, 4363-4366.

Kang, A. S., Jones, T. M. & Burton, D. R. (1991b). Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. *Proc Natl Acad Sci U S A* **88**, 11120-11123.

Kato, H., Kato, N., Watanabe, K., Ueno, K., Sakata, Y. & Fujita, K. (1996). Relapses or reinfections: analysis of a case of *Clostridium difficile*-associated colitis by two typing systems. *Curr Microbiol* **33**, 220-223.

Kato, H., Kato, N., Watanabe, K. Iwai N, Nakamura H, Yamamoto T, Suzuki K, Kim SM, Chong Y, &Wasito EB (1998). Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. *J Clin Microbiol* **36**, 2178-2182.

Kelly, C. P., Becker, S., Linevsky, J. K., Joshi, M. A., O'Keane, J. C., Dickey, B. F., LaMont, J. T. & Pothoulakis, C. (1994a). Neutrophil recruitment in *Clostridium difficile* toxin A enteritis in the rabbit. *J Clin Invest* **93**, 1257-1265.

Kelly, C. P., Pothoulakis, C. & LaMont, J. T. (1994b). *Clostridium difficile* colitis. *N Engl J Med* **330**, 257-262.

Kelly, C. P., Pothoulakis, C., Vavva, F., Castagliuolo, I., Bostwick, E. F., O'Keane, J. C., Keates, S. & LaMont, J. T. (1996). Anti-*Clostridium difficile* bovine immunoglobulin concentrate inhibits cytotoxicity and enterotoxicity of C. *difficile* toxins. *Antimicrob Agents Chemother* **40**, 373-379.

Kelly, C. P., Chetham, S., Keates, S., Bostwick, E. F., Roush, A. M., Castagliuolo, I., LaMont, J. T. & Pothoulakis, C. (1997). Survival of anti-*Clostridium difficile* bovine immunoglobulin concentrate in the human gastrointestinal tract. *Antimicrob Agents Chemother* **41**, 236-241.

Kikuchi, Y., Uno, S., Kinoshita, Y., Yoshimura, Y., Iida, S., Wakahara, Y., Tsuchiya, M., Yamada-Okabe, H. & Fukushima, N. (2005). Apoptosis inducing bivalent single-chain antibody fragments against CD47 showed antitumor potency for multiple myeloma. *Leuk Res* **29**, 445-450.

Kim, P. H., Iaconis, J. P. & Rolfe, R. D. (1987). Immunization of adult hamsters against *Clostridium difficile*-associated ileocecitis and transfer of protection to infant hamsters. *Infect Immun* **55**, 2984-2992.

Kink, J. A. & Williams, J. A. (1998). Antibodies to recombinant *Clostridium difficile* toxins A and B are an effective treatment and prevent relapse of C. *difficile*-associated disease in a hamster model of infection. *Infect Immun* **66**, 2018-2025.

Kipriyanov, S. M., Moldenhauer, G. & Little, M. (1997). High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures. *J Immunol Methods* **200**, 69-77.

Kipriyanov, S. M., Moldenhauer, G., Schuhmacher, J., Cochlovius, B., Von der Lieth, C. W., Matys, E. R. & Little, M. (1999). Bispecific tandem diabody for tumor therapy with improved antigen binding and pharmacokinetics. *J Mol Biol* **293**, 41-56.

Knappik, A. & Pluckthun, A. (1995). Engineered turns of a recombinant antibody improve its in vivo folding. *Protein Eng* **8**, 81-89.

Knappik, A., Ge, L., Honegger, A. Pack P, Fischer M, Wellnhofer G, Hoess A, Wolle J, Pluckthun A, & Virnekas B (2000). Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J Mol Biol* **296**, 57-86.

Kohler, G. & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495-497.

Kotloff, K. L., Wasserman, S. S., Losonsky, G. A., Thomas, W., Jr., Nichols, R., Edelman, R., Bridwell, M. & Monath, T. P. (2001). Safety and immunogenicity of increasing doses of a *Clostridium difficile* toxoid vaccine administered to healthy adults. *Infect Immun* **69**, 988-995.

Kristensen, P. & Winter, G. (1998). Proteolytic selection for protein folding using filamentous bacteriophages. *Fold Des* **3**, 321-328.

Krivan, H. C., Clark, G. F., Smith, D. F. & Wilkins, T. D. (1986). Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Gal alpha 1-3Gal beta 1-4GlcNAc. *Infect Immun* **53**, 573-581.

Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J. L. & Mignotte, B. (1995). The biochemistry of programmed cell death. *Faseb J* **9**, 1277-1287.

Kyne, L., Warny, M., Qamar, A. & Kelly, C. P. (2000). Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. *N Engl J Med* **342**, 390-397.

Kyne, L., Warny, M., Qamar, A. & Kelly, C. P. (2001). Association between antibody response to toxin A and protection against recurrent *Clostridium difficile* diarrhoea. *Lancet* **357**, 189-193.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lah, M., Goldstraw, A., White, J. F., Dolezal, O., Malby, R. & Hudson, P. J. (1994). Phage surface presentation and secretion of antibody fragments using an adaptable phagemid vector. *Hum Antibodies Hybridomas* **5**, 48-56.

Landolfi, N. F. (1991). A chimeric IL-2/Ig molecule possesses the functional activity of both proteins. *J Immunol* **146**, 915-919.

Li, R., Hoess, R. H., Bennett, J. S. & DeGrado, W. F. (2003). Use of phage display to probe the evolution of binding specificity and affinity in integrins. *Protein Eng* **16**, 65-72.

Libby, J. M., Jortner, B. S. & Wilkins, T. D. (1982). Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. *Infect Immun* **36**, 822-829.

Limaye, A. P., Turgeon, D. K., Cookson, B. T. & Fritsche, T. R. (2000). Pseudomembranous colitis caused by a toxin A(-) B(+) strain of *Clostridium difficile*. *J Clin Microbiol* **38**, 1696-1697.

Linevsky, J. K., Pothoulakis, C., Keates, S., Warny, M., Keates, A. C., Lamont, J. T. & Kelly, C. P. (1997). IL-8 release and neutrophil activation by *Clostridium difficile* toxin-exposed human monocytes. *Am J Physiol* **273**, G1333-1340.

Lorimer, I. A., Keppler-Hafkemeyer, A., Beers, R. A., Pegram, C. N., Bigner, D. D. & Pastan, I. (1996). Recombinant immunotoxins specific for a mutant epidermal growth factor receptor: targeting with a single chain antibody variable domain isolated by phage display. *Proc Natl Acad Sci U S A* **93**, 14815-14820.

Lowman, H. B., Bass, S. H., Simpson, N. & Wells, J. A. (1991). Selecting high-affinity binding proteins by monovalent phage display. *Biochemistry* **30**, 10832-10838.

Lyerly, D. M., Lockwood, D. E., Richardson, S. H. & Wilkins, T. D. (1982). Biological activities of toxins A and B of *Clostridium difficile*. *Infect Immun* **35**, 1147-1150.

Lyerly, D. M., Saum, K. E., MacDonald, D. K. & Wilkins, T. D. (1985). Effects of *Clostridium difficile* toxins given intragastrically to animals. *Infect Immun* **47**, 349-352.

Lyerly, D. M., Phelps, C. J., Toth, J. & Wilkins, T. D. (1986). Characterization of toxins A and B of *Clostridium difficile* with monoclonal antibodies. *Infect Immun* **54**, 70-76.

Lyerly, D. M., Bostwick, E. F., Binion, S. B. & Wilkins, T. D. (1991). Passive immunization of hamsters against disease caused by *Clostridium difficile* by use of bovine immunoglobulin G concentrate. *Infect Immun* **59**, 2215-2218.

Mackay, D. J. & Hall, A. (1998). Rho GTPases. *J Biol Chem* **273**, 20685-20688.

Mahida, Y. R., Makh, S., Hyde, S., Gray, T. & Borriello, S. P. (1996). Effect of *Clostridium difficile* toxin A on human intestinal epithelial cells: induction of interleukin 8 production and apoptosis after cell detachment. *Gut* **38**, 337-347.

Makvandi-Nejad, S., McLean, M. D., Hirama, T., Almquist, K. C., Mackenzie, C. R. & Hall, J. C. (2005). Transgenic tobacco plants expressing a dimeric single-chain variable fragment (scfv) antibody against Salmonella enterica serotype Paratyphi B. *Transgenic Res* **14**, 785-792.

Mantyh, C. R., Maggio, J. E., Mantyh, P. W., Vigna, S. R. & Pappas, T. N. (1996). Increased substance P receptor expression by blood vessels and lymphoid aggregates in *Clostridium difficile*-induced pseudomembranous colitis. *Dig Dis Sci* **41**, 614-620.

Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D. & Winter, G. (1991). By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J Mol Biol* **222**, 581-597.

Marks, J. D., Griffiths, A. D., Malmqvist, M., Clackson, T. P., Bye, J. M. & Winter, G. (1992). By-passing immunization: building high affinity human antibodies by chain shuffling. *Biotechnology (N Y)* **10**, 779-783.

Mathieu, R., Lim, J., Simpson, P., Prasannan, S., Fairweather, N. & Matthews, S. (2003). Resonance assignment and topology of a clostridial repetitive oligopeptide (CROP) region of toxin A from *Clostridium difficile*. *J Biomol NMR* **25**, 83-84.

Maynard, J. & Georgiou, G. (2000). Antibody engineering. *Annu Rev Biomed Eng* **2**, 339-376.

McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**, 552-554.

McCafferty, J., Jackson, R. H. & Chiswell, D. J. (1991). Phage-enzymes: expression and affinity chromatography of functional alkaline phosphatase on the surface of bacteriophage. *Protein Eng* **4**, 955-961.

McCafferty, J., Fitzgerald, K. J., Earnshaw, J., Chiswell, D. J., Link, J., Smith, R. & Kenten, J. (1994). Selection and rapid purification of murine antibody fragments that bind a transition-state analog by phage display. *Appl Biochem Biotechnol* **47**, 157-171; discussion 171-153.

McElhiney, J., Drever, M., Lawton, L. A. & Porter, A. J. (2002). Rapid isolation of a single-chain antibody against the cyanobacterial toxin microcystin-LR by phage display and its use in the immunoaffinity concentration of microcystins from water. *Appl Environ Microbiol* **68**, 5288-5295.

McFarland, L. V., Surawicz, C. M., Rubin, M., Fekety, R., Elmer, G. W. & Greenberg, R. N. (1999). Recurrent *Clostridium difficile* disease: epidemiology and clinical characteristics. *Infect Control Hosp Epidemiol* **20**, 43-50.

McFarland, L. V., Brandmarker, S. A. & Guandalini, S. (2000). Pediatric *Clostridium difficile*: a phantom menace or clinical reality? *J Pediatr Gastroenterol Nutr* **31**, 220-231.

McFarland, L. V., Reiber, G. E. & Norman, J. E. (2005). Recruitment of Medicaid and dual-enrolled Medicare beneficiaries with diabetes mellitus into a randomized controlled trial. *Am J Manag Care* **11**, 443-448.

Meulemans, E. V., Slobbe, R., Wasterval, P., Ramaekers, F. C. & van Eys, G. J. (1994). Selection of phage-displayed antibodies specific for a cytoskeletal antigen by competitive elution with a monoclonal antibody. *J Mol Biol* **244**, 353-360.

Milstein, C. & Cuello, A. C. (1983). Hybrid hybridomas and their use in immunohistochemistry. *Nature* **305**, 537-540.

Mogg, G. A., George, R. H., Youngs, D., Johnson, M., Thompson, H., Burdon, D. W. & Keighley, M. R. (1982). Randomized controlled trial of colestipol in antibiotic-associated colitis. *Br J Surg* **69**, 137-139.

Munro, S. & Pelham, H. R. (1986). An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46**, 291-300.

Murphy, C., Vernon, M. & Cullen, M. (2006). Intravenous immunoglobulin for resistant *Clostridium difficile* infection. *Age Ageing* **35**, 85-86.

Mylonakis, E., Ryan, E. T. & Calderwood, S. B. (2001). *Clostridium difficile*--Associated diarrhea: A review. *Arch Intern Med* **161**, 525-533.

Neuberger, M. (1996). Generating high-avidity human Mabs in mice. *Nat Biotechnol* **14**, 826.

Nieroda, C. A., Milenic, D. E., Carrasquillo, J. A., Scholm, J. & Greiner, J. W. (1995). Improved tumor radioimmunodetection using a single-chain Fv and gamma-interferon: potential clinical applications for radioimmunoguided surgery and gamma scanning. *Cancer Res* **55**, 2858-2865.

Nisonoff, A. & Mandy, W. J. (1962). Quantitative estimation of the hybridization of rabbit antibodies. *Nature* **194**, 355-359.

Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D. & Winter, G. (1994). Antibody fragments from a 'single pot' phage display library as immunochemical reagents. *Embo J* **13**, 692-698.

Nobes, C. D. & Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Nord, C. E. & Edlund, C. (1990). Impact of antimicrobial agents on human intestinal microflora. *J Chemother* **2**, 218-237.

Nowakowski, A., Wang, C., Powers, D. B. Amersdorfer P, Smith TJ, Montgomery VA, Sheridan R, Blake R, Smith LA, & Marks JD. (2002). Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody. *Proc Natl Acad Sci U S A* **99**, 11346-11350.

Pack, P., Kujau, M., Schroeckh, V., Knupfer, U., Wenderoth, R., Riesenberg, D. & Pluckthun, A. (1993). Improved bivalent miniantibodies, with identical avidity as whole antibodies, produced by high cell density fermentation of *Escherichia coli*. *Biotechnology (N Y)* **11**, 1271-1277.

Pack, P., Muller, K., Zahn, R. & Pluckthun, A. (1995). Tetravalent miniantibodies with high avidity assembling in *Escherichia coli*. *J Mol Biol* **246**, 28-34.

Parmley, S. F. & Smith, G. P. (1988). Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. *Gene* **73**, 305-318.

Parsons, H. L., Earnshaw, J. C., Wilton, J., Johnson, K. S., Schueler, P. A., Mahoney, W. & McCafferty, J. (1996). Directing phage selections towards specific epitopes. *Protein Eng* **9**, 1043-1049.

Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K. & Hall, A. (1990). Microinjection of recombinant p21rho induces rapid changes in cell morphology. *J Cell Biol* **111**, 1001-1007.

Pelaez, T., Alcala, L., Alonso, R., Rodriguez-Creixems, M., Garcia-Lechuz, J. M. & Bouza, E. (2002). Reassessment of *Clostridium difficile* susceptibility to metronidazole and vancomycin. *Antimicrob Agents Chemother* **46**, 1647-1650.

Perelson, A. S. (1989). Immune network theory. *Immunol Rev* **110**, 5-36.

Perisic, O., Webb, P. A., Holliger, P., Winter, G. & Williams, R. L. (1994). Crystal structure of a diabody, a bivalent antibody fragment. *Structure* **2**, 1217-1226.

Persky, S. E. & Brandt, L. J. (2000). Treatment of recurrent *Clostridium difficile*-associated diarrhea by administration of donated stool directly through a colonoscope. *Am J Gastroenterol* **95**, 3283-3285.

Pfeifer, G., Schirmer, J., Leemhuis, J., Busch, C., Meyer, D. K., Aktories, K. & Barth, H. (2003). Cellular uptake of *Clostridium difficile* toxin B. Translocation of the N-terminal catalytic domain into the cytosol of eukaryotic cells. *J Biol Chem* **278**, 44535-44541.

Philippa M. O'Brien, R. A. (2002). Antibody Phage Display. 7-8.

Pluckthun, A. (1994). *Escherichia coli* producing recombinant antibodies. *Bioprocess Technol* **19**, 233-252.

Poljak, R. J., Amzel, L. M., Avey, H. P., Chen, B. L., Phizackerley, R. P. & Saul, F. (1973). Three-dimensional structure of the Fab' fragment of a human immunoglobulin at 2,8-A resolution. *Proc Natl Acad Sci U S A* **70**, 3305-3310.

Pothoulakis, C., Sullivan, R., Melnick, D. A., Triadafilopoulos, G., Gadenne, A. S., Meshulam, T. & LaMont, J. T. (1988). *Clostridium difficile* toxin A stimulates intracellular calcium release and chemotactic response in human granulocytes. *J Clin Invest* **81**, 1741-1745.

Pothoulakis, C., Karmeli, F., Kelly, C. P., Eliakim, R., Joshi, M. A., O'Keane, C. J., Castagliuolo, I., LaMont, J. T. & Rachmilewitz, D. (1993). Ketotifen inhibits *Clostridium difficile* toxin A-induced enteritis in rat ileum. *Gastroenterology* **105**, 701-707.

Pothoulakis, C. (1996). Pathogenesis of *Clostridium difficile*-associated diarrhoea. *Eur J Gastroenterol Hepatol* **8**, 1041-1047.

Pothoulakis, C. & Lamont, J. T. (2001). Microbes and microbial toxins: paradigms for microbial-mucosal interactions II. The integrated response of the intestine to *Clostridium difficile* toxins. *Am J Physiol Gastrointest Liver Physiol* **280**, G178-183.

Poxton, I. R., McCoubrey, J. & Blair, G. (2001). The pathogenicity of *Clostridium difficile*. *Clin Microbiol Infect* **7**, 421-427.

Prepens, U., Just, I., von Eichel-Streiber, C. & Aktories, K. (1996). Inhibition of Fc epsilon-RI-mediated activation of rat basophilic leukemia cells by *Clostridium difficile* toxin B (monoglucosyltransferase). *J Biol Chem* **271**, 7324-7329.

Pugsley, A. P. (1993). The complete general secretory pathway in gram-negative bacteria. *Microbiol Rev* **57**, 50-108.

Qa'Dan, M., Spyres, L. M. & Ballard, J. D. (2000). pH-induced conformational changes in *Clostridium difficile* toxin B. *Infect Immun* **68**, 2470-2474.

Rajewsky, K. (1996). Clonal selection and learning in the antibody system. *Nature* **381**, 751-758.

Rauchenberger, R., Borges, E., Thomassen-Wolf, E. Rom E, Adar R, Yanlv Y, Malka M, Chumakov I, Kotzer S, Resnitzky D, Knappik A, Reiffert S, Prassler J, Jury K, Waldherr D, Bauer S, Kretzschmar T, Yayon A, & Rothe C. (2003). Human combinatorial Fab library yielding specific and functional antibodies against the human fibroblast growth factor receptor 3. *J Biol Chem* **278**, 38194-38205.

Reisner, Y. & Dagan, S. (1998). The Trimera mouse: generating human monoclonal antibodies and an animal model for human diseases. *Trends Biotechnol* **16**, 242-246.

Riechmann, L. & Holliger, P. (1997). The C-terminal domain of TolA is the coreceptor for filamentous phage infection of *E. coli*. *Cell* **90**, 351-360.

Riegler, M., Sedivy, R., Pothoulakis, C. Hamilton G, Zacherl J, Bischof G, Cosentini E, Feil W, Schiessel R, & LaMont JT (1995). *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic epithelium in vitro. *J Clin Invest* **95**, 2004-2011.

Riley, T. V. (1996). Antibiotic-associated diarrhoea. A costly problem. *Pharmacoeconomics* **10**, 1-3.

Roberts, A. K. & Shone, C. C. (2001). Modification of surface histidine residues abolishes the cytotoxic activity of *Clostridium difficile* toxin A. *Toxicon* **39**, 325-333.

Roberts, B. L., Markland, W., Siranosian, K., Saxena, M. J., Guterman, S. K. & Ladner, R. C. (1992). Protease inhibitor display M13 phage: selection of high-affinity neutrophil elastase inhibitors. *Gene* **121**, 9-15.

Roberts, M. C., McFarland, L. V., Mullany, P. & Mulligan, M. E. (1994). Characterization of the genetic basis of antibiotic resistance in *Clostridium difficile*. *J Antimicrob Chemother* **33**, 419-429.

Rubinstein, J. L., Holt, L. J., Walker, J. E. & Tomlinson, I. M. (2003). Use of phage display and high-density screening for the isolation of an antibody against the 51-kDa subunit of complex I. *Anal Biochem* **314**, 294-300.

Rupnik, M., Avesani, V., Janc, M., von Eichel-Streiber, C. & Delmee, M. (1998). A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J Clin Microbiol* **36**, 2240-2247.

Salcedo, J., Keates, S., Pothoulakis, C., Warny, M., Castagliuolo, I., LaMont, J. T. & Kelly, C. P. (1997). Intravenous immunoglobulin therapy for severe *Clostridium difficile* colitis. *Gut* **41**, 366-370.

Sambol, S. P., Merrigan, M. M., Lyerly, D., Gerding, D. N. & Johnson, S. (2000). Toxin gene analysis of a variant strain of *Clostridium difficile* that causes human clinical disease. *Infect Immun* **68**, 5480-5487.

Sambrook, J., E. F. Fristsch, and T. Maniatis (1989). Molecular cloning : a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Sping Harbor, N. Y.

Samore, M. H. (1993). Epidemiology of nosocomial *Clostridium difficile* infection. *Compr Ther* **19**, 151-156.

Sandhu, J. S. (1992). Protein engineering of antibodies. *Crit Rev Biotechnol* **12**, 437-462.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463-5467.

Sauerborn, M., Leukel, P. & von Eichel-Streiber, C. (1997). The C-terminal ligand-binding domain of *Clostridium difficile* toxin A (TcdA) abrogates TcdA-specific binding to cells and prevents mouse lethality. *FEMS Microbiol Lett* **155**, 45-54.

Savidge, T. C., Pan, W. H., Newman, P., O'Brien, M., Anton, P. M. & Pothoulakis, C. (2003). *Clostridium difficile* toxin B is an inflammatory enterotoxin in human intestine. *Gastroenterology* **125**, 413-420.

Schier, R., McCall, A., Adams, G. P. Marshall KW, Merritt H, Yim M, Crawford RS, Weiner LM, Marks C, & Marks JD. (1996). Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J Mol Biol* **263**, 551-567.

Schlenzka, J., Moehler, T. M., Kipriyanov, S. M. Kornacker M, Benner A, Bahre A, Stassar MJ, Schafer HJ, Little M, Goldschmidt H, & Cochlovius B (2004). Combined effect of recombinant CD19 x CD16 diabody and thalidomide in a preclinical model of human B cell lymphoma. *Anticancer Drugs* **15**, 915-919.

Schroeder, M. S. (2005). *Clostridium difficile*-associated diarrhea. *Am Fam Physician* **71**, 921-928.

Segal, D. M., Perez, P., Karpovsky, B. & Titus, J. A. (1986). Targeting of cytotoxic cells with cross-linked antibody heteroaggregates. *Mol Immunol* **23**, 1211-1214.

Selvakumar, D., Miyamoto, M., Furuichi, Y. & Komiyama, T. (2006). Inhibition of fungal beta-1,3-glucan synthase and cell growth by HM-1 killer toxin single-chain anti-idiotypic antibodies. *Antimicrob Agents Chemother* **50**, 3090-3097.

Settle, C. D. & Wilcox, M. H. (1996). Review article: antibiotic-induced *Clostridium difficile* infection. *Aliment Pharmacol Ther* **10**, 835-841.

Shadidi, M. & Sioud, M. (2001). An anti-leukemic single chain Fv antibody selected from a synthetic human phage antibody library. *Biochem Biophys Res Commun* **280**, 548-552.

Shearman, C. W., Pollock, D., White, G., Hehir, K., Moore, G. P., Kanzy, E. J. & Kurrle, R. (1991). Construction, expression and characterization of humanized antibodies directed against the human alpha/beta T cell receptor. *J Immunol* **147**, 4366-4373.

Sheets, M. D., Amersdorfer, P., Finnern, R. Sargent P, Lindquist E, Schier R, Hemingsen G, Wong C, Gerhart JC, & Marks JD. (1998). Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. *Proc Natl Acad Sci U S A* **95**, 6157-6162.

Silva, J., Jr. (1989). Update on pseudomembranous colitis. *West J Med* **151**, 644-648.

Skerra, A. & Pluckthun, A. (1988). Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* **240**, 1038-1041.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**, 1315-1317.

Soehn, F., Wagenknecht-Wiesner, A., Leukel, P., Kohl, M., Weidmann, M., von Eichel-Streiber, C. & Braun, V. (1998). Genetic rearrangements in the pathogenicity locus of *Clostridium difficile* strain 8864--implications for transcription, expression and enzymatic activity of toxins A and B. *Mol Gen Genet* **258**, 222-232.

Stockwin, L. H. & Holmes, S. (2003). Antibodies as therapeutic agents: vive la renaissance! *Expert Opin Biol Ther* **3**, 1133-1152.

Strachan, G., McElhiney, J., Drever, M. R., McIntosh, F., Lawton, L. A. & Porter, A. J. (2002). Rapid selection of anti-hapten antibodies isolated from synthetic and semi-synthetic antibody phage display libraries expressed in *Escherichia coli*. *FEMS Microbiol Lett* **210**, 257-261.

Strieter, R. M., Koch, A. E., Antony, V. B., Fick, R. B., Jr., Standiford, T. J. & Kunkel, S. L. (1994). The immunopathology of chemotactic cytokines: the role of interleukin-8 and monocyte chemoattractant protein-1. *J Lab Clin Med* **123**, 183-197.

Stubbe, H., Berdoz, J., Kraehenbuhl, J. P. & Corthesy, B. (2000). Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing *Clostridium difficile* toxin A damaging of T84 monolayers. *J Immunol* **164**, 1952-1960.

Surawicz, C. M., McFarland, L. V., Greenberg, R. N. Rubin M, Fekety R, Mulligan ME, Garcia RJ, Brandmarker S, Bowen K, Borjal D, & Elmer GW (2000). The search for a better treatment for recurrent *Clostridium difficile* disease: use of high-dose vancomycin combined with Saccharomyces boulardii. *Clin Infect Dis* **31**, 1012-1017.

Tang, P. M., Foltz, L. A., Mahoney, W. C. & Schueler, P. A. (1995). A high affinity digoxin-binding protein displayed on M13 is functionally identical to the native protein. *J Biol Chem* **270**, 7829-7835.

Tang-Feldman, Y., Mayo, S., Silva Jr, J., Jr. & Cohen, S. H. (2003). Molecular analysis of *Clostridium difficile* strains isolated from 18 cases of recurrent clostridium *difficile*-associated diarrhea. *J Clin Microbiol* **41**, 3413-3414.

Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. & Galeffi, P. (1993). Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. *Nature* **366**, 469-472.

Tomizuka, K., Shinohara, T., Yoshida, H., Uejima, H., Ohguma, A., Tanaka, S., Sato, K., Oshimura, M. & Ishida, I. (2000). Double trans-chromosomic mice: maintenance of two individual human chromosome fragments containing Ig heavy and kappa loci and expression of fully human antibodies. *Proc Natl Acad Sci U S A* **97**, 722-727.

Traunecker, A., Schneider, J., Kiefer, H. & Karjalainen, K. (1989). Highly efficient neutralization of HIV with recombinant CD4-immunoglobulin molecules. *Nature* **339**, 68-70.

Triadafilopoulos, G., Pothoulakis, C., Weiss, R., Giampaolo, C. & Lamont, J. T. (1989). Comparative study of *Clostridium difficile* toxin A and cholera toxin in rabbit ileum. *Gastroenterology* **97**, 1186-1192.

Tucker, K. D., Carrig, P. E. & Wilkins, T. D. (1990). Toxin A of *Clostridium difficile* is a potent cytotoxin. *J Clin Microbiol* **28**, 869-871.

Tucker, K. D. & Wilkins, T. D. (1991). Toxin A of *Clostridium difficile* binds to the human carbohydrate antigens I, X, and Y. *Infect Immun* **59**, 73-78.

Valiquette, L., Low, D. E., Pepin, J. & McGeer, A. (2004). *Clostridium difficile* infection in hospitals: a brewing storm. *Cmaj* **171**, 27-29.

van den Berg, R. J., Claas, E. C., Oyib, D. H., Klaassen, C. H., Dijkshoorn, L., Brazier, J. S. & Kuijper, E. J. (2004). Characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping. *J Clin Microbiol* **42**, 1035-1041.

van Dijk, M. A. & van de Winkel, J. G. (2001). Human antibodies as next generation therapeutics. *Curr Opin Chem Biol* **5**, 368-374.

Van Ewijk, W., de Kruif, J., Germeraad, W. T., Berendes, P., Ropke, C., Platenburg, P. P. & Logtenberg, T. (1997). Subtractive isolation of phage-displayed single-chain antibodies to thymic stromal cells by using intact thymic fragments. *Proc Natl Acad Sci U S A* **94**, 3903-3908.

van Kuppevelt, T. H., Dennissen, M. A., van Venrooij, W. J., Hoet, R. M. & Veerkamp, J. H. (1998). Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney. *J Biol Chem* **273**, 12960-12966.

Vaughan, T. J., Williams, A. J., & Pritchard, K. (1996). Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat Biotechnol* **14**, 309-314.

von Eichel-Streiber, C., Harperath, U., Bosse, D. & Hadding, U. (1987). Purification of two high molecular weight toxins of *Clostridium difficile* which are antigenically related. *Microb Pathog* **2**, 307-318.

von Eichel-Streiber, C., Laufenberg-Feldmann, R., Sartingen, S., Schulze, J. & Sauerborn, M. (1990). Cloning of *Clostridium difficile* toxin B gene and demonstration of high N-terminal homology between toxin A and B. *Med Microbiol Immunol (Berl)* **179**, 271-279.

von Eichel-Streiber, C., Laufenberg-Feldmann, R., Sartingen, S., Schulze, J. & Sauerborn, M. (1992a). Comparative sequence analysis of the *Clostridium difficile* toxins A and B. *Mol Gen Genet* **233**, 260-268.

von Eichel-Streiber, C., Sauerborn, M. & Kuramitsu, H. K. (1992b). Evidence for a modular structure of the homologous repetitive C-terminal carbohydrate-binding sites of *Clostridium difficile* toxins and Streptococcus mutans glucosyltransferases. *J Bacteriol* **174**, 6707-6710.

von Eichel-Streiber, C., Boquet, P., Sauerborn, M. & Thelestam, M. (1996). Large clostridial cytotoxins--a family of glycosyltransferases modifying small GTP-binding proteins. *Trends Microbiol* **4**, 375-382.

Vora, S., Glikman, D., Kahanna, M. & Daum, R. S. (2006). Severe diarrhea and shock. *Pediatr Infect Dis J* **25**, 279, 284-275.

Voth, D. E. & Ballard, J. D. (2005). *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin Microbiol Rev* **18**, 247-263.

Wang, X., Campoli, M., Ko, E., Luo, W. & Ferrone, S. (2004). Enhancement of scFv fragment reactivity with target antigens in binding assays following mixing with anti-tag monoclonal antibodies. *J Immunol Methods* **294**, 23-35.

Ward, R. L., Clark, M. A., Lees, J. & Hawkins, N. J. (1996). Retrieval of human antibodies from phage-display libraries using enzymatic cleavage. *J Immunol Methods* **189**, 73-82.

Ward, S. J., Douce, G., Dougan, G. & Wren, B. W. (1999a). Local and systemic neutralizing antibody responses induced by intranasal immunization with the nontoxic binding domain of toxin A from *Clostridium difficile*. *Infect Immun* **67**, 5124-5132.

Ward, S. J., Douce, G., Figueiredo, D., Dougan, G. & Wren, B. W. (1999b). Immunogenicity of a Salmonella typhimurium aroA aroD vaccine expressing a nontoxic domain of *Clostridium difficile* toxin A. *Infect Immun* **67**, 2145-2152.

Warny, M., Vaerman, J. P., Avesani, V. & Delmee, M. (1994). Human antibody response to *Clostridium difficile* toxin A in relation to clinical course of infection. *Infect Immun* **62**, 384-389.

Warny, M., Fatimi, A., Bostwick, E. F., Laine, D. C., Lebel, F., LaMont, J. T., Pothoulakis, C. & Kelly, C. P. (1999). Bovine immunoglobulin concentrate-*clostridium difficile* retains C *difficile* toxin neutralising activity after passage through the human stomach and small intestine. *Gut* **44**, 212-217.

Watanabe, T., Ohashi, K., Matsui, K. & Kubota, T. (1997). Comparative studies of the bactericidal, morphological and post-antibiotic effects of arbekacin and vancomycin against methicillin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* **39**, 471-476.

Weiner, C., Pan, Q., Hurtig, M., Boren, T., Bostwick, E. & Hammarstrom, L. (1999). Passive immunity against human pathogens using bovine antibodies. *Clin Exp Immunol* **116**, 193-205.

Weiner, L. M. (2006). Fully human therapeutic monoclonal antibodies. *J Immunother* **29**, 1-9.

Weis, W. I. & Drickamer, K. (1996). Structural basis of lectin-carbohydrate recognition. *Annu Rev Biochem* **65**, 441-473.

Welschof, M., Terness, P., Kolbinger, F. Zewe M, Dubel S, Dorsam H, Hain C, Finger M, Jung M, & Moldenhauer G (1995). Amino acid sequence based PCR primers for amplification of rearranged human heavy and light chain immunoglobulin variable region genes. *J Immunol Methods* **179**, 203-214.

Whitlow, M., Filpula, D., Rollence, M. L., Feng, S. L. & Wood, J. F. (1994). Multivalent Fvs: characterization of single-chain Fv oligomers and preparation of a bispecific Fv. *Protein Eng* **7**, 1017-1026.

Wilcox, M. H., Cunniffe, J. G., Trundle, C. & Redpath, C. (1996). Financial burden of hospital-acquired *Clostridium difficile* infection. *J Hosp Infect* **34**, 23-30.

Wilcox, M. H., Fawley, W. N., Settle, C. D. & Davidson, A. (1998). Recurrence of symptoms in *Clostridium difficile* infection--relapse or reinfection? *J Hosp Infect* **38**, 93-100.

Wilcox, M. H. (2003). Gastrointestinal disorders and the critically ill. *Clostridium difficile* infection and pseudomembranous colitis. *Best Pract Res Clin Gastroenterol* **17**, 475-493.

Wilcox, M. H. (2004). Descriptive study of intravenous immunoglobulin for the treatment of recurrent *Clostridium difficile* diarrhoea. *J Antimicrob Chemother* **53**, 882-884.

Williamson, P. & Matthews, R. (1999). Development of neutralising human recombinant antibodies to pertussis toxin. *FEMS Immunol Med Microbiol* **23**, 313-319.

Winter, G., Griffiths, A. D., Hawkins, R. E. & Hoogenboom, H. R. (1994). Making antibodies by phage display technology. *Annu Rev Immunol* **12**, 433-455.

Winter, G. (1998). Synthetic human antibodies and a strategy for protein engineering. *FEBS Lett* **430**, 92-94.

Wren, B. W., Russell, R. R. & Tabaqchali, S. (1991). Antigenic cross-reactivity and functional inhibition by antibodies to *Clostridium difficile* toxin A, Streptococcus mutans glucan-binding protein, and a synthetic peptide. *Infect Immun* **59**, 3151-3155.

Wu, S., Ke, A. & Doudna, J. A. (2007). A fast and efficient procedure to produce scFvs specific for large macromolecular complexes. *J Immunol Methods* **318**, 95-101.

Wu, T. T. & Kabat, E. A. (1970). An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J Exp Med* **132**, 211-250.

Yokota, T., Milenic, D. E., Whitlow, M. & Schlom, J. (1992). Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res* **52**, 3402-3408.

Young, G. P., Ward, P. B., Bayley, N., Gordon, D., Higgins, G., Trapani, J. A., McDonald, M. I., Labrooy, J. & Hecker, R. (1985). Antibiotic-associated colitis due to *Clostridium difficile*: double-blind comparison of vancomycin with bacitracin. *Gastroenterology* **89**, 1038-1045.

Zaccolo, M., Griffiths, A. P., Prospero, T. D., Winter, G. & Gherardi, E. (1997). Dimerization of Fab fragments enables ready screening of phage antibodies that affect hepatocyte growth factor/scatter factor activity on target cells. *Eur J Immunol* **27**, 618-623.

Zeitlin, L., Olmsted, S. S., Moench, T. R. Co MS, Martinell BJ, Paradkar VM, Russell DR, Queen C, Cone RA, & Whaley KJ. (1998). A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nat Biotechnol* **16**, 1361-1364.

Zhang, M. X., Bohlman, M. C., Itatani, C., Burton, D. R., Parren, P. W., St Jeor, S. C. & Kozel, T. R. (2006). Human recombinant antimannan immunoglobulin G1 antibody confers resistance to hematogenously disseminated candidiasis in mice. *Infect Immun* **74**, 362-369.

Zhong, R. K., Loken, M., Lane, T. A. & Ball, E. D. (2006). CTLA-4 blockade by a human MAb enhances the capacity of AML-derived DC to induce T-cell responses against AML cells in an autologous culture system. *Cytotherapy* **8**, 3-12.

Zhou, K., Wang, Y., Gorski, J. L., Nomura, N., Collard, J. & Bokoch, G. M. (1998). Guanine nucleotide exchange factors regulate specificity of downstream signaling from Rac and Cdc42. *J Biol Chem* **273**, 16782-16786.